STRUCTURAL AND MOLECULAR BIOLOGY OF PROTEASE FUNCTION AND INHIBITION

Organizers: Christine Debouck and Charles Craik

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Structural and Molecular Biology of Protease Function and Inhibition

Overview of Protease Classes

S 001 THE THREE-DIMENSIONAL STRUCTURE AND FUNCTION OF ASPARTIC PROTEINASES, Tom L Blundell, Venugopal Dhanaraj, Chris Dealwis, Carlos Aguilar, Kunchur Guruprasad, Jon Cooper, Stephen P Wood, Alan Mills, Mark Crawford, and Mohammed Badasso, Laboratory of Molecular Biology, and ICRF Unit of Structural Molecular Biology, Department of Crystallography, Birkbeck College, University of London, Malet Street, London WC21 7HX.

Three-dimensional structures have been defined for eight pepsin-like aspartic proteinases from fungal and mammalian sources. These include high and medium resolution X-ray analyses of human recombinant renin, mouse submandibular renin, and yeast proteinase A complexed with small peptide inhibitors. We have also prepared crystals of the protein inhibitor IA3 of yeast proteinase A. There are also more than thirty crystal structures of fungal aspartic proteinase inhibitor complexes with enzymes from Endothia parasitica, Mucor pusillus and Trichoderma reesei. The detailed structure analyses will be used as a basis to discuss the catalytic mechanism and the varying specificities of the aspartic proteinases. The structures and mechanism of the enzymes will be compared with those for the retroviral proteinases.

S 002 SERINE PROTEINASES AND THE CONVERGENCE OF ACTIVE SITE GEOMETRIES AMONG THE FOUR CLASSES OF PROTEOLYTIC ENZYMES, Michael N.G. James, Medical Research Council of Canada Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada.

Thus far, proteolytic enzymes can still be placed into one of four separate categories originally defined in the 1960's by Brian Hartley, namely the serine, aspartic, metallo- and cysteine proteinases. On a structural basis, however, it has been necessary to increase the numbers of subfamilies over the years. In this way it has still been possible to include many varieties of serine proteinase such as: a) eukaryotic enzymes having a fold similar to pancreatic chymotrypsin, b) bacterial enzymes with a pancreatic-like fold (e.g. SGPB), c) bacterial enzymes with a subtilisin fold or d) eukaryotic enzymes with a subtilisin fold (e.g. KEX2). Structural subfamilies are also required for the metalloproteinases and the aspartic proteinases. Further sub-categories maybe required for newly discovered proteolytic activities. All of these enzymes have a common substrate, i.e. the peptide bond. In spite of differences in structure and in the nature of the groups at the active site responsible for the catalysis, these proteolytic enzymes have converged on a common active site geometry. The serine and cysteine proteinases have covalently attached acyl-enzyme intermediates on their reaction pathways whereas the aspartic and metalloproteinases catalyze peptide bond hydrolysis without involving covalently attached intermediates. In all four classes, catalysis involves an attack by an appropriate nucleophile on the carbonyl-carbon atom of the substrate with the concommitant formation of a transitory tetrahedral intermediate. Generation of the attacking nucleophile from water or an amino-acid side chain is assisted by a general base that subsequently transfers the abstracted proton to the nitrogen of the leaving group. Electrophilic assistance for the nucleophilic attack is also associated with the stabilization of the developing negative charge on the carbonyl-oxygen atom of the tetrahedral intermediate. The detailed stereochemistry of the formation and breakdown of the tetrahedral transition state of the hydrolytic reaction is thus established. Comparisons of the structures of the enzymes in the four proteinase classes show that the serine, aspartic and metalloproteinases have converged on analogous hydrolytic mechanisms in which nucleophilic attack of the peptide carbonyl-carbon atom is on the *re* face. In contrast, the available evidence for inhibitor binding to the cysteine proteinases favors an interpretation that has the thiolate attack on the *si* face of the peptide bond. Since these complexes involve alkylation of the cysteine sulfur atom, the presently known structures are not good transition-state mimics. Only serine or cysteine residues and water molecules have been used by the proteinases to generate the attacking nucleophiles. Imidazole rings of histidine residues and carboxylate groups from aspartate and glutamate residues are the general bases. Electrophilic assistance to hydrolysis is provided by a wide variety of functional groups; even metal cofactors (e.g. Zn^{2+}) have been recruited to this role.

S 003 OVERVIEW OF METALLOPROTEASES, William N. Lipscomb, Harvard University, Cambridge, MA 02138

Progress will be described on structures and inhibitory complexes of metalloproteases of known three-dimensional structures from various laboratories. These known structures include carboxypeptidase A (1 Zn^{+2}, CPA)[1], carboxypeptidase B (1 Zn^{+2}, CPB)[2], thermolysin (1 Zn^{+2}, TLN)[3], a D-Ala-D-Ala carboxypeptidase (1 Zn^{+2}, PEP G, Zn^{-} G peptidase, peptidase G)[4], a neutral protease (1 Zn^{+2}, NEU)[5], an elastase of *Pseudomonas aeruginosa* (1 Zn^{+2}, PAE)[6], astacin (1 Zn^{+2})[7], leucine aminopeptidase (2 Zn^{+2}, LAP)[8], and methionene aminopeptidase (2 Co^{+2}, MAP)[9]. These structures will be related to unpublished or unknown metallopeptidases. The inhibitory complexes will include substrate or "transition state" analogues especially for carboxypeptidase A (P. A. Bartlett's phosphonate with a K_d of 10^{-14}M), thermolysin, and leucine aminopeptidase (bestatin and amastatin). Also, a description of current views of catalytic mechanisms in these enzymes will lead to description and interpretation of mechanism-based inhibitors. Extrapolations will be described for design of inhibitors for other metalloproteases, such as the dipeptidase, angiotensin-converting enzyme.[10,11,12]

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S 004 OVERVIEW OF THIOL PROTEINASES, Andrew C, Storer, Pharmaceutical Biotechnology Sector, Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montréal, Québec, Canada.

The exponential growth in the size of the gene and protein sequence databases has made possible the extraction, from multiple sequence alignments, of important information regarding the relationship between the primary structure and properties of a given enzyme class. For example, over 100 sequences are now available for proteins belonging to the papain superfamily of cysteine proteinases. A multiple alignment coupled with a phylogenetic analysis of these sequences has yielded information on the evolutionary history of the superfamily and made it possible to infer the existence of cysteine proteinases that have not yet been isolated or sequenced.In addition, coupled with site directed mutagenesis experiments it has provided detailed information on the catalytic mechanism of these enzymes. This insight is particularly valuable for the understanding of how cysteine proteinases work since although their physical-chemical properties have been characterized extensively with regards to structure, mechanism, specificity and stability the molecular basis of the linkages between these properties is still not clearly established. From a closer inspection of the papain like proteinase sequence alignments it can be seen that, with the exception of cystine residues, only six residues are entirely conserved. These residues are (papain numbering): Gln19, Cys25, His159, Asn175, Trp177, and Gly185 and based on the available X-ray crystallographically determined structures all six are found in the active site of these enzymes. The functional roles of these and other residues in the catalytic mechanism of cysteine proteinases have been investigated using site directed mutagenesis of papain, and cathepsins B and S. For example, Gln19 assists in stabilizing the catalytic transition state through its interaction with the carbonyl oxygen of the substrates scissile bond (1). The side chains of Cys25, His159, and Asn175 have long been known to comprise the catalytic triad of these enzymes, however, the catalytic role of Asn175 in this triad had not previously been satisfactorily explained. From site directed mutagenesis experiments it is suggested that this residue plays a dual role in that it stabilizes the Cys25-His159 thiolate-imidazolium ion pair required for activity of the enzyme and also the overall stability of the protein. The side chain of Trp177 also functions to stabilize the active site ion pair.

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Intracellular Processes

S 005 SECRETION-LEADER PEPTIDASE, Ross E. Dalbey, Ohio State University, Columbus.

Amino-terminal cleavable leader peptides target proteins for secretion and are removed by leader peptidase, a membrane-bound proteolytic enzyme. Leader peptidase plays an important role in protein export and is essential for cell growth. The substrate specificity of this protein requires small uncharged apolar residues at -3 (P3) and -1 (P1). This requirement is more strict at the P1 than the P3 position. Recently, our laboratory as well as others have shown that leader peptidase does not belong to the four "standard" protease groups (serine, cysteine, aspartic acid, and metalloproteases). Rather, it is a member of an unusual class of serine protease that utilizes a lysine residue instead of an histidine residue. We speculate that leader peptidase carries out catalysis using a serine/lysine dyad, not the standard serine/histidine/aspartic acid triad. Efforts are now being initiated to crystallize an active soluble fragment, which would lead to a structure of the protein by X-ray crystallography. This would facilitate the determination of the mechanism of action of this enzyme.

S 006 REGULATORY PROTEINS OF THE PROTEASOME, George N. DeMartino and Clive A. Slaughter, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

The proteasome is a 700,000-dalton protease distributed from archaebacteria to humans. This protease is comprised of about 28 subunits that, in higher eukaryotes, represent the products of at least 13 different, but homologous genes. The proteasome displays a characteristic cylinder-shaped morphology in which the subunits are arranged as a stack of four rings, each containing six to seven subunits. The proteasome is a multicatalytic enzyme with three, and probably more, distinct proteolytic activities. These activities, however, are latent in one form of purified enzyme that exists within intact cells. We have recently identified, purified, and characterized a variety of proteins that specifically regulate the proteolytic activities of the proteasome. Two of these regulatory proteins function as activators of the latent proteasome and have been termed PA28 and PA700.

PA28 is a 28,000-dalton protein with no homology to any proteasome subunit. PA28 appears to be a homohexamer or homoheptamer under native conditions. PA28 activates three distinct peptidase activities of the proteasome by increasing maximum reaction velocities and by decreasing the concentration of substrate required for half-maximal reaction velocities. Thus, PA28 may function as a positive allosteric effector of the proteasome. Electron microscopy of PA28 shows it to be a ring-shaped structure that forms regulatory caps on one or both ends of the cylinder-shaped proteasome. Binding of PA28 to the proteasome is required for activation and binding requires the last several amino acids of the carboxyterminus of PA28. An 18 amino acid peptide corresponding to the carboxyterminus of PA28 binds to the proteasome but does not activate it. These results indicate that PA28 contains separate functional domains involved in binding to and activation of the proteasome.

PA700 is a 700,000-dalton proteasome activator composed of about 16 electrophoretically distinct subunits with molecular weights from 20,000-110,000. PA700 activates three distinct peptidase activities of the proteasome. PA700 activation of the proteasome requires ATP hydrolysis, and ATP-dependent activation is closely linked to the formation of a high molecular weight proteasome/PA700 complex. PA700 subunits represent distinct gene products, although several are homologous to one another. PA700 and PA28 may bind to the same sites on the proteasome because the 18 amino acid carboxyterminal peptide of PA28 competitively blocks PA700 activation of the proteasome. These results indicate that cells contain multiple proteins that regulate the function of the proteasome, and that proteasome-containing complexes with distinct regulatory compositions may have specific cellular functions.

S 007 THE MULTICATALYTIC PROTEINASE COMPLEX (PROTEASOME). STRUCTURE, CATALYTIC COMPONENTS AND FUNCTION, Marian Orlowski, Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029.

The multicatalytic proteinase complex (MPC), a high molecular mass (~700 kDa) cytoplasmic and nuclear particle is composed of 28 low molecular mass subunits (21 to 34 kDa) of which 13 to 15 are nonidentical. The subunits are organized into four stacked rings surrounding a central water-filled tunnel. They can be dissociated in acidic solutions and isolated by HPLC. The primary structures of most of the subunits has been obtained from the nucleotide sequences of cDNA clones. Most of the subunits show some sequence similarity in the N-terminal region, indicating that they are derived from a related family of genes. No sequence homology has been found to any of the four mechanistic classes of proteolytic enzymes. Incubation of the MPC with ^{14}C-3,4-dichloroisocoumarin (DCI), a general serine protease inhibitor, leads to labeling of seven nonidentical subunits, and three additional 'silent' subunits become labeled when the MPC is exposed to DCI in the presence of activating peptides. Assuming that DCI reacts with residues in the active centers, these observations suggest that most of the subunits are involved in the catalytic process, and that a serine residue is involved in the bond-breaking process. Dissociation of the MPC, leads to an irreversible loss of activity indicating that integrity of the MPC and interaction between subunits is necessary for the expression of proteolytic activity. Initial work in our laboratory has led to the identification of three distinct catalytic components, designated as trypsin-like (T-L), chymotrypsin-like (ChT-L), and peptidylglutamyl-peptide hydrolyzing (PGPH), based on the structure of the residue in the P_1 position. Kinetic experiments indicate that the PGPH and ChT-L components are expressed by at least two distinct components. Two additional catalytic components, one cleaving preferentially bonds between small neutral amino acids, the other cleaving bonds on the carboxyl side of branched chain amino acids, have been recently identified in our laboratory. Evidence indicates that the branched chain amino acid preferring (BrAAP) component is the major factor responsible for the protein-degrading activity of the MPC, the other components being probably involved in the breakdown of peptide products derived from protein breakdown catalyzed by the BrAAP component. Specificity studies have shown that a Pro residue in the P_3 position and a branched chain amino acid in the P_1 position are necessary for activity of the BrAAP component and that the Pro residue in P_3 directs the substrate away form the catalytic site of the ChT-L component even in the presence of a hydrophobic residue in the P_1 position. A series of inhibitors of the BrAAP component were synthesized and shown to inhibit the activity of the MPC toward both synthetic and protein substrates. Experiments with intact cells show that the inhibitors interfere with incorporation of thymidine into DNA and inhibit cell growth. The MPC is present in all eukaryotic cells in both the cytoplasmic and nuclear compartments, and represents up to between 0.5 and 1% of the soluble fraction of tissue homogenates. Available evidence indicates that the MPC functions in both ubiquitin dependent and ubiquitin independent pathways of intracellular proteolysis, that it is involved in antigen processing, that it is necessary for cell mitosis, apparently because of its function in degradation of cyclins and oncogene products, and that it is absolutely necessary for cell survival and proliferation.

Proteases in Cell Proliferation and Cancer

S 008 MATRIX METALLOPROTEINASE INHIBITORS: SAR's AND ENZYME STRUCTURE, DO THEY TELL THE SAME STORY?

Paul A Brown[1], Neera Borkakoti[1], Kevin M Bottomley[1], Michael J Broadhurst[1], Alan D'Arcy[2], Trevor J Hallam[1], William H Johnson[1], Geoffrey Lawton[1], Edward J Lewis[1], Edward J Murray[1], John S Nixon[1], David H Williams[1] and Fritz Winkler[2]. [1]Roche Products Ltd, Welwyn Garden City, Herts, AL7 3AY, U.K, [2] Hoffmann La Roche, CH-4002 Basle, Switzerland.

The matrix metalloproteinases (MMP's) collagenase, stromelysin and gelatinase are a group of enzymes with the combined ability to break down all of the structural components of articular cartilage. Under normal physiological conditions this breakdown is tightly controlled to allow normal tissue remodelling to occur. However, in diseases such as rheumatoid arthritis and osteoarthritis excessive cartilage degradation occurs leading to joint destruction.

The lecture will describe the design and synthesis of potent collagenase, stromelysin and gelatinase inhibitors. The SAR data will be compared with the three dimensional information obtained from molecular modelling and x-ray crystallographic studies of human recombinant collagenase complexed to a synthetic inhibitor.

S 009 HIGH RESOLUTION CRYSTALLOGRAPHIC STUDIES OF HUMAN MATRILYSIN, Michelle F. Browner, Molecular Structure Department, Discovery Research, Syntex, Palo Alto CA 94303.

Matrix metalloproteases are critically involved in the degradation of extracellular-matrix both in normal metabolic processes and in disease states. There are at least nine members of the matrix metalloprotease family; biochemically these enzymes are characterized by expression as pro-enzymes that are activated by various organomercurials. Members of this enzyme family also have a relatively obvious domain structure as characterized at the primary sequence level. Matrilysin (formally known as PUMP) is the smallest member of the family, containing only the pro-peptide domain and the conserved catalytic domain. Matrilysin cleaves a wide selection of substrates including fibronectin, laminin and collagen IV. There is evidence for the involvement of matrilysin in both normal cellular invasive processes, as well as, in tumor metastasis. X-ray crystallographic studies of the mature form of matrilysin have been initiated. Hexagonal crystals of the mature enzyme in the presence of peptide mimetic inhibitors were grown; X-ray diffraction data extended beyond 1.9 Å. The first inhibited matrilysin structure was solved by molecular replacement and has been refined to an R-factor of 19%, including all data between 6.0-1.9 Å. In addition to the active site zinc, there are three additional bound metals (one zinc and two calcium ions). The bound inhibitor is a hydroxamic acid and the mode of interaction at the active site will be discussed.

S 010 PROTEOLYTIC MATURATION OF TRANSFORMING GROWTH FACTOR-α (TGF-α), Erika Cappellutti and Robert B. Harris, Dept. Biochem. & Molec. Biophys., Virginia Commonwealth Univ., Richmond, VA 23298.

TGF-α, a mitogenic peptide produced by tumor cells and by virally and chemically transformed cells in culture, is derived from its precursor protein (pro-TGF-α) by limited endoproteolysis and the putative processing sites within pro-TGF-α closely resemble elastase or thermolysin-like cleavage sequences. However, the physiologically relevant processing enzyme(s) of pro-TGF-α are unknown. We previously showed[1] that transformed rat liver epithelial or Schwann cells contain elastase-like enzymes which we suggested may be involved in processing pro-TGF-α at the plasma membrane. We now report isolation of the elastase-like enzyme from MDA and MCF human breast cancer cells, cells of epithelial origin. Like their previously characterized counterparts, the MDA and MCF enzymes are inhibited by MeO-succ-AAAPV-CH_2Cl and α_1-antiproteinase (α_1-AT), two well characterized elastase enzyme inhibitors. α_1-AT also significantly reduces anchorage-independent colony formation of MCF-7 cells[2]. Irradiation of MDA or MCF cells up-regulates pro-TGF-α and EGF receptor mRNA and protein levels (and down regulates the level of estrogen receptor mRNA), but the activity of the elastase-like enzyme remains unchanged compared with the non-irradiated cells. Hence, inhibition of the protease may be important in regulating the tumorigenic potential of these cells. Finally, we report that a family of cephalosporin-based compounds developed as inhibitors of elastase are differential inhibitors of the cellular enzyme; that is, the most potent inhibitor of pancreatic elastase (IC_{50} 4.5 ng) is the least potent inhibitor of the cellular enzyme and conversely, the best inhibitor of the cellular enzyme (800 $min^{-1} \cdot M^{-1}$) is the poorest inhibitor of pancreatic elastase. Thus, an appropriate derivative of these compounds can be used to label the cell-surface enzyme in cultured cells.

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S 011 CATHEPSIN B AND PROGRESSION OF HUMAN TUMORS, Bonnie F. Sloane[1], Mansoureh Sameni[1], Isabelle M. Berquin[1], Jurij Rozhin[1], Grace Ziegler[1], Michael R. Buck[2], Sandra Rempel[3], Mark L. Rosenblum[3], Akhouri Sinha[4], and Daniel Visscher[1], [1]Wayne State University, Detroit, MI 48201, [2]National Cancer Institute, Bethesda, [3]Henry Ford Medical Center, Detroit and [4]VA Medical Center, Minneapolis.

Increases in expression (mRNA, protein, and activity) and altered trafficking (localization/secretion) of the lysosomal cysteine protease cathepsin B have been found to correlate with malignancy of murine and human tumors. Our working hypothesis has been that the alterations in expression and trafficking of cathepsin B seen in malignant cells are responsible in part for enhanced invasion of tumor cells. We have shown that both normal and tumor cathepsin B can degrade the extracellular matrix proteins laminin, fibronectin and type IV collagen at neutral as well as acidic pH. Thus, secreted cathepsin B might facilitate the invasion of tumor cells. Staining of human tumor specimens with monospecific antibodies to cathepsin B or with probes for cathepsin B mRNA has provided further evidence that cathepsin B might participate in tumor invasion as the staining for cathepsin B is most intense in tumor cells at the leading, invasive edges of bladder and prostate tumors. In bladder tumors, there is an inverse correlation between cathepsin B staining and laminin staining, linking cathepsin B to the degradation of this basement membrane protein. In order to identify the cellular or stromal component responsible for cathepsin B activity, we isolate tumor cells, invasive edges of tumors, tumor stroma and normal epithelium from frozen sections of human colon and breast carcinomas by microdissection. Using this technique, we have been able to show that cathepsin B activity is elevated in the invasive edges of colon and breast tumors. In colon carcinoma, high levels of tumor cell staining for cathepsin B correlate inversely with patient survival, suggesting a possible functional role for cathepsin B in progression of colon carcinoma. Increased expression of cathepsin B appears to be associated with malignant progression of human gliomas as well. The abundance of cathepsin B transcripts and the intensity of staining for cathepsin B protein increase progressively in astrocytoma, anaplastic astrocytoma and malignant glioblastoma. In glioblastoma cell lines which invade through Matrigel *in vitro*, levels of expression (mRNA, protein and activity) and altered trafficking of cathepsin B parallel their invasive abilities. Furthermore, in the most invasive line, the cathepsin B transcripts contain an additional exon, exon 2', observed previously in cDNAs isolated from a highly anaplastic gastric adenocarcinoma. Recently, we have established that the alterations in the trafficking of cathepsin B seen in malignant tumor cells occur at early stages of progression of MCF-10 human breast epithelial cells from preneoplastic to neoplastic, i.e., at the point of transition between the pre-neoplastic and neoplastic state and coincident with acquisition of the ability to invade through Matrigel *in vitro*. Vesicles staining for mature cathepsin B are found peripherally, often adjacent to the cell surface and in microvilli. We hypothesize that having a vesicular compartment containing mature cathepsin B poised at the cell surface may be an important component of the preneoplastic phenotype of these cells and a prelude to the induction of invasive processes in neoplastic lesions. The fact that similar patterns of redistribution are observed for lysosomes in activated osteoclasts and macrophages indicates that transport of lysosomes to the cell periphery may be a mechanism common to cells that engage in local degradative and invasive processes.

Proteases in Inflammation and Immunity

S 012 MECHANISMS OF ANTIGEN PROCESSING BY CATHEPSIN B AND REGULATION OF ANTIGENIC PEPTIDE PRESENTATION BY INVARIANT CHAIN, Nobuhiko Katunuma, Institute for Health Sciences, Tokushima Bunri University, Tokushima 770, Japan

The immunogenic ligands to T cells must be presented as a complex with MHC class II in antigen presenting cells. The processing of exogenous antigens is an intralysosomal events. However, identity of special protease responsible for the processing of specified antigens remains largely unknown.

We reported recently that lysosomal cathepsins B of macrophages play an essential roles in processing of exogenous antigens to present with MHC class II. Cellular and also humoral immune-responses to vaccines of hepatitis B and rabies as antigens were suppressed by specific inhibitors of cathepsin B, anti-cathepsin B antibody F(ab') and specific substrate of cathepsin B. The antigenic peptides of these vaccines show strong proliferative response (^{3}H-thymidine incorporation by rechallenge of these antigenic peptides) to the splenocyte primed by these vaccines. However, the responses to these antigenic peptides were not inhibited by the cathepsin B inhibitors. These findings suggest that cathepsin B inhibitors dose not inhibit any other processes of the immune-responses than that of proteolitic processing of antigens. Therefore, the suppression of these immune-responses by cathepsin B inhibitors is not due to the inhibition of invariant chain (γ-chain) degradation.

The antigenic peptides of these vaccines are processed by cathepsin B and the antigenic fragments are capable of binding and presenting with desetope of MHC class II, β-chain. Because, one of the active domains of cathepsin B, $VN_{217-222}$ shares highly homology with a part of desetope, VN_{57-62} of MHC class II, β-chain.

We found that the invariant chain shares about 40% homology with cystatin family which are the endogenous inhibitors of cysteine proteases. The invariant chain belongs to the cystatin family and practically the invariant chain synthesized by recombinant cDNA *inhibits cathepsin* family and cathepsin L is inhibited especially strong by the invariant chain. The invariant chain may participate in the regulation of antigenic peptide presentation and also antigen processing by cathepsin B.

S 013 PROTEIN PROCESSING BY MAMMALIAN AND PARASITE ASPARTIC PROTEINASES, John Kay, Department of Biochemistry, University of Wales, P.O. Box 903, Cardiff CF1 1ST, Wales, U.K.

Five aspartic proteinases are known to exist at present in the human body. Of these, two (cathepsin D and cathepsin E) are intracellular, with the former being identified as a lysosomal enzyme in many cell types while cathepsin E has a non-lysosomal distribution and is present in certain cells only (e.g. gastric epithelium and red blood cells).

Activation of helper T lymphocytes involves the recognition of fragments of an extracellular antigen complexed with Class II histocompatibility antigens (MHC) on the surface of antigen presenting cells. Processing of antigens such as ovalbumin by the murine antigen presenting cell, A20, (which is a B-cell lymphoma cell line) is prevented by pepstatin. Identification of the intracellular aspartic proteinase responsible was carried out using more specific inhibitors which permitted distinction between cathepsin D and cathepsin E. Processing was unequivocally carried out by cathepsin E which was demonstrated to be located in an endosomal compartment. Further aspects of antigen processing by such systems will be discussed.

Cathepsin E is also present in human red blood cells. During the intraerythrocytic stages of the life cycle of the malarial parasite, *Plasmodium falciparum*, vast quantities (as much as 100 grammes) of haemoglobin are catabolised in the (lysosome-like) food vacuole of the parasite. The initial stages of this processing in the vacuole can be blocked completely by pepstatin.

Characterisation of the members of the aspartic proteinase superfamily present in this human parasite will be described. Development of specific inhibitors targetted against such parasite proteinases but which have no effect on the cathepsin E also present in human red blood cells, nor on any of the other four known human enzymes, might offer a novel therapeutic approach to the treatment of malaria. In 1989, 110 million cases of malaria were estimated world-wide.

S 014 INTERLEUKIN-1ß CONVERTING ENZYME, Nancy A. Thornberry[1], Merck Research Laboratories, Rahway, New Jersey 07065

Interleukin-1ß converting enzyme (ICE) is a cysteine proteinase in monocytes that is essential for the proteolytic activation of interleukin-1ß, an important mediator of inflammation. The enzyme catalyzes the cleavage of the 31 kDa interleukin-1ß precursor at Asp^{116}-Ala^{117} to generate the mature, biologically active 17.5 kDa cytokine. Purification and cloning indicated that the enzyme is a heterodimer composed of two subunits of 20 and 10 kDa, both of which are required for catalytic activity, and both of which are proteolytically derived from an inactive 45 kDa proenzyme by a mechanism that is, at least in part, autocatalytic. The murine enzyme is highly homologous to its human counterpart (72%), and is virtually identical with respect to subunit structure and substrate specificity. The proenzyme is the predominant form (> 99%) of the enzyme in both activated and resting monocytes. Electronmicroscopy has localized the proenzyme to the cytoplasm and the active enzyme to the plasma membrane. Regarding catalytic properties, the enzyme is a unique cysteine proteinase, displaying an unusual requirement for Asp in the P_1 position in both peptide and macromolecular substrates, and lacking sequence homology to any other known enzyme in this class. The catalytic Cys^{285} is located within a highly conserved sequence of amino acids in the 20 kDa subunit. Classic peptide-based reversible and irreversible cysteine proteinase inhibitors, when designed with the appropriate peptide recognition sequence (Ac-Tyr-Val-Ala-Asp), are potent and highly selective, and have been used to prove that the enzyme is essential to the production of interleukin-1ß in monocytes. Reversible inhibitors include the tetrapeptide aldehyde (K_i = 0.76 nM), nitrile (K_i = 60 nM), and phenylbutylketone (K_i = 37 nM). Tetrapeptide acyloxymethylketones, which irreversibly inhibit the enzyme through formation of a thiomethylketone with Cys^{285}, have second-order inactivation rates that are limited by diffusion ($\sim 1 \times 10^6\ M^{-1}s^{-1}$). The only known macromolecular inhibitor of the enzyme is a 38,000 kDa protein (crmA) from cowpox virus, a member of the serpin superfamily.[2] This inhibitor, which is potent and selective, is the first example of a serpin whose target enzyme is a cysteine proteinase.

[1] This abstract describes the work of the Merck Research Laboratory ICE Project Team

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S 015 ANTIGEN PROCESSING GENES IN THE HUMAN MHC, John Trowsdale, Monica Belich, Richard Glynne, Adrian Kelly, Stephen Powis, Stephan Beck. Imperial Cancer Research Fund, 44 Lincolns Inn Fields, Holborn, LONDON WC2A 3PX, UK.

The MHC encompasses several clusters of genes (class I and class II) with related functions in antigen presentation. Interestingly, the class II region also contains a cluster of loci which have provided considerable insight into antigen processing pathways: *LMP2-TAP1-LMP7-TAP2*. The *TAP* genes are traffic ATPases which transport peptides from the cytoplasm into the lumen of the endoplasmic reticulum. The *LMP* gene products are interferon-inducible proteasome beta-subunits. The finding of these genes close to the TAPs implicate a role for the proteasome in antigen processing in the cytoplasm, to provide petides for transport into the ER. The products of *LMP2* and *LMP7* play a subtle role in antigen processing, by altering peptide cleavage specificity. We have also cloned two *LMP*-related genes which are not in the MHC and we propose that their products can substitute for the MHC-encoded proteins on the proteasome when *LMP2* and *LMP7* are absent. This finding helps to explain the minimal phenotype of cells in which the MHC-encoded LMPs are deleted. The sequences of the four proteasome components lead to the conclusion that the MHC-encoded loci are not derived, as was previously thought, from a recent duplication event, as *LMP2* and *LMP7* are each more similar to their non-MHC partners than they are to each other. This raises some interesing evolutionary questions.

Proteases in Blood Processes

S 016 PROTEOLYTIC ACTIVATION OF THE COMPLEMENT CASCADE, Tony E. Hugli, Richard G. DiScipio, Marleen Kawahara and James Travis* Dept. of Immunol, Scripps Research Institute, La Jolla, CA. 92037 *Dept of Biochem, U. of GA, Athens, GA 30602

Components C3 and C5 are pivotal proteins of the blood complement cascade. Their activation by limited proteolysis is central to the physiologic role of complement in host defense mechanisms. The smaller fragments C3a and C5a (called anaphylatoxins) are 9-11 kDa factors that promote chemotaxis, leukocyte and monocyte metabolic activation, spasmogenesis and exhibit immunoregulatory functions while the larger fragments C3b and C5b enhance phagocytosis and initiate cytolytic events. C3a and C5a participate in proinflammatory activities and when generated in excess can be pathobiologic. Possible examples are acute infections accompanied by a progressive inflammatory state such as adult periodontitis.The condition of advanced gingival tissue injury is promoted by anaerobic bacteria and their enzymatic products.The arginine-specific cysteine proteinase from *Porphyromonas gingivalis* (called Gingipain-1) cleaves both C3 and C5 with high specificity. A functional anaphylatoxin (i.e. C5a) is generated only from C5. Cleavage of human C3 by Gingipain-1 is selective and scission occurs at or near the C3a/C3b scission site on the C3 α chain; however C3a activity is not detected in the digest. We concluded that the C3a (9 kDa and 77 residues) is rapidly degraded by the proteinase once it is released. The primary cleavage site for Gingipain-1 in C5 occurs between residues 715-716 of the α-chain and a secondary cleavage occurs more slowly between residues 74-75 releasing the C5a. Since the C5a molecule appears to be more resistance to Gingipain-1 than does C3a, it survives digestion and is demonstrated functionally as an intact factor. A similar series of experiments have been carried out using a lysine-specific cysteine proteinase from *P. ginivalis* and this proteinase cleaves C3, but not at the C3a/C3b site.The cleavage pattern indicates that C3 α chain is degraded extensively while the more resistant β chain is partially degraded. An intial cleavage probably occurs near the mid-portion of the α chain generating two fragments of approximately 60 kDa. One of these fragments is further degraded while the other appears to be resistant. C5 is also digested by lysine-Gingipain. We will discuss implications of proteinases produced in the anaerobic microbiota of the infected gingiva and their effects on plasma components capable of releasing potent inflammatory factors at the site of injury.

S 017 α_2-MACROGLOBULIN: CAN YOU TEACH AN OLD DOG NEW TRICKS? Salvatore V. Pizzo, Charleen T. Chu, Gayle C. Howard and Uma K. Misra, Duke University Medical Center, Durham, NC 27710

The α-macroglobulin/complement superfamily evolved over 600,000,000 years ago. Gene duplication and divergence eventually resulted in two separate groups of proteins with distinct properties: the α-macroglobulins, which bind proteinases, and the complement components, which lyse cells. Over 200,000,000 years ago, a cellular receptor evolved which binds proteinase-treated but not native α-macroglobulins. This cellular receptor also recognizes α-macroglobulins in which the internal β-cysteinyl-γ-glutamyl thioesters have been directly attacked by primary amines. α-macroglobulins exhibit a wide range of specificity in binding proteinases of each of the four mechanistic classes, however, there are few situations in which α-macroglobulins function as the primary inhibitor of particular proteinases. We propose that α-macroglobulins, like the complement system, function as proteinase activated sensors for situations in which coordinated cellular responses are critical. At least two of these functions are antigen delivery to macrophages and signal transduction. During proteinase activation, α_2-macroglobulin (α_2M) is able to entrap and covalently bind non-proteolytic proteins. Using MHC-restricted T cell hybridoma cells, we have shown that α_2M-lysozyme complexes are presented by macrophages more rapidly and to a greater extent than free lysozyme, requiring 200-250 times less complex than free lysozyme for effective presentation to T cells. *In vivo* studies indicated that complexes of α_2M and antigen could elicit a greater primary and secondary IgG response than free antigen and could generate antibody titers comparable to those elicited by emulsification of the antigen in complete Freund's adjuvant. In addition to its role in antigen presentation, we have also shown that α_2M plays a role in signal transduction. Application of receptor recognized α_2M (α_2M*), but not native α_2M, to macrophages elicits an increase in intracellular Ca^{2+} concentration and the generation of cAMP and inositol phosphates. Further studies of the signalling effects of α_2M* suggest that the signalling mechanism involves two or more G proteins, and that it may represent a second receptor for α_2M distinct from the previously described α_2M/low density lipoprotein receptor-related protein which has been shown to bind and endocytose α_2M and various other proteins.

Proteinaceous Protease Inhibitors

S 018 THE STRUCTURE OF CYSTATINS AND THEIR INTERACTIONS WITH COGNATE CYSTEINE PROTEINASES, Wolfram Bode[1], Milton Stubbs[1], Djordje Musil[1], Bernd Laber[1], Richard Engh[1], Robert Huber[1], Vito Turk[2], [1]Max-Planck-Institute of Biochemistry, D-82152 Martinsried/Munich, Germany, [2] Department of Biochemistry, Jozef-Stefan-Institute, SLO-61000 Ljubljana, Slovenia.

The cystatins are tight and reversibly binding inhibitors of papain-like cysteine proteinases. They form a superfamily which includes the stefin, the cystatin and the kininogen families. The crystal structure of chicken egg white cystatin revealed a five stranded antiparallel β-pleated sheet wrapped around a straight α-helix, and an appending segment of α-helical geometry. The wedge-shaped edge of this sheet was shown to be complementary to the active-site cleft of cysteine proteinases. The subsequently determined crystal structure of a papain complex formed with stefin B confirmed the proposed interaction mechanism, which is fundamentally different to that observed for the "canonically" binding serine proteinase inhibitors. The structures of the inhibitors and of the complexes will be shown and their interaction mechanism will be discussed considering more rescent results obtained from new crystal structures and by NMR techniques.

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S 019 THREE-DIMENSIONAL STRUCTURE OF UNCLEAVED ANTICHYMOTRYPSIN: IMPLICATIONS FOR SERPIN FUNCTION.
David W. Christianson, Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104-6323.

The first three-dimensional structure of an uncleaved, inhibitory serpin (serine protease inhibitor) has been determined by X-ray crystallographic methods at 2.5 Å resolution. Specifically, the structure of a variant of human antichymotrypsin engineered to be an inhibitor of human neutrophil elastase reveals an uncleaved reactive loop with a distorted helical conformation. The reactive loop is held above the protein scaffolding by two peptide stalks: the stalk on the P1' side of the reactive loop leads to strand s1C of β-sheet C, and the stalk on the P1 side of the reactive loop leads directly to strand s5A of β-sheet A. Contrary to much prior speculation, there is no pre-insertion of the P1-side stalk as strand s4A in β-sheet A of the native serpin. Comparison of the intact reactive loop conformation with the canonical binding conformations of protease-bound inhibitors such as the third domain of turkey ovomucoid in complex with chymotrypsin, or the bovine pancreatic trypsin inhibitor complexed with trypsin, suggests that the reactive loop of antichymotrypsin must uncoil in order to optimize the association of P3-P3' residues with the corresponding subsites of a target protease. It is possible that this conformational transition is triggered only by the association of serpin with protease, and phenomena related to reactive loop uncoiling and strand s4A insertion may determine whether a serpin behaves as an inhibitor or a substrate of a particular serine protease. Finally, a comparison of the structures of uncleaved and cleaved antichymotrypsin highlights significant conformational changes throughout the protein scaffolding which are triggered by reactive loop cleavage: in addition to the characteristic rearrangement of β-sheet A allowing strand s4A insertion, β-sheet C anneals and several flanking α-helices and loop segments reorient with improved hydrogen bond geometry. Based on the experimentally-determined structure of the intact serpin and a model of the serpin-protease complex, a model for the mechanism of serpin function will be presented.

S 020 MOLECULAR CHELATION OF TRYPSIN BY ECOTIN, Mary E. McGrath, Thorsten Erpel, Christopher Bystroff, and **Robert J. Fletterick**, Department of Biochemistry & Biophysics, University of California, San Francisco, San Francisco, CA.

Ecotin is a dimeric serine protease inhibitor found between the inner and outer membranes of E. coli. Each 142 amino acid monomer can inhibit one protease molecule. Ecotin is a highly effective inhibitor of trypsin, chymotrypsin, elastase, and additional proteases. For example, ecotin inhibits the blood coagulation protease Factor Xa with a low picomolar Ki. The 2.4Å X-ray crystal structure of ecotin was solved in a complex with trypsin to assess the structural basis for this inhibitor's potency, and especially to determine the characteristics which allow ecotin to inhibit proteases with different substrate specificities. Interactions between the reactive site of ecotin and the protease active site were seen to be comparable with those noted for other protease:inhibitor complexes. The reactive site P1 residue of ecotin, Met84, is bound at the trypsin active site. The Met84 side chain is located in the substrate binding pocket and makes few interactions with the protease, while the carbonyl oxygen of the scissile bond is bound by trypsin's oxyanion hole. The geometry at the active site indicates formation of a distorted Michaelis complex. We noted that ecotin contacts the protease at a second, distal site facilitated by the dimer structure. Presumably, the additional contacts produce binding energy which compensates for non-optimal primary site interactions.

S 021 INTERACTIONS BETWEEN TISSUE INHIBITORS OF METALLOPROTEINASES, TIMPS-1 AND -2 AND THE MATRIX METALLOPROTEINASES, Gillian Murphy[1], Frances Willenbrock[2], Mark Cockett[3], Mark O'Shea[1], Quang Nguyen[1] and Andrew Docherty[3], [1] Strangeways Research Laboratory, Cambridge CB1 4RN, UK, [2] Queen Mary and Westfield College, London, [3]Celltech Ltd, Slough, SL1 4EN, U.K.

TIMPs-1 and -2 are made up of 6 loops defined by disulphide bonds, forming a tertiary structure that is essential for inhibitory activity against the active forms of matrix metalloproteinases (MMPs). Mutagenesis and expression studies to remove sections of the TIMPs have shown that two structurally distinct domains exist. The N-terminal domain (loops 1-3) can fold independently of the C-terminal domain to give a functional inhibitor as long as the disulphide bonds are intact. The MMPs are also made up of definable domains of different function, including a propeptide, a catalytic domain and a C-terminal disulphide bonded domain. The gelatinases A and B have a further domain related to the collagen binding domain of fibronectin. Mutants of recombinant MMPs with systematic domain deletions can also be prepared. We have compared the binding between TIMPs-1 and -2 and genetically forms that consist of their N-terminal domains and pro and active forms of gelatinase A, gelatinase B, collagenase and stromelysin-1 and their mutants by kinetic methods. Interactions between TIMP-2 and progelatinase A and TIMP-1 and progelatinase B are mediated by their respective C-terminal domains. The N-terminal domain of the TIMPs binds to the catalytic domain of the active form of all the MMPs. However, the C-terminal domain of the TIMPs plays an important regulatory role in determining the rate of complex formation with the active MMPs. The TIMP-1 regulatory domain appears to have at least two different binding-sites, one of which interacts with the C-terminal domain of gelatinase A and another which binds to the N-terminal domain of stromelysin-1. In the case of TIMP-2 the additional binding site on the pro form of gelatinase A enhances the rate of active gelatinase A-TIMP-2 complex. It is possible that a similar mechanism exists in the interaction of active gelatinase B with TIMP-1. Systematic site-directed mutagenesis of TIMP-1 has been carried out and it has been found that His7 and Gln9 modifications result in a form of inhibitor that has the kinetic characteristics of the N-terminal domain alone. These data will be discussed in terms of a general model of TIMP-MMP interactions.

S 022 REACTION PATHWAY FOR INHIBITION OF FACTOR Xa by rTAP, Jules A. Shafer, Jin Huang, and Shi-Shan Mao, Biological Chemistry Department, Merck Research Laboratories, West Point, PA 19486.

Recombinant tick anticoagulant peptide (rTAP) is a specific, reversible, tight-binding, competitive inhibitor of factor Xa. The inhibition of human factor Xa by rTAP was shown to be a two-step process wherein a weak enzyme-inhibitor complex initially forms and then rearranges to a more stable complex. Studies of the interaction of rTAP with human factor Xa in the presence of the fluorescent active-site probe *p*-aminobenzamidine or active-site blocked factor Xa indicated that rTAP can bind factor Xa through a site distinct from the active site.

Site-directed mutagenesis revealed two regions were important for inhibition of human factor Xa, an N-terminal region (residues 1-10) and a second region near C-terminus (residues 41-54). Certain mutations in the N-terminal region that increase the hydrophobicity (Tyr1 --> Trp, Y1W) or the cationic character (Asp10 --> Arg, D10R) of this region increased inhibitory potency toward factor Xa. The double mutant Y1W/D10R showed a synergistic improvement of inhibitory potency for factor Xa. The increased inhibitory potency of the double mutant was less toward dog factor Xa than human factor Xa. The observed increase in inhibitory potency of this rTAP variant was demonstrated to reflect a decrease in the rate constant for dissociation of the rTAP:factor Xa inhibitory complex. The mutant Y1E (Tyr1 --> Glu) showed reduced inhibitory potency and a slower rate (than that of wild type rTAP) for inhibition of factor Xa. This reduction in inhibitory potency could be attributed to a lower propensity for formation of the initial rTAP:factor Xa complex.

Although the sequence in the 41-54 region of rTAP is homologous to a sequence in the factor Xa substrate prothrombin, mutations in rTAP that increase the homology of this region to its counterpart in prothrombin yielded inhibitors that were less potent than wild type rTAP. The lowered potency of the rTAP mutant H43K could be accounted for by a lowered rate constant for rearrangement of the initially formed complex to a more stable complex. A peptide (P41-54) containing residues 41-54 of rTAP was shown to be a non-competitive inhibitor for human factor Xa-catalyzed chromogenic substrate hydrolysis. Interestingly, P41-54 did not displace the active-site probe *p*-aminobenzamidine from the active site of factor Xa. These results suggest that the prothrombin-like domain of rTAP interacts with factor Xa at an exosite that is spatially distinct from the active site.

Viral Proteases

S 023 **STRUCTURAL ANALYSIS OF HEPATITIS A VIRUS (HAV) PROTEINASE SPECIFICITY, Bruce A. Malcolm[1,2], Marc Allaire[1], Katherine S. Bateman[1], Maia M. Chernaia[1] and Michael N.G. James[1], Department of Biochemistry[1] and Department of Medical Microbiology and Infectious Diseases[2], University of Alberta, Edmonton, Alberta T6G 2H7, Canada.**

The recent determination of the structure of a mutant HAV-3C proteinase by Allaire *et al.* (submitted) provides a framework for the detailed understanding of the specificity of 3C proteinases from all picornaviruses. Using Insight II (Biosym Technologies, San Diego, CA) a substrate peptide was modeled into the active site of this chymotrypsin-like cysteine proteinase, based on coordinates from the high resolution structure of the complex of α chymotrypsin with its inhibitor, turkey ovomucoid [Fujinaga *et al.*, J. Mol. Biol. (1987) 195, 397]. A model of the active site cleft of the rhinovirus 3C proteinase, based on the structurally conserved regions, was generated using Homology (Biosym Technologies, San Diego, CA) and fitted with an ideal substrate peptide in an analogous fashion. A comparison of the specificity of both HAV-3C and rhinovirus 3C, in light of the structural data and models, will be presented. Research funded by ARPA and the MRC of Canada.

S 024 FLAVIVIRUS PROTEINASES, Charles M. Rice[1], Arash Grakoui[1], Thomas J. Chambers[1], Chao Lin[1], Sean M. Amberg[1], Alexander A. Kolykhalov[1], Yevgenii V. Agapov[1], Karen Reed[1], Ann C. Nestorowicz[1], David W. McCourt[1], Czeslaw Wychowski[2] and Stephen M. Feinstone[2]. [1]Dept. of Mol. Microbiol., Washington Univ. Sch. of Med., Box 8230, St. Louis, MO; [2]Division of Virology, FDA/CBER, Bethesda, MD, USA.

Based on their genome organizations and virion properties, the flaviviruses, the pestiviruses, and the hepatitis C viruses (HCV) have been classified as three genera in the family *Flaviviridae*. Properties shared by these three groups include a lipid envelope, conferring sensitivity to organic solvents, and a single-stranded positive-polarity RNA genome containing a long open reading frame which encodes the viral polypeptides. The structural proteins are located in the N-terminal portion of the polyprotein followed by the putative nonstructural replicase components. Mature protbins are produced by a combination of host and viral proteinases located in both the cytosol and in subcellular vesicular compartments. The viral proteinases encoded by these three genera will be compared and contrasted. In the case of flaviviruses like yellow fever virus (YF), the functional viral proteinase complex is composed of the hydrophobic NS2B protein and a serine proteinase domain found in the N-terminal one-third of the NS3 protein. This proteinase is responsible for at least seven cleavages in the YF polyprotein, including one cleavage which produces the C terminus of the mature virion capsid protein and six cleavages in the nonstructural region. The amino acid residues surrounding these cleavage sites are highly conserved and usually include a pair of basic residues (Arg or Lys) at the P2 and P1 positions and a Gly or Ser residue at the P1' position of the scissile bond. For pestiviruses such as bovine viral diarrhea virus (BVDV), the p80/p125 proteins also contain a serine proteinase domain responsible for multiple cleavages in the nonstructural region. Interestingly, cleavage at the N-terminal boundary of the proteinase domain (to produce p80) in found only in cytopathogenic BVDV strains isolated from animals with fatal mucosal disease. For HCV, two viral proteinases are required for nonstructural region processing. The homologous serine proteinase domain is necessary for downstream processing at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites. Upstream (NS2) sequences are not required for this activity. Surprisingly, cleavage at the 2/3 site appears to be autocatalytic and mediated by a novel overlapping proteinase encompassing the region encoding NS2B and the NS3 serine proteinase domain. Cleavage sites for both HCV proteinases have been localized by N-terminal sequence analysis and their substrate specificity studied by site-directed mutagenesis.

S 025 POLIOVIRUS PROTEINASES SERVE MULTIPLE DISPARATE FUNCTIONS IN THE VIRAL LIFE CYCLE, Eckard Wimmer, Xuemei Cao, Kevin S. Harris, Christopher U.T. Hellen, Akhteruzzaman Molla, Aniko V. Paul, and Wenkai Xiang, State University of New York at Stony Brook, NY 11794

Picornaviruses, a large family of pathogenic viruses, synthesize only one polypeptide, the viral polyprotein (vPP). Proteolytic processing of the vPP to functional viral proteins commences co-translationally, and it is catalyzed by proteolytic activities embedded in the vPP. Poliovirus vPP contains two principal proteolytic activities: $2A^{pro}$ (18 kD) and $3C^{pro}$ (20 kD), two sulfhydryl proteinases with a proposed serine protease structure. A third activity is $3CD^{pro}$, the precursor to $3C^{pro}$ and the RNA polymerase $3D^{pol}$. $3CD^{pro}$ is responsible for cleavage of the capsid precursor P1. In fact, $3CD^{pro}$ may carry out all 3C-related cleavages. The amino acids of the catalytic triad of these enzymes have been determined. They catalyze 11 cleavages of which 10 are essential: a Y*G cleavage by $2A^{pro}$, severing structural from non-structural proteins, and 9 Q*G cleavages by $3CD^{pro}$ and/or $3CD^{pro}$. The 11th cleavage at a Y*G in $3D^{pol}$, catalyzed by $2A^{pro}$; can be eliminated by genetic manipulation without any effect on viral replication. Sequences surrounding the scissile bonds influence the kinetics of cleavage as shown by biochemical and by kinetic analyses. We have developed a genetic system whereby the virus, for its survival, selects less favorable cleavage signals at a specific scissile bond in the vPP. Although random cleavages of vPP would be expected to produce 77 different products, less than 30 have been detected. Some cleavage intermediates have long half lives indicating functions distinct from their cleavage products (e.g. 2BC, 3AB, and 3CD). Genetic separation of the vPP at different cleavage sites, by insertion of a heterologous picornavirus internal ribosomal entry site (IRES) into cleavage sites of the ORF, has produced viable dicistronic polioviruses. Analysis of different dicistronic constructs has led to the surprising observation that most cleavages within the vPP appear to occur in *cis*. The only clear-cut exceptions are P1 processing by $3CD^{pro}$, and the non-essential cleavage of $3D^{pol}$ by $2A^{pro}$. Genetic and biochemical analyses by us and others have uncovered different functions for the proteinases. These functions include: (i) the *cis* cleavage of vPP, (ii) shut-off of host cell protein synthesis, (iii) *trans*-activation of viral translation, and (iv) genome replication for $2A^{pro}$; furthermore (i) proteolysis of vPP, (ii) degradation of cellular transcription factors, and (iii) genome replication for $3CD^{pro}$ (and $3C^{pro}$). The involvement of $3CD^{pro}$ in genome replication includes an interaction of this proteinase with the 5'-terminal clover leaf of the viral RNA. We have discovered co-factors essential for the binding of $3CD^{pro}$ to the clover leaf, and we will discuss parameters of this reaction.

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Proteases in Alzhiemer's Disease

S 026 PROCESSING OF THE ALZHEIMER AMYLOID PROTIEN PRECURSORS, Todd E. Golde, X. Dan Cai, Tobun T. Cheung, and Steven G. Younkin. Division of Neuropathology, Department of Pathology, Case Western Reserve University, Cleveland, Ohio, USA 44106

The amyloid deposited in Alzheimer's disease (AD) is composed of a 39-43 residue polypeptide (amyloid ß protein, Aß) that is derived from a set of 695-770 residue precursors referred to as the amyloid ß protein precursor (ßAPP). Strong evidence that amyloid deposition plays an important role in AD has come from the identification of familial AD (FAD) kindreds in which the AD phenotype cosegregates with mutations in the ßAPP gene. Three of the FAD-linked ßAPP mutations convert the val located three residues carboxyl to Aß43 , to ile (ΔI), phe (ΔP), or gly (ΔG). A fourth mutation (ΔNL) alters the lys-met located immediately amino to Aß1 to asn-leu. To test the hypothesis that these mutations cause AD by altering ßAPP processing in a way that is amyloidogenic, we examined human neuroblastoma (M17) cells expressing normal or mutant ßAPP695. The cells expressing mutant ßAPPΔNL showed a 5-fold increase in the relative amount of the ~11.4 kD Aß-bearing carboxyl-terminal ßAPP derivative that has Aß at its amino terminus, and they released 6-fold more 4 kD Aß into the medium (1). Recently, Seubert et al. (2) reported that the secreted amino-terminal derivatives (sßAPP) generated from full length ßAPP end not only at Aß15 or Aß16 as reported previously (α secretase) but also at the met preceeding Aß (ß secretase), a cleavage that could generate the 11.4 kD carboxyl-terminal fragment with Aß at its amino terminus. To examine the possibility that the ΔNL mutation increases Aß production by enhancing cleavage at the ß-secretase site, we introduced the ΔNL mutation into two deletion constructs ßAPP438 and ßAPP423 that we had previously examined (3), and we compared M17 cells transfected with wild type deletion constructs versus those with the ΔNL or ΔI mutations. Examination of the shortened sßAPP produced by transfected M17 cells showed that the sßAPP produced migrated as a doublet. The larger sßAPP band appeared to be sßAPPα because it was immunoprecipitated by anti-Aß1-40 while the smaller sßAPP band appeared to be sßAPPß based on its size, the fact that anti-Aß1-40 failed to immunoprecipitate it, and that antisera specific for sßAPPß recognize it. Quantitative analysis of the sßAPP in these transfected cell lines indicated that the ratio of sßAPPα to sßAPPß was ~3:1 for 438, ~1:1 for 423 and ~1: 2 for both 438ΔNL and 423ΔNL These data provide strong evidence that ßAPPΔNL increase Aß production by enhancing cleavage at the ß secretase site. However quite unexpectedly ßAPP423 also enhanced ß secretase cleavage. The ßAPP438 and 423 deletion constructs differ by 15 amino acids that lie completely within exon 15 of the ßAPP gene. Exon 15 is spliced out of some APP transcripts (referred to as LAPP) and it contains a putative O-linked glycosylation site. Interestingly exclusion of this site in the 423 constuct appears to effect glycosylation of the cell associated ßAPP holoprotein. We are currently exploring whether the enhanced ß-secretase cleavage in ßAPP423 is due to exclusion of exon 15, whether glycosylation effects ß-secretase activity, and if LAPP which lacks this putative glycosylation site shows enhanced cleavage by ß-secretase.

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S 027 CELLULAR MECHANISMS FOR β-AMYLOID PRODUCTION AND SECRETION, Sukanto Sinha, Athena Neurosciences, Inc., South San Francisco, CA 94080.

The recent recognition that β-amyloid secretion is a normal cellular process has led to a re-evaluation of the previous hypothesis that β-amyloid formation is essentially an aberrant process, pathogenetic for Alzheimer's disease. It is becoming increasingly accepted that a pathway in the normal cellular metabolism of the APP leads to the formation and secretion of the β-amyloid protein. The availability of cell culture systems which allow for the quantitative measurement of β-amyloid production has direct implications for the identification of inhibitors which selectively block the secretion of β-amyloid, a key milestone in the development of therapeutic approaches to this disease. In addition, such systems afford insight into the cellular mechanism of β-amyloid production, in which the key step appears to be mediated by a cellular protease, β-secretase. β-secretase is distinct in its activity and response to pharmacological agents from α-secretase, the enzymatic activity which mediates the release and secretion of the extracellular domain of APP. A crucial role of β-secretase is additionally exemplified by a rare, disease-causing, double mutation in APP, which changes the P_2P_1 residues (for β-secretase) from Lys-Met to Asn-Leu, leading to greatly increased β-amyloid production. This increased production is mechanistically correlated with increased cleavage by β-secretase at the $P_1P_{1'}$ Leu-Asp site in this mutated protein. Furthermore, inhibition of β-secretase activity completely blocks the production of β-amyloid. Thus, selective inhibition of the β-secretory pathway of APP processing promises to be a pharmacologically attractive therapeutic approach to the treatment of Alzheimer's disease.

Microbial and Parasitic Proteases

S 028 AN OUTER MEMBRANE PROTEASE THAT DETERMINES THE INVASIVE CHARACTER OF PLAGUE. Y. V. B. K. Subrahmanyam and Jon D. Goguen University of Massachusetts Medical School, Worcester, Massachusetts 01655.

Virulent strains of *Yersinia pestis*, the causative agent of plague, contain a 9.5 kilobase plasmid which encodes a 33 kDa outer membrane protease designated Pla. Pla is closely related to the OmpT protease of *E. coli*, and similar proteases found in *Salmonella typhi* and *Salmonella typhimurium*. Although no physiological function has been demonstrated for the proteases of these latter three species, specific inactivation of the *pla* gene in *Y. pestis* dramatically reduces the severity of disease observed following subcutaneous infection of mice, resulting in a million-fold increase in mean lethal dose. Rather than developing fatal systemic infection typically caused by Pla^+ strains, *pla* mutants cause a well-localized subcutaneous abcess at the site of inoculation. This lesion contains many more inflammatory cells than are present in the injection site lesions caused by virulent the Pla^+ strain. Despite their low virulence following subcutanius infection, *pla* mutants remain highly virlent when injected intravenously.

The important physiological substrates of the protease are unknown, although two potentially important activities have been identified: Pla has plasminogen activator activity, and is also capable of cleaving and causing the release of properdin from the alternative complement pathway C3 convertase complex assembled on the bacterial surface. Purification and charaterization of Pla have yielded a series of interesting observations: (1) Pla can be expressed at very high levels in *E. coli*, constituting more than 50% of total outer membrane protein, with little deleterious effect. (2) Extraction of the protease from bacterial membranes causes the pH optimum to drop from 7.6 to 5.3. (3) Pla activity is not inhibited by any of a wide variety of protease inhibitors tested, and thus cannot be assigned to any of the recognized proteases classes on the basis of inhibitor specificity. (4) The kinetics of plasminogen activation by membrane-bound Pla are unusual in that both the apparent Km (~100nM) and k_{cat} (0.0035 s^{-1}) are very low, indicating high substrate affinity but slow catalysis. (5) Pla has negligable activity against all chromogenic peptide substrates tested, included those cleaved by other plasminigen activators. (6) Both determination of sites cleaved by Pla in protein substrates, and competitive inhibition of Pla plasminogen activator activity with slected dipeptides indicate specificity for a basisc residue followed either a second basic residue, or a residue with a small non-polar R-group (gly, ala, val). (7) Inhibition of the plasminogen activator activity of membrane-bound Pla by dipeptides yields distinctly non-linear inhibition plots that can be explained by models in which Pla and plasminogen interact at two distinct sites. The lysine binding site of plasminogen does not appear to play an important role in these interactions. (8) The migration of Pla on denaturing gels is substantially reduced by exposure to 95°C during sample preparation. This has revealed that there are two forms of the enzyme present in bacterial membranes. They can be distinguished by differential susceptibility to this "heat modification" phenomenon, as well as by their chromatographic behavior.

Work currently in progress is focused on the effect of Pla on the complement system, and on identification of the Pla active site.

S 029 CRYSTAL STRUCTURE OF CRUZAIN COMPLEXED WITH Z-PHE-ALA-FMK, **Mary E. McGrath**, Ann E. Eakin, Sarah A. Gillmor, Charles S. Craik, and Robert J. Fletterick, Departments of Biochemistry & Biophysics, and Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA.

The 2.35Å X-ray crystal structure of cruzain, the major cysteine protease from *Trypanosoma cruzi*, was determined in the presence of Z-Phe-Ala-FMK. The general structure of cruzain especially the two domains, one characterized by helices and the other containing two beta sheets, strongly resembles papain, to which it is 35% identical. The root mean square deviation in common carbon alpha positions between cruzain and papain is 0.72Å. The differences in the structures are most easily seen in the surface loop regions. The active site is located, as expected, between the two domains, and the catalytic triad residues superimpose with those of papain. Our structure was compared with the papain:Z-Phe-Ala-CMK structure, and alkylation of the protease by the inhibitor and conformation of the P1 Ala is very similar between papain and cruzain. However, 2 hydrogen bonds made between backbone atoms of the inhibitor and papain are not made in the cruzain structure. This is probably because the active site cleft is 0.6Å wider in cruzain than papain. The P2 Phe sidechain is also bound differently in cruzain than in papain due to a complete change in the sequence of residues which make up the S2 site in the protease. The S2 site in papain appears to bind the Phe sidechain more effectively, while less favorable contacts are made between cruzain and the inhibitor at P2. This probably stems from the additional specificity of cruzain for Arg sidechains at P2. Thus, comparison of these two inhibitor-bound cysteine proteases indicates areas of the active site which could be exploited in designing better cruzain inhibitors and provides a wealth of information regarding specificity determinants in cysteine proteases.

S 030 THE DIVERSITY OF PROTEASE FUNCTION IN PARASITES, James H. McKerrow, University of California, San Francisco and Veterans Administration Medical Center, San Francisco, CA 94121.

Protozoa and helminth (worm) parasites utilize a variety of proteases to infect and replicate in their human host. Invasive larvae of the schistosome parasite penetrate through intact human skin utilizing a serine protease with broad activity against a variety of extracellular matrix and basement membrane macromolecules. Computer modeling of the active site has been achieved by homology-based structure prediction. Inhibitors predicted from the active site modeling confirm that inhibition of the enzyme blocks the invasive cercariae from entering skin. The capacity of the enzyme to degrade fibrillar macromolecules appears in part related to an extended active site with specificity at P_2 - P_4'. Cysteine proteases have been identified as key enzymes in the metabolism of protozoan parasites, as well as their transformation between morphologically distinct stages of the parasite life cycle. Specific inhibitors have been used to identify key roles played by these enzymes. These include hemoglobin degradation, cell remodeling, and cell rupture. From studies performed both *in vitro* and in animal models of disease, specific inhibitors of these enzymes have been shown to have promise as potential chemotherapeutic agents.

S 031 IgA1-PROTEASES OF PATHOGENIC BACTERIA: SYNTHESIS AND ACTIVITY IN HUMAN SECRETIONS, Andrew G. Plaut[1], Qiu, Jiazhou[1] and Andrew Wright[2]. [1]Dept. of Medicine, New England Med. Ctr. Hospital, and [2]Dept. of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111.

IgA proteases are endopeptidases produced by infectious bacteria including *Haemophilus influenzae, Streptococcus pneumoniae, Neisseria gonorrhoeae* and *N. meningitidis*, and bacteria causing periodontal disease(1). Non-pathogenic Haemophilus and Neisseria are IgA1-protease negative. The only known substrate is human IgA1 immunoglobulin, one of two IgA antibody subclasses in secretions on mucosal surfaces. Each enzyme cleaves after one of several proline residues in the extended hinge region of IgA1, separating the Fab (antigen-binding) and Fc domains. Human IgA2 has a shorter hinge region, and is not a substrate. IgA proteases are encoded by chromosomal *iga* genes and are secreted extracellularly; there is no known gene regulation, and enzyme is constituitively expressed *in vitro*. Secretion by Gram negative species Haemophilus and Neisseria is by a novel pathway (2) in which the carboxy-proximal portion of a 190 kD precursor creates an outer membrane pore through which the remainder of the precursor, which contains the active enzyme, reaches the extracellular environment. The enzyme is finally processed and released by autoproteolysis. Despite shared specificity for human IgA1 substrate the proteases are very dissimilar in structure and catalytic mechanism, with serine-, metallo- and thiol-types all being represented (3). Inhibiting antibodies are present in human secretions. The role of IgA proteases in the infectious process is not yet clear, but has recently been explored by culturing wild type *Haemophilus influenzae* and *iga-* mutant cells in normal human milk whey, a prototype of an IgA-rich human secretion (4). Milk IgA antibodies completely block the activity of the secreted enzyme, but only marginally block the final processing step leading to enzyme release. In milk, dividing wild-type cells aggregate but mutants do not, suggesting that milk constituents (?antibody ?lipid) interact with the enzyme precursor, or with the retained, pore-like helper section remaining on the microbial surface.

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2. Pohlner,J; Halter,R; Beyreuther,K; Meyer,TF. *Nature* 325; 458, 1987.
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S 032 THE ROLE OF BACTERIAL PROTEINASES IN THE DEVELOPMENT OF PERIODONTAL DISEASE, James Travis, Takahisa Imamura, Jan Potempa, and Robert Pike, Department of Biochemistry, University of Georgia, Athens, Georgia 30602.

Periodontitis is a major inflammatory disease primarily initiated by the presence of opportunistic, anaerobic pathogens within the dento-gingival crevice. One of these organisms, *Porphyromonas gingivalis*, is the most common bacterium associated with the disease and is characterized by its ability to produce and secrete significant quantities of "trypsin-like" proteinases. We have isolated the major enzymes responsible for this activity and, contrary to previous reports, found two cysteine-activated proteinases referred to as Arg-gingipain and Lys-gingipain, which cleave specifically after arginine and lysine residues, respectively. Arg-gingipain occurs in two forms, one of which is free and the other in complex with a hemagglutinin. Both forms can activate complement C5 to release the potent neutrophil chemotactic factor C5a. Furthermore, each can rapidly convert prekallikrein to kallikrein, thus activating the kallikrein/kinin pathway. Lys-gingipain, which is present in a single form in complex with hemagglutinin, can also activate the kallikrein/kinin pathway and, in addition, cause hemolysis of red blood cells to provide iron for bacterial growth. Significantly, neither enzyme appears to be homologous with other, known cysteine-activated proteinases. Taken together, both enzymes appear to be involved in *i)* the recruitment of neutrophils to the dento-gingival cavity, *ii)* the increase in crevicular flow which occurs during periodontitis due to edema, *iii)* the degradation of components of the coagulation pathway which would result in bleeding, and *iv)* the lysis of erythrocytes. Intervention with specific inhibitors of these two proteinases could thus prove to be useful in controlling the development of this inflammatory disease.

Banquet Address

S 033 PROTEOLYTIC ENZYMES PAST AND PRESENT - THE SECOND GOLDEN ERA, Hans Neurath, University of Washington, Seattle, WA 98195

The first golden era of research on proteolytic enzymes was heralded by the isolation of crystalline proteases and their inhibitors, and by the application of emerging techniques for the molecular characterization of proteins (chromatography, amino acid composition and sequence analysis, electrophoresis, x-ray diffraction, etc.) These have led to the elucidation of the classical, digestive proteases in terms of active sites, mechanism of action, zymogen activation, protease-inhibitor interactions, homology and evolution, and several related phenomena. Together, they have established the basis of our current understanding of protease structure and function. In the present, second golden era, our knowledge has been extended to the regulatory proteases which are the product of evolutionary recombinations of catalytic and regulatory domains, by a wealth of structural information derived by x-ray and NMR analyses, and by the application of methods of molecular biology to the synthesis of mutants of proteases and their inhibitors of predetermined design and properties. Having had the good fortune of witnessing and contributing to some of these developments, I shall present a historical, somewhat anecdotal view of the past and gaze at future developments of research on proteolytic enzymes.

Engineering in Proteases and Inhibitors

S 034 CATALYTIC ANTIBODIES WITH ESTROLYTIC AND AMIDOLYTIC ACTIVITY, Stephen J. Benkovic, The Pennsylvania State University, Department of Chemistry, University Park, Pennsylvania.

Efforts to obtain antibodies capable of cleaving ester and amide linkages will be reviewed. Various haptens have been designed and synthesized to induce catalytic antibodies with the desired hydrolytic properties. These haptens include: 1) phosphoryl containing derivatives that mimic the tetrahedral transition state or intermediates encountered in hydrolytic reactions: 2) compounds that create a volume for binding of a metal ion in juxtaposition to the scissile bond and: 3) complex analogues which contain constructs for more than one tetrahedral species within the same structural framework. In addition, attempts have been undertaken to improve existing antibodies by site-specific mutagenesis based on a mechanistic analysis of how these entities function. One of the more active catalytic antibodies (43C9) has been shown to possess many of the characteristics of a primative esterase in the sense of possessing an active site nucleophile derived from histidine and an oxyanion hole derived from arginine. This antibody in particular has been the object of site-specific mutagenesis with the intent now to introduce a metal ion binding site capable of promoting more efficient cleavage of ester and amide substrates. Efforts in that direction will also be be described.

S 035 HIRULOG™: DESIGN OF A POTENT ANTITHROMBIN USING HIRUDIN AS A MODEL, John M. Maraganore, Biogen, Inc., Cambridge,MA.

Thrombin is a central enzyme in thrombotic processes as it: i.) catalyzes cleavages of A-alpha and B-beta chains in fibrinogen forming fibrin monomer; ii.) amplifies blood coagulation through activation of Factors VIII and V; and, iii.) activates a specific platelet and cellular receptor through a novel proteolytic mechanism. Whilst active toward numerous physiologic substrates, thrombin action is through limited proteolysis, and specificity for thrombin action is mediated by dependence for efficient Michaelis complex formation on unique substrate interaction areas distant from the catalytic site.Hirudin, a 65 amino acid protein isolated from the saliva of the medicinal leech *Hirudo medicinalis*, is a potent and highly specific inhibitor of thrombin. Structure-function and kinetic studies on hirudin-thrombin interactions showed that, in addition to interactions with thrombin's catalytic site, hirudin binds to thrombin's anion-binding exosite (ABE). X-ray crystallographic studies of the hirudin-thrombin complex have confirmed these extensive interactions with the ABE. Peptide fragments (e.g., "hirugen") corresponding to the COOH-terminal dodecapeptide segment of hirudin bind to thrombin's ABE with µM affinity and neutralize thrombin's action toward physiologic substrates without blocking active site function. In order to replicate the high affinity and specific interactions of hirudin for thrombin, a series of bivalent peptides were designed. "Hirulog™" is a synthetic eicosapeptide inhibitor of thrombin comprised of three segments: i.) a COOH-terminal region corresponding to residues 53-64 in hirudin; ii.) a NH2-terminal tetrapeptide segment capable of binding to thrombin's active site; and, iii.) an intervening oligoglycyl linker segment which serves as a molecular bridge linking catalytic site- and ABE-binding domains. Hirulog™ was found to inhibit thrombin-catalyzed hydrolysis of tripeptidyl substrates (Ki = 1-2 nM), and, in plasma coagulation assays, showed essentially identical activity to hirudin. X-ray crystallographic studies on the thrombin complexes with Hirulog™ and derivatives confirmed bivalent interactions with thrombin. Pharmacologic and clinical studies with Hirulog™ have been performed demonstrating both this agent's activity and tolerability. In addition to serving as a locus for targetting novel thrombin inhibitors, the ABE serves as a key recognition area for thrombin interactions with its platelet and cellular receptor. Synthetic peptide fragments of the ABE inhibit competitively thrombin activation of the receptor. Engineering of this site serves the basis for a novel class of thrombin receptor antagonists.

HIV-1 Protease as a Therapeutic Target

S 036 KINETIC ANALYSIS OF MUTANT HIV-1 PROTEINASES: FUNCTIONAL ANALYSIS OF DRUG-RESISTANT MUTANTS, **Ben M. Dunn[1],Wichet Leelamanit[1], Daniel Raterman[1], Yangzhang Lin[2], Xinli Lin[2], and Jordan Tang[2], [1]Department of Biochemistry & Molecular Biology, University of Florida College of Medicine, Gainesville, FL 32610-0245, [2]Protein Studies Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.**

HIV proteinase [HIV PR], a member of the aspartic proteinase family, is an essential enzyme in the life cycle of the human immunodeficiency virus. During viral assembly, the enzyme is required to process the precursor proteins, gag and gag-pol, to produce the various fragments which assemble into the infectious particle. Therefore, HIV PR has become a prime target for active drug design. Based on prior observations of the development of drug resistance of the retroviral reverse transcriptase, it is not surprising that anti-proteinase drug resistance has also been observed[1]. When virus is grown in culture with high but non-lethal doses of anti-proteinase drugs, mutation of the viral genome eventually produces variant enzymes that exhibit some resistance to the drug. We have established a collaborative study to develop information regarding the enzymatic properties of these mutant enzymes. In our initial study, we have examined the catalytic properties of HIV-1 PR molecules with variation in the amino acids at positions 45, 48, and 82. These positions are within the active site cleft and are expected to have influence upon the substrate and inhibitor specificity of the enzymes. We have employed a panel of oligopeptide substrates (based on the sequence Lys-Ala-Arg-Val-Leu*Nph-Glu-Ala-Nle-Gly-NH_2) to determine the catalytic efficiency of the mutant enzymes, which were prepared by site-directed mutagenesis, expression, and purification. In addition, we have studied the inhibition of these mutant forms of HIV-1 PR by a panel of anti-proteinase drugs. In order for a mutant enzyme form to lead to a successful resistant virus, the enzyme must retain efficient catalytic properties while demonstrating reduced inhibition. We propose this criteria as a method of classification of mutant proteinase species. **This work was supported by NIH grants AI28571 (to BMD) and AI26762**

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S 037 THE DESIGN AND SYNTHESIS OF ORALLY BIOAVAILABLE HIV-1 PROTEASE INHIBITORS. J. R. Huff, J. P. Vacca, B. D. Dorsey, W. J. Thompson, A. K. Ghosh, R. W. Hungate, H. Y. Lee, P. L. Darke, E. A. Emini, W. A. Schleif, J. H. Lin, I-W. Chen, M. K. Holloway, P. S. Anderson. Merck Research Laboratories, West Point, PA, 19486.

The HIV protease (HIVPR) has been identified as a potentially important target for the development of chemotherapeutic agents for the treatment of HIV infection and AIDS. Although a number of potent HIVPR inhibitors have been reported, few have been evaluated in man because of poor oral bioavailability. We have designed a new class of HIVPR inhibitors using computer modeling techniques in conjunction with X-ray structures of enzyme-inhibitor complexes. Several members of this class potently inhibited the enzyme *in vitro* with IC_{50} values of <1 nM and blocked virus replication in cell culture with IC_{95} values of <100 nM. One of those, L-735,524, exhibited good oral bioavailability and an acceptable plasma half life in laboratory animals when administered in a clinically acceptable formulation. The compound has been evaluated in man for oral bioavailability. The design, biological profile, and limited clinical data for L-735,524 will be discussed.

S 038 SYMMETRY-BASED INHIBITORS OF HIV PROTEASE: ORAL BIOAVAILABILITY AND RESISTANCE EMERGENCE
Dale J. Kempf[1], Charles Flentge[1], Kennan C. Marsh[1], Edith Mcdonald[1], Terry Robins[1], Sudthida Vasavanonda[1], Chih-ming Chen[1], Norman E. Wideburg[1], Chang Park[1], Xiangpeng Kong[1], Jon Denissen[1], David. D. Ho[2], Martin Markowitz[2], Takuo Toyoshima[2], Mandaleshwar K. Singh[2], John Erickson[3], Hing L Sham[1], Kent Stewart[1], Brian E. Green[1], Mary Turon[1], Ayda Saldivar[1], Daniel W. Norbeck[1]; [1]Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL; [2]Aaron Diamond AIDS Research Center, New York, NY; [3]Structural Biochemistry Program, Frederick Biomedical Supercomputing Center, National Cancer Institute, Frederick, MD

The essential role of HIV protease in the proteolytic processing of the *gag* and *gag-pol* gene products of HIV offers the opportunity for chemotherapeutic intervention of AIDS through the design of specific inhibitors. While a wide variety of inhibitors have been described, the generally poor oral absorption and/or rapid biliary clearance of most HIV protease inhibitors remains a significant obstacle for clinical development. We recently reported the structure-based protease inhibitors A-77003 (Kempf, et al. *Antimicrob. Agents Chemother.*, **1991**, *35*, 2209-2214) and A-80987 (Norbeck, et al. VII International Conference on AIDS, Amsterdam, The Netherlands, July 19-24, 1992), the design of which was based upon consideration of the C_2-symmetric nature of the active site of HIV protease. Although the oral bioavailability of A-77003 was low, administration of A-80987 to three animal species resulted in plasma levels well in excess of its *in vitro* IC_{50}. Consideration of the metabolic pathways for A-77003 and A-80987 and systematic structure-activity studies led to the discovery of a series of inhibitors with markedly improved pharmacokinetic properties, culminating in the identification of the second generation inhibitor A-84538. The K_i for A-84538 against purified, recombinant HIV protease measured 15 pM. The EC_{50} for inhibition of HIV in MT4 cells was 0.03 µM, with cytotoxic effects observed only at >50 µM. Following a single 10 mg/kg oral dose in three species, plasma concentrations exceeded the *in vitro* EC_{90} for >10 hours, and the calculated oral bioavailability exceeded 70%. The X-ray crystal structure of A-84538 bound to the active site of HIV-1 protease provided a rationale for the enhanced potency relative to A-77003 and A-80987. The *in vitro* activity of A-84538 against mutant virus and mutant protease with decreased sensitivity to A-77003 and A-80987 was evaluated and analyzed on the basis of the X-ray crystal structures of each inhibitor.

S 039 HIV PROTEINASE INHIBITOR Ro 31-8959: SELECTIVITY, RESISTANCE AND CLINICAL STATUS, Noel A Roberts, Roche Products Limited, Welwyn Garden City, Hertfordshire, UK.

Ro 31-8959 is a rationally designed inhibitor of HIV proteinase which has potent *in vitro* antiviral activity. It is a transition-state mimetic based on a cissile Phe-Pro bond. This design basis was chosen because such cleavages are a unique feature of retroviral proteinases being essentially unknown in mammalian biochemistry. This strategy has produced a compound which has a highly selective inhibitory profile, not only with respect to related mammalian proteinases, but also other retroviral proteinases. Hence, or otherwise, Ro 31-8959 has an extremely good tolerance profile when administered long term to animals and man at doses which maintain plasma levels of drug well in excess of its antiviral IC_{90}. The compound is in Phase II clinical trials in HIV infected patients, some having now been dosed for well in excess of one year. This is allowing a study of the potential for the emergence of virus resistant to the drug during long term clinical dosing. An update on this compound will be presented with respect to new *in vitro* data supporting its mode of action, resistance studies and its clinical status.

Posters Relevant to Oral Sessions 1 and 2

S 100 THE THREE-DIMENSIONAL STRUCTURE OF HEPATITIS A VIRUS 3C PROTEINASE. Marc Allaire†, Maia M. Chernaia†, Bruce A. Malcolm†‡ and Michael N.G. James†, Department of Biochemistry† and Department of Medical Microbiology and Infectious Diseases‡, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada

The family of picornaviruses includes a large number of human and agricultural pathogens. All picornaviral proteins are expressed by direct translation of the genomic RNA into large polypeptide precursors. The cysteine 3C proteinase, encoded by the genetic material of the viruses, is essential in the proteolytic processing of the viral polyprotein. A double mutant of hepatitis A virus 3C proteinase in which both cysteine residues were replaced (C24S and the active site C172A) has been crystallized from polyethylene glycol with salt additives. Diffraction data to a resolution of 2.3Å were collected on synchrotron radiation at the Photon Factory, Tsukuba, Japan. The "phase problem" has been solved by the technique of multiple isomorphous replacement with seven heavy atom derivatives. Crystallographic refinement is in progress with a current R-factor of 20.9% and a root mean square deviation on the bond lengths of 0.024Å and on the bond angles of 3.5°. The molecular structure of HAV-3C (C24S/C172A) consists of an NH_2-terminal and COOH-terminal domain. Each domain is mainly made up of β-strands which fold into two orthogonally packed antiparallel β-sheets. The overall architecture of each domain is similar and approximates an antiparallel β-barrel. As proposed before[1,2], the three-dimensional structure of HAV-3C (C24S/C172A) is remarkably similar to the chymotrypsin-like serine proteinases. Details of the structure as well as comparison with the chymotrypsin-like serine proteinases will be described.

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S 101 CHARACTARIZATION OF PROTEASES IN CHO CULTURE FLUID, Payman Amiri and Mary B. Sliwkowski, Department of Cell Culture and Fermentation R&D, Genentech Inc. S. San Francisco, CA 94080-4918

Chinese hamster ovary (CHO) cells are used routinely for production of heterologous proteins of human therapeutic value. CHO cell lines used for expression of such proteins are mutants, lacking the dihydrofolate reductase (DHFR) gene. Coamplification of the gene of interest along with the DHFR gene can be achieved by selection in the presence of methotrexate, a DHFR inhibitor. These CHO cell lines are then adapted to grow in suspension culture in the presence or absence of serum, which is thought to be a source of growth factors, as well as proteases and protease inhibitors. During cultivation of these CHO cell lines the recombinant proteins are secreted into the culture fluid, and by an unknown mechanism proteases get released as well. This can lead to proteolytic degradation of valuable therapeutic proteins, and therefore reduce the yield and quality of desired products. When the type and function of protease activities in CHO culture fluid are known, it will be easier to design an efficient process for expression of recombinant proteins by eliminating the undesirable proteolysis.

We have begun to characterize the major proteases of CHO culture fluid with the goal of preventing their detrimental action. Thus far, we have identified three classes of proteases: a serine proteinase with an optimum pH of 8, a cysteine proteinase with an optimum pH of 4, and a carboxypeptidase with activity at pH 8. Based on the substrate specificity of the serine protease, we have designed a specific inhibitor. When this inhibitor was added to CHO cultures, a significant decrease in measurable activity was observed without any signs of toxicity. Efforts are underway to purify these CHO proteases using immobilized inhibitors and to design specific peptide inhibitors that can be used in cell culture.

S 102 NEUROPEPTIDE PRECURSOR PROCESSING 'PROHORMONE THIOL PROTEASE' (PTP) IS A NEW MEMBER OF THE FAMILY OF CYSTEINE PROTEASES, Azaryan A.V. and Hook V.Y.H., Dept. of Biochemistry, Uniformed Services University of Health Sciences, Bethesda, MD 20814

A novel prohormone thiol protease (PTP) involved in processing the enkephalin precursor has been described (Krieger and Hook, 1991). PTP is a single-chain 33 kDa glycoprotein with a pI 6.0, requires DTT for activity, and is inhibited by iodoacetate, p-CMB & mercuric chloride. PTP converts the enkephalin precursor (pH optimum of 5.5) to multiple NH_2-terminal-containing fragments similar in M_r to those in vivo. Importantly, PTP generates the product (Met)-enkephalin from BAM-22P, peptides E and F (Krieger and Hook,1991; Krieger et al., 1992; Hook et al.,1993) by cleaving at dibasic and monobasic sites. To place PTP in the family of cysteine proteases, selective active site-directed cysteine protease inhibitors were tested. E-64c was most potent with K_{2nd} 6,710,000 $M^{-1}s^{-1}$. Inhibition of PTP by Z-Arg-Leu-Val-Gly-CHN_2(1) and Z-Leu-Val-Gly-CHN_2 (2) (K_{2nd} 308,000 and 220,000$M^{-1}s^{-1}$, respectively) indicates a high affinity of the enzyme for Val-Gly in the P_2-P_1 positions. Peptides 1 & 2 correspond to the important sequences of enzyme binding region of human cystatin C, another selective cysteine protease inhibitor. These results are in agreement with PTP cleavage of peptide E and BAM-22P at the Gly-Arg bond within the Val-Gly-Arg-Pro sequence. Data obtained provide evidence that PTP belongs to the papain family of cysteine proteases.

S 103 CRYSTALLOGRAPHIC RE-INVESTIGATION INTO THE STRUCTURE OF SGPA REVEALS A POSSIBLE ACYL-ENZYME INTERMEDIATE, Helen Blanchard and Michael N.G. James, Medical Research Council of Canada Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, T6G 2H7, Canada

The structure of the bacterial serine proteinase from *Streptomyces griseus*, SGPA, was determined initially at 2.8Å resolution [1] and has been refined at 1.5Å resolution to an R-value of 12.6% [2]. The residual electron density in the active site was interpreted at that time as 13 hydrogen-bonded waters. In an attempt to understand anion binding to SGPA in the tetragonal crystals, a reinvestigation of this structure has been undertaken. The results of the present work suggest that the origin of the rather continuous active site density is that of a peptide forming an acyl intermediate on the enzyme's catalytic reaction pathway. The electron density is most adequately explained by a tetrapeptide incorporating the unusual amino acid β-hydroxy aspartic acid as the P_1 residue, with the complete peptide amino acid sequence being interpreted as Ac-Gly-Ala-Ser-(β-OH Asp). The resultant interatomic distance between the active site Ser195 Oγ and the carbonyl carbon of the peptide P_1 residue after unrestrained refinement is 1.7Å. The carbonyl oxygen of the ester is located in the oxyanion pocket and participates in hydrogen bonds with Gly193 NH (2.6Å) and Ser195 NH (3.0Å). Further, there is a peak of electron density which resides at a distance from the P_1 carbonyl carbon of 2.7Å. From our present refinement results this density most likely represents the hydrolytic water (W-221) which would be involved in the deacylation step of catalysis.

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S 104 DISSOCIATION AND REASSOCIATION OF PROTEASOMES (PROSOMES) FROM CALF LIVER CELLS.

Yves Briand, Wolfgang Tomek*, Jacques Buri**, Marie Noelle Pouch, Saloua Badaoui, Gerard Boissonnet, Mariele Briand and Peter Schmid. Universite Blaise Pascal-Clermont II, Laboratoire de Biochimie F-63177 AUBIERE Cedex (France), *Biologisches Institut der Universität, Pfaffenwaldring 57, D-7000 Stuttgart 80, FRG. **Station de Virologie et d'Immunologie, INRA, F-78350 Jouy en Josas (France)

Proteasomes appear to function as enzymatic core of the ATP dependent degradation of ubiquitinated proteins. Proteasome expression has been found abnormaly high in tumor cells. Others reported that deletion of certain proteasomal proteins was lethal in yeast. Finally we have shown that proteasomes inibit the in vitro translation of viral mRNA but not of cellular mRNA.

Proteasomes (prosomes) are large particles with sedimentation coefficients of about 19S. They have a typical cylinder shaped structure and consist of a specific set of proteins which band in SDS-Laemmli gels in the range of 19000-35000 daltons. Some of them eg. the 27000 dalton protein were highly conserved during evolution where others vary from species to species. All proteasomal proteins are glycosylated. A matter of controversity is the content of RNA at least a population of these particles contain small RNA.

Here we present a new method which is usefull to separate and study well defined subcomponents of proteasomes as well as the incorporation of small RNA molecules.

At a concentration of 8M urea proteasomes dissociated partially and eluted from superose 6 (Pharmacia-FPLC) as single protein components and a 120-200 kDa complex with a specific set of 6-8 proteins. After elimination of urea, proteasomes reassembled totally integrating labelled proteasomal RNA but not phe-tRNA. Native as well as reassociated proteasomes resist to a concentration of 1% Lauroyl sarcosyl-Na.

The proteolytic activity of reassociated particles will be discussed.

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Horsch A, Martin de Sa C, Dineva B, Spindler E, Schmid H P (1984) FEBS Letters 246, 131-136

S 105 EVIDENCE THAT THE NATURE OF AMINO ACID RESIDUES IN THE P_3 POSITION OF SUBSTRATES DETERMINES CLEAVAGE AT DISTINCT CATALYTIC SITES OF THE MULTICATALYTIC PROTEINASE COMPLEX (PROTEASOME),

Christopher Cardozo, Alexander Vinitsky, and Marian Orlowski, Department of Pharmacology and Medicine, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029.

The multicatalytic proteinase complex (MPC), a multisubunit intracellular particle involved in ubiquitin-dependent and ubiquitin-independent pathways of cytoplasmic and nuclear proteolysis, exhibits at least five distinct activities, cleaving peptide bonds on the carboxyl side of basic, acidic and neutral amino acids in synthetic and natural peptides. Three initially identified activities have been designated as trypsin-like (T-L), chymotrypsin-like (ChT-L) and peptidylglutamyl-peptide hydrolyzing (PGPH) based on the nature of residues providing the carbonyl group to the scissile bond. Two additional recently identified components of the complex cleave peptide bonds between small neutral amino acids (small neutral amino acid preferring; SNAAP) and after branched chain amino acids (branched chain amino acid preferring; BrAAP). Both the ChT-L and BrAAP components cleave peptide bonds after hydrophobic amino acids, but differ with respect to the nature of preferred residues around the scissile bond. Thus, the ChT-L component cleaves peptide bonds after both branched chain and aromatic amino acids and also amino acid arylamide bonds. By contrast the BrAAP component shows a strong preference toward bonds after branched chain amino acids but is inactive toward amino acid aryl amide bonds. The finding that the BrAAP component represents the major factor responsible for the protein-degrading activity of the MPC posed the need for identification of those structural elements that determine the involvement of either the ChT-L or the BrAAP components of the complex in cleavage of substrates. This led us to synthesize a series of substrates to probe the role of residues beyond those flanking the scissile bond in directing substrates to a defined catalytic site. The data indicate that a proline residue in P_3 directs the substrate to the catalytic site of the BrAAP component and that replacement of this residue by hydrophobic residues shifts the activity to the catalytic site of the ChT-L component. Introduction of the small neutral amino acid glycine makes the substrate susceptible to cleavage by both the BrAAP and ChT-L components. The presence of a proline residue in P_3 interferes with binding to the catalytic site of the ChT-L activity even in the presence of a phenylalanine residue in P_1. Such substrates are poorly cleaved by both the BrAAP and ChT-L components further supporting the preference of the former for branched chain amino acid residues in P_1. Supported by a KO8 award HL02835.

S 106 MODULATION OF THE MULTICATALYTIC PROTEINASE COMPLEX ACTIVITY,

José G. Castaño, Paz Arizti, Inmaculada Ruiz de Mena, Esther Mahillo and Joaquin Arribas. Departamento de Bioquímica e Ins. Investigaciones Biomédicas CSIC. Facultad de Medicina de la UAM. 28029 Madrid. Spain.

The multicatalytic proteinase (MCP) or proteasome, is a high molecular weight proteinase composed of 10-15 subunits ranging from 21-32 kDa. The MCP is able to hydrolyze proteins (protease activity) and small peptides whose carboxyl side contain basic, hydrophobic and acid aminoacids (peptidase activity); being involved in the cytoplasmic and nuclear protein turnover by ubiquitin-dependent and independent pathways. An overview of our studies aim to understand the modulation of the MCP activity will be presented, focused in three main aspects :1) modulation of the activity of the MCP by lipids, showing that cardiolipin, sulfatides and gangliosides modulate the peptidase and protease activity of the MCP complex; 2) modulation by proteolytic processing of MCP subunits, presenting data on the relationship between limited proteolysis of the C2 component of the MCP complex and the generation of the so-called active form of the enzyme and 3) modulation by inter-conversion of MCP subunits, showing that casein kinase II copurifies with rat liver MCP, being this kinase responsible of the phosphorylation in Ser residues of two subunits of the complex, which have been identified and demonstrated to be also phosphorylated in vivo. All these data suggest that MCP complex activity, is probably under tight and multi-factorial control in vivo.

S 107 CLONING OF THE GENE CODING FOR A NEW *E.COLI* OUTER MEMBRANE PROTEASE.

Elizabeth A. DiBlasio-Smith, Kathleen L. Grant, Neil Wolfman, Edward R. LaVallie, Lisa A. Racie and John M. McCoy, Genetics Institute, Inc., Cambridge, MA 02140

Specific proteolysis was observed during the purification of a thioredoxin-BMP2 fusion protein overproduced in *E.coli*. Specific cleavage occurred between the lysine-8 and arginine-9 residues at the N-terminus of Bmp2. Deletion of ompT, which encodes for an *E.coli* outer membrane serine protease possessing cleavage specificity for dibasic residues, did not alleviate proteolysis of the fusion. This poster describes the cloning of the gene encoding a new protease, designated 'OmpX', responsible for this cleavage and its preliminary characterization. The nucleotide sequence predicts a 315-amino acid polypeptide (including a putative 17-amino acid signal sequence) that is 70% identical to OmpT and 47% identical to both the E protein of *S.typhimurium* and Pla (Plasminogen activator) of *Y.pestis*. Attempts to map the cloned ompX sequence utilizing the Kohara gene mapping membrane failed. Subsequent southerns determined the absence of ompX sequence from *E.coli* strain W3110, the strain used to generate the Kohara membrane. Several other *E.coli* strains were analyzed to determine the prevalence of this new protease in commonly used strains.

S 108 PROHORMONE CONVERTASE PC2: ITS PRESENCE IN HUMAN BRAIN AND EXPRESSION BY BACULOVIRUS, Margaret Fahnestock and Weijia Zhu, Department of Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada L8N 3Z5.

PC2 is a subtilisin-like serine protease belonging to a family of mammalian enzymes sharing extensive homology with the protease furin. Furin is expressed in many tissues and may be responsible for the proteolytic activation of a wide variety of precursor proteins. PC2 has been shown to be expressed in neuronal and endocrine tissues, particularly in the CNS of both mouse and rat. In coexpression studies, PC2 is able to process mouse proopiomelanocortin (POMC) at appropriate cleavage sites. Thus, PC2 may be involved in neuroendocrine prohormone processing.

We report here the presence of PC2 transcripts in human frontal cortex. mRNA was isolated from postmortem brain tissue and PC2 sequences demonstrated by RT-PCR. The presence of PC2 in human frontal cortex tissue suggests that PC2 may be responsible for processing cortex-specific prohormones in man. In order to study the activity and specificity of PC2, we need to express large quantities of the protein. We have used a baculovirus expression system to produce insect cells containing human PC2. These cells will be useful for the further study of PC2 action.

S 109 CRYSTAL STRUCTURE OF ATROLYSIN C AND THE FUNCTIONAL PROPERTIES OF THE NON-PROTEINASE DOMAINS (PRO-, DISINTEGRIN-LIKE AND HIGH CYS) IN SNAKE VENOM METALLOPROTEINASES (REPROLYSINS)
Jay W. Fox[1], Li-Guo Jia[1], Tibor Gyorfi[1] and Jon B. Bjarnason[2], Edgar Meyer[3] [1]University of Virginia Health Sciences Center, Charlottesville, VA 22908, [2]Science Institute, Reykjavik, Iceland and [3]Texas A & M University, College Station, TX.
The atrolysins are hemorrhagic zinc metalloproteinases isolated from the venom of *C. atrox*. Atrolysins B, C, E and F are low molecular mass (~24kDa) proteins belonging to the P-I class whereas atrolysin A has a higher mass (~64kDa) and belongs to the P-III class. All the atrolysins are members of the Reprolysin subfamily of metalloproteinases which include venom as well as mammalian reproductive proteins (1). Atrolysin C is represented by the toxins previously termed ht-c and d. The crystal structure of atrolysin Cd is now solved and shows an active site region very similar to that observed in the astacin and matrix metalloproteinase subfamilies. Binding of a natural inhibitor in the active site of atrolysin C suggests a lock and key mechanism. The atrolysins are synthesized as zymogens of which the pro- sequence has a P-K-M-M-C-G-V-T consensus sequence reminiscent of the cysteine switch in the MMPs. We have shown that peptides with these sequences are effective inhibitors of the atrolysins. Atrolysin A has a disintegrin-like and a high cys domains in addition to the homologous proteinase domain shared by all atrolysins. Recombinant disintegrin-like domain is a potent inhibitor of platelet aggregation and here we report the use of synthetic peptides to localize in the domain the GPIIbIIIa interaction site. Furthermore, this domain appears to be capable of disrupting cell adhesion. The biological activity of this domain is likely to modulate the activity of atrolysin A and studies with recombinant atrolysin A are ongoing to understand this relationship.
(1) Bjarnason, J.B. and Fox, J.W., (1994), in press.

S 110 DECAPOD CHYMOTRYPSIN, Fernando L. García-Carreño, Martha P. Hernández- Cortés and Norman F. Haard', Centro de Investigaciones Biológicas. PO Box 128, La Paz, BCS, México 23000. Fax (112) 54710 and 'University of California. Davis, CA.

The controversy over the presence of chymotrypsin in the digestive system of decapods was resolved using a dual technique including specific substrate and inhibition assays for serine-proteinases. Chymotrypsin was found in both fresh water and marine decapods. The enzymes were either slightly sensitive or insensitive to an inhibitor for vertebrate chymotrypsins, showing that decapod chymotrypsins have a unique mechanism of catalysis which circumvents the reduction of activity by TPCK, a mighty vertebrate chymotrypsin inhibitor. Additional catalytic properties exclusive to decapod enzymes were: 1) substrate specificity was lower than mammalian enzyme, 2) *Km* was two orders of magnitude higher than vertebrate enzyme (porcine chymotrypsin), 3) there was a notable difference between fresh water and marine enzyme *Vmax* and 4) as result of 2) and 3) catalytic efficiency (*Vmax/Km*) of decapod enzymes was lower than mammalian preparations. A zymogram of decapod extracts exhibited several active zones with caseinolytic activity. Some of them were suppressed by the inhibitor PMSF, including one resembling the molecular weight of the mammal chymotrypsin. Further investigation of the molecular, three-dimensional and catalytic properties of decapod chymotrypsin must be achieved to determine divergences in the decapod serine-proteinase family.

S 111 METHOD FOR CONVERTING A PEPTIDE SUBSTRATE OF A PROTEINASE TO A FLUOROGENIC SUBSTRATE,
Kieran F. Geoghegan, Central Research Division, Pfizer Inc, Eastern Point Road, Groton, CT 06340
Proteinases are most conveniently and successfully assayed by optically-based methods, but the necessary substrates can be difficult to produce. In particular, when the specificity of the enzyme precludes placing a chromophore near the scissile bond, it is necessary to turn to fluorescence-based approaches. There is increasing reliance on "energy-transfer" substrates, in which a peptide is doubly tagged (usually at its termini) with chromophores that form an energy donor-acceptor pair. In the intact substrate, the fluorescence of the donor is quenched by the nearby acceptor group. Enzymatic cleavage of the peptide abolishes the quenching, and the resulting fluorescence increase is used to follow this activity. This poster reports a relatively simple way in which an unmodified peptide containing a sequence cleaved by a proteinase can be converted to a fluorogenic substrate that permits direct and continuous measurement of the enzymatic activity. The method consists of three steps, and originates with strategic design of the starting peptide to facilitate the chemistry. The peptide must have N-terminal Ser, C-terminal Lys, and no other Lys or source of an amino group in its sequence. The three steps of chemistry are (i) periodate oxidation of the N-terminal Ser to generate an α-*N*-glyoxylyl moiety at the N-terminus, which (ii) becomes the locus of reaction with the hydrophilic fluor Lucifer Yellow CH. (iii) In the third and final step, the amino group of C-terminal Lys is allowed to react with 5-carboxytetramethylrhodamine succinimidyl ester, to incorporate a suitable quencher of Lucifer Yellow fluorescence. Substrates of this type have been provided for human renin and collagenase (MMP-1). Each was characterized by structural and spectroscopic methods, and furnished a continuous fluorescence-based assay for its respective proteinase. The chemistry should be readily applicable to providing substrates for other proteinases. As this method begins with an unmodified peptide, it can be performed by any lab equipped with HPLC and is not restricted to the laboratory in which the peptide has been made.

S 112 CALPAIN ACTIVITY INCREASES IN HEPATOCYTES FOLLOWING ADDITION OF ATP: DEMONSTRATION BY A NOVEL FLUORESCENT APPROACH, Gregory J. Gores, Barry G. Rosser and Stephen P. Powers, Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, MN 55905

Our aim was to measure calpain protease activity during increases in cytosolic free calcium (Ca^{++}_i) after addition of extracellular ATP. The calpain protease substrate Boc-Leu-Met-7-amino-4-chloromethyl-coumarin (Boc-Leu-Met-CMAC) was synthesized. Nonfluorescent Boc-Leu-Met-CMAC diffuses into the cell where it is conjugated to glutathione forming Boc-Leu-Met-MAC-SG. The nonfluorescent, membrane impermeant Boc-Leu-Met-MAC-SG accumulates in the cell. Intracellular proteolytic hydrolysis of Boc-Leu-Met-MAC-SG releases and unquenches the fluorescence of MAC-SG. Intracellular fluorescence of MAC-SG was quantitated in single, cultured rat hepatocytes using digitized video fluorescent microscopy. Enhancement of intracellular fluorescence generation by increases in Ca^{++}_i and inhibition by a calpain inhibitor indicated the probe was a calpain substrate. After addition of ATP, calpain protease activity increased to 156±13% of basal concurrent with a 3-fold rise of Ca^{++}_i for 2-4 minutes. Thereafter, Ca^{++}_i decreased to values of 1.5 fold above basal and protease activity returned to normal. Incubation of cells in Ca^{++}-free buffer abolished the rise in Ca^{++}_i and calpain protease activity. Calpain protease activity increases concomitantly with increases of Ca^{++}_i supporting the hypothesis that calpain proteases participate in Ca^{++}-mediated signal transduction.

S 113 INHIBITION OF HUMAN CALPAIN I BY NOVEL QUINOLONE CARBOXAMIDES

Todd L. Graybill,† Roland Dolle,† Irennegbe K. Osifo,† Stanley J. Schmidt,† Jill S. Gregory,‡ Alex L. Harris,‡ Matthew S. Miller‡, Departments of †Medicinal Chemistry and ‡Neuroscience, Sterling Winthrop Pharmaceuticals Research Division, 1250 South Collegeville Rd., Collegeville, PA 19426.

Calcium-activated proteolysis of cytoskeletal proteins by the cysteine proteinase, calpain, is an important event in the process of post-ischemic neuronal cell death. Therapeutic neuroprotection might be obtained by post-ischemic treatment with a potent and selective inhibitor of this proteinase. We describe here the synthesis and biological evaluation of several novel non-peptide inhibitors of human calpain I.

S 114 AMINO ACID SEQUENCE COMPARISON OF PSYCHROPHILIC COD TRYPSIN AND CHYMOTRYPSIN WITH THEIR MESOPHILIC MAMMALIAN ANALOGS, Ágústa Guðmundsdóttir[1], Elín Guðmundsdóttir[1], Ann E. Eakin[2], Charles S. Craik[2] and Jón B. Bjarnason[1], [1]Science Institute, University of Iceland, Dunhagi 3, 107 Reykjavík, Iceland and [2]Department of Pharmaceutical Chemistry, University of California, San Francisco

Our primary interest is to investigate the molecular mechanisms underlying cold-adaptation of enzymes. Trypsin and chymotrypsin isolated from the Atlantic cod have been shown to be psychrophilic (1). The cDNAs encoding two different anionic forms of cod trypsinogen and one form of chymotrypsinogen have been cloned and sequenced (2, 3). The amino acid identity between the cod and bovine trypsins and chymotrypsins is 60% and 68% respectively. The cod trypsins are one amino-acid-residue shorter than most mammalian trypsins, presumably due to a deletion of a conserved proline at position 152. In addition, the highly conserved proline 28 is substituted in both cod enzymes as is proline 124 in cod chymotrypsin. All the prolines are localized at structurally important sites. We postulate that these proline substitutions increase the flexibility of the cod enzyme structures resulting in increased catalytic efficiency.

1. Ásgeirsson, B. Fox, J.W., and Bjarnason, J.B. (1989), Eur. J. Biochem. 180, 85-94
2. Gudmundsdóttir, Á., Gudmundsdóttir, E., Óskarsson, S., Bjarnason, J.B., Ekin, A.E., and Craik, C.S. (1993) Eur. J. Biochem. (in the press)
3. Gudmundsdóttir, Á., Óskarsson, S., Eakin, A.E., Craik, C.S. and Bjarnason, J.B. (1994), Eur. J. Biochem. (submitted)

S 115 CLONING AND CHARACTERIZATION OF THIOL PROTEASES FROM THE FALSE COLORADO POTATO BEETLE, Karl Guegler, Sherry Capitant, Liliana Scarafia, Victor Chan, Raymond Fucini, Wolfgang Seghezzi and Camille DeLuca-Flaherty, Sandoz Agro, Inc., 975 California Avenue, Palo Alto, CA 94304-1104

The growth of various insects can be inhibited by ingestion of thiol protease inhibitors (1). In order to develop inhibitors which are effective on an agricultural pest, the False Colorado Potato Beetle (FCPB, *Leptinotarsa texana*) we have undertaken a study of the target proteases. Using a zymogram assay we can visualize several distinct proteolytic activities in the gut contents of the FCPB. A substantial fraction of these gut-derived proteolytic activities can be suppressed with *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64), an irreversible inhibitor of the thiol class of proteases. Four clones encoding specific thiol proteases have been isolated from a FCPB larvae cDNA library and the nucleic acid sequences determined. The predicted amino acid sequences of each of the four FCPB clones align well with those of papain and related thiol proteases. One FCPB protease is similar in primary structure to vertebrate cathepsin L proteins. This protease does not appear to be present in gut tissues but may be sequestered in the hemolymph of FCPB larvae. Further characterization of additional proteases is in progress. From Northern hybridization analysis of each of the four FCPB cDNAs there is evidence that expression of the individual proteases is developmentally regulated.

1. Murdock, L.L., Brookhart, G., Dunn, P.E., Foard, D.E., Kelley, S., Kitch, L., Shade, R.E., Schuckle, R.H. and Woolfson, J.L. (1987) Comp. Biochem. Physiol. 87B: 793.

S 116 **α_1-ANTICHYMOTRYSIN INHIBITION OF A PROHORMONE PROCESSING ENZYME.** V.Y.H. Hook*, A.V. Azaryan, and T.J. Krieger. Dept. of Biochemistry, Uniformed Services Univ., Bethesda, MD.

Evidence is presented showing that α_1-antichymotrypsin (ACT)-like protein inhibits and is colocalized with the novel 'prohormone thiol protease' (PTP) involved in processing the enkephalin precursor. These results are the first demonstration that neuropeptide precursor processing may be regulated by an endogenous protease inhibitor. ACT immunoreactivity was colocalized with PTP within secretory vesicles of bovine adrenal medulla and posterior pituitary. The purified 60 kDa bovine pituitary ACT-like protein was a potent inhibitor of both PTP and chymotrypsin in the nanomolar range. Typical of serpin protease inhibitors, the bovine pituitary ACT-like protein formed SDS-stable complexes with chymotrypsin, and PTP formed SDS-stable complexes with human liver ACT. PTP cleavage of enkephalin-containing peptides at the NH_2-terminal side of paired basic residues (Lys-Arg, Arg-Arg, Lys-Lys) that flank the COOH-terminus of (Met)enkephalin (Tyr-Gly-Gly-Phe-Met), indicates methionine at the P_1 position of the cleavage site. These results showing PTP processing at a Met residue resemble the allowable P_1 specificity of ACT and are compatible with inhibition of PTP by ACTs. The estimated molar ratio of PTP/ACT-like protein of 2-3 within secretory vesicles of adrenal medulla suggests that PTP in vivo may be partially inhibited; this is consistent with the presence in vivo of a high degree of incompletely processed proenkephalin.

S 117 MITOCHONDRIAL INTERMEDIATE PEPTIDASE (MIP), A MEMBER OF THE THIMET PEPTIDASE FAMILY, IS ESSENTIAL FOR OXIDATIVE METABOLISM IN *S. CEREVISIAE*, Grazia Isaya, Yale University School of Medicine, New Haven, CT 06520.

Rat MIP (EC 3.4.24.93D) cleaves N-terminal octapeptides (FXXSXXXX) in the maturation pathway of mitochondrial enzymes imported to the matrix or the inner membrane. We have pursued the molecular characterization and genetic inactivation of the yeast homolog of rat MIP as a mean to study the function of this peptidase *in vivo*. A novel yeast gene, *YMIP*, was isolated by screening a *S. cerevisiae* genomic library with a rat MIP cDNA probe. *YMIP* encodes a protein with 54% similarity and 31% identity to rat MIP. Rat and yeast MIP contain the identical sequence LFHEMGHAMHSMLGR, including a Zinc-binding motif. Genetic inactivation of *YMIP* resulted in a mutant yeast strain unable to grow on non-fermentable carbon sources and exhibiting multiple respiratory chain complex defects. This *ymip*$^-$ mutant failed to process the nuclear-encoded precursors for cytochrome oxidase subunit IV (CoxIV) and the iron-sulfur protein (Fe/S) of the bc_1 complex to mature-size proteins; it rather accumulated octapeptide-containing intermediate-size polypeptides. Transformation of the *ymip* disruption mutant with a single copy *CEN* plasmid carrying wild type *YMIP* restored formation of mature CoxIV and mature Fe/S along with oxidative metabolism competence.

When rat and yeast MIP were used as a profile to search the sequence data bases for related sequences, all of the known thiol-dependent metallopeptidases were identified and clearly distinguished from unrelated proteins. Multiple comparisons indicate that MIP evolved independent of the other members within the thimet peptidase family, and that the gene duplication leading to the presence of MIP and thimet oligopeptidase EC 3.4.24.15 in mammals took place at an earlier evolutionary stage than the eukaryotic microorganisms.

S 118 **A NOVEL APPROACH TO RATIONAL PROTEIN ENGINEERING BASED ON ANALYSIS OF SERINE PROTEASES,** ***Nilofer G. Jiwani* and Michael N. Liebman*,#*** *Loyola University of Chicago, Department of Molecular and Cellular Biochemistry, Maywood, Illinois; #Amoco Technology, Bioinformatics, Naperville, Illinois

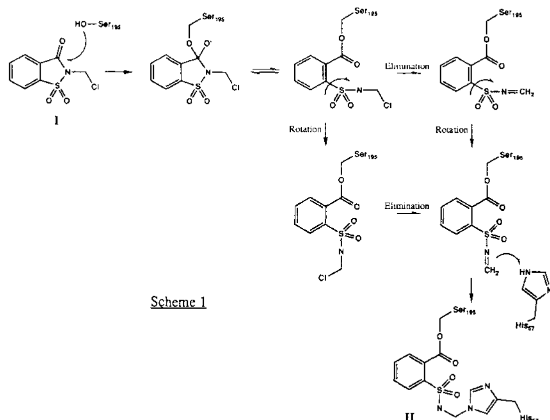
We are studying the relationships among computable structural and physical characteristics of serine proteases and their correlation with function. Serine proteases are a class of proteolytic enzymes exhibiting unique abilities to function as independent enzymes as well as to display interactive behavior within an enzyme cascades. The evolutionary basis of this unique functional characteristic is not known. It is our hypothesis that they exhibit similar characteristics related to their common catalytic mechanism and distinguishable characteristics unique to their function within a given pathway or environment. Preliminary evaluation of fundamental characteristics (such as structure, sequence and physico-chemical properties) of macromolecules supports this hypothesis. Our analysis indicates that both sequence and structure conservation has evolved along with the evolution of function of serine proteases. Analysis of physical properties, including hydrophobicity, van der Waals volume and accessible surface area, indicates that an amino acid will exhibit a cassette of properties which may mimic different amino acids dependent on their conformations. This could explain how similar conformations may result from different sequences. Our observations thus provide a novel approach to examine sequence variability of structural moieties and to develop rational protein engineering. Our preliminary data thus provide insight into conformational and environmental effects in protein and to critical aspects in the relationship between sequence and structure. Currently, we are mapping this information onto alignments of proteins involved in an enzyme cascade (e.g., blood coagulation) to understand potential coevolution of the pathways.

S 119 RESPONSE OF THE ACTIVE CENTER TO VARIOUS CLASSES OF SERINE PROTEASE INHIBITORS: EVIDENCE FROM NUCLEAR MAGNETIC RESONANCE, Frank Jordan, Khadijah Haghjoo, Zhixiang Hu, Sheng Zhong, Department of Chemistry, Rutgers University, Newark, New Jersey 07102.

The active center triad of serine proteases can interact with a number of different classes of inhibitors, such as protein-protease inhibitors, boronic acids, aldehydes, organophosphorus compounds, peptide ketones, etc. Multinuclear magnetic resonance studies have been used to study this interaction for all classes by a variety of groups, including ours. Such studies will be of ultimate benefit as diagnostics of the type of inhibition present and have the promise of aiding drug design. We have been intrigued by the wealth of information that protons bonded to the active center His imidazole nitrogen(s) can provide in both the native and the inhibitor-bound enzymes. Such resonances are located at 14-19 ppm downfield from silanes and can be observed using a variety of water suppression techniques. Well-resolved spectra are obtained for native chymotrypsin, trypsin and subtilisin, as well as on these enzymes in the presence of several classes of inhibitors. For example, the spectra in the presence of peptideboronic acids clearly indicate binding to Ser195, since resonances corresponding to both $HN^{\delta 1}$ and $HN^{\epsilon 2}$ are clearly visible. For such inhibitors, it is also clear that the His pK is very much elevated compared to that in the native enzymes, probably due to the negative charge on boron in the tetrahedral hybridization state (deduced from ^{11}B results in Zhong et al. *JACS*, **1991**). This pK elevation is similar to that observed in the monoisopropylphosphoryl, but not in the diisopropylphosphoryl, derivatives of these enzymes (Adebodun & Jordan, *J. Cell. Biochem.* **1989**). More recently, such studies have been extended to an examination of active centers in the presence of aldehyde inhibitors and protein protease inhibitors. Apparently, the active center reacts differently to these various classes of inhibitors, even though many are termed "transition state analogs". An interesting new class of inhibitors is constituted by the pro-sequence of secreted proteases. In particular, the interaction of the pro-peptide corresponding to the pro-sequence of subtilisins with the mature active proteases is also being evaluated to determine whether these inhibitors fall into any of the previously found classes. Several inhibitors being examined fall into the "slow-binding" kinetic regime (some of the peptideboronic acids and the pro-peptide of subtilisins), and an attempt is being made to discern the alterations induced in the active center by these slow-binding inhibitors compared to the rapid-equilibrium types. Supported by the Rutgers Busch Fund and Ciba-Geigy, Ardsley, NY.

S 120 INHIBITION OF SERINE PROTEASES BY N-CHLOROMETHYL-1,2-BENZISOTHIAZOLE-1,1,3-TRIONE: QUANTUM MECHANICAL CALCULATIONS. Paul J. Kowalczyk, Biophysics and Computational Chemistry, Sterling Winthrop Pharmaceuticals Research Division, Collegeville, PA 19426

Ab initio quantum mechanics calculations have been used to model the pathway of serine protease inhibition by N-chloromethyl-1,2-benzisothiazole-1,1,3-trione (**I**). A unique feature of this inhibition is the formation of two covalent bonds between the inhibitor and the serine protease (**II**); one bond forming between Oγ of the active site serine and the carbonyl carbon of the inhibitor and the second bond forming between Nε of the active site histidine and the exocyclic methylene carbon of the inhibitor. The formation of the first covalent bond follows the "classical" pathway of tetrahedral intermediate to acyl enzyme. Two paths are proposed for the formation of the second covalent bond. These are outlined in Scheme 1. Our calculations map the energetic profile of this inhibition and discriminate between the pathways presented in Scheme 1.



Scheme 1

S 121 RECOMBINANT BOVINE ENTEROKINASE: EXPRESSION, PURIFICATION, AND COMPARISON WITH THE NATIVE HETERODIMERIC ENZYME, Edward R. LaVallie, Alnawaz Rehemtulla, Lisa A. Racie, Elizabeth A. DiBlasio-Smith, Cathy Ferenz, Albert Light*, and John M. McCoy, Department of Microbiology, Genetics Institute, Inc., Cambridge, MA 02140 and the *Department of Chemistry, Purdue University, West Lafayette, IN 47907

Enterokinase (enteropeptidase) is a heterodimeric serine protease which is produced in mammalian duodena and initiates the digestion cascade by activating trypsinogen via a highly specific cleavage following the pentapeptide recognition sequence $(Asp)_4$-Lys. A cDNA was isolated which encodes the catalytic (light) chain of bovine enterokinase, which predicts a 235 amino acid polypeptide with a high degree of similarity to a variety of trypsin-like serine proteases involved in digestion, coagulation, and fibrinolysis. The recombinant enterokinase light chain cDNA was successfully expressed in mammalian cell culture, and a high yield single-step purification of the enzyme from conditioned media was devised. The recombinant single-chain form of the enzyme is capable of cleaving peptides, polypeptides, and trypsinogen with the same specificity exhibited by the heterodimeric form of the enzyme. Interestingly, recombinant enterokinase light chain activates trypsinogen poorly relative to the native heterodimer, but is superior in its ability to cleave fusion proteins containing an $(Asp)_4$-Lys recognition sequence.

S 122 CHARACTERIZATION OF A SECRETED FORM OF HUMAN FURIN PROTEASE AND SEARCH FOR ITS "P-DOMAIN", Richard Leduc, Gaël Vidricaire and Jean-Bernard Denault, Department of Pharmacology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec, CANADA J1H 5N4

The subtilisin-related proprotein convertases (SPCs) are a new family of Ca^{+2}-dependent serine proteases that cleave a variety of precursor proteins ranging from serum factors, growth factors, cell adhesion molecules, receptors, proteolytic enzymes, viral envelope glycoproteins and peptidergic hormones. There are now six mammalian SPCs that process proproteins at specific pairs of basic amino acids into their biologically active forms. The ubiquitously distributed furin, also called PACE or SPC1, is the first member of the mammalian SPCs to be identified. Furin appears to be responsible for the conversion of many types of proproteins, cleavage taking place at a furin consensus cleavage site, Arg-X-X-Arg. The most infamous precursor which is matured in such a fashion by furin, is gp160 of human immunodeficiency virus, HIV.

Due to its hydrophobic transmembrane domain it was thought that furin may exclusively be localized to trans-Golgi membranes. We have detected furin-like proteolytic activity not only in the membrane fractions of BSC40 cells infected with a vaccinia virus recombinant expressing human furin but also in the media of these cells. Using anti-furin antibodies, proteins, secreted into the media and labeled with $[^{35}S]$ Met/Cys were immunoprecipitated. Analysis by SDS-PAGE indicates a distinct band having a molecular mass of 80 kDa. Our results suggest that a processing step in the Golgi membranes enables furin to become a soluble form which can follow a secretory route. Thus, possible translocation of furin from Golgi membranes to cell surface to outright secretion may indicate other roles being played by furin notably in the processing of bacterial toxins or in the shedding of receptors.

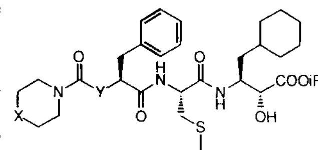
We have also focused on the "P-domain" of human furin which confers critical enzymatic activity to the protease. To that end we constructed the hFUR582 mutant and still found furin-like enzymatic activity. This signifies that residues 583-794 of the native enzyme are not necessary for enzymatic activity but may have other functions.

S 123 POTENT INHIBITORS OF HUMAN RENIN WITH HIGH ORAL BIOAVAILABILITY. B. A. Lefker, D. J. Hoover, R. L. Rosati, W. F. Holt, W. R. Murphy, M. L. Mangiapane, M. R. Nocerini, M. J. Gumkowski, W. A. Hada, M. P. Carta, M. L. Gillaspy, S. S. Ellery, J. T. MacAndrew, K. A. Simpson, J. Wentland, Central Research Division, Pfizer Inc., Groton, Connecticut, 06340.

Renin is an aspartyl protease which converts angiotensinogen (A0) to angiotensin I (AI), the first and rate limiting step in the renin-angiotensin system (RAS). Since renin is an extremely specific enzyme, it's inhibition may have therapeutic advantages over ACE inhibitors by more effectively blocking the RAS.

We report herein the discovery of a series of potent renin inhibitors as potential antihypertensive agents which have high oral bioavailability and good plasma pharmacokinetics. These are tripeptide-like substances derived by modification of the P4-P1' sequence of the renin substrate, angiotensinogen. Previously reported renin inhibitors **1** (**X**=O, **Y**=NH) and **2** (**X**=C=O, **Y**=NH) are orally active compounds which suffer from low aqueous solubility and poor oral bioavailability in animal models. Reductive amination of **2** with various amines produced a series of water soluble inhibitors with improved i.v. and oral efficacy and significant oral bioavailability. Subsequent exploration in this series gave **3** (**X**=CH-NHMe, **Y**=CH_2), having high bioavailability and oral efficacy in various species. The discovery, in vitro, and in vivo characterization of this series of inhibitors will be discussed.

Our peptidomimetic approach to the design of inhibitors of human renin has resulted in a series of compounds with excellent in vitro potency, aqueous solubility, and stability to common digestive enzymes such as chymotrypsin.



S 124 PROHORMONE CONVERTASE 1 : SPONTANEOUS CONVERSION TO AN ACTIVE 66 KDA FORM, Iris Lindberg and Yi Zhou, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70112

The prohormone convertase PC1 (also known as PC3) is a calcium-activated subtilisin-like serine proteinase predominantly expressed in neuroendocrine tissues. We have purified recombinant PC1 from overexpressing CHO cells and demonstrated that this enzyme is secreted as a mature form of 87 kDa. Recombinant PC1 possesses low specific activity but is extremely thermostable. Recent experiments have shown that purified PC1 can catalyze the removal of its own carboxyl terminus, thus resulting in the generation of a 74/66 kDa PC1 proteins. The amount of 74/66 kDa protein produced was not dependent on the amount of 87 kDa protein incubated, perhaps suggesting an intramolecular cleavage mechanism. The production of the 74/66 kDa proteins was inhibited in the presence of the dibasic fluorogenic substrate. The 74/66 kDa enzyme was repurified on Mono Q and was demonstrated to represent a catalytically active entity; the two molecular weight forms could not be separated. Like the parent 87 kDa form, 74/66 kDa PC1 is calcium-dependent and exhibits an acidic pH optimum. However, 74/66 kDa PC1 is most active at higher concentrations of calcium and within a narrower pH range than the 87 kDa form; it was also more susceptible to inhibition by a peptide chloromethyl ketone as well as by PMSF. Both forms of PC1 were capable of cleaving proenkephalin to the same 15- 27 kDa peptides (during the incubation with proenkephalin, however, 87 kDa PC1 was concurrently converted to the 74/66 kDa forms). These data indicate that both the truncated and full-length PC1 forms are capable of participating- though possibly at different subcellular sites- in the physiological cleavage of prohormone precursors to bioactive peptides.

Supported by NIDA 05084; I.L. was supported by an RCDA from NIDDK.

S 125 YEAST MUTANTS DEFICIENT IN ER DEGRADATION **Ardythe A. McCracken, James E. Ernaga, and Igor V. Karpichev. Department of Biology, University of Nevada, Reno, NV 89557.**

Recent evidence indicates a quality control mechanism for the identification and selective degradation of abnormal proteins in the endoplasmic reticulum (ER). To investigate the molecular mechanisms of this process, we used a genetic approach to identify the relevant proteases and accessory proteins. Mutants deficient in ER degradation have been isolated from a *S. cerevisiae* strain that expresses the Z variant of human alpha-1-proteinase inhibitor (A1PiZ) from a 2μ plasmid vector. This strain, which accumulates A1PiZ in the ER for rapid degradation (McCracken and Kruse, 1993, Mol.Biol.Cell 4:729-736), was mutagenized and screened for degradation-deficient clones by a colony blot immunoassay. Selected clones accumulating A1PiZ were cured of the A1PiZ-containing vectors and were then transformed with A1PiZ and other "ER degradation" substrates. The degradation-deficient phenotype present in the transformed cured cells established that the mutation directing this trait was in the host genome. The degradation rate of A1PiZ in selected mutant strains was decreased, as measured by pulse-chase radiolabelling, confirming that these mutants were deficient in A1PiZ degradation. Analysis of invertase secretion by these mutant strains indicated that, for most mutants, the defect in degradation was not due to a secretion defect. Genetic analyses revealed a group of mutations which were inherited in a recessive manner and others which were semidominant. The number of complementation groups is being determined. These mutants will be used to identify the genes directing the degradation defects. (Supported by ACS grant MV#551)

S 126 MECHANISM OF CYSTEINE PROTEASE INHIBITION BY PEPTIDYL METHYLKETONE DERIVATIVES: AN INVESTIGATION USING SITE-DIRECTED MUTAGENESIS, Robert Ménard, Céline Plouffe, Euridice Carmona and Andrew C. Storer, Biotechnology Research Institute, National Research Council of Canada, Montréal, Canada H4P 2R2 and Allen Krantz and Roger A. Smith, Syntex Research (Canada).

Because of their role in many biological processes and their pathophysiological significance, cysteine proteases constitute prime targets for the design of specific inhibitors. A number of compounds are known to irreversibly inhibit cysteine proteases, including many peptidyl methylketone derivatives (chloro- fluoro- and acyloxy-methylketones). However, the mechanisms by which these compounds alkylate cysteine proteases remain unknown. Using site-directed mutagenesis, we have shown that these inhibitors can make use of the catalytic machinery (i.e. the oxyanion hole) of cysteine proteases in different manners. In addition, although these inhibitors differ only by the nature of their "leaving group", they display very different pH-dependencies for their reaction with papain and papain mutants. A model has been derived that offers an explanation for these observations. Interpretation of the data according to this model suggests that mainly two properties of the inhibitor must be considered to explain the results: the ability to form a stable intermediate with papain and the inherent chemical reactivity of the inhibitor. Alternative mechanisms in agreement with our results will be presented. The knowledge gained from this study allows us to highlight the features of these inhibitors that are essential for their reactivity towards cysteine proteases.

S 127 EXPRESSION OF E-24.18 IN COS-1 CELLS: MEMBRANE TOPOLOGY AND ACTIVITY, Pierre-E. Milhiet, Denis Corbeil, Valérie Simon, Philippe Crine and Guy Boileau, Département de Biochimie, Université de Montréal, C.P. 6128, Montréal, Canada, H3C3J7

Endopeptidase-24.18 (EC 3.4.24.18, endopeptidase-2, meprin, E-24.18) is a metallopeptidase of the astacin family highly expressed in kidney brush border membranes of rodents. Rat E-24.18 is an heterotetrameric protein composed of two disulphide-linked α/β dimers. In order to investigate the molecular mechanisms of assembly and the importance of each subunit in the enzymatic process, the cloned cDNAs for the rat α and β subunits were used to independently transfect or cotransfect COS-1 cells. Immunoblotting analysis of expression products present in cell extracts and culture media showed that, when expressed alone, the α subunit is secreted while the β subunit is membrane-bound. In cells transfected with both subunits, α and β proteins were membrane-bound demonstrating that the β subunit is necessary for the plasma membrane expression of the α subunit. Papain or DTT treatment of intact cells expressing both subunits released α proteins in the supernatant. These results are consistent with a model of topology of E-24.18 in which the β subunit is anchored in the plasma membrane and the α subunit is disulphide-linked to the β subunit. Both the α and β subunits expressed in COS-1 cells showed little degradation activity towards azocasein used as a substrate. However, activity could be unmasked for both subunits and α/β dimers upon mild trypsin digestion suggesting that the enzymes are synthesized in COS-1 cells as zymogens. Finally, using an α subunit inactive mutant we show that in the α/β dimer both subunits participates in the enzymatic process.

S 128 **CARBOXYPEPTIDASE Y MUTANTS WITH ALTERED SUBSTRATE SPECIFICITIES.**

Kjeld Olesen and Klaus Breddam.
Department of Chemistry, Carlsberg Laboratory, Gamle Carlsbergvej 10, DK-2500 Copenhagen Valby, Denmark

Carboxypeptidase Y of yeast *Saccharomyces cerevisiae* (CPD-Y) exhibits a pronounced substrate preference with respect to the C-terminal and especially the penultimate amino acid of peptide substrates (P_1 and P_1' respectively). By studying the three dimensional structure of a homologous enzyme from wheat (CPD-WII) and an alignment of the primary structures of the two enzymes a number of amino acid residues of CPD-Y were hypothesized to form the binding pockets (S_1 and S_1' respectively) for the two most C-terminal amino acid residues of a peptide substrate. In an attempt to produce CPD-Y mutants with changed substrate preferences toward the P_1 residue the entire S_1 subset of residues were subjected to saturation random mutagenesis followed by phenotypic screens. It was found that leucine 178 plays an important role for the substrate preference of CPD-Y. To further investigate the importance of Leu178 a number of mutants were constructed by site directed mutagenesis in which this residue had been replaced by either Trp, Phe, Ala, Ser, Cys, Asn, Asp or Lys. These mutants have been characterized kinetically with substrates of the general formula FA-Xaa-Ala-OH, where Xaa is either Phe, Leu, Val, Ala, Ser, Glu, Arg or Lys. We find that substitution of Leu178 leads to a number of pronounced changes in substrate preference. The most significant changes are found with the enzyme L178D. With this enzyme the activity towards FA-Lys-Ala-OH is increased by a factor of 100 (as compared to the wild type enzyme) while the activity towards FA-Val-Ala-OH has decreased by a factor of 2400. Thus the relative activity towards the two substrates has changed by a factor of $2.4x10^5$.

S 129 PROLYL OLIGOPEPTIDASE: A REPRESENTATIVE OF A NEW SERINE PROTEASE FAMILY, László Polgár, Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, P.O. Box 7, H-1518, Hungary
Prolyl oligopeptidase, dipeptidyl peptidase IV, acylaminoacyl peptidase, and other peptidases constitute a new family of serine peptidases (Rawlings, N.D., Polgár, L. and Barrett, A.J., Biochem. J. 279, 907, 1991). In distinction from the enzymes of the trypsin and subtilisin families, prolyl oligopeptidase has two reactive forms, displays an unusual secondary specificity and pH-rate profile, and has a rate-limiting step that is physical rather than chemical in nature. By comparing the amino acid sequences, a structural relationship between lipases and the enzymes of the prolyl oligopeptidase family can be demonstrated.

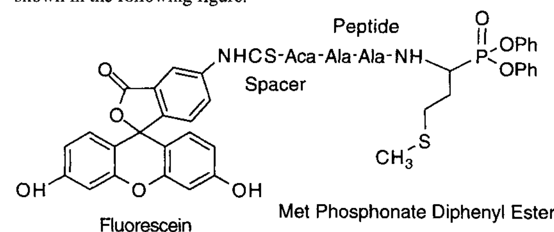
S 130 CLONING OF A DIBASIC PROCESSING ENDOPROTEASE FROM DIROFILARIAE IMMITIS: A CANDIDATE PROTEASE FOR PROCESSING THE POLYPROTEIN ALLERGEN OF NEMATODES, Catherine Poole, Dong Ma, Larry McReynolds, New England Biolabs, Beverly, MA 01915

Nematode polyprotein allergens (NPA)[1] have been cloned from filarial parasites that infect animals and humans worldwide. Filarial parasites cause elephantiasis and blindness in humans. The filarial parasite of dogs, D.immitis (dog heartworm), causes severe damage to the heart and other vital organs.

The D.immitis NPA gene, Di5 is composed of 25-50 direct tandem repeats of a 399bp monomer. Metabolic labeling studies of adult parasites showed that Di5 is synthesized as a large precusor that is cleaved producing a protein ladder from 14 to >200 kDa with steps about 15 kDa apart. N-terminal sequencing of the 14.3 kDa protein monomer revealed the tetrabasic site, RRKR, a common processing motif of eukaryotic dibasic processing endoproteases. The concensus sequence, RX(K/R)R is conserved among all the NPA genes sequenced so far. Using degenerate oligonucleotide probes based on the catalytic domains of the dibasic endoproteases, a fragment was amplified from adult D.immitis RNA. Sequencing of this fragment revealed 81%, 63% and 54% identity with the homologous regions of furin, blisterin and kexin respectively. Currently, adult cDNA D.immitis libraries are being screened for full length clones. Characterization of this protease may aid our understanding of the role of the parasite NPA family whose function is unknown. It might also serve as a target for chemotherapeutic intervention.

[1] McReynolds et al. (1993) Parasitology Today (9) 403-406

S 131 FLUORESCENT LABELED PHOSPHONATE INHIBITORS OF SERINE PROTEASES, James C. Powers and Ahmed S. Abuelyaman, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332-0400, Dorothy Hudig and Susan Woodard, Department of Microbiology, University of Nevada, Reno, NV 89557-0046.
Alpha-aminoalkyl phosphonate diphenyl esters are irreversible inhibitors of serine proteases. The inhibition mechanism involves formation of a stable enzyme phosphonyl derivative by reaction with the active site Ser-195. The phosphonate inhibitors are stable in aqueous solution and plasma with half-lives of >10 h. The inactivated serine protease derivatives are also stable and usually regain no activity after standing for several days. The phosphonates are quite specific and require the proper peptide sequence in order to inhibit their targeted serine protease. We have synthesized a number of fluorescent labeled phosphonate inhibitors in order to specifically label serine proteases in biological experiments. The phosphonates have a fluorescein moiety or a Texas Red moiety attached to a spacer and a specific peptide sequence with a C-terminal phosphonate inhibitor moiety. A representative structure is shown in the following figure.



Derivatives with specificity toward chymotrypsin-like, trypsin-like, and elastase-like enzymes have been synthesized. Similar derivatives with Texas Red labels are underway. The inhibitors have been used to specifically label serine proteases in natural killer cell granules.

S 132 PROCESSING OF THE PROPROTEIN OF THE E144S MUTANT NEUTRAL PROTEASE FROM *Bacillus cereus* IS SECRETED UNPROCESSED, Rodney S. Roche and Diana R. Wetmore, Department of Biological Sciences, Division of Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 1N4

We have shown that Glu144 is essential for the enzyme activity of the thermolysin-like neutral protease secreted by *B. cereus,* Cnp. The mutation of this residue to Ser (E144S) provides strong evidence that the processing of wild-type proCnp is autocatalytic. Perhaps the most surprising result of the E144S studies is that the unprocessed pro-protein is secreted. This is the first reported evidence that pro-protein processing may not be a requirement for release of the pro-protein from the cell membrane after secretion. Finally, we have provided strong evidence to suggest that the processing of the pro-protein occurs in a sequential manner via at least one intermediate.

S 133 COMPARATIVE PROPERTIES OF THE PRO-PROTEIN CONVERTASES PC5 AND PACE4, Nabil G. Seidah, Robert Day, Weija Dong, Mieczyslaw Marcinkiewicz, Didier Vieau, Suzanne Benjannet and Michel Chrétien, IRCM, Montréal, P.Q., Canada, H2W 1R7.

Specific cleavage of precursors at either single or pairs of basic residues by one or more specific convertase(s) leads to the excision of their active component(s). Furin, PC1/PC3, PC2, PACE4, PC4, and PC5/PC6 are six distinct mammalian convertases recently identified. The analysis of the gene regulation, ontogeny, tissue and cellular distribution and precursor cleavage specificity demonstrated a unique pattern for each enzyme. Overexpression of PC1, PC2 and furin allowed the characterization of their biosynthetic rates and their cleavage selectivity with a number of precursors both *in vitro* and in cell lines. In general, studies on processing efficiency, cellular and subcellular localization suggest that PC1 and PC2 preferentially cleave pro-proteins negotiating the regulated secretory pathway. In contrast, furin is mostly involved in the cleavage of precursors routed to the constitutive secretory pathway. So far, not much is known about the properties of the newly discovered PACE4 and PC5 which exhibit a 70% sequence identity within their catalytic domain, and a very similar C-terminal five times repeat of a Cys-rich segment (Lusson et al. PNAS **90**, 6691, 1993). Northern blot analysis, immunocytochemistry and *in situ* hybridization histochemistry was used to analyze the comparative distribution of PC5 and PACE4 in the rat central nervous system and in the periphery. The data demonstrate that neither of these convertases is ubiquitously expressed and that each enzyme exhibits a unique tissue and cellular distribution profile which is distinct from all other convertases known so far. The available data suggest that PC5 and PACE4 are each expressed in a unique subset of endocrine and non-endocrine tissues. Furthermore, the mRNA levels of each convertase are regulatable, attesting to the plasticity of their expression. Results will also be presented dealing with the cellular co-expression of each convertase with potential substrates and the analysis of the processed products.

S 134 RAPID AND QUANTITATIVE INVESTIGATION OF PROTEOLYSIS USING HIGH-SPEED HPLC MICROCOLUMNS.

Christopher Southan, SmithKline Beecham Pharmaceuticals, Welwyn, Herts, AL6 9AR, U.K.

Following proteolysis by reverse-phase (RP) HPLC is an important technique for investigating proteases. The activity can be monitored of either a novel protease against a known substrate or a known protease against an untested substrate. Large substrates with multiple cleavage sites produce a complex peptide map wheras single peptide substrates can be followed as a peptidolytic assay. The disadvantages of conventional RP-HPLC are the time reqired and amount of protein consumed in the analysis. In this work the analysis time has been greatly reduced by using large-pore column packings with steep gradients and horizontal flow velocities up to ten-fold higher than conventional RP-HPLC. Detection sensitivity has been increased by home-packing glass or plastic capillary columns of less than 1mm i.d.and by using A 205nm for detection. For example a 0.65 X 50mm glass capillary packed with Poros R 10µ m particles can be run at 0.5ml/min and eluted with gradients of 5-70% CH3CN in 5 minutes. Under these conditions a low-resolution tryptic peptide map can be obtained from 2ug of lactoglobulin with only 7 minutes between injections. An important advantage of RP-HPLC is the ability to quantify any protein or peptide .The decrease in lactoglobulin peak height provided a direct measurement of the progress of digestion. Using run times as short as 4 minutes the protein concentrations of lactoglobulin and trypsin solutions were assayed directly by peak height. This allows accurate E:S ratios to be used. for cleavage studies. By the use of flow progamming a peptidolytic assay was developed for the measurment of a peptide precursor and two cleavage products with only 8 min between injection times. These high-speed, micro-HPLC conditions can be adapted follow cleavage by different protease/substrate combinations, quantitatively and in real time.

S 135 BOVINE KIDNEY LEUCINE AMINOPEPTIDASE cDNA: COMPARISON WITH THE LENS ENZYME AND USE TO DEMONSTRATE TISSUE-SPECIFIC EXPRESSION OF TWO mRNAs, REGULATION OF EXPRESSION, INDUCTION BY INTERFERON GAMMA AND HOMOLOGIES WITH OTHER AMINOPEPTIDASES Allen Taylor, Tufts Human Nutr. Res. Ctr., Boston, MA 02111

Aminopeptidases appear to be involved in myriad physiological processes, including protein turnover, immune responses, regulation of hormone levels, etc. Leucine aminopeptidase (LAP) from bovine lens is the best characterized aminopeptidase. We isolated a bovine kidney LAP cDNA and compared its deduced amino acid sequence to the published amino acid sequence for bovine lens LAP. The sequences are highly conserved. However, the kidney LAP cDNA indicates a 26 amino acid extension at the amino terminus which is not found in the mature purified lens LAP. The cDNA also indicates an additional octapeptide in the C-terminal region not indicated in the published lens LAP amino acid sequence but which was required for best fit of crystallographic data regarding bovine lens LAP. Several other single amino acid changes were also noted. There are significant homologies between many aminopeptidases. Lens epithelial tissue showed only one LAP transcript (2.4 kb), whereas two transcripts (2.0 and 2.4 kb) were observed in cultured lens cells (BLEC) derived from epithelial tissue and in kidney tissue. LAP mRNA levels correlated with changes of LAP activity in aging lens tissue and in progressively passaged BLEC used to simulate aging *in vitro*. No differences were found in LAP mRNA levels in epithelial tissue from old and young lenses. LAP mRNA concentrations are regulated in a manner consistent with the transient increases in LAP activity and in intracellular proteolytic activity in passaged BLEC. LAP transcripts of 2.0 and 2.4 kb arise by differential splicing of a common precursor RNA, and they code for similar but distinct proteins. Gamma-interferon induces LAP mRNA in human cell lines. Funded in part with funds from the USA, ARS under contract #53-3K06-0-1 and NIH #EYO8566.

S 136 CLONING AND SEQUENCING OF MURINE TRIPEPTIDYL PEPTIDASE II, Birgitta Tomkinson, Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Biomedical Center, Box 575, S-751 23 Uppsala, Sweden

Tripeptidyl peptidase II (TPP II) is a cytosolic high molecular weight exopeptidase with a widespread tissue- and species distribution. The peptidase has a broad substrate specificity and is believed to serve a general role in intracellular protein catabolism. The human enzyme has previously been cloned and sequenced, and it could be demonstrated that it is a serine peptidase with an active site of the subtilisin-type.

The cloning and sequencing of the corresponding murine enzyme is described. Different cDNA-clones encoding TPP II was isolated from a mouse mastocytoma cDNA-library, and sequenced. The deduced amino acid sequence shows a remarkably high overall identity, i. e. 96%, when compared to human TPP II. This is also true for the nucleotide sequence which shows 90% and 77% identity for translated and untranslated sequences respectively. It is therefore concluded that not only the catalytic domain, but also the other parts of this unusually large peptidase, must be of functional importance.

It was demonstrated earlier that the human TPP II was encoded by two different mRNAs, which probably result from the utilization of two different polyadenylation sites. This has been confirmed for the murine TPP II, i. e. two different cDNA clones containing the long and the short untranslated 3'-end, respectively, have been isolated.

Finally, one of the isolated cDNA clones contains an extra 39 bp, encoding 13 amino acids, which is not present in the other cDNA clones. This probably represents an extra exon included as a result of alternative splicing. The physiological importance of the extra domain remains to be investigated.

S 137 CHARACTERIZATION AND CLONING OF CRAB COLLAGENOLYTIC SERINE PROTEASE 1: IDENTIFICATION OF THE BOVINE TYPE I COLLAGEN CLEAVAGE SITES, Christopher A. Tsu, John J. Perona, Volker Schellenberger and Charles S. Craik, Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0446

Collagenolytic serine protease 1 from *Uca pugilator* (fiddler crab) is the first known serine protease capable of cleaving native, triple helical collagen. The crab collagenase also possesses trypsin, chymotrypsin and elastase-like activities. The amino acid sequence has been determined at the cDNA level, unambigously verifying this versatile enzyme as a member of the pancreatic serine protease family. Affinity-based purification and characterization of the collagenolytic serine protease 1 from fiddler crab hepatopancrei shows that the enzyme cleaves the native bovine α1(I) collagen chain carboxy-terminal to several Gln and Arg residues, adjacent to the vertebrate metallo-collagenase cleavage site. Cleavage carboxy-terminal to Leu residues is observed in the α2(I) chain and at a secondary site in α1(I). These sites correlate with the preferences observed towards peptidyl *p*-nitroanilide substrates varying at the P1 position, for which the specificity (k_{cat}/K_m) is Arg > Leu, Phe, Lys > Gln > Ala. Crab collagenase cleaves adjacent to Leu and Gln at the P1 more efficiently than does trypsin, chymotrypsin or elastase. Further, the high activity manifested toward P1- Arg substrates indicates that the unusual Gly 189/Asp 226 binding pocket geometry can sustain catalysis at a level comparable to Asp 189/Gly 226 in trypsin. Crystals of crab collagenase have been grown in complex with the protein inhibitor BPTI. These crystals diffract to 2.9 Å resolution and belong to the space group $P3_121$ or $P3_221$ with unit cell dimensions of a=b=126.1 Å, c=181.0 Å.

S 138 INHIBITORS OF THE PROTEOLYTIC COMPONENT OF THE MULTICATALYTIC PROTEINASE COMPLEX (19S PROTEASOME), Alexander Vinitsky, Christopher Cardozo and Marian Orlowski, Department of Pharmacology, Mount Sinai School of Medicine of the City University of New York, New York, NY 10029

Evidence indicates that the multicatalytic proteinase complex (MPC), a large (~ 700 kDa), multisubunit cytosolic and nuclear enzyme, is involved in ubiquitin-dependent and ubiquitin-independent pathways of intracellular proteolysis. The complex exhibits five distinct endopeptidase activities cleaving bonds after basic, acidic and neutral amino acids in synthetic and natural substrates. A recently identified component that preferentially cleaves bonds after branched-chain amino acids (BrAAP) is a major factor responsible for the protein-degrading activity of the MPC. A series of peptidyl aldehydes was tested for inhibition of the proteolytic component of the MPC using synthetic peptides and proteins such as β-casein and histone H_3 as substrates. Peptidyl aldehydes containing branched-chain amino aldehydes in the P_1 position were shown to be effective inhibitors of the proteolytic component of the MPC. The most potent of the inhibitors containing a leucinal residue inhibits the BrAAP component in a competitive manner with a K_i of 1.5 μM, and causes an 80 to 90% inhibition of histone and β-casein degradation at a concentration of 50 μM. Replacement of the leucinal residue by a phenylalaninal causes a 30-fold increase in K_i. The leucinal containing peptidyl aldehydes also inhibit the components of the MPC that preferentially cleave bonds after small neutral amino acids (SNAAP) and after acidic residues (PGPH). An inhibitor in which the leucinal residue is replaced by a valinal residue becomes a more effective inhibitor of the SNAAP component than of the BrAAP component. Studies on the structural determinants necessary for inhibitor binding to the active site of the BrAAP component showed that the nature of residues in the P3 and P4 position greatly affect binding and inhibitory potency. The BrAAP component was inhibited with the same K_i both before and after activation of this component by exposure of the MPC to 3,4 dichloroisocoumarin. Similarly the same IC_{50} was obtained for the PGPH activity both before and after activation of the MPC by exposure to low concentrations of sodium dodecylsulfate. These results indicate that the peptidyl-aldehyde is an effective inhibitor of both the overt and latent activities of the MPC.

This work was supported by Aaron Diamond Postdoctoral Fellowship RO1813 (A.V.), NIH grant DK 25377 (M.O.), NRSA fellowship HL 08254 (C.C.)

Posters Relevant to Oral Sessions 3 and 4

S 200 C6 GLIOBLASTOMA CELLS EXPRESS A NEW METALLOPROTEASE WHICH SEEMS TO PLAY AN IMPORTANT ROLE IN TUMOR INVASION, Verena R. Amberger and Martin E. Schwab, Brain Research Institute, University of Zurich, 8029 Zurich, Switzerland.
Oligodendrocytes and CNS white matter express two proteins which are inhibitory for cell migration of e.g. astrocytes and neurite outgrowth (Schwab and Caroni, 1988). C6 glioblastoma cells, however, are able to overcome this inhibitory substrate property and infiltrate the brain in a very diffuse manner. This cellular behaviour is correlated with the expression of a proteolytic activity by the C6 cells which is able to inactivate the myelin associated inhibitors. Using various known protease blockers the activity was identified as a metalloprotease (Paganetti et al., 1988). We developed a specific peptide degradation assay to characterize the protease. The metalloprotease is tightly bound to the plasma membrane and can be solubilized only with detergents, e.g. Chaps or Triton X-114. An active fragment can be cleaved from the membrane with a short trypsin treatment. The protease is insensitive to blockers of the serin-, aspartyl- and cysteineproteases, but the activity can be destroyed with chelating agents and restored with cobalt and partly with zinc. The C6 protease is highly sensitive to phosphoramidon, but not to thiorphan which distinguishes it clearly from the metallo-endopeptidases 24.11 and 24.18. Another important characteristic of the C6 metalloprotease is that it can be stabilised with dithiothreitol unlike the dithiothreitol-sensitive endothelin converting enzyme. The enzyme is active in a pH range from 5.5 to 6.5. Several newly developed metalloprotease blocker show strong blocking effect for the cell spreading of C6 glioblastoma cells on purified CNS myelin. Current work is focused on the purification of the C6 metalloprotease, on the study of blocker effects on C6 cell migration in in vitro and in vivo, and on the development of effective protease blockers with respect of a future medical application.

S 201 ISOLATION OF CELL MUTANTS DEFECTIVE IN proTGFα PROCESSING. Joaquin Arribas & Joan Massagué, Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center and Howard Hughes Medical institute, New York, NY 10021.

The extracellular domain of several transmembrane molecules can be released into the cell media by regulated proteolytic cleavage. Proteins that undergo regulated secretion of ectodomain include several membrane anchored growth factors, some cytokine receptors, ectozymes, cell adhesion molecules and the β-amyloid precursor. We are using proTGFα as a model to study this process. The secretion of TGFα in a variety of cell lines is activated by several compounds such as serum, calcium ionophores and activators of protein kinase C. It has been seen proposed that the regulated cleavage of proTGFα requires a proteolytic system that acts at the cell surface and one or more components that allow responsiveness to different stimuli.
In order to obtain cell lines deficient in proTGFα processing, Chinese hamster ovary cells expressing proTGFα tagged with the HA1 epitope from the human influenza virus were mutagenized with ethyl methane sulfonate. Cells that failed to cleave the tagged proTGFα in the presence of activators, were selected by flow cytometry using an anti HA1 monoclonal antibody. Characterization of these cells indicate that they have lost the function of factor(s) acting downstream of the different activation pathways that regulate proteolytic cleavage of proTGFα. The use of mutants defective in proTGFα processing will provide a useful tool for the elucidation of the molecular mechanisms regulating cell surface cleavage events.

S 202 ALTERNATIVE SPLICING IN 5' OF TRANSCRIPTS COULD MEDIATE POST-TRANSCRIPTIONAL REGULATION OF EXPRESSION OF HUMAN TUMOR CATHEPSIN B, Isabelle M. Berquin and Bonnie F. Sloane, Wayne State University, Detroit, MI 48201
The activity of cathepsin B is regulated at various steps, including expression, activation and processing, intracellular trafficking and inhibition. Increased cathepsin B activity, secretion or plasma membrane association mediated by alterations in each of these steps has been correlated with tumor malignancy. Gong *et al.* (DNA and Cell Biol. 12: 299-309, 1993) have reported alternative splicing species of cathepsin B transcripts lacking exons 2 or 2 and 3. The latter species may yield a truncated protein which can be refolded *in vitro* to a proteolytically active enzyme (Gong and Frankfater, FASEB J., p. A1208, 1993). We have previously isolated four full-length human cathepsin B cDNA clones from a gastric adenocarcinoma cell line, AGS 1-6-39-1 (Cao *et al.*, Gene, in press, 1993). Two of these clones contain an additional exon, designated exon 2', located between exons 2 and 3. Exon 2' may be tumor-specific and could alter transcript stability or translation efficiency. Translation is normally initiated from an ATG codon in exon 3, thus exons 1, 2 and 2' are untranslated. Although there is a potential ATG codon in exon 2, it is followed directly by a stop codon. Interestingly, two ATG codons are present in exon 2'. One of the ATG codons is not in frame, whereas the other is followed six codons later by a stop codon in exon 3. The presence of these upstream initiation codons could play a regulatory role by altering the efficiency of translation initiation at the correct site or mRNA stability. If alternative splicing species of cathepsin B mRNA are found at different ratios in tumors than in normal cells, they could contribute to elevated activity of the protease and facilitate invasion. To test this, we designed primers for reverse transcription-polymerase chain reaction (RT-PCR) experiments using RNA isolated from normal and tumor cells or surgical samples. The size of amplification products indicates which combinations of exons 2, 2' and 3 are present. U87, an invasive, tumorigenic glioblastoma cell line, expresses transcripts which contain exons 2, 2' and 3, whereas two other glioblastoma cell lines of lower invasive potential only show transcripts with exons 2 and 3. The consequence of this phenomenon for the expression of cathepsin B is now under study.

S 203 EFFECT OF CARDIAC OUTFLOW TRACT-CONDITIONED MEDIUM ON NEURAL CREST CELL-PLASMINOGEN ACTIVATOR ACTIVITY AND CELL MIGRATION, Philip R. Brauer and Manisha Agrawal, Department of Biomedical Sciences, Creighton University, Omaha, NE 68178.
Evidence suggests that cell-cell and cell-extracellular matrix interactions between migrating neural crest (NC) cells and the cardiac outflow tract are required for normal heart development and may be mediated through specific growth factors. Previously, we have shown that isolated avian cardiac-outflow tract segments secrete latent transforming growth factor-beta (TGF-ß) that NC cells activate by plasminogen-dependent mechanisms. Active TGF-ß can alter plasminogen activator (PA) activity and mediate cell migration in various cells. We hypothesize that NC cell migration into the heart is limited to the outflow tract region because newly activated cardiac growth factors decrease protease activity required for NC cell migration. Here, we determined if TGF-ß or outflow tract-conditioned medium (OTCM) inhibited NC cell migration in the presence or absence of plasminogen and if any OTCM effects required NC activation of latent cardiac TGF-ß. NC cells were incubated with or without 1 ng/ml TGF-ß1 or OTCM for 24 hr and their migratory capacity then measured in a fibronectin chemotactic assay. Our results show that addition of plasminogen enhanced NC cell migration and that this effect was blocked by aprotinin. After treatment with TGF-ß or OTCM, NC cell migration was not different from untreated cells and the plasminogen effect was lost. Similar results were obtained in the absence of a chemotactic gradient and could not be attributed to differences in initial cell attachment, therefore, the effects reflected changes in NC cell-motility. PA activity was significantly decreased within 6 hr after TGF-ß or OTCM treatment as compared to untreated NC cells and in OTCM-treated cells was due to activation of TGF-ß since TGF-ß neutralizing antibodies blocked the inhibitory effect on PA activity. TGF-ß antibody returned NC PA-activity levels in CM-treated cells to that of untreated cells but did not return NC migration levels to that of untreated cells. This suggests that some factor(s) other than newly activated TGF-ß in OTCM may alter plasminogen-dependent NC migration in vitro. Further work will be necessary to explore this possibility but our results suggest that the cardiac environment inhibits plasminogen-dependent NC cell migration and may restrict NC cell migration to this region of the heart. Supported by NIH #HL50397, AHA-Nebraska, & Health Futures Foundation.

S 204 THE ROLE OF DIPEPTIDYL DIPEPTIDASE-IV (DPP-IV, E.C.N.3.4.14.5) CD 26 ENZYMATIC ACTIVITY IN REGULATION OF HEMATOPOIETIC AND EARLY T CELL PROLIFERATION IN VITRO AND IN VIVO .

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The cell surface serine protease, DPP-IV, has been identified as the thymocyte activating molecule (THAM) in the mouse and the thymocyte co-stimulating molecule in the rat. We have previously shown that CD 26 mediates co-stimulatory activity on granulocyte and monocyte lineages in bone marrow colony assays in vitro. By the use of a potent slow-binding inhibitor, Pro-boro-pro, we demonstrate that it is the CD26/DPP-IV enzymatic activity rather than other mechanisms that are involved in mediating the regulatory role of CD 26 peptidase. Studies have been carried out to test the effect of Pro-boro-pro in vivo and to analyze the target molecules as well as the populations that are affected. These studies in addition to those which examine hematolymphoid cell development in a CD 26 deficient rat strain, suggest that CD 26/DPP-IV plays an important role in peptidase regulation in hematopoietic and intrathymic microenvironments.

S 205 STRUCTURE AND FUNCTION RELATIONSHIPS OF MURINE CYTOTOXIC CELL PROTEINASE-1,

Antonio Caputo¶, Rosemary S. Garner¶, Ulrike Winkler§, Dorothy Hudig§ and R. Chris Bleackley¶, from the ¶ Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G2H7, Canada and the § Cell and Molecular Biology Graduate Program and Department of Microbiology, University of Nevada, Reno, Nevada 89557

Murine Cytotoxic Cell Proteinase-1 (CCP1/granzyme B) is a member of a family of novel serine proteinases that have been implicated to participate in destruction of target cells by cytotoxic T lymphocytes. Comparison of the amino acid sequence of CCP1 deduced from the cDNA with that obtained by sequencing CCP1 isolated from lytic granules of cytotoxic lymphocytes indicated that this proteinase may be synthesized as a preproenzyme having an amino-terminal signal peptide of eighteen amino acids followed by a putative activation dipeptide Gly-Glu which immediately precedes Ile-1 of the mature enzyme. Additionally, molecular modeling of CCP1 indicated that Arg-208 occupies a position in which the side chain partially fills the specificity pocket of the enzyme, thereby predicting a preference for substrates containing acidic side chains at the P1 site. The preferred substrate for CCP1 has subsequently been shown to contain an aspartyl residue at P1. This substrate specificity is unique among eukaryotic serine proteinases although interleukin-1β-converting enzyme, a cysteine proteinase, also shares this substrate specificity. In our studies of the structure and function relationships of CCP1 we have shown that the activation dipeptide regulates the activity of this enzyme in hydrolysis of its preferred substrate *tert*-butyloxycarbonyl-Ala-Ala-Asp-thiobenzyl ester. Lysates of COS cells transfected with a vector expressing unmodified CCP1 were unable to hydrolyze this substrate, whereas lysates of cells transfected with a construct in which the activation dipeptide codons had been deleted were able to hydrolyze this substrate. Western blotting of these lysates indicated equivalent amounts of this proteinase, the predominant form of which had an apparent molecular weight of 27,000. Higher molecular species with apparent molecular weights of 33,000 and 36,000 were also detected and may reflect glycosylation of CCP1. The role of Arg-208 in determination of the substrate specificity of CCP1 is being investigated by site directed mutagenesis of this enzyme. The results of these experiments will be presented at the conference.

S 206 α_2-MACROGLOBULIN: A PROTEINASE-ACTIVATED ANTIGEN CAPTURE AND DELIVERY SYSTEM,

Charleen T. Chu, Tim D. Oury, Jan J. Enghild, & Salvatore V. Pizzo, Department of Pathology, Duke University, Durham, NC 27710

The proteinase "inhibitor" α_2–macroglobulin (α_2M) can entrap and form covalent linkages with diverse proteins during a transient proteinase-activated state. Neutrophil elastase, pancreatic elastase, plasmin, trypsin, Serratia proteinase, and potentially any proteinase that is inhibited by α_2M, can initiate complex formation. These complexes are rapidly endocytosed after binding to receptors present on macrophages, dendritic cells, and other cells. We have previously shown that compared to free hen egg lysozyme (HEL), α_2M-complexed HEL undergoes enhanced macrophage uptake and presentation to T-hybridoma clones *in vitro*. Since T-hybridoma responses may not accurately reflect primary immune responses *in vivo*, we examined the effects of complexing antigens to human or rabbit α-macroglobulins (αM) upon antibody production in rabbits. Thirty-two rabbits were injected s.c. with adjuvant-free preparations of free HEL or porcine pancreatic elastase (PPE), human or rabbit αM-HEL-PPE complexes, or mixtures of the uncomplexed proteins. Complexing the antigens to the αM resulted in >200-fold higher IgG titers compared to uncomplexed controls. Injection of antigens complexed to either human α_2M or rabbit αM resulted in levels of anti-HEL IgG comparable to those elicited by emulsification in complete Freund's adjuvant. Thus, α_2M activated by either host or microbial proteinases can potentially mediate enhanced antigen delivery into antigen presenting cells *in vivo*, resulting in augmented Ag processing and antibody production. We further suggest that, like the thioesters of the related complement proteins C3 and C4, the thioesters of α_2M may function to covalently tag foreign targets for clearance and processing by organismic defense systems.

S 207 ELEVATED LEVELS OF A COLLAGENASE IV ACTIVATING FACTOR IN C6 ASTROCYTOMA CELLS

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A collagenase IV activating factor (CAF) released by C6 astrocytoma cells has been identified. Characterization of the CAF was carried out by comparison with the endothelial cell stimulating angiogenesis factor (ESAF), the latter obtained from mammalian pineal glands. ESAF activates collagenase IV by cleavage of the procollagenase IV and/or by dissociation of the TIMP-collagenase IV complex. The conditioned media of a rat glioma cell line (C6) and a human glioma cell line (U251) were assessed in terms of the levels of activating factor and collagenase IV activity. The total basal collagenase IV activity of C6 astrocytoma cells was 4X greater than U251 cells. Using a procollagenase IV assay, C6 conditioned media contained 10X greater levels of the collagenase IV activating factor compared to the U251 media. In either cell line the levels of the activating factors was related to the levels of collagenase IV activity. Addition of either CAF or ESAF increased the total basal collagenase IV activity of U251 by 5X and 4X respectively, to comparable levels of basal C6 collagenase IV activity. Neither factor effected the total collagenase IV activity from C6 cells. The CAF appears to be characteristic of an ESAF-like molecule. C6 cells represent a rich source of this ESAF-like molecule. In the human glioma cell line collagenase IV activity appears to be under stricter control than in the rat counterpart. The lower levels of ESAF from U251 cells suggests that in certain human tumors an alternate mechanism may exist for activating collagenase IV in vivo.

S 208 CHARACTERIZATION OF SERINE AND METALLO-PROTEINASE ACTIVITIES FROM STIMULATED RAT NEUTROPHILS AND RAT ALVEOLAR MACROPHAGES.

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Proteinases produced by human neutrophils and alveolar macrophages have been extensively characterized. At the same time, several models of acute inflammation in the rat have been shown to have proteolytic enzyme-mediated components of injury. While these models are generally assumed to be relevant to human disease, much less work has been done to characterize the proteases of rat cells. It was of interest, therefore, to characterize the serine and metalloproteinase activities expressed by the neutrophils and alveolar macrophages of the rat. Neutrophils were obtained from the peritoneum by elicitation with glycogen and from peripheral blood by Ficoll-Hypaque centrifugation followed by dextran sedimentation. Alveolar macrophages were obtained by bronchoalveolar lavage. In addition to unstimulated controls, the cells were stimulated with a variety of agonists. In comparison to human neutrophils, rat neutrophils contained less elastase and more cathepsin G activity. In addition, rat neutrophils contained both 92 kD and 72 kD gelatinase activity but little, if any, activity against native type I collagen. The rat alveolar macrophages contained no detectable elastase, cathepsin G or metalloproteinase activity when stimulated for 90 minutes. This is not surprising given that macrophages do not store significant amounts of enzymes in pre-formed granules as do neutrophils. When cultured for 18 hours or longer, alveolar macrophages from rat did produce a significant quantity of 92 kD gelatinase, but no elastase or cathepsin G activity. These data generally support the applicability of rat models to human disease, but suggest caution in a too rigorous interpretation regarding the role of individual effector molecules.

S 209 GELATINASE B PRODUCING CELLS IN MULTIPLE SCLEROSIS LESIONS,

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Proteolytic activity could play a major role in the inflammatory tissue damage in the demyelinating disease multiple sclerosis (MS). Elevated levels of the metalloproteinase gelatinase B (type IV collagenase), are present in the cerebrospinal fluid of MS patients and the enzyme degrades myelin basic protein, one of the major protein components of the myelin sheath, in vitro *(Gijbels et al., J Neuroimmunol 41:29-34, 1992; Gijbels et al., J Neurosci Res 36:432-440, 1993).* We used antibodies against gelatinase B (kind gift of Dr. W.G. Stetler-Stevenson, NIH, Bethesda, MD) on formalin fixed paraffin embedded CNS tissue to examine whether gelatinase B is produced in the MS lesion itself and to identify the producing cell type(s). Macrophages, microglial cells, endothelial cells and astrocytes in the lesions were positive. Some cortical neurons, ependymal cells and vascular smooth muscle cells showed gelatinase B immunoreactivity, even in normal controls. Astrocytes were strongly positive in older demyelinated lesions, even without inflammatory cells in the vicinity. Immunohistochemical staining for type IV collagen was reduced to absent around the capillaries in these lesions. These findings demonstrate that gelatinase B is produced in the MS lesion by cells from both the immune and the nervous system. Ongoing production of this enzyme by astrocytes in older lesions could prevent remyelination. Degradation of type IV collagen in the basement membrane surrounding capillaries indicates a role for gelatinase B in the breakdown of the blood-brain barrier as occurs in MS.

S 210 DESIGN AND ACTIVITY OF LINEAR AND CYCLIC STATINE - BASED INHIBITORS OF CATHEPSIN D,

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Cathepsin D is a highly abundant lysosomal aspartic proteinase widely distributed in various mammalian tissues. In addition to normal physiological functions, cathepsin D has been associated with several biological processes of possible therapeutic significance including antigen processing, connective tissue diseases and breast cancer. We have recently solved the crystal structure of the cathepsin D complex with a naturally-occurring inhibitor of aspartic proteinases, pepstatin A, at 2.5 Å resolution. A series of linear and cyclic inhibitors of cathepsin D were designed and synthesized based on the crystal structure information. The inhibition constants of these compounds were measured to be in the nanomolar to subnanomolar range for cathepsin D and were compared with several other human aspartic proteinases, including cathepsin E, pepsin and gastricsin.

S 211 INFLUENCE OF PROTEINASE INHIBITOR THERAPY ON THE RELEASE OF CELLULAR PROTEINASES, CYTOKINES AND SOLUBLE ADHESION MOLECULES IN ACUTE INFLAMMATION,

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Acute inflammation is characterized by a complex network of interacting cellular and humoral defence mechanisms. Of the numerous inflammatory mediators/effectors investigated hitherto proteolytic enzymes of lysosomal origin (PMN elastase, macrophage-derived cathepsin B) as well as of plasma cascade systems (thrombin, plasmin, etc.) have turned out to be highly destructive thus initiating and maintaining organ dysfunctions in severe posttraumatic and postoperative courses. Such proteolysis-induced disorders are especially rendered possible by the concurrently arising consumption of inhibitory regulators (e.g. α_1-proteinase inhibitor = α_1PI, antithrombin III = AT III) via complex formation and /or proteolytic/oxidative inactivation. Besides the well-known destructive potency against protein substrates, proteinases (active or in complex with serpin inhibitors such as α_1PI and AT III) are also supposed to increase the inflammatory reaction by inducing cytokine release or shedding of soluble adhesion molecules from various inflammatory cells (PMNs, monocytes/macrophages, endothelial cells). Those cell-derived mediators may provoke further cell activation through an autocrine/paracrine loop thereby increasing significantly the proteinase burden at the inflammatory focus. Thus, to ameliorate the inflammatory process in vivo this noxious circle should be interrupted by a convenient proteinase inhibitor therapy. To verify this hypothesis, high doses of AT III were applied in controlled randomized studies on surgical patients suffering from multiple trauma or severe sepsis. We could clearly demonstrate the sequential appearance of lysosomal proteinases (PMN-elastase, cathepsin B), proteinase/serpin-complexes (Eα_1PI, TAT), cytokines (IL-8, IL-6) and soluble intercellular adhesion molecules (ICAM, ELAM) in the circulation. These factors turned out to be a helpful tool for early diagnosis and prognosis of forthcoming organ dysfunctions. In AT III-treated patients a significantly reduced release/production of the inflammatory mediators became obvious concomitantly with an improved clinical course.

S 212 A SIMPLE *IN VIVO* MODEL OF COLLAGEN DEGRADATION USING COLLAGEN-GELLED COTTON BUDS - THE EFFECTS OF COLLAGENASE INHIBITORS AND OTHER PROTEINASE INHIBITORS

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A simple *in vivo* model of collagen degradation has been used to investigate the effects of proteinase inhibitors. [^{14}C]- or [^{3}H]-acetylated Type I collagen was gelled within cotton buds that were then subcutaneously implanted into rats to provoke a chronic inflammatory response. Over a two week period, the radiolabelled collagen was progressively removed as granulomatous tissue invaded the cotton bud. To guarantee their bio-availability, all the agents tested were continuously infused into the collagen-gelled cotton buds for 7 days, using subcutaneously implanted osmotic mini-pumps. Radiolabelled collagen degradation was inhibited using collagenase inhibitors containing thiol or hydroxamate moieties predicted to act as active-site zinc ligands, but inhibitors with phosphonic or phosphinic acid moieties were inactive in this particular model system, despite being potent *in vitro* inhibitors *versus* human collagenase. Inhibitors of other classes of proteinases were inactive. We believe this model to be a useful system for investigating *in vivo* collagen catabolism.

S 213 **PRODUCTION OF RECOMBINANT HUMAN PROCATHEPSIN B IN *E. coli* AS A FUSION PROTEIN TO GLUTATHIONE S-TRANSFERASE,**

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The proteolytic enzyme, cathepsin B, has been implicated in a variety of pathophysiological processes, such as inflammatory and autoimmune diseases, neurodegenerative diseases accompanying aging, muscular dystrophy, and the malignant progression of tumors. In non-pathological situations, this protease is generally found inside cells, i. e. within the endosomal/lysosomal compartment. It has a broad activity spectrum degrading most proteins in a pH range from 4.0 to 8.0. During the progression of many malignant tumors (melanoma, breast, gastrointestinal, and lung carcinomas) cathepsin B has been found to be secreted either as active enzyme or as proenzyme. It might thus, represent a potential new diagnostic or prognostic marker for these malignancies.

The quantitative assay of cathepsin B activity in complex biological samples is difficult due to the presence of endogenous inhibitors and activators of enzyme activity. Attempts were made in the past by several groups, including ours, to produce mouse monoclonal antibodies directed against native human cathepsin B or its proenzyme, but without success. In the light of our present knowledge, two main properties of the native proenzyme may be at the origin of this result: 1. The proenzyme undergoes rapid autolysis at physiological pH and thus, its half-live in mice might be too short to elicit a strong immunological response. 2. The protein is N-glycosylated and carries the lysosomal recognition marker, mannose 6-phosphate. It might thus be rapidly taken up by monocytic cells expressing high levels of mannose 6-phosphate receptors at their surfaces.

To circumvent these problems, we have produced recombinant human procathepsin B in bacteria as a fusion protein to glutathione S-transferase. For this, the nearly full length human procathepsin B cDNA, from phCB79-1, was subcloned into the expression vector pGEX-2T. Clones, one positive and one negative, were selected by colony hybridization, plasmid mini-prep analysis, SDS-PAGE analysis after IPTG-induction, and Western blotting using polyclonals to human liver cathepsin B. After induction by IPTG, the 60 K fusion protein (28 K glutathione S-transferase + 32 K procathepsin B) represents 11 % of total bacterial proteins. About 20 % only is soluble and can be isolated from total cell lysates in a single step by GSH-Sepharose 4B affinity chromatography. The engineered cleavage site for thrombin is shown to be accessible within the purified fusion protein, thereby allowing to separate the proenzyme from the carrier protein. N-terminal sequencing and activation of recombinant human procathepsin B will be presented.

S 214 ACTIVATION MECHANISM AND DOMAIN FUNCTIONS OF HUMAN NEUTROPHIL COLLAGENASE

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Human neutrophil procollagenase was activated by HOCl, stromelysin-1 and stromelysin-2. It was demonstrated that stromelysin-1 and -2 were able to generate active neutrophil collagenase displaying high specific collagenolytic activity thus demonstrating superactivation of the enzyme. This was due to the cleavage of Gly^{78}-Phe^{79} peptide bond at end of the propeptide domain of neutrophil collagenase. In contrast, oxidative and mercurial activation resulted in autoproteolytic generation of Met^{80} or Leu^{81} N-terminal collagenase. The specific collagenolytic activity of the mercurially or oxidatively activated enzyme was considerably lower when compared to stromelysin-1 or -2 activated collagenase.

The active neutrophil collagenase was not stable, and fragmentation into two major products was observed by cleavage of the Gly^{242}-Leu^{243} and Pro^{247}-Ile^{248} peptide bonds at the end of the catalytic domain. This cleavage was inhibited by TIMP-1 and -2 demonstrating autoproteolytic processing of the enzyme. Separation of the fragments by hydroxamic acid-Sepharose chromatography allowed investigation of domain functions of human neutrophil collagenase. The investigation of the substrate specificity of the catalytic domain and intact active enzyme revealed that the C-terminal domain is important for collagenolysis, since the catalytic domain failed to cleave the specific substrate type I collagen. In contrast, α_1-PI, C1-inhibitor, α_2-AP, α_1-ACT, gelatin and synthetic peptide substrates were hydrolysed by the catalytic domain and the intact collagenase, thus showing that the C-terminal domain has no influence on mediating general proteolysis. Human aggrecan was cleaved by human neutrophil collagenase and the catalytic domain at the Asn^{341}-Phe^{342} and Asp^{441}-Leu^{442} peptide bonds within the interglobular G1-G2 domain. This might be of physiological relevance during rheumatoid arthritis.

S 215 ENTRY OF HIV INTO CD4+ CELLS REQUIRES A T-CELL ACTIVATION ANTIGEN, CD26,

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The CD4 molecule is essential for binding HIV particles to target permissive cells but by itself, is not sufficient for efficient viral entry and infection. This and the potential requirement for the cleavage of the third hypervariable domain (the V3 loop) in the surface glycoprotein of HIV have suggested the involvement of a cell surface protease besides the CD4 molecule in the mechanism of HIV entry.

Here we provide evidence to indicate that this coreceptor which may interact and cleave the V3 loop is dipeptidyl peptidase IV (DPP IV) also referred to as CD26. DPP IV is an integral serine-protease that binds collagen and adenosine deaminase, and is characterized by a catalytic activity specific to proline residues, cleaving synthetic peptides with motifs GP, RP, KP, AP. In the V3 loop, the RP motif is conserved for HIV-1, HIV-2 and related simian isolates whereas the GP motif is more than 90 % conserved among HIV-1 isolates. Consequently, entry of HIV-1 LAI into T lymphoblastoid CEM, Jurkatt and MOLT4 cells, and into monocytoid U937 cells, is 80-90 % inhibited, either by a specific monoclonal antibody against DPP-IV or a specific peptide inhibitor of this protease. Other peptides containing the GP, RP or KP motif can also inhibit both viral entry and enzyme activity. Interestingly, such inhibitory agents also blocked infection of CEM cells by HIV-2 EHO which is completely unrelated to HIV-1 LAI, thus indicating that the requirement of DPP IV for viral entry is a general phenomenon for different isolates of HIV-1 and HIV-2. The observation that peptide inhibitors do not affect the binding of HIV surface glycoprotein to CD4 expressing cells, suggested that the inhibitory mechanism is a post-binding event. These observations provide the potential for the development of new and potent inhibitors of HIV infection.

S 216 INDUCTION OF CD10/NEUTRAL ENDOPEPTIDASE 24.11 IN TUMORIGENIC VARIANTS OF EARLY STAGE MELANOMA, Michelle Letarte, Adonna Greaves, Sonia Vera and Robert S. Kerbel, Division of Immunology and Cancer, Hospital for Sick Children and Division of Cancer Research, Sunnybrook Health Science Centre,Toronto, Canada, M5G 1X8
Co-injection into *nude* mice of early stage melanoma cell lines and Matrigel led to the generation of tumorigenic variants, characterized by the acquisition of a multicytokine-resistance phenotype (Kobayashi et al., Am. J. Pathol. in press). We analyzed by flow cytometry the expression of CD10 and of several adhesion molecules on two radial growth phase early melanoma lines, WM35 and WM1341B, and on the sublines derived from tumors excised after one, two and three passages *in vivo*. The WM35 cell line which does not express CD10 showed a gradual increase in this cell surface metallopeptidase as cells were serially passaged *in vivo*; the induced expression of CD10 is constitutive and stable in culture. The WM 1341B line already expresses high levels of this enzyme and no changes occurred with the development of more tumorigenic lines. Both lines undergo an increase in $\alpha3\beta1$ integrin with passages *in vivo* but no major changes in other integrins are observed. The rate of growth of the sublines in culture was higher than that of the parental lines. Morphologically, the tumorigenic variants have elongated processes and can form clusters. CD10 was distributed all over the surface of the WM35 tumorigenic variants in a punctated fashion. CD44, a hyaluronic receptor, was expressed at very high levels on these cell lines and could be seen in the long processes of the tumorigenic variants and in imprints left on the matrix by the migrating cells. The CD10+ variants of WM35 were not more adhesive to fibronectin, laminin, collagen type IV and to monolayers of endothelial cells. CD10, an enzyme which cleaves small peptides such as fMLP, might be required to regulate levels of peptides which stimulate haptotactic migration and/or invasion by the melanoma cells. The expression of CD10 would be required early on in these processes and WM35 might represent a cell at an earlier stage of differentiation than the WM1341B line, which has yet to acquire this enzymatic function, normally associated with an intermediate stage of differentiation of melanocytes. Future studies will address the role of CD10 and its inhibitors in haptotactic migration and/or invasion assays and will also analyse CD10 expression in other early melanoma lines and their variants to confirm the potential role of neutral endopeptidase 24.11 in melanoma progression.

S 217 HYPERACTIVITY OF NEUTROPHILS AND INCREASED LEVELS OF α1-ANTICHYMOTRYPSIN IN THE BLOOD OF PATIENTS WITH SENILE DEMENTIA OF THE ALZHEIMER'S TYPE, Federico Licastro, M. Cristina Morini, L. Jane Davis, Raffaele Parente, Cristina Melotti, and Domenico Cucinotta. Dept. of Exp. Pathol. and Dept. of Biochem., University of Bologna. Cli. Lab. and Dept. of Geriatrics, USL 27, Bologna, I-Italy.
Infections are often a complication observed in patients with SDAT and an increased morbidity has been reported during the terminal phase of the disease. However, data concerning the efficiency of granulocyte responsiveness in patients with SDAT are scanty and conflicting. The aim of the present study was to investigate functional efficiency of circulating neutrophils (PMN) from patients with SDAT and discriminate the effects of the disease and age on this immune response. The PMN activity from patients with SDAT was investigated by a chemiluminescence (CL) assay. PMN from SDAT patients showed a higher and faster CL emission than those of old and young healthy controls when activated *in vitro* by autologous or heterologous sera. A positive linear correlation was present between the score of mini mental state evaluation and the CL response of granulocytes activated by autologous serum in patients with SDAT. Sera from patients with SDAT depressed the CL emission of PMN from young donors. Serum levels of α1-antichymotrypsin (α1-ACT) were also determined by an ELISA assay and were found higher in demented subjects than in old and young controls. These data indicated that: 1) peripheral and systemic indexes of inflammation were present in the disease and were associated with mental deterioration; 2) this form of dementia could be considered a systemic disease affecting tissues other than the brain. Studies are in progress to clarify whether the "systemic acute phase response" might be a secondary event or intrinsically associated with SDAT.

S 218 **NEW ASSAY SUBSTRATES AND A KINETIC ANALYSIS OF HUMAN INTERLEUKIN-1β CONVERTING ENZYME,** David J. Livingston, Scott A. Raybuck and Michael D. Mullican, Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge MA, 02139-4211 USA.
Interleukin-1β converting enzyme (ICE) is an intracellular cysteine protease that is structurally distinct from previously reported cysteine proteases. ICE processes the 33 kD interleukin-1β precursor (pro IL-1β) to active 17 kD IL-1β, specifically cleaving the protein at several Asp-X sequences. We have developed new UV-visible and fluorescence assays for ICE, utilizing modified tetra- and octapeptide substrates, respectively. The synthesis of these substrates will be described. Proteolytic cleavage of the UV-visible Suc-Tyr-Val-Ala-Asp-p-nitroanilide susbstrate releases the pNA chromophore which absorbs at 400 nm. The substrate is hydrolyzed at 37°C with a k_{cat} of 25 min^{-1} and a K_m of 50 μM. The assay can be run in an automated format with 96-well microtiter plates and a kinetic UV/visible reader. We have used these assays for inhibitor screening and kinetic analysis. The pH dependence of k_{cat} and the inhibitor sensitivity of ICE distinguishes this enzyme from other cysteine proteases and suggest a unique catalytic site. Further experiments are in progress to characterize the reaction mechanism.

S 219 N-GLYCOSYLATION OF CYSTEINE PROTEINASE INHIBITORS: A POSSIBLE CONTROL MECHANISM FOR THE DETERMINATION OF MALIGNANT POTENTIAL, Rose A. Maciewicz*#, Jos W.J. van der Stappen, and Christos Paraskeva, Dept. of Pathology and Microbiology, and *Muscle and Collagen Research Group, University of Bristol, Bristol BS8 1TD U.K. (# present address Zeneca Pharmaceuticals, Alderley Park, Macclesfield, Cheshire SK10 4TG U.K.)
Structural alterations in N-linked glycosylation have been found to be associated with neoplastic transformation of human tumour cells and with advanced stages of human carcinomas. Expression of these N-linked oligosaccharides is required for efficient tumour metastasis as glycosylation mutants have decreased metastatic potential. Alteration in glycosylation may affect the cellular physiology in many ways. However, since invasion and metastasis are thought to be facilitated by the secretion of proteinases it is plausible that N-linked glycosylation events might somehow regulate matrix proteolysis and therefore determine the cell's malignant potential. Study of the role of extracellular cysteine proteinases in tumour progression in colorectal carcinogenesis, using media from human epithelial cell lines that represent an *in vitro* model of the adenoma to carcinoma sequence, indicated that the level of active cysteine proteinases positively correlated with the malignant potential of the tumour cells. However, an inverse correlation was observed for heat-stable cysteine proteinase inhibitors. Further characterisation of these inhibitors indicated that as the malignant potential of the tumour cell increased they acquired N-linked oligo-saccharides. As these inhibitors are not normally found to contain the N-linked glycosylation motif, Asn-X-(Ser/Thr), the observation suggests that an amino acid mutation must have occurred in these inhibitors during the process of malignant transformation. These results suggest that N-linked glycosylation of cysteine proteinase inhibitors decreases their inhibitory capacity toward cysteine proteinases. This, in turn, may increase the cells' ability to invade an extracellular matrix and be responsible for the increased tumourigenicity of these cells. Survey of other tumour cell lines indicated that this N-glycosylated inhibitor was only observed in carcinoma cell lines and thus may be a useful marker of the metastatic phenotype.

S 220 MOLECULAR BASES OF THE FUNCTIONAL PROPERTIES OF THE MOUSE DIPEPTIDYL PEPTIDASE IV (MOUSE CD26).

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The mouse dipeptidyl peptidase IV (DPP IV, CD26) is a serine protease abundantly expressed on the surface of a variety of cells. During the last years, the structural identity between DPP IV and CD26 has been established in human, mouse and rat (1). It is a surface differentiation marker involved in the transduction of mitogenic signals. The interaction of CD26 with cell matrix elements has also been described (2).

In an attempt to define the role of mouse CD26 in T cell development, we have first explored the structural bases of the enzymatic activity in relation to the other properties of the molecule. By site directed mutagenesis, we have clearly stablished the implication of Ser ^{624}, Asp ^{702}, and His^{734} as the critical residues of the catalytic site of DPP IV (3). This also comfirms the notion that DPP IV-related family displays a catalytic triad superimposable with that of the lipase/esterase family. Further characterization is based on the analysis of its genomic organization. The study of the promoter region of mouse CD26 is also being discuse. Moreover, we have developed a system to produce wild-type and catalytical deffective mutants of soluble DPP IV molecules in an aim to analyse DPP IV molecular interactions and to identify physiological substrates in the thymus microenvironment.

1) Hagen *et al.* J. Immunol. **144**, 2908; Vivier *et al.* J. Immunol. **147**, 4101; Marguet *et al.* J. Biol. Chem. **267**, 2200; Gorrel *et al.* Cell. Immunol. **134**, 205.
2) Hanski *et al.* Exp. Cell Res. **178**, 64; Piazza *et al.* Biochem. J. **262**, 327
3) David *et al.* J. Biol. Chem. **268**, 17247.

S 221 A NOVEL TRYPSIN-LIKE ENZYME IN HUMAN BREAST CANCER.

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The involvement of proteases in the establishment and progression of breast cancer has been extensively documented. We have conducted a clinical study examining trypsin-like proteolytic activities in breast tumour biopsy samples, and compared the levels to those found in benign breast conditions and normal breast tissue. The biopsy material (31 tumour samples, 16 benign and 22 normal) was snap frozen in liquid nitrogen and then homogenized and assayed using the fluorimetric substrates Z-Gly-Gly-Arg-MCA and Boc-Val-Pro-Arg-MCA. The latter was hydrolysed approximately 6-fold more effectively than was Z-Gly-Gly-Arg-MCA. The level of trypsin-like activity in the malignant tumour tissue was approximately 3-fold higher than in the benign tissue. Levels in the normal and malignant levels were comparable, although the normal tissue was taken from cancerous breasts and hence may have been under paracrine stimulation. A similar activity was also detected in several breast cancer cell lines. Use of a novel biotinylated affinity label, Bio-Arg-Gln-Arg-CMK (CMK=chloromethylketone), has allowed selective detection of this protease using SDS-PAGE followed by Western Blotting. An extensive inhibition study was carried out in order to characterize the activity, and we tentatively suggest that the enzyme may be related to the tryptase family of serine proteinases.

S 222 IMMUNOCOLOCALIZATION OF COMPLEMENT C1s AND MATRIX METALLOPROTEINASE 9 (92kDa GELATINASE/ TYPE IV COLLAGENASE)IN THE DEVELOPING CARTILAGE.

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The main physiological function of the first component of complement, C1s is to cleave C2 and C4 to form C3 convertase. Resently we have found that activated C1s degrades Types I, II and IV collagen which are major constituents of cartilage matrix (Yamaguchi et al., FEBS Lett, 268, 206, 1990). In order to understand the role of C1s in the development and resoption of the cartilage, the expression of C1s was examined in the ossification center where the matrix has to be removed and replaced by bone marrow. In the primary ossification center of the 9, 10 and 12-week-old human embryo femur, hypertrophic but not proliferating chondrocytes were stained by anti-C1s monoclonal antibody PG11. Endothelium and hematogenous elements in the capillary buds were also intensely stained by the antibody. The synthesis of C1s by chondrocytes was confirmed by in situ and Northern blot hybridization using the hamster tibia. The expression of C1s RNA and the secretion of C1s into the culture medium increased with differentiation of chondrocytes. We also found other novel function of C1s to activate zymogen of matrix metalloprotease 9 (MMP-9, 92kDa geratinase /typeIV collagenase). MMP-9 was immunocolocarized with C1s in hypertrophic chondrocytes, mesenchymal cells in primitive bone marrow and the cartilage matrix adjacent to the marrow. These observations suggest that C1s and MMP-9 coodinately participate in the matrix degradation in the cartilage.

S 223 DESIGN AND SYNTHESIS OF FLUORESCENT RESONANCE ENERGY TRANSFER SUBSTRATE FOR ASSAYING INTERLEUKIN 1ß CONVERTING ENZYME

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Interleukin 1ß converting enzyme (ICE) is responsible for processing an inactive 31 kD precursor to the active mature 17 kD Il-1ß with cleavage occurring between the Asp^{116}-Ala^{117} amide bond. A series of truncated peptide substrates which have been tailored around the precursor processing site have been conveniently used to assay ICE (1). We have prepared a peptide substrate which positions the protease cleavage site between two fluorophores located at the termini of the molecule. Upon cleavage of **DABCYL-Tyr-Val-Ala-Asp-Ala-Pro-Val-EDANS** (DABCYL-ICE-EDANS), an increase in fluorescence is observed at the EDANS emission wavelength of 490 nm permitting a continuous assay of ICE which is useful in the screening of inhibitory compounds. The K_m and k_{cat} results for hydrolysis of DABCYL-ICE-EDANS by ICE were 11.4±1.6 μM and 0.79±0.4 s^{-1}. The second order rate constant for hydrolysis of this substrate k_{cat}/K_m=7.0±1.3 x 10^4 $M^{-1}s^{-1}$ is comparable to that for the cleavage of the previously described fluorogenic substrate, Ac-Tyr-Val-Ala-Asp-AMC (6.4 x10^4 $M^{-1}s^{-1}$ (1).

1. Thornberry, N.A. et al. (1992) Nature **356**, 768-774.

S 224 REGULATION OF 92-kDa GELATINASE RELEASE IN HL-60 LEUKEMIA CELLS: TUMOR NECROSIS FACTOR-α AS AN AUTOCRINE STIMULUS FOR BASAL AND PHORBOL ESTER INDUCED SECRETION.
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Matrix metalloproteinase 9 (MMP-9), also known as 92-kDa type IV collagenase/gelatinase, is believed to play a critical role in tumor invasion and metastasis because of its ability to degrade type IV collagen, a major structural component of basement membranes. Here we report, that MMP-9 was constitutively released from the human promyelocytic cell line HL-60 as determined by zymographic analysis. Tumor necrosis factor-α (TNF-α) or the phorbol ester 12-O-tetra-decanoylphorbol-13-acetate (TPA) enhanced the enzyme release 3-4- or 4-6-fold, respectively. Neutralizing monoclonal antibodies to TNF-α (anti-TNF-α) decreased the basal MMP-9 release of these cells. In addition, these antibodies also significantly interfered with the TPA-induced enzyme release. Agents that inhibit TNF-α expression in HL-60 cells such as pentoxifylline and dexamethasone, completely abrogated both the constitutive and TPA-evoked MMP-9 release. Diethyldithiocarbamate (DDTC), which is known to stimulate TNF-α production in HL-60 cells exerted a positive effect on MMP-9 release in untreated cells but was inhibitory in TPA-induced HL-60 cells. The protein kinase C (PK-C) inhibitor staurosporine at low concentrations (100 ng/ml) caused a significant augmentation of MMP-9 release in untreated cultures, that was blocked by the addition of anti-TNF-α. High concentrations (2 μM) of staurosporine completely abolished the extracellular enzyme activity both in untreated and TPA-stimulated cells. These results suggest, that TNF-α is required for basal and PK-C-mediated MMP-9 release in HL-60 leukemia cells. Thus, MMP-9 secretion may be regulated by TNF-α not only in a paracrine but also in an autocrine fashion, possibly potentiating the matrix degradative capacity of immature leukemic cells in the processes of bone marrow egress and the evasion of these cells into peripheral tissue.
This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Pe 258-10/2) and from GSF (Fe 71971).

S 225 Endogenous Vascular Elastase (EVE): Characterization and Mechanism of Release from Vascular Smooth Muscle Cells, Marlene Rabinovitch, Division of Cardiovascular Research, The Hospital for Sick Children, 555 University Avenue, M5G 1X8, Toronto, Ontario, Canada
We have previously shown increased elastase activity in pulmonary arteries associated with the development and progression of experimental pulmonary hypertension in rats. Furthermore, inhibition of elastase activity in the vessel with a variety of serine proteinase inhibitors, such as alpha-1 antitrypsin, prevented the development and progression of pulmonary hypertension and associated vascular changes. Using degenerate oligonucleotides to conserved regions of other serine proteinases, such as human leukocyte elastase and porcine pancreatic elastase, together with cDNA reversed transcribed from polyA+ RNA from rat pulmonary artery, we isolated a cDNA which, when used to screen rat pulmonary artery cDNA expression library, isolated the sequence for rat adipsin. An antibody to recombinant adipsin was used to purify the endogenous vascular elastase (EVE) and the enzyme was further resolved on an elastase substrate gel as a 20kD species. N-terminal sequencing of the isolated enzyme is currently being carried out to determine whether it represents a truncated elastolytically active form of the serine proteinase, adipsin. We have further shown that EVE is released from vascular smooth muscle cells by serum and endothelial-conditioned medium, and this release is mediated by the elastin binding protein and requires tyrosine kinase activity.

S 226 INTERMOLECULAR, AUTOCATALYTIC PROCESSING OF THE PROENZYME OF IL-1β CONVERTING ENZYME (ICE) IN BACULOVIRUS-INFECTED CELLS, A. M. Rolando*, O. C. Palyha¶, G. J.-F. Ding¶, A. D. Howard¶, and S. M. Molineaux*, Departments of Molecular Immunology* and Biochemical and Molecular Pathology¶, Merck Research Laboratories, Rahway, New Jersey 07065.
Interleukin-1β is a proinflammatory cytokine that is processed by IL-1β converting enzyme (ICE) prior to release from activated monocytes. ICE is a heterodimeric cysteine protease with a requirement for an Asp in the P_1 position of its cleavage site. The mature subunits of ICE (p22/p20:p10) are derived from a 45 kDa precursor consisting of a 119 aa prodomain, a p22/p20 subunit, a 19 aa linker, and a p10 subunit by proteolytic processing at four Asp-X sites. While the majority of ICE in intact monocytes is in the p45 form, when expressed in a Baculovirus system, p45 undergoes efficient intracellular processing to mature ICE. Analysis of a series of point mutations shows that an intermediate, p35, is converted to active p20:p10 by a series of autocatalytic cleavages. A mutation that leaves the linker connected to the p20 subunit or p10 subunit greatly reduces IL-1β cleavage activity. A proenzyme mutant lacking all four autoprocessing sites can cleave an active site mutant to p20:p10 upon co-infection or when lysates from each mutant are mixed *in vitro*. A high concentration of the biotinylated aculoxymethylketone inhibitor (10 μM), 1000-fold higher than that required to affinity label p20:p10, labels the proenzyme and inhibits processing *in vitro*. This suggests that intermolecular, autocatalytic processing of p45 is required to generate active ICE in intact cells.

S 227 TRYPTASE IS A POTENT GROWTH FACTOR FOR HUMAN KERATINOCYTES, Christian P. Sommerhoff, Beate Feser, and Hans Fritz, Abt. Klin. Chemie and Klin. Biochemie, LMU München, Nußbaumstr. 20, D-80336 Munich, Germany

Psoriasis vulgaris, a common skin disease of unknown etiology, is characterized by an increased proliferation and a decreased differentiation of keratinocytes. Already in the evolving psoriatic lesion the number of mast cells and their degranulation are found to be increased. Tryptase, a major proteinase of mast cell secretory granules, has been demonstrated extracellularly in psoriatic lesions by immunofluorescence and enzyme histochemistry.

To determine whether tryptase may influence the proliferation of keratinocytes we studied the effect of isolated human tryptase on human keratinocytes in culture. Tryptase markedly stimulated the proliferation of keratinocytes in a concentration-dependent fashion with a threshold concentration of $<10^{-11}$ M. A saturation of the response was not observed in the range of concentrations used (10^{-12} - 3×10^{-8} M); tryptase at a concentration of 3×10^{-8} M increased the cell number by 85 ± 7% (mean ± SEM; n = 6) above control (supplemented MCDB153, i.e. a medium optimized for the growth of keratinocytes). Incubation of cells with other proteinases (chymotrypsin, trypsin, thrombin, elastase; 10^{-8} M each) had no effect. Only catalytically active, tetrameric tryptase is a growth factor for keratinocytes: no effect was observed in the presence of inhibitors of tryptase or in the absence of heparin which is required for the stability of the tryptase-tetramer.

These results demonstrate that tryptase is a potent growth factor for human keratinocytes; they suggest that tryptase may play an important role in the pathogenesis of psoriasis.

Supported by project G-11 of the SFB 207 of the University of Munich.

S 228 SYNTHESIS AND CHARACTERISTIC LOCALIZATIONS OF HUMAN NEUTROPHIL PROTEINASE 3, A SERINE PROTEASE INVOLVED IN THE DEVELOPMENT OF AN AUTOIMMUNE DISEASE. Christof H. Szymkowiak, Elena H. Csernok, Jörn Kekow and Wolfgang L. Gross. Dept. of Rheumatology, Medical University of Lübeck, 23538 and Rheumaklinik Bad Bramstedt, 24572 Bad Bramstedt, Germany.

Human neutrophil proteinase 3 (PR3) is a proteolytic enzyme secreted by polymorphonuclear leukocytes (PMN) in response to a variety of inflammatory stimuli. PR3 has been recently described as a multifunctional protein and specially as target antigen of circulating antineutrophil cytoplasmic antibodies (ANCA). The aim of our studies was to investigate the effect of proinflammatory cytokines (TNFα, TGFβ, IL-8) on the intracellular localization, translocation and release of PR3 in PMN by flow cytometry (FACS), electron microscopy (EM), and ELISA. In order to determine if PR3 is newly produced upon stimulation with cytokines or if it is distributed from PMN storage particles, the synthesis of PR3 mRNA was measured by nuclease S1 mapping experiments.

We could demonstrate that PR3 is located in the azurophil granules of human resting PMNs. In vitro, the treatment of PMN with both TNFα and IL-8 leads to a time dependent translocation of PR3 from the intracellular loci to the cell surface. TGFβ alone also induces translocation of PR3 to the plasma membrane. The extracellular release of PR3 in response to TNFα and IL-8 reaches a maximum from 38.5% of the total amount within 20 minutes. In addition, we could detect PR3 on the plasma membrane of PMN from patients with ANCA-associated diseases (e.g. Wegener's granulomatosis) and sepsis. Preliminary data show that PR3 mRNA is newly synthesized upon activation. Our results provide strong evidence of the involvement of PR3/ANCA in the development and progression of an autoimmune disease.

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S 229 IN VITRO FOLDING AND AUTOPROCESSING OF ACTIVE INTERLEUKIN-1β CONVERTING ENZYME FROM AN INACTIVE RECOMBINANT PRECURSOR
John A. Thomson, Jo-Anne F. Black, Stephen P. Chambers, Joyce T. Coll, Mark A. Fleming, Ted Fox and John R. Fulghum. Vertex Pharmaceuticals Incorporated, 40 Allston Street, Cambridge, MA 02139-4211 U.S.A.

Mature interleukin-1β is a potent modulator of immune and inflammatory responses, and is generated by the proteolytic cleavage of an inactive precursor. This proteolytic activation appears to be catalyzed *in vivo* by a specific and novel cysteine protease, recently named IL-1β converting enzyme (ICE). Small amounts of active ICE have been isolated from cultured human monocytic cells. The active enzyme is a heterodimer whose subunits (M_r ~ 20,000 & 10,000) are probably excised from a larger inactive precursor via a multi-step autoproteolytic process. We report here, the production of active human ICE from the *in vitro* autoprocessing of an inactive precursor, over-expressed in *E. coli*. We also show that the enzyme is extremely vulnerable to autolytic degradation and destruction.

The cDNA encoding the 30 kDa polyprotein precursor component of the human ICE gene was overexpressed in *E.coli*, using an inducible promoter system. The expression product accumulates in high abundance as insoluble aggregates, which are solubilized in urea buffer and subjected to size-exclusion chromatography. The purified 30 kDa precursor is then refolded by the removal of urea, concentrated, and then autoproteolytically processed, to yield a mixture of mature, active enzyme and inactive degradation products (approx. ratio 1:4). This material can be used directly for the enzymatic screening of potential inhibitors, giving high activities both in pre-IL-1β cleavage assays, and in spectrophotometric/spectrofluorometric assays using smaller peptidyl substrates. Alternatively, it can be complexed with an appropriate inhibitor and prepared for high resolution structural analysis.

S 230 AN EXTRACELLULAR PROTEASOME (EP) FROM C6 ASTROCYTOMA CELLS WITH ASPARTYL, SERINE AND METALLOPROTEASE ACTIVITY, **Indrasen S. Vaithilingam, Warren McDonald, Rolando F. Del Maestro, Brain Research Laboratories, Experimental Research Unit, Department of Clinical Neurological Sciences, Division of Neurosurgery, University of Western Ontario, Victoria Hospital, 375 South Street, London, Ontario, Canada N6A 4G5.**

An extracellular proteasome (EP) has been isolated from serum free media conditioned by C6 astrocytoma cells. C6 cells represent a model for studying the modulation of angiogenesis and the interstitial microenvironment by glial tumor proteases. EP has a native molecular weight of 1000-kDa and is composed of two subunits, 70-kDa and 65-kDa. The extracellular proteasome degraded collagen I, collagen IV, α-casein and β-insulin. Proteolysis of each of these substrates was inhibited by a different class of protease inhibitors. Collagenase I activity was inhibited by the aspartic protease inhibitor pepstatin, collagenase IV activity was inhibited by the serine protease inhibitor 3,4 dichloro-isocoumarin, and digestion of α-casein and β-insulin was inhibited by the metalloprotease inhibitors TIMP-1, TIMP-2 or EDTA. Degradation of interstitial collagen I may confer invasive properties to the glial tumor. Collagen IV composes a minimum of 60% of the basement membrane. Collagenase IV activity indicates a role for the extracellular proteasome in the initiation of angiogenesis. Breakdown of the additional protein substrates, α-casein and β-insulin suggests the further potential for the extracellular proteasome in degrading a significant portion of the proteins either in the interstitial space and/or within the basement membrane. The release by a tumor cell of a proteasome with multiple activities, each under specific regulation represents an efficient mechanism for tumor progression. Different proteolytic activities suggests that multiple inhibitor therapy may be required for complete inhibition of tumor growth.

S 231 SERINE AND METALLOPROTEINASES IN HUMAN SKIN: RELATION TO LOSS OF EPIDERMAL COHESION IN RETINOIC ACID-TREATED ORGAN-CULTURED SKIN. James Varani, Bill Burmeister, Robert G. Sitrin, Susan B. Shollenberger, Douglas F. Gibbs and Kent Johnson. Departments of Pathology and Internal Medicine, University of Michigan, Ann Arbor, MI 48109.

Neonatal human foreskin obtained at circumcision was cut into 2x2 mm pieces and placed in organ culture. Culture medium consisted of a serum-free, growth factor-free basal medium containing either 0.15 mM Ca2+ or 1.4 mM Ca2+. Some cultures were left as control while others were treated with 3 μM all-trans retinoic acid (RA). In the presence of RA, epidermal cohesion was disrupted and the upper layers separated from the viable epidermis beneath. This effect was most pronounced under low-Ca2+ conditions. Epidermal separation was partially inhibited in the presence of either soybean trypsin inhibitor or aprotinin. Metalloproteinase activity was detected using a synthetic substrate and by gelatin zymography. Zymography indicated major bands of activity at 92 kD and 72 kD and both were eliminated in the presence of EDTA. Metalloproteinase activity was decreased slightly in the presence of RA. No significant activity was detected when synthetic peptide substrates that measure elastase and cathepsin G activity were used. However, a synthetic substrate assay for plasminogen activator detected measurable amounts of activity in all groups but total activity was significantly higher in culture fluids from RA-treated tissues. Enyzme-linked immunosorbant assays revealed that the total measured activity was due to presence of both urokinase-type plasminogen activator and tissue-type plasminogen activator. Taken together, these data suggest that a variety of proteolytic enzymes are produced during organ culture of human skin and that these proteases may influence the structural integrity of the tissue.

S 232 SYNTHESIS AND CHARACTERIZATION OF HUMAN NEUTROPHIL ELASTASE INHIBITORS DERIVED FROM AROMATIC DIESTERS OF PHENYLALKANOIC ACIDS, **M. Wieczorek[1], L. W. Spruce[1], J. Oleksyszyn[1], G. P. Kirschenheuter[1], T. M. Kloppel[1], S. E. Mangold[1], S. E. Ross[1], M. K. Newman[1], J. C. Cheronis[1], Q. L. Ying[2] and S. R. Simon[2]. [1]Cortech Inc., Denver, CO 80221, [2]Pathology Dept., SUNY, Stony Brook, NY 11794.**

A series of 4-(alkylsulfonyl)phenyl diesters of 2,2'-phenylenedibutyric and 2,2'-phenylenediisobutyric acids was synthesized and evaluated as potential inhibitors of human leukocyte elastase (HLE). The sulfonyl compounds inhibited HLE with IC_{50} values (corrected for substrate competition) in the range of 0.2 - 10 nM. These inhibitors seem to be alternative substrates which combine with HLE at rates on the order of 10^5 - 10^6 M^{-1} s^{-1} to form covalent (acyl-enzyme) intermediates. The intermediates decomposed at rates on the order of 10^{-3} s^{-1} to regenerate active enzyme and hydrolyzed inhibitor. The 2,2'-phenylenediisobutyrate derivatives were somewhat weaker but more selective inhibitors of HLE than the 2,2'-phenylenedibutyrate derivatives. One diester, selected for clinical trials, has not shown acute toxicity in the rat and dog. Low molecular weight, diester inhibitors of HLE may become useful drugs in the treatment of adult respiratory distress syndrome, cystic fibrosis, and/or myocardial ischemia-reperfusion injury.

S 233 TUMOR INVASIVE BEHAVIOR AND EXPRESSION OF PLASMINOGEN ACTIVATOR AND METALLOPROTEASE ACTIVITIES IN HUMAN PROSTATE TUMOR CELL LINES GROWN IN NUDE MICE, Michael J. Wilson, VA Medical Center and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55417

Changes in tissue organization that occur in tumor invasion and metastasis are generally associated with increased expression of plasminogen activators (PA) and metalloproteases (MP). We examined PA and MP activities in human prostate tumor cell lines of low (Du-145), modest (PC-3), and high (1-LN-PC-3-1A) metastatic behavior grown subcutaneously in nude mice. The 1-LN-PC-3-1A line (1-LN) is a variant derived from a lymph node metastasis of PC-3 grown in nude mice (Ware et al., J. Urol. 128:1064, 1982). The PA activities of the Du-145, PC-3, and 1-LN cell lines respectively were 0.5 ±0.1 (±S.D.), 0.9 ±0.3, and 2.6 ±0.1 mU/mg protein in extracts of cells from culture and 1.7 ±1.3 , 6.2 ±2.8, and 11.5 ±4.2 mU/mg protein in extracts of tumors grown in nude mice. Urokinse was the molecular form of PA expressed in tumors of all cell lines based on molecular weight in zymograms (predominantly 54 kDa with a minor activity of 33 kDa) and a strong sensitivity to amiloride inhibition. Prominent MP activities of about 68, 76, and 96 kDa and lesser activities of 56, 59, 63, 84, 155, 165, and 180 kDa were found in 1-LN tumors using zymography. In contrast, only MP activities of 59, 68, and 96 kDa were detected in PC-3 tumors and much less active 59 and 96 kDa forms in the Du-145 tumors. The lack of MP activities in Du-145 tumors did not appear to be due to inhibitor activities since MP activities of 1-LN tumors were not affected when mixed with Du-145 extracts. No metastases were detected from the subcutaenously injected tumor cells, but 1-LN tumors were invasive into the underlying muscle. It has not been established whether the source(s) of the induced MP activities in 1-LN tumors are tumor cells or stromal cells. If it is in the stroma (mouse origin), then a potent factor(s) must be produced by 1-LN cells to induce the formation of these MP activities since such an induction was absent in Du-145 tumors. On the other hand, pronouced expression of certain MP activities by 1-LN cells may be an indicator of a further step in prostate tumor progression since all tumors expressed PA but only the more invasive tumors expressed substantial MP activities. (Supported by the General Research Funds Dept. Veterans Affairs)

S 234 INTERFERON-γ INDUCIBLE PROTEASOME SUBUNITS REPLACE CONSTITUTIVELY EXPRESSED SUBUNITS

Young Yang , Klaus Früh, and Per A. Peterson

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The proteasome is thought to be the cytoplasmic protease responsible for the generation of peptides presented by MHC class I molecules. Two subunits, LMP2 and LMP7, of the proteasome are encoded in the MHC class II region of the genome and the expression of LMP2 and LMP7 can strongly be induced by IFN-γ. We have observed previously that this induction not only leads to the enhanced presence of LMP2 and LMP7 in the proteasomal complex, but also to the disappearance of certain other subunits. We have now further characterized the events taking place during the incorporation of LMP2 and LMP7 into the proteasome. Our data indicate that both LMP2 and LMP7 replace a disinct other subunit. The respective subunits are constitutively expressed and highly homologous to the MHC-encoded ones. The results will be discussed with regard to proteasome function and antigen processing.

S 235 A RECOMBINANT HUMAN STROMELYSIN CATALYTIC DOMAIN IDENTIFIES TRYPTOPHAN DERIVATIVES AS HUMAN STROMELYSIN INHIBITORS, Qi-Zhuang Ye, Linda L. Johnson, Ian Nordan, Donald Hupe, and Lynn Hupe, Departments of Biochemistry and Biotechnology, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105

The human stromelysin catalytic domain (SCD) has been expressed in *E. coli* and purified to homogeneity. We have used this recombinant SCD for inhibitor screening and identified tryptophan derivatives as competitive inhibitors of SCD. Both Cbz-L-Trp-OH (IC_{50} 2.5 µM, K_i 2.1 µM) and Boc-L-Trp-OH (IC_{50} 10 µM, K_i 8 µM) showed good inhibitory activity. Modification at the indole nitrogen with formyl group (IC_{50} 34 µM, K_i 28 µM) or mesitylene-2-sulfonyl group (IC_{50} 63 µM, K_i 52 µM) showed reduced activity. The amide Cbz-L-Trp-NH_2 was not active, but esters Cbz-L-Trp-OSu (IC_{50} 13 µM, K_i 11 µM) and Boc-L-Trp-OSu (IC_{50} 102 µM, K_i 84 µM) showed activity. Aromatic amino acid derivatives Cbz-L-Tyr-OH (IC_{50} 24 µM, K_i 20 µM) and Cbz-L-Phe-OH (IC_{50} 40 µM, K_i 33 µM) were also active, but other amino acid derivatives had no activity. Although Cbz-D-Trp-OH (IC_{50} 86 µM, K_i 71 µM) was active, the L-configuration is consistently preferred for inhibitory activity. Some of the SCD inhibitors were tested on full length human stromelysin purified from cultured human cells, and they showed the same potency rank order. These results demonstrate the usefulness of recombinant DNA technology in generating the authentic human protein with improved properties for drug discovery.

Posters Relevant to Oral Sessions 5, 6, 8, 9 and 11

S 300 ENGINEERING TISSUE-TYPE PLASMINOGEN ACTIVATOR TO BE RESISTANT TO PLASMA PROTEASE INHIBITORS, Murtaza Alibhai, Alice M. Chow, Hung V. Nguyen, Bruce A. Keyt, William F. Bennett, and Nicholas F. Paoni, Cardiovascular Research, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080

Human tissue-type plasminogen activator is inactivated in human plasma by various fast acting and slow acting protease inhibitors. Studies with wild type t-PA indicate that at pharmacological concentrations (1 to 10 µg/mL), approximately 20% of the initial activity of t-PA remains after 4 hours of incubation in human plasma. While the inhibition by slow acting proteases in human plasma is not a problem in administering wild type t-PA, which has a short half life and is administered as an infusion, it can reduce the effectiveness of long half life variants, which are designed to be active for extended periods following bolus administration. We have identified a mutation in the t-PA protease domain, A473S, which imparts significant resistance to inactivation in human plasma. This resistance of the A473S variant to plasma proteases is not mediated through resistance to plasminogen activator inhibitor -1 (PAI-1) as the levels of t-PA used in these studies were chosen to mimic pharmacological levels, and far exceed the concentration of PAI-1 in plasma. Furthermore, variants which are resistant to inactivation by PAI-1 are rapidly inactivated in this assay system. Site-directed mutagenesis has been utilized to study the structural requirements and mechanism of action of A473S, and to determine if this mutation is compatible with mutations that are designed to reduce the clearance rate of t-PA.

S 301 THROMBIN ENGINEERING: PRODUCTION OF SINGLE POINT MUTANTS OF RECOMBINANT HUMAN PRETHROMBIN-2, Rosaria Arcone, M. Gabriella Pagliuca, Alberto Chinali, and Concetta Pietropaolo, Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli "Federico II", Via S. Pansini 5, I-80131-Napoli, Italy.

Thrombin (EC 3.4.21.5) is a serine protease involved in hemostasis control as well as in thrombosis (1). Besides the enzymatic properties, thrombin is also a chemiotactic factor for macrophages and monocytes, and a fibroblast growth factor. Furthermore, thrombin is involved in neuronal differentiation (2). Structural and functional studies of thrombin and its mutants might clarify the molecular mechanism governing such a broad range of biological activities. Prethrombin-2, a natural precursor from which the activated thrombin can be obtained, has been produced in our laboratory (3), using stable transfectant CHO-DXB11 clones. A human prethrombin-2 cDNA has been engineered to obtain a secreted protein (4). Recombinant prethrombin-2 shows, as expected, a relative mobility of 37 kD and its catalytic activity (amidolytic and coagulant) is comparable to that of the purified human thrombin. Prethrombin-2 mutants have been obtained by site directed mutagenesis. Single amino acid substitutions have been introduced: i) in the catalytic site (His43, Asp99, Ser205); ii) disrupting a saline interaction site (Gly203, Asp204); and iii) in the anion recognition exosite I (Arg73). Most mutants are efficiently produced by transient transfection both in HepG2 and in HeLa cells. The structural and functional characterization of the expressed mutant proteins is in progress.

1) Fenton J. W. (1988) Semin. Thromb. Hemostasis 14, 234-240.
2) Cunningham , D. D. , *et al.* (1986) Annals N. Y. Acad. Sci. 485, 240-248
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4) Russo, G. *et al.* manuscript in preparation.

S 302 PRODUCTION AND CHARACTERISATION OF DELETION AND SUBSTITUTION MUTANTS OF CYSTATINS AS WELL AS OF CYSTATIN DIMERS, Ennes A. Auerswald, Dorit K. Nägler, Meta Bernd, Werner Machleidt*, Richard A. Engh# and Hans Fritz, Abtl. Klin. Chemie und Klin. Biochemie, LMU München, Nußbaumstr. 20, D 80336 München, * Inst. Physiolog. Chemie, Physikal. Biochemie und Zellbiologie, LMU München and # Max-Planck-Institut für Biochemie, D 82152 Martinsried, Germany

About 30 mutants of chicken cystatin, carrying deletions or substitutions, as well as dimeric inhibitors, were constructed, expressed in *E.coli*, purified and characterized by SDS/PAGE, IEF, RP-HPLC, partial amino acid sequencing and inhibiton assays. Some selected variants were described further by binding assays, NMR, CD, fluorescence and mass spectroscopy. Furthermore intuitive structural model building was used to explain protein-protein interactions between some variants and the cysteine proteinases papain, actinidin and cathepsin B.

Among the deletion variants we identified molecules with different functional and structural behaviour, one group of molecules could not be expressed in an active form, a second group of variants bound cysteine proteinases weaker than wildtype (greater K_i values), a third group of variants showed similar inhibition behaviour as wildtype but the variants analysed, melted at lower GdmHCl concentrations and a fourth group of variants showed temporary inhibition followed by cleavage during interaction with papain. A model for this interaction will be presented.

Functional active double headed inhibitors as cystatin-cystatin dimers or cystatin-kunin dimers were expressed in different amounts. The analysis of their inhibition behaviour is under progress and latest results will be presented.

The inhibition behaviour of the "hairpin loop 1" substitution variants indicated differences in the interaction with structural related cysteine proteinases, but this effects can not be explained on the basis of the currently available structural information. Data on substitution variants harbouring amino acid regions of the kininogen domain 2 binding aerea will be discussed.

S 303 PIGMENT EPITHELIUM-DERIVED FACTOR: A SERPIN WITH NEUROTROPHIC ACTIVITY, S. Patricia Becerra, Sally S. Twining* and Gerald J. Chader, Laboratory of Retinal Cell and Molecular Biology, NEI, NIH, Bethesda, MD 20892 and *Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI 53226

Pigment epithelium derived-factor (PEDF), secreted by human fetal retinal pigment epithelial cells, promotes neurite outgrowth in human retinoblastoma cells. The amino acid sequence derived from a fetal human PEDF cDNA shares identity of its primary structure (~30%) with the serine protease inhibitor (serpin) family, preserving 90% of the residues essential for the structural integrity of serpins. However, recombinant PEDF does not inhibit the serine proteases trypsin, chymotrypsin, elastase or cathepsin G. A natural target for PEDF has not yet been identified. We have analyzed proteins from the interphotoreceptor matrix (IPM), the space between the retinal pigment epithelium and the retina, by immunodetection on Western blots with antibodies raised against rPEDF and by zymography in gels containing casein as a proteolytic substrate. Our results show that bovine IPM contains a stable, glycosylated PEDF polypeptide (50,000 Mr) at about 2µg per eye. Limited proteolysis of bovine PEDF produced a polypeptide of 46,000 Mr with trypsin, subtilisin, chymotrypsin and elastase, suggesting a globular structure with a hinge region susceptible to proteolytic cleavage. On the other hand, casein SDS-PAGE zymography revealed low protease activity in the IPM which migrated as a doublet of about 80,000 ± 5,000 Mr. The caseinolytic activities were inhibited 100% with 1µg/ml aprotinin, 10mM PMSF and 15µg/ml α_1-antitrypsin added to the gel mixture, but were not affected by E64 or EDTA. Importantly, IPM proteins did not react with antibody against plasminogen, a serine protease of about 80,000 Mr. When rPEDF protein was added at 1µg/ml, the signal for these caseinolytic activities, as well as another serine protease activity of unknown origin, diminished by about 50%. Our results suggest the IPM as a natural extracellular site for novel serine proteases and the serpin PEDF, both present at ≤1% of the total protein. Analysis of these activities and interactions with PEDF will be presented.

S 304 METALLOPROTEINASES FROM SNAKE VENOMS (REPROLYSINS), STRUCTURE, FUNCTION, AND RELATIONSHIP TO MAMMALIAN REPRODUCTIVE PROTEINS, Jón Bragi Bjarnason and Jay William Fox, Science Institute, University of Iceland, Dunhagi 3, 107 Reykjavík, Iceland, and Department of Microbiology, University of Virginia Medical School, Charlottesville, 22908 Virginia

Over the past 15 years of investigation it has become evident that the primary factors responsible for snake venom induced hemorrhage are metalloproteinases present in the venoms of these snakes. The biochemical basis for their activity appears to be the proteolytic destruction of basement membrane and extracellular matrix surrounding capillaries and small vessels. The protelytic activities of four of the *Crotalus atrox* toxins have been invetigated using isolated extracellular matrix proteins. The proteinase toxins were shown to hydrolyse fibronectin, laminin, collagen IV and nidogen (entactin). Sequence analysis of the digestion products reveal that the alpha-1(IV) chains of collagen IV are cleaved in a triplet interruption region of the triple helix at position Ala(219)-Gln(220) while the alpha-2(IV) chain is cleaved in the same region at the Thr(228)-Leu(229) peptide bond. Structural studies have shown that these proteinases are synthesized as zymogens and are processed at both the amino and carboxy termini to give the mature protein. The variety of hemorrhagic toxins found in snake venoms is due to structurally related proteins comprised of various domains. The type of domains found in each toxin plays an important role in the hemorrhagic potency of the protein. The cDNAs encoding the toxins demonstrate that there are at least three different classes of mRNA precursors. Thus the large and potent hemorrhagic toxin HT-a (atrolysin A, EC 3.4.24.1) has a signal sequence, a zymogen domain, a proteinase domain, a small spacer, a disintegrin-like domain and a final cysteine-rich domain. Hemorrhagic toxin e (atrolysin E, EC 3.4.24 44) represents the medium sized class, which lacks the final cysteine-rich domain but possesses all the other elements. The low molecular weight class, whose members include HT-b, c and d (atrolysins B and C, EC 3.4.24. 41 and 3.4.24. 42), lack both the final cysteine-rich domain and the disintsgrin-like domain. Recently, structural homologs to the venom hemorrhagic proteinases have been identified in several mammalian reproductive systems.

S 305 LOCALIZATION OF AN EPITOPE IN ANTITHROMBIN EXPOSED ON INSERTION OF THE REACTIVE-BOND-LOOP INTO THE A β-SHEET DURING BINDING OF TARGET PROTEINASES. Ingemar Björk and Kerstin Nordling, Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Considerable evidence indicates that the reactive bond of antithrombin and other inhibitory serpins is located in an exposed loop analogous to that in the non-inhibitory serpin, ovalbumin. Cleavage of the reactive bond of antithrombin, or partial denaturation of the inhibitor, results in insertion of the N-terminal segment of this loop into the main β-sheet of the protein, the A sheet. A synthetic peptide with the sequence of this segment can be similarly inserted into the β-sheet of intact antithrombin. Loop insertion by any of these means results in exposure of antigenic determinants not present in native antithrombin. The same determinants are exposed on complex formation with thrombin or Factor Xa, indicating that the conformational change leading to trapping of target proteinases also involves insertion of the reactive-bond loop into the β-sheet. CNBr degradation of bovine antithrombin, followed by SDS electrophoresis and Western blotting, allowed identification of a peptide with an apparent molecular mass of ~6000 kDa that reacted strongly with a polyclonal antiserum specific for the epitopes exposed on loop insertion. The N-terminal sequence of this peptide was Gly-Leu-Glu-Asp-Leu-Phe-. Degradation of reactive-bond-cleaved bovine antithrombin gave a similarly reacting peptide with ~2500 kDa lower apparent molecular mass. These results indicate that an epitope exposed on insertion of the reactive-bond-loop into the A β-sheet of bovine antithrombin is located within a 55-residue stretch of the protein from Gly-340 to Arg-394 of the reactive bond.

S 306 **CRYSTALLOGRAPHIC ANALYSIS OF MOUSE PRO-RENIN CONVERTING ENZYME**

Michael Blaber, Institute of Molecular Biology, University of Oregon, Eugene, OR 97403

Mouse pro-renin converting enzyme (PRECE) is a member of the glandular kallikrein gene family. This family of proteases is involved in the processing of a variety of important bioactive peptides from larger inactive precursor molecules. PRECE cleaves inactive mouse prorenin (*Ren-2*) at a dibasic sequence to yield mature active renin. Renin, an aspartyl protease, regulates the formation of angiotensin I from angiotensinogen. Angiotensin I is a potent vasoactive peptide and plays a role in the regulation of blood pressure.

A crystallographic study was initiated in order to better understand the structural basis of the substrate specificity of PRECE. Purified protein was subjected to a 'fast screen' analysis to determine possible crystallization conditions. Hexagonal crystals (space group $P6_4$) were obtained from solutions containing polyethylene glycol as a precipitant. Although the crystals were small (0.2 x 0.05 x 0.05 mm), it was possible to collect a 3Å data set. The method of molecular replacement, using the related rat tonin structure (~62% amino acid identity), was successfully utilized to determine the location of the molecule in the unit cell and to provide a starting model for refinement. At the present time the structure has been refined to an R factor of 26% and acceptable geometry using data to 3Å. The current status of the PRECE model will be presented and structural features which might influence specificity will be discussed.

S 307 SURFACE ACTIVE VARIANTS OF SUBTILISIN BPN': INTERFACIAL HYDROLYSIS, Philip F. Brode III, Christopher R. Erwin, Deborah S. Rauch, Ellen S. Wang, James M. Armpriester, Bobby L. Barnett, Mark D. Bauer, Phillip R. Green, Deborah A. Thaman, and Donn N. Rubingh, The Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, OH 45239-8707

Site-directed as well as random mutagenesis were used to produce variants of subtilisin BPN' (*Bacillus amyloliquefaciens*) that were evaluated for their surface adsorption properties. The level of adsorption on a model substrate consisting of a peptide covalently bound to a surface, and the rate of hydrolysis of this surface-bound peptide were measured for these variants. While most variants adsorb at a level very similar to native BPN', several variants were identified which adsorb either more or less.

The observation that the catalyzed rate of hydrolysis of *soluble* substrates increases linearly with enzyme concentration is a fundamental principle incorporated into Michaelis-Menten kinetics. Likewise, we have shown that for *insoluble* substrates a similar dependence is seen between substrate hydrolysis and the concentration of adsorbed enzyme. Based on this knowledge we hypothesized that by designing variants which adsorb at a higher level on an insoluble peptide substrate, we could achieve enzymes that hydrolyze the insoluble substrate faster.

Contrary to the hypothesis, the variants that adsorb more on the surface with covalently bound peptide, hydrolyze this substrate slower. In addition, variants of BPN' which adsorb at a lower level than native BPN', hydrolyze the surface-bound substrate faster. Enzyme adsorption and the subsequent peptide hydrolysis has been shown to be affected by substituting amino acids that alter the surface charge or hydrophobicity of the native enzyme. This effect is most dramatic when the changes are made at surface exposed sites around the binding-pocket/active-site of the enzyme. One mechanism which is consistent with the data is based on the link between the level of adsorption and the enzyme's affinity for the surface. This mechanism will be presented along with the adsorption/hydrolysis data on a series of BPN' variants.

S 308 ENGINEERING THE S_2 SUBSITE SPECIFICITY OF HUMAN CATHEPSIN S TO A CATHEPSIN L- AND CATHEPSIN B -LIKE SPECIFICITY,
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The primary specificity of papain-like proteinases is determined by S_2-P_2 site interactions. By using site-directed mutagenesis, the substrate specificity of the S_2 binding site of human cathepsin S has been altered to a cathepsin L- and cathepsin B-like specificity. The most crucial residue determining a cathepsin L-like S_2 subsite specificity is residue 133. The replacement of Gly133 in cathepsin S by a cathepsin L-analog alanine residue results in a specificity pattern which is similar to cathepsin L for the tested substrates. A further increase in size and hydrophobicity of residue 133 (mutants Gly133Val, and Gly133Leu) alters more dramatically the specificity towards hydrophobic P_2 residues. The Gly133Val mutant is highly specific for a phenylalanine residue at P_2. This mutant displays a ca.100-fold higher activity towards Z-Phe-Arg-MCA than to Z-Leu-Arg-MCA whereas cathepsin L utilizes the phenylalanine-containing substrate only 2-times better than the leucine-containing substrate. The replacement of other residues constituting the S_2 subsite cleft (mutants Val157Leu and Phe205Ala) does not change the specificity of cathepsin S.

A single mutation in position 205 of cathepsin S (Phe205Glu) changes the specificity towards a cathepsin B-like specificity. The second-order rate constant for the hydrolysis of cathepsin B specific substrate Z-Arg-Arg-MCA increases 77-fold compared to the wild-type enzyme. This is one of the highest values reported in protein engineering studies with proteinases. Similar to cathepsin B the activity of the Phe205Glu mutant of cathepsin S towards the dibasic substrate is modulated by a group with pK_a of 5.7.

S 309 PURIFICATION AND PARTIAL CHARACTERIZATION OF A MEMBRANE - ASSOCIATED CYSTEINE PROTEINASE INHIBITOR FROM HEPA-CL9 CELLS.
Catharine C.Calkins, Grace Ziegler, Kamiar Moin, Bonnie F. Sloane, Department of Pharmacology, Wayne State University, Detroit, MI 48201.
Alterations of the balance between proteinases and their endogenous inhibitors have been postulated to be important in the malignant progression of metastatic tumor cells. Evidence has been accumulating implicating the low molecular weight cysteine proteinase inhibitor stefin A as playing a role in tumor progression. Hawley-Nelson *et al.* (Mol. Carcinogenesis, **1**:202-211, 1988) have demonstrated that stefin A mRNA is underexpressed in some malignant murine skin tumors relative to benign tumors or normal epidermis. In addition Lah et al. (Biochem. Biophys. Acta, **993**:63-73, 1989) have shown that stefin A purified from human sarcoma has a reduced inhibitor activity against human cysteine proteinases. Our laboratory has observed the localization of the CPI activity in the highly malignant B16a amelanotic melanoma and in an invasive murine hepatoma not only to be in the cytosol but also in the membrane fraction. We are presently purifying CPI activity from the total membrane fraction of Hepa-cl9 (a murine hepatoma) cells grown in culture. A seven step purification protocol was used to isolate the CPI activity from the total membrane fraction of the Hepa-cl9 cells grown in culture: 1) homogenization of the cell pellet, 2) Ultracentrifugation at 108,000 x g for 60 min. to isolate the total membrane fraction, 3) solubilization of CPIs from the membranes with detergent (0.5%), 4) alkaline treatment (pH 11.5 for two hr at RT) to dissociate possible enzyme-inhibitor complexes, 5) affinity chromatography on CM-papain-Affigel 10. 6) gel filtration chromatography using two Superose 12 columns in tandem to isolate the low Mr CPI activity, and 7) ion exchange using a mono Q column as a final stage of purification based on charge difference. When the isolated membrane-associated CPI was compared to purified human recombinant stefin A (isolated by the same protocol) on an isoelectric focusing gel 4-6.5 similar bands were detected at pI values of 4.76 and 4.9. However, a primary band at 5.15 in the stefin A sample was absent from the membrane associated CPI sample. This result suggests that the membrane-associated CPI may be an isoform of stefin A.

S 310 SITE-DIRECTED MUTAGENESIS OF THE REACTIVE CENTER OF RECOMBINANT PROTEIN C INHIBITOR,
Scott T. Cooper, Jeanne E. Phillips, Elizabeth E. Potter and Frank C. Church. Center for Thrombosis & Hemostasis, University of North Carolina School of Medicine, Chapel Hill, NC 27599.
Protein C inhibitor (PCI) is a member of the family of plasma serine proteinase inhibitors called serpins. The specificity of a serpin for its target proteinases is in large part determined by the residues in the reactive center. Mutagenesis of this region results in changes in inhibitor activity and specificty. PCI inhibits a broad range of serine proteinases and is thought to be the major physiological regulator of activated protein C (APC) in plasma. We have inserted the reactive centers of seven other serpins into recombinant PCI and measured the changes in activity and specificity of these mutant PCI molecules with different proteinases. The cDNA for PCI was PCR amplified from HepG2 cells and was inserted into baculovirus DNA for expression of recombinant protein in Spodoptera frugipera (Sf9) cells.

The residues FRS (P2-P1-P1') constitute the reactive center of PCI, with the R-S bond being cleaved by the proteinase. Recombinant PCI was not significantly different from plasma PCI in inhibiting thrombin, APC, trypsin or urokinase. However, there was a shift in the optimum heparin concentration for inhibition with both thrombin and APC, with the recombinant requiring less heparin for maximum inhibition. Changing the P2 F→P generated a mutant with a reactive center like antithrombin which was better at inhibiting thrombin, but less active with APC or trypsin. Changing this same residue to alanine (P2F/A) generates a protease nexin I-like molecule which was a poor inhibitor of thrombin. Insertion of a proline (P2F/P) made an inhibitor that was very active with thrombin. Generation of a double mutant in which FR→PL resulted in a heparin cofactor II-like molecule which was a poor inhibitor of thrombin. Changing P1 R→M generated a reactive center similar to α_1–proteinase inhibitor which was a very poor inhibitor of thrombin and APC, but still inhibited trypsin and urokinase. Changing the P1' S→M generated a plasminogen activator inhibitor-1 reactive center, this protein inhibits thrombin, APC, trypsin and urokinase to the same degree as wildtype PCI.

S 311 NON-FIBRINOGEN RELATED SPECIFICITY OF TISSUE PLASMINOGEN ACTIVATOR FOR PLASMINOGEN
David R. Corey[1], Edwin L. Madison[2], Gary Coombs[1], [1]Howard Hughes Medical Institute, Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235 and [2]The Scripps Research Institute, La Jolla, California 92037

Tissue-type plasminogen activator (t-PA) is highly specific for cleavage of a single protein, plasminogen (Plg), and is highly specific for cleavage of a single peptide bond within plasminogen, Arg^{560}-Val^{561}. Part of this specificity is due to a ternary interaction between fibrin, t-PA, and Plg which reduces the K_m of t-PA for plasminogen. However, even in the absence of fibrin, t-PA remains specifically activated towards Plg when compared to trypsin, a highly homologous enzyme. We have localized the interactions responsible for specificity by assaying truncated versions of t-PA consisting of either the protease domain alone or the protease domain and the kringle 2 domain. Their activity profile is similar to that of t-PA, suggesting that the protease domain is inherently specific for Plg. We also conducted assays with peptide substrates that demonstrate that this specificity is not due to recognition of a continuous linear cleavage sequence but is instead due to an interaction which depends on the tertiary structure of Plg, and that t-PA is highly deactivated towards these substrates in comparison to trypsin.

S 312 IDENTIFICATION, PURIFICATION AND cDNA CLONING OF A NOVEL SERINE PROTEINASE INHIBITOR, Paul Coughlin, Sun Jiuru, Loretta Cerruti, Hatem Salem, Phillip Bird, Department of Medicine, Monash University, Box Hill Hospital, Box Hill, Australia, 3168

We have identified and purified a novel serine proteinase inhibitor present in extracts from human placentas and in the cytosolic fraction of the leukemic cell line, K562. Extracts from these tissues exhibited time-dependent inhibition of the serine proteinase, thrombin. The activity corresponded to a protein which formed a 67 kDa complex with ^{125}I-thrombin which was stable on reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis. We have given this protein the operational name of placental thrombin inhibitor (PTI).

A cDNA encoding the inhibitor has been obtained and characterised. Amino acid sequence comparisons show that PTI is a member of the serpin family with a reactive center P_1-$P_{1'}$ at arginine 341-cysteine 342. It lacks a classical amino terminal signal sequence and contains oxidation-sensitive residues adjacent to the reactive site. The PTI cDNA was expressed in rabbit reticulocyte lysate and in COS-7 cells and a 40 kDa protein was produced. Recombinant PTI formed a 67 kDa complex when incubated with thrombin. Analysis of human tissue mRNA indicated that PTI is expressed widely. Immunohistological survey indicates that the inhibitor is present in endothelial cells, fibroblasts and smooth muscle.

The physiological role of PTI is unknown and the significance of its interaction with thrombin is unclear. Its importance is indicated by its wide distribution, intracellular location and the presence of an unusual, oxidation sensitive region around the reactive site.

S 313 THE BINDING SITE FOR CALPASTATIN WITHIN MILLI-CALPAIN, Dorothy E. Croall and Kevin S. McGrody, Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, Orono, ME 04401-5735

The calpains (EC 3.4.22.17) are intracellular, non-lysosomal, calcium-dependent, cysteine endoproteinases. There are two well characterized mammalian isoforms of the enzyme; milli(m)- and micro(μ)-calpain. Each heterodimeric enzyme has a distinct catalytic subunit (approximately 80kDa) and an identical smaller subunit (approximately 30kDa). The C-terminal domain of each subunit has homology to the calmodulin family of Ca^{2+}-binding proteins and to each other. Calpains are regulated by a variety of mechanisms including Ca^{2+}-binding, autoproteolytic processing and an endogenous inhibitory protein called calpastatin. It is well known that the interaction between calpain and calpastatin is Ca^{2+}-dependent but the binding site for calpastatin is uncharacterized. Calpastatin contains four functional domains and each contains a highly conserved, central consensus sequence. EKLGERDDTIPPEYRELLEKKTGV was synthesized to mimic this functional region of calpastatin and shown to specifically inhibit both calpains as a competitive inhibitor. This inhibitory peptide was cross-linked to m-calpain from bovine heart using the heterobifunctional reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester. Cross-linking occurred preferentially in the presence of Ca^{2+} and the cross-linked products were analyzed by random chemical cleavage at cys-residues using the method of Nefsky and Bretscher (Proc. Natl. Acad. Sci. 86, 3549). Calpain fragments were identified by immunoblotting with an N-terminal specific antibody and by fluorography; ^{14}C was incorporated (from [^{14}C]N) into non-amino terminal fragments. These data localize the putative site of cross-linking and suggest that the functional interaction between m-calpain and calpastatin occurs in domain III of calpain's catalytic subunit. This is in contrast to the conclusions of others (J. Biol. Chem. 266, 11842; 1991) who suggested that calpastatin binds to the calmodulin-like regions.

S 314 SUBCLONING AND EXPRESSION OF TWO BARLEY SERPINS IN *E. COLI*

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Protein Z from barley (*Hordeum vulgare* L.) seed belongs to the serpin superfamily of serine proteinase inhibitors and is the only known plant serpin. Two immunologically distinct forms of protein Z (Z4 and Z7 encoded by genes on barley chromosome 4 and 7, respectively) have been isolated. As for ovalbumin no inhibitory activity has been assigned to Z4 or Z7, which are abundant in the endosperm where they may function as storage proteins without regulatory functions (1). cDNA clones of Z4 and Z7 (unpublished) and a genomic clone encoding Z4 (2) have been characterized. The sequence of a third genomic clone (Z5114) belonging to a new gene family, has recently been published (3). However, the gene product of Z5114 (protein Zx) has not yet been identified. The cDNA encoding protein Z4 has now been inserted into a pTrc99A expression vector (Pharmacia) using a *Nco*I restriction site at the initiation codon. Expression of soluble Z4 in *E. coli* upon induction with IPTG and isolation of native Z4 has been verified by Western blotting. The Z5114 gene has also been inserted into the pTrc99A vector after removal of a 971 bp intron.

Recombinant protein Z4 and Zx and site directed mutants of these proteins will be used to examine the requirements for inhibitory activity and to probe interactions, that are essential to the thermal stabilisation of serpins upon cleavage of the reactive site loop.

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2. Brandt, A., Svendsen, I. & Hejgaard, J. (1990). *Eur. J. Biochem.*, **194**, 499-505.
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S 315 TISSUE FACTOR•FACTOR VIIa INHIBITORS DESIGNED BY PHAGE DISPLAY OF KUNITZ DOMAINS, Mark S. Dennis and Robert A. Lazarus, Department of Protein Engineering, Genentech, Inc. 460 Point San Bruno Boulevard, South San Francisco, CA 94080

Potent inhibitors of human Factor VIIa (FVIIa) complexed with tissue factor (TF) have been designed from monovalent phage display of 3 separate APPI Kunitz domain libraries on the surface of the filamentous phage M13. The libraries comprised 8 randomized positions on the extended binding loop of APPI (P_5 through P_4') and 2 positions on a supporting loop; all 20 amino acids were substituted at 4 to 5 amino acid positions per library. Following 4 rounds of selection on FVIIa associated with immobilized TF, sequences from each library were determined. Several variants were expressed in *E. coli*, purified, and characterized for their ability to inhibit TF•FVIIa; apparent equilibrium dissociation constants (Ki*) generally ranged from 10 to 100 nM. This data was then used to generate TF7I-C, a consensus sequence mutant differing from APPI by 4 residues. TF7I-C inhibited TF•FVIIa with a Ki* of 2 nM, resulting in an increase in binding affinity of more than 160-fold relative to APPI. As expected, TF7I-C prolonged the clotting time in PT assays. In addition, TF7I-C prolonged the clotting time in APTT assays, indicative of inhibition of the intrinsic pathway of coagulation. TF7I-C is also a potent inhibitor of Factor XIa and plasma kallikrein as well as a moderate inhibitor of plasmin and FXa; TF7I-C did not inhibit activated protein C, thrombin, or Factor XIIa. These results indicate that phage display of Kunitz domains can be used to generate potent inhibitors of TF•FVIIa which may be useful in the management of thrombotic disease.

S 316 EVIDENCE THAT ASPARAGINE[542] OF NEUTRAL ENDOPEPTIDASE 24.11 IS INVOLVED IN SUBSTRATE BINDING BY MAKING A HYDROGEN BOND,

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Neutral endopeptidase (E.C. 3.4.24.11, NEP) is a Zn-metallopeptidase involved in the degradation of biologically active peptides, e.g. enkephalins and atrial natriuretic peptide. The substrate specificity and catalytic activity of NEP resemble those of thermolysin (TLN), a crystallized bacterial zinc-metalloprotease. Despite little overall homology between the primary structures of TLN and NEP, many of the amino acid residues involved in catalysis, as well as zinc and substrate binding, are highly conserved. Most of the active site residues of NEP have their homologues in TLN and have been characterised by site-directed mutagenesis. Furthermore, hydrophobic cluster analysis has revealed some other analogies between the NEP and TLN sequences (Benchetrit *et al.*, 1988, *Biochemistry* **27**, 592-596). According to this analysis the role of Asn^{542} in the NEP active site would be analogous to Asn^{112} of TLN which is to bind the substrate. Site-directed mutagenesis has been used to change Asn^{542} to Gly or Gln residues. The effect of these mutations on substrate catalysis and inhibitor binding was examined with a series of thiorphan-like compounds containing various degrees of methylation at the P'_2 residue. In both cases, determination of kinetic parameters with [D-Ala^2,Leu^5]enkephalin as substrate, showed that the large decrease in activity was attributable to an increase in K_m (14 to 16-fold) while k_{cat} values were only slightly affected (2 to 3-fold). This is in agreement with a role of substrate binding for Asn^{542} rather than being directly involved in catalysis as proposed above. Finally, the IC_{50} curves for thiorphan-like inhibitors strongly suggest that Asn^{542} binds the substrate at the amino terminal part of the P'_2 residue by making a hydrogen bond.

S 317 USE OF SYNTHETIC PEPTIDE LIBARIES TO SCREEN FOR PROTEINASE INHIBITORS,

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Bowman-Birk inhibitors (BBIs) are bi-headed proteinase inhibitors found in various plant sources. The three-dimensional structure of these inhibitors reveals a highly disulphide linked molecule in which the two independent heads are at opposite ends of the structure [1]. The structure itself shows considerable symmetry, and is presumed to arise from a primordial gene duplication event. The reactive sites of the inhibitor are each encompassed within a 9-residue disulphide-linked loop, and the main specificity determinant, the P1 residue, is located within this loop. In many BBIs, the two inhibitory loops have different P1 residues, giving rise to differing specificities. For example, in the soybean inhibitor, the two P1 residues are Lys and Leu. This results in inhibition of both trypsin and chymotrypsin, respectively. The encapsulation of inhibition within this 9-residue sequence has been demonstrated by the ability of disulphide-linked peptides based on this loop sequence to retain inhibitory activity [2, 3].

To probe the specificity of inhibition, and to assist in the design of novel inhibitors, we have used mixed libraries of peptide sequences based on the BBI reactive loop. Using a library of sequences in which the P1 residue was randomised to incorporate 20 different amino acids, we have isolated variants that are able to bind to immobilised trypsin, chymotrypsin and elastase. It was found that different subfractions of the library bound to the different proteinases, and amino acid sequencing was used to identify which sequences were compatible with inhibition of various proteinases. Initial results show that only Lys and Arg P1 variants bind to trypsin, whereas the hydrophobic amino acids Phe, Met, Leu and Norleucine can be selected by immobilised chymotrypsin. The use of such semi-random libaries provides a rapid and cost-effective means of screening for inhibitory function, and for optimizing peptide sequences.

References

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2 Terada *et al.* (1978) *FEBS Lett.* **90**, 89.
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S 318 REGULATION OF A SERINE PROTEASE INHIBITOR, PROTEASE NEXIN-1, BY THE EXTRACELLULAR MATRIX,

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Recent studies have shown that serine proteases and serine protease inhibitors can be regulated in their activity, specificity and location by glycoprotein or extracellular matrix (ECM) co-factors. Protease Nexin-1 (PN-1) is a member of the SERPIN superfamily of serine protease inhibitors and can rapidly inhibit thrombin, urokinase and plasmin. PN-1 is known to bind tightly to the ECM and to be regulated in its target protease specificity by the ECM. PN-1 bound to the ECM is accelerated in its inhibition of thrombin, and is blocked in its inhibition of urokinase and plasmin. Previous work has shown that heperan sulfate proteoglycan is largely responsible for the acceleration of thrombin inhibition by PN-1. Our current studies indicate that collagen IV can alter PN-1 target specificity such that PN-1 becomes a less efficient inhibitor of urokinase and plasmin, but is unaltered in its ability to inhibit thrombin. Collagen IV can decrease the formation of SDS stable complexes between urokinase or plasmin and PN-1 without affecting formation of complexes between thrombin and PN-1. The second order rate constant for inhibition of urokinase by PN-1 was markedly decreased with increasing collegen IV, whereas the second order rate constant for inhibition of thrombin by PN-1 was unaffected by addition of collagen IV up to 1μm. Other ECM components, collagen type I, vitronectin, fibronectin, and heat denatured collagen IV were unable to affect complex formation or the rate of inhibiton of proteases by PN-1, indicating that these effects were specific to collagen IV. Binding of PN-1 to immobilized collagen IV was demonstrated using an enzyme linked immunoabsorbant assay, and the concentration of PN-1 necessary to obtain 50% saturation of the immobilized collagen IV binding sites was *approximately* 15nm. It is of interest to note that collagen IV is found only in basement membranes. Immunohistochemical staining for PN-1 in the brain localized PN-1 around blood vessels and capillaries where collagen IV is present as a major constituent of the blood brain barrier basement membrane. It has been hypothesized that PN-1 acts as a protective barrier against extravasated thrombin during injury to the brain. Regulation of PN-1 target specificity by collagen IV, changing PN-1 to a more specific thrombin inhibitor, supports this hypothesis.

S 319 THE C-TERMINUS OF THE SERPIN C1 INHIBITOR CONTAINS CRUCIAL RESIDUES THAT PREVENT SPONTANEOUS INSERTION OF THE REACTIVE CENTRE LOOP,

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A screen for mutations in exon 8 of the C1 inhibitor gene in patients suffering from hereditary angioedema yielded a clustering of pointmutations in the region encoding the C-terminal fragment. Some of these have been found at equivalent positions also in α1-antitrypsin and antithrombin III. In all cases, the mutations result in defective secretion, either because of retention in the ER, or because of a blockade at a later stage in transport. Three mutant proteins were however partially secreted upon transient transfection into COS cells, and we have examined their functional and structural properties. The mutant proteins (V451M, F455S and P476S) had greatly diminished capacity to form complexes with C1s and did not behave as substrates. Immunoprecipitation demonstrated that intact, uncleaved mutants bind to a mAb which is specific for complexed or cleaved normal C1 inhibitor. Heat-stability appeared to be increased compared to the normal S-form. The reactive centre loop of the mutant proteins was less susceptible to cleavage by trypsin. Finally, gel filtration studies showed that the V451M and P476S mutant proteins were prone to multimerization at ambient temperature.

These results strongly indicate a conformational change as a result of the mutations, such that a segment of the reactive centre loop is intrinsically inserted into the central ß-sheet A, yielding a complex-like structure. Interestingly, the tendency of C1 inhibitor mutants to multimerize coincides with decreased heparin affinity of antithrombin III mutated at equivalent positions (Olds et al., Blood 5, 1206, 1992, and Lane et al., JCI 90, 2422, 1992). We conclude that these highly conserved residues in serpins, which are in close proximity in the 3D-structure, serve to anchor the reactive centre loop at the C-terminal side and to counteract the tendency of the loop to insert in ß-sheet A.

S 320 Study of different based Bowman and Birk peptides inhibitors.
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The Bowman-Birk Inhibitor (BBI), is a double headed inhibitor, coming from a large family of proteases (BBPI), found generally in *leguminosae* plants (garden bean. lima bean. soybean for example). It differs through particular features from the other natural inhibitors by having a low molecular weight (6 to 9 kda), a high proportion in cystine residues and by being biheaded: there are two tricyclic domains which display independent and usually heterologous antiproteinase activity. The high content in cystine groups enables a tight structure in the active conformation of the protein, which explains the unusual stabilities of the inhibitor towards heat, acid and proteolytic digestion. It is supposed that the BBI evolved from a single headed ancestral inhibitor by internal gene duplication.
Several sequences of peptide inhibitors corresponding to the antitryptic loop of the BBI, were described (1-3), two single cross linked peptides and one double cross linked. A comparison of each of them, regarding their stabilities and inhibition properties has been carried out. It was found that slight changes in the amino acid sequences lead to dramatic changes in stabilities to enzyme cleavage of the peptides inhibitors. The results provide an insight to protein design for serine protease inhibitors.

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S 321 SERPINS AS INHIBITORS OR SUBSTRATES: EVIDENCE THAT OVALBUMIN CAN UNDERGO LOOP-TO-SHEET INSERTION, Peter G.W. Gettins, James A. Huntington, and Philip A. Patston, Department of Biochemistry, and Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232

Serpins are a class of structurally-related proteins with molecular masses in the range 44-100kDa. Most serpins are inhibitors of serine proteinases and are thought to undergo a major conformational change upon complex formation with proteinase that involves partial insertion of the reactive center loop into a β-sheet of the inhibitor. Such loop insertion greatly increases the stability of the serpin. Ovalbumin, although a serpin, is not an inhibitor of serine proteinases. It has been proposed that this deficiency arises from the presence of a charged residue, arginine, at a critical point in the reactive center region, which prevents loop insertion into the β-sheet and thus precludes inhibitory properties. To test this hypothesis we have examined the properties of two forms of ovalbumin; the native protein and S-ovalbumin. From comparison of the susceptibility of the reactive center region of each protein to proteolysis by subtilisin Carlsberg we concluded that the native-to-S transformation specifically affected the reactive center region. Calorimetric measurements showed that S-ovalbumin was significantly more stable than native ovalbumin. CD spectra indicated that S-ovalbumin had less α-helix and more β-sheet than native ovalbumin. These data support a structure for S-ovalbumin in which part of the reactive center loop has inserted into β-sheet A to give a more stable conformation. Relative rates of dephosphorylation of serine 344, however, showed no change between native and S-ovalbumins, suggesting that insertion is limited to a few residues. We conclude that a charged residue at position P14 is insufficient to prevent loop insertion and that action of a serpin as an inhibitor may depend critically on the rate of loop insertion.
Supported by NIH grant HL49234.

S 322 PARTIALLY MIXED NON-COMPETITIVE INHIBITION OF DIPEPTIDYL PEPTIDASE IV BY THE HIV-1 TAT PROTEIN, William G. Gutheil, George R. Flentke and William W. Bachovchin, Department of Biochemistry, Tufts University School of Medicine, Boston MA, 02111
Dipeptidyl Peptidase IV (DP IV) is a serine exopeptidase found on the surface of $CD4^+$ T-cells which has been implicated in the regulation of the immune system. We have found that the HIV-1 Tat protein inhibits DP IV and have kinetically characterized this inhibition. The inhibition is strongly NaCl concentration dependent. The affinity of Tat for DP IV varies from 24 pM to 11 nM, and the activity of the Tat-DP IV complex varies from 13% to 33%, as the NaCl concentration is varied from 0 to 40 mM. The inhibition data were analyzed in terms of a partially mixed non-competitive inhibition model using a combination of methods including the analysis of full progress curves by numerical integration. These methods may be of interest for the study of other protease-inhibitor systems.

S 323 MOLECULAR CHARACTERIZATION OF PROTEASE NEXIN 1 REGULATION BY DEXAMETHASONE, Denis C. Guttridge and Dennis D. Cunningham, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717

We are interested in studying the regulation of Protease Nexin 1 (PN-1), a member of the SERPIN family and an inhibitor of thrombin. Previously, we reported that 1 μM concentrations of the glucocorticoid, dexamethasone (DXM), resulted in the down-regulation of PN-1 activity in cultured medium from normal human fibroblasts. This decrease in protein activity correlated with a repression in cytoplasmic PN-1 mRNA. Treatment with cycloheximide confirmed that the DXM affect was independent of protein synthesis, and results from nuclear run-on experiments suggested that the mechanism of regulation was mainly at the transcriptional level. In conjunction with these findings we are currently interested in examining the precise mechanism by which DXM transcriptionally represses PN-1 production. Regulation of gene expression by glucocorticoids is known to be mediated by their respective receptors. However, several lines of evidence by other investigators indicate that receptor-mediated regulation may not necessarily occur through protein-DNA interactions at the 5' flanking regions of genes. To investigate precisely how the DXM receptor represses PN-1 transcription, we are in the process of isolating the PN-1 promoter element. Our cloning strategy has been based on the information provided by McGrogan and co-workers, who have reported that the first intron in the PN-1 gene lies approximately 121 base pairs downstream from the 5' end of the cDNA. We have therefore utilized a short exon 1-specific oligo to screen a human lung fibroblast genomic library. From approximately 7.5×10^5 Lambda phage clones, 8 were strongly recognized by the oligo according to Southern blot analysis. In addition, 4 out of 8 clones were also hybridized by a riboprobe corresponding to the first 94 bases of the PN-1 cDNA. We are in the process of further characterizing these 4 clones and obtaining sequence information.

S 324 **Active - Site Binding Loop Stabilization in the Serine Proteinase Inhibitor Eglin c**

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Eglin c, naturally occuring in the leech *Hirudo medicinalis*, is a 70 amino acid, potent serine proteinase peptide inhibitor. As is known from its X-ray crystal structure in complex with subtilisin, eglin c has a compact core and an extended, solvent exposed binding loop, stabilized by a network of non-covalent interactions. Less is known, however, how these internal electrostatic interactions contribute to the high inhibitory potency of eglin c without affecting residues of the enzyme. — This intriguing question was addressed by preparing specifically designed mutants of eglin c.

Structural and functional studies on these mutants show a good correlation between differences in free binding enthalpy, as inferred from kinetic experiments, and changes in non-covalent core - binding loop interactions, as inferred from the X-ray crystal structures in complex with a proteinase. The X-ray crystal structures of uncomplexed wild-type eglin and one mutant with drastically reduced inhibitory potency imply that for the uncomplexed inhibitor, the system of core - binding loop interactions is not sufficient for a significant stabilization of the active-site binding loop. Hence, an activation of the core - binding loop interactions to their full strength, as observed in the complex structures, must occur upon formation of the complex with the target proteinase. — A flexible binding loop, as observed for uncomplexed eglin, is advantageously since it allows one inhibitor to interact with a wide range of proteinases. "Induced binding loop stabilization", as observed in the complex structures of eglin, might explain that a flexible binding loop does not become disadvantageously due to large entropic terms which have to be reduced upon binding.

S 325 MOLECULAR CLONING REVEALS ISOFORMS OF ALPHA1-ANTICHYMOTRYPSIN IN BOVINE, Shin-Rong Hwang, Andrea B. Kohn, and Vivian Y.H.Hook, Dept. of Biochem., USUHS, MD 20814
The bovine ACT cDNA of 1.5 kb was obtained by screening a bovine liver cDNA library with the human liver α1-antichymotrypsin cDNA. The deduced primary sequence indicated that the 1456 base pair cDNA encoded a protein of 416 amino acids that contains a high degree of homology (55%) with human ACT; both bovine and human ACT share common sequences in the reactive site domain. Importantly,the reactive site of bovine ACT possesses serine in the P1 position of the reactive site, whereas human ACT contains leucine in the P1 position. This is the first demonstration of ACT possessing serine in the P1 position of the reactive site that usually is involved in determining specificity of the protease inhibitor towards target protease(s).
Comparsion of bovine ACT with mouse or rat contrapsin (rodent counterparts of ACT) shows that the reactive site domain of these serpins possess homologous amino acid sequences. However, bovine, human, and rodent ACTs differ in the P1 residue at the reactive site. These differences in P1 residues among several species suggests evolutionary divergence of the ACT-like gene at the reactive site. Genomic blots demonstrated the presence of multiple copies of the bovine ACT gene. Primary sequences of another partial cloned bovine ACT-like protein which shared 68% identinity to the first copy of bovine ACT confirmed the results of the genomic blot. Therefore, multiple copies of bovine ACT gene represent similar, but not identical genes. ACT immunoreactivity has recently been demonstrated in bovine neuroendocrine tissues of adrenal medulla and pituitary; it is not known whether they are identical to either of the two cloned liver ACTs or represent other related ACTs. It will be important to define in future how multiple bovine ACT genes are structurally related.

S 326 MOLECULAR EVOLUTION OF SERINE PROTEASES WITH KRINGLE STRUCTURES, Kazuho Ikeo[1)], Kei Takahashi[2)], and Takashi Gojobori[1)], [1)]DNA Research Center, National Institute of Genetics, Mishima 411, Japan. [2)]Shimane Medical University, Izumo, Japan.
A group of serine proteases, which are involved in the cascade of blood coagulation and fibrinolysis, consist of several functional domains. A 'kringle' is one of such functional domains. It is composed of approximately 80 amino acids and three disulfide bonds. Recently, the kringle structures have been found in some other proteins which do not have the function as proteases. They include are apolipoprotein(a), hepatocyte growth factor (HGF), and a cell surface protein called ROR. ROR is a kind of tyrosine kinase. To study the evolutionary process and the origin of kringle structures, we constructed the phylogenetic trees by use of the nucleotide sequences of kringle structures, serine protease domains, and tyrosine kinase domains. The phylogenetic tree obtained shows that the ancestral gene of kringle structures appeared about 500 million years. Then, the kringle structure was duplicated and were inserted into other genes. Thus, our molecular evolutionary analysis shows that the domains, such as kringle structures, play an important role as one of evolutionary and functional units.

S 327 STRUCTURE-FUNCTION STUDIES ON HUMAN TISSUE FACTOR, Robert F. Kelley, Kimberly Costas, Mary Wessinger, and Robert A. Lazarus, Protein Engineering Dept., Genentech, 460 Pt. San Bruno Blvd., South San Francisco, CA 94080
Tissue factor (TF) is a membrane bound glycoprotein that serves as a cofactor for coagulation factor VIIa (FVIIa) in the initiation of the extrinsic pathway of blood coagulation. We are performing structure-function studies on human TF with the aim of developing inhibitors of the TF•FVIIa interaction for use as potential antithrombotic agents. The TF extracellular domain, residues 1-219, has been expressed at high levels by secretion from *E. coli* and purified by using immunoaffinity chromatography. Surface plasmon resonance (SPR) on a Pharmacia BIAcore system was used to determine the kinetics (k_{assoc} = 1.3 x 10^5 M^{-1} s^{-1}; k_{diss} = 3.0 x 10^{-4} s^{-1}) and equilibria (K_D = 2.3 nM; ΔG = −11.8 kcal/mol) for human FVIIa binding to immobilized TF (1-219). Microcalorimetric measurements of the TF(1-219)•FVIIa interaction indicate a large enthalpy change upon binding (ΔH = −30 kcal/mol), and therefore an unfavorable TΔS of −18.2 kcal/mol. These data are consistent with a conformational change upon binding as suggested by others. A soluble version of the K165A:K166A double mutant TF, which is functional for FVIIa binding, is shown to lack procoagulant activity with human plasma and acts as a specific inhibitor of TF-dependent clotting. A series of additional TF mutants are currently being analyzed for FVIIa binding and cofactor function.

S 328 KINETIC STUDIES OF BM 06.022, A PLASMINOGEN ACTIVATOR MUTEIN PRODUCED IN E.COLI,

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BM 06.022 (r-PA) is a t-PA deletion variant comprising only the kringle-2 and protease domains. Production of rPA in E.coli leads to formation of inactive inclusion bodies which are refolded by a large-scale in vitro process.

Amidolytic activity (on a molar basis) and lysine affinity are similar for BM 06.022 and t-PA derived from CHO cells. Both plasminogen activators have the same plasminogen activation activity in the absence of any stimulator ($k_{cat}/K_m = (1.7\pm0,1)\cdot 10^{-3}\ l\cdot s^{-1}\cdot \mu mol^{-1}$), but in the presence of CNBr fragments of fibrinogen (Fbg) or fibrin monomer as the stimulator, the in vitro catalytic efficiency of BM 06.022 is lower by a factor of 4 or 2, respectively ($k_{cat}/K_m = 3.3\cdot 10^{-1}$ and $5.3\cdot 10^{-1}\ l\cdot s^{-1}\cdot \mu mol^{-1}$ vs. 1.25 and $1.05\ l\cdot s^{-1}\cdot \mu mol^{-1}$) so that higher stimulator concentrations are required for maximal activity (e.g., 50% stimulation at 43 vs. 11 µg CNBr fragments/ml) as compared to CHO-t-PA. BM 06.022 also has a lower in vitro affinity for fibrin, presumedly due to the lack of the finger domain supposed to be important for the high-affinity interaction of t-PA with fibrin.

However, animal studies have demonstrated that treatment of rabbits with equipotent doses of BM 06.022 and t-PA leads to a similar decrease of Fbg and α_2-antiplasmin plasma concentrations indicating sufficient fibrin specificity of BM 06.022 in vivo [ref.1].

BM 06.022 is now in phase III clinical trials as a thrombolytic agent in acute myocardial infarction.

Ref.1. Martin, U. et al. (1991) Thromb. Haemostas. 65, 560-564.

S 329 ECOTIN: A POTENT ANTICOAGULANT AND REVERSIBLE TIGHT-BINDING INHIBITOR OF FACTOR Xa,

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Ecotin, a serine protease inhibitor found in the periplasm of *Escherichia coli*, is an extremely potent anticoagulant and reversible tight-binding inhibitor of human Factor Xa (FXa). The ecotin gene was cloned by PCR, highly expressed in *E. coli*, and purified from the periplasm. The binding of ecotin to FXa was stoichiometric with an equilibrium dissociation constant K_i of 54 pM. The association rate constant was $1.35 \times 10^6\ M^{-1}s^{-1}$ and the dissociation rate constant, measured in the presence of human leukocyte elastase (HLE) to prevent reassociation of ecotin with FXa, was $6.5 \times 10^{-5}\ s^{-1}$. Ecotin prolonged clotting time ca. 10-fold at 0.3 µM and at 2 µM in APTT and PT assays respectively. Ecotin did not effectively inhibit human thrombin, tissue factor(TF)•Factor VIIa, Factor XIa, activated protein C, plasmin, or tissue plasminogen activator (t-PA); however it did potently inhibit Factor XIIa, plasma kallikrein, HLE, and bovine trypsin and chymotrypsin. Incubation of ecotin and FXa at 10 µM each resulted in a $(ecotin)_2\bullet(FXa)_2$ complex as determined by gel filtration. Dimerization of ecotin alone was measured by fluorescence titration which yielded a K_d of ~390 nM. FXa cleaved ecotin slowly at pH 4.0 between M84 and M85. Replacement of the P_1 M84 residue with Arg and Lys led to FXa inhibitors with K_i values of 11 and 21 pM, respectively. The P_1 Arg and Lys mutants also significantly inhibited thrombin, Factors XIa and XIIa, activated protein C, plasmin, kallikrein, bovine trypsin and chymotrypsin, but not TF•Factor VIIa, t-PA, or HLE.

S 330 CHYMOTRYPSIN INHIBITOR 2 (CI2) DISPLAY ON THE SURFACE OF BACTERIOPHAGE,

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CI2 is a small molecule (M_r 9200), isolated from Barley seeds, which is a potent inhibitor of the microbial serine protease subtilisin and chymotrypsin. The N-terminal end of the protein forms a structural scaffold, whereas the C-terminal end comprises of a reactive loop which interacts non-covalently with the specificity pocket of serine proteases.

One of the potential goals of protein engineering is the introduction of novel inhibitor specificities. A powerful strategy for selecting a desired protein, from a mixture of variants, together with the gene encoding it is the expression of protein molecules on the surface of bacteriophage. Phage display is being used to determine the relevance of specific CI2 - protease interactions.

Bacteriophage fd is a filamentous phage which infects male *E.coli*. Adsorption to the host cell pilus occurs via the gene III protein (gIIIp) produced at the tip of the virion. CI2 was displayed at the surface of phage by fusion to the N-terminus of gIIIp. The fusion product gIIIp-CI2 was identified using Western blotting and silver staining of SDS-PAGE. Titration inhibition curves with subtilisin indicated that phage harbouring the gIIIp-CI2 fusion were kinetically active with a Ki approximately 200-fold higher than native CI2. The weaker association may be due to the attachment of the fusion to the large phage molecule, or due to the introduction of a G83D mutant.

A different phagemid secretion system is currently being used to assist the monovalent production of random peptide libraries, with the aim of selecting CI2-phage with desired binding characteristics.

S 331 SELECTION FOR PROTEASE INHIBITORS USING THE BACTERIOPHAGE-DISPLAY TECHNOLOGY.

William Markland, Bruce L. Roberts, Edward Cannon and Robert C. Ladner. Protein Engineering Corporation, Cambridge, MA 02138.

Proteases play a major role in many disease states; inhibitors to such enzymes could be valuable therapeutic agents. There is a need for small, stable recombinant inhibitor molecules to be used in the treatment of chronic and acute diseases of protease origin. We have previously demonstrated the usefulness of the bacteriophage-display system in the development of effective protease inhibitors, producing the most potent human neutrophil elastase inhibitor known to date (PNAS 89 pp2429-2433, 1992). In that work we used the well characterised trypsin inhibitor, aprotinin, as the parental molecule for variegation and selection. This has been extended by the use of human homologues of aprotinin which, via the method of directed evolution, have been screened against other human serine proteases of clinical relevance allowing for the generation of new families of protease inhibitors. The sequences of the selected variants and their kinetic properties will be presented, together with a discussion of the general applicability of this methodology to protease and protease inhibitor research.

S 332 FOLDING OF WILD TYPE AND A DISULFIDE MUTANT OF BOVINE PRETHROMBIN-2 OVER-EXPRESSED IN E.COLI. Muriel C. Maurer, Elsie E. DiBella, and Harold A. Scheraga, Baker Laboratory of Chemistry, Cornell University, Ithaca, NY 14853-1301.

A recombinant E.coli system has been developed for overproducing wild type prethrombin-2 (PT-2) and a mutant in which the cysteines involved in a disulfide bond between the A and B chain portions of PT-2 are replaced with alanines [PT-2 (C22A, C168A)]. These single-chain proteins have been purified, refolded, and subsequently activated with E. carinatus snake venom. They will be used in ongoing structural studies to characterize the specificity of fibrinopeptide binding. PT-2 is being overexpressed in an E.coli T7 system, isolated as inclusion bodies (80-120 mg/l), resolubilized, reversibly sulfonated, and then purified by reverse phase HPLC. Refolding is accomplished by incubating sulfonated PT-2 in the presence of GdnHCl, EDTA, and glutathione for 24 hrs, followed by slow dialysis to remove the denaturant and the oxidizing/reducing agents. High concentrations of denaturant are needed to keep sulfonated PT-2 soluble and, as a result, folding may be occurring by a predominantly random process. The dialyzed refolding mixture binds specifically to a heparin column and 3 protein peaks are eluted off the resin at increasing NaCl concentration. The protein from each peak was incubated with E. carinatus snake venom to activate the PT-2. The cleaved protein originating from the third eluted peak was the only one capable of hydrolyzing the synthetic substrate peptide S-2238 and of clotting fibrinogen. This fraction is proposed to contain PT-2 folded in the native conformation. Refolding experiments reveal that the same number of species are eluted off the heparin column at the same NaCl concentrations for both wild type PT-2 and disulfide mutant PT-2 (C22A, C168A).

S 333 CLEAVAGE OF ATRIAL NATRIURETIC FACTOR (ANF) RECEPTOR BY MEMBRANE-BOUND METALLOENDO-PEPTIDASE: POSSIBLE ROLE IN RECEPTOR REGULATION, Kunio S. Misono, Department of Cardiovascular Biology, Cleveland Clinic Foundation Research Institute, Cleveland, OH 44195

ANF is a peptide hormone secreted by the heart atrium which has potent natriuretic and vasorelaxant activities. The actions of ANF at its target organs, kidney, adrenal, and vascular beds, are mediated by cell surface receptor coupled to guanylate cyclase (GCase). The receptor consists of a single 130 kDa polypeptide containing an extracellular ANF binding domain, a single transmembrane sequence, and an intracellular GCase domain. Incubation of partially purified adrenal membranes at acidic pH (pH 4 to 5.6) followed by neutralization resulted in rapid cleavage of the 130 kDa ANF receptor to yield a 65 kDa ANF-binding protein as detected by photoaffinity labeling using azidobenzoyl-^{125}I-ANF and SDS-PAGE. SDS-PAGE without reduction gave a affinity labeled 130 kDa band but no 65 kDa band, indicating that polypeptide fragments generated by the cleavage were still held together by a disulfide bond(s). The results also suggest that the cleavage occured at a limited number of sites. The cleavage of the 130 kDa receptor to the 65 kDa protein was complete in 20 - 30 min, and was inhibited by EDTA, but not by PMSF, NEM, or pepstatin. The cleavage of the 130 kDa receptor during the incubation at acidic pH was accompanied with parallel loss of membrane-associated GCase activity. The cleaved receptor also showed relaxed ligand structure requirement for binding as determined by competitive protection experiments. Inclusion of EDTA, which prevented the cleavage, abolished these effects, indicating that the cleavage by a metalloendopeptidase caused inactivation of GCase and relaxed binding specificity. A peptide containing potential proteolysis site in the extracellular region of the receptor sequence was synthesized. Cleavage of this peptide was observed upon incubation with adrenal membrane preparations at acidic pH and was inhibited by EDTA. I propose that, upon ligand binding, ANF receptor may be internalized together with membrane-bound metalloendopeptidase into the endosomes, and cleaved by the enzyme following acidification of the endosomal lumen. The proteolytic cleavage of the receptor that occurs with concomitant loss of GCase activity and change in binding specificity may be involved in ligand-induced receptor down-regulation or desensitization.

S 334 SYNERGISTIC INHIBITION OF COMPLEMENT ACTIVATION BY SOLUBLE CR1 AND A LOW M_R PROTEASE INHIBITOR, BRL24894A, Danuta E. Mossakowska[1], Susanne M. Scesney[2], Henry C. Marsh[2] and Richard A. G. Smith[1], Department of Biotechnology, SmithKline Beecham, Harlow, Essex CM19 5AD, UK[1] and T-Cell Sciences Inc., Cambridge, MA 02139.

Soluble complement receptor 1 (sCR1, BRL 55730), a multidomain glycoprotein (Mr 244,000), is a potent and specific inhibitor of both classical and alternative pathways of complement activation [CA] (IC_{50} 10 & 90 ng/ml). The 4-amidinophenyl ester BRL 24894A (APAN) has been shown to be an inhibitor of serine proteases acting by reversible acyl transfer to active center serine and is used in the synthesis of the acyl-enzyme thrombolytic anistreplase (Eminase ™). In hemolytic assays of CA, APAN inhibited the classical pathway with an $IC_{50} \sim 8$ μM and the alternative pathway with an IC_{50} of ~30 μM. Addition of APAN (4 μM, $<IH_{50}$) to sCR1 reduced the classical pathway IC_{50} of sCR1 from 10 to 1 ng/ml. Statistical and graphical analysis of potentiation at multiple APAN concentrations showed the effect to be truly synergistic. sCR1 inhibition of alternative pathway CA was potentiated by APAN additively not synergistically. APAN was inactive against CA measured at the C3 convertase level (C3a RIA), implying inhibition at the C5 convertase or membrane attack complex assembly steps. APAN inhibited Factor I cleavage of C3b in the presence of sCR1 but at concentrations > 10X those required for synergy with sCR1. These observations suggest that combinations of specific protein agents and less specific low-M_r inhibitors can be used to potentiate inhibition of CA and modulate pathway specificity.

S 335 THE RAPID DISCOVERY OF SUBSTRATES FOR THE METALLOPROTEASE MATRILYSIN USING A PHAGE DISPLAY SYSTEM. Marc Navre, Matt Smith and Lihong Shi. Affymax Research Institute, 4001 Miranda Avenue, Palo Alto, CA 94304.

The characterization of the peptide substrates of a protease is an important part of protease characterization the design of inhibitors of therapeutic interest. We have used peptide phage display libraries to find optimal peptide substrates for proteases. To **critically** assess the method, we have chosen the matrix metalloprotease (MMP) matrilysin as a target. The MMPs represent a family of enzymes that recognize at least 6 amino acids in their subsites. Additionally, only a limited amount of information is available for matrilysin, a protease strongly implicated in tumor invasiveness. The goal of this work is to seek substrates without making any *a priori* assumptions about the specificity or true "physiological substrate" of the enzyme.

Using polyvalent phage, a hexamer library of $2x10^8$ recombinants was prepared in which the randomized N_6 target domain was located between an NH_2-terminal constant region (carrying a pair of epitopes for monoclonal antibodies [mAbs]) and the rest of the gene III protein. The library was treated with a limiting amount of protease in solution, and the uncleaved phage were separated from cleaved using the mAbs and a protein-A resin. The cleaved phage were then added to bacteria for amplification followed by reselection or analysis. We have developed a rapid assay for determining whether the clones isolated do indeed carry good substrates, by analyzing the products of protease treatment of the phage particles. Using these methods, over 40 "hit" sequences were obtained, and used to build consensus substrate sequences that yielded peptides with k_{cat}/K_M values 3-10-fold better than current literature standards. In contrast to traditional methods, only 8 peptides substrates needed to be prepared synthetically over the course of these experiments.

Matthews and Wells (*Science* (1993) 260:1113) have pioneered the use of monovalent phage libraries for discovering protease substrates. The polyvalent phage method presented here has enabled us to screen larger libraries and characterize "hits" more quickly, allowing, for the first time, the building of consensus sequences from the hits. The advantages and disadvantages of each method will be discussed.

S 336 ANALYSIS OF ACTIVE-SITE MUTANTS OF BLOOD CLOTTING FACTOR VIIa: IMPORTANCE OF K_{192} IN COAGULANT ACTIVITY, Pierre F. Neuenschwander and James H. Morrissey, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

Clotting factor VIIa (fVIIa) is the serine protease responsible for catalyzing the initial proteolytic activation reaction in the extrinsic pathway of the clotting cascade. When bound to the cell-surface receptor/cofactor, tissue factor, the enzymatic activity of fVIIa is dramatically enhanced, both in cleavage of macromolecular protein substrates (*i.e.* factors VII, IX and X), and in cleavage of small tripeptidyl substrates. Comparison of the fVIIa amino acid sequence with that of other blood clotting enzymes revealed that while factors IXa, Xa, thrombin and activated protein C have either a Q (factors IX and X) or an E (activated protein C and thrombin) at the 192 position (numbered as per chymotrypsin), fVIIa is unique in bearing a K at this position. Since residue 192 has been shown to be of great import in determining the specificity and activity of thrombin (Le Bonniec *et al.*, *Proc. Natl. Acad. Sci. USA*, (1992) **88**:7371-5), it was of particular interest to examine the potential role of this analogous residue in fVIIa. With this in mind, two mutants of fVII were made where K_{192} was mutated to either Q or E ($fVII_{K192Q}$ and $fVII_{K192E}$). The mutants were expressed in human kidney 293 cells, and purified by affinity chromatography. Analysis by SDS-PAGE showed that both mutants were secreted as single chain zymogens. Although both $fVII_{K192Q}$ and $fVII_{K192E}$ were found to be cleaved to the two-chain form by factor Xa at rates comparable to that of wild-type fVII, when analyzed in a single-stage clotting assay $fVII_{K192Q}$ was only 40% as active as wild-type fVII, while $fVII_{K192E}$ was completely ineffective. These results were essentially paralleled when examined in factor X activation in a pure system using the two-chain forms of both mutants. When examined for amidolytic activity, however, the two-chain form of $fVII_{K192Q}$ was found to have retained full activity while $fVII_{K192E}$ was roughly 60% active. These results suggest an important role of K_{192} in the proteolytic *versus* enzymatic acitivity of fVIIa, and may effect the substrate/inhibitor preferences of fVIIa.

S 337 NMR STUDIES ON UROKINASE-TYPE PLASMINOGEN ACTIVATOR, Ursula K. Nowak *, Richard A.G. Smith † and Christopher M. Dobson *. * New Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QT, U.K. and † SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, U.K.

NMR studies have been performed on urokinase-type plasminogen activator (u-PA), a multidomain fibrinolytic protein consisting of an EGF-like, a kringle and a serine protease domain. Despite the high molecular weight of 46 kDa, well resolved NMR spectra can be obtained. This has been attributed to the independent dynamic mobility of the individual domains. Unfolding studies show that isolation of the domains does not affect their stability. The protease domain itself shows a complex unfolding behaviour with three transitions, which becomes more cooperative after inactivation with a specific peptide chloromethyl ketone (EGRcmk). A major part of the N-terminal subdomain of the protease domain has been shown to be of especially high stability. This region has been isolated by limited proteolysis with thermolysin under denaturing conditions where only the N-terminal subdomain remains folded and resistant against proteolysis. The isolation of such stable units can be an approach to study and characterise the structure and stability of proteins. Furthermore, they may give insight into understanding processes of assembly and folding of proteins. The structural properties of the isolated fragment of the serine protease domain of u-PA have been characterised by NMR and CD studies.

S 338 ROLE OF THE CATALYTIC SERINE IN THE INTERACTIONS OF SERINE PROTEINASES WITH INHIBITORS OF THE KUNITZ AND SERPIN FAMILIES. S.T.Olson*, P. E. Bock+, I. Björk#, J. Kvassman*, J. D. Shore*, D. Lawrence†, and D. Ginsburg†. *Henry Ford Hospital, Detroit, MI, +Vanderbilt University, Nashville, TN, #Swedish University of Agricultural Sciences, Uppsala, Sweden, and †University of Michigan, Ann Arbor, MI

Trypsin and anhydrotrypsin (AH-trypsin), in which the catalytic serine residue is converted to dehydroalanine, were used to distinguish between noncovalent and covalent mechanisms of interaction with inhibitors of the Kunitz and serpin families. AH-trypsin effectively competed with trypsin for inhibition by the Kunitz inhibitors, aprotinin and soybean trypsin inhibitor, when all components were equimolar (10 nM), and essentially blocked the inhibition of active enzyme at a 10-fold molar excess. In contrast, AH-trypsin had little or no detectable effect on the interaction of trypsin with the serpins, α_1-proteinase inhibitor (α_1PI), antithrombin(AT) or AT-heparin complex, up to a 100-fold molar excess, under the same conditions. However, significant competition was observed in the case of the PAI-1 reaction at the highest level of AH-trypsin (1 μM). Kunitz and serpin inhibitors all displaced the active-site fluorescence probe, p-aminobenzamidine, from trypsin in a stoichiometric manner, consistent with the formation of tightly-bound complexes ($K_D < 10^{-8}$ M). Similarly, the two Kunitz inhibitors also showed a stoichiometric displacement of the probe from AH-trypsin, in agreement with the established ability of AH-trypsin to form a complex with these inhibitors of stability comparable to that of trypsin. In contrast, either no displacement (α_1PI or AT) or a less than stoichiometric displacement of the probe (AT-heparin complex or PAI-1) resulted when AH-trypsin was titrated with the serpin inhibitors up to 20 μM, indicating weak interactions, with K_Ds of ~1 and ~10 μM measurable for PAI-1 and AT-heparin complex. Contrasting these K_Ds, apparent K_Ds of 7×10^{-12}, 1×10^{-10}, 2×10^{-11}, and 5×10^{-12} M were measured for the interactions of trypsin with α_1PI, AT, AT-heparin complex, and PAI-1, respectively, from the ratio, k_{off}/k_{on}, for the inhibition reactions. These results indicate that noncovalent interactions of the reactive-site loop of inhibitors with the active-site region of proteinases account for the high affinity of Kunitz inhibitor-proteinase interactions but not serpin-proteinase interactions, suggesting that a covalent interaction involving the catalytic serine of the proteinase is essential for the tight binding of the serpins.

S 339 ***alpha*-Hyrdoxy Phosphinyl Based Transition State Analog Renin Inhibitors: Minimal Strucutral Requirements For Good Inhibitory Activity**

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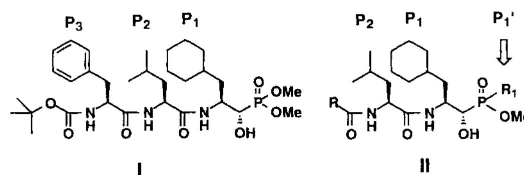

Incorpororation of the novel transition state analog *alpha*-hydroxy phosphonate group in a P_3-P_1 tripeptidic framework led to the discovery of potent inhibitors of the aspartyl protease, renin (I, I_{50} = 10 nM). With the objective of enhancing oral bioavailability, a reduction in the peptidic nature for this class of compounds was desired. Simple P_3 truncation of **I** to dipeptide analogs **II** led to a substantial reduction in activity. Efforts were undertaken to compensate for this loss by incorporating R_1 residues on the phosphinyl moiety that may be capable of mimicking the P_1' side chains (**II**, R_1 = P_1' group). In addition, new analogs encompassing the P_1-P_2' region were also synthesized. Preparation of these truncated hydroxy phoshinyl analogs, and their biological results will be described.

S 340 THE SOLUTION STRUCTURE OF THE *ACTIVE* REACTIVE SITE LOOP OF α1-ANTITRYPSIN BY NMR METHODS

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Serpins are a family of related proteins which act as specific inhibitors of serine proteinases. The details of the inhibitory mechanism of serpins are not well understood, in part, due to the little direct structural information about the active form of any serpin. The application of multidimensional NMR methods to the structural determination of the reactive site loop (RSL) of α1-antitrypsin (AT), in its active conformation, is reported. Elastase inhibitory activity of AT has been conferred to the cytokine interleukin-1ß (IL-1ß) by replacing residues E50-E51-S52-N53 with the ten amino acid serpin RSL segment, EAIPMSIPPE(1). The IL-1ß ß-barrel portion of the chimeric protein, AT/IL, is structurally indistinguishable from the wild type IL-1ß. Sharp new ^{1}H resonances appear on the AT/IL spectra which correspond to the ten amino acid insertion. The narrow linewidths of these resonances suggest that the loop is mobile relative to the rest of the protein. However, the three prolines do not appear to undergo cis/trans isomerism. Residues E-A-I extend the ß-strand of the native IL-1ß, V41-G49. Measurements of ϕ angles are being used to further constrain the backbone of the elastase inhibitory loop. The solution structure of the *active* α1-antitrypsin reactive site loop is described. This work is supported by grants from the Johnson & Johnson Focused Giving Program and by the NSF.

1. Wolfson et al. *Prot. Eng.* 4, 313 (1991).

S 341 ENGINEERING THE SUBSTRATE SPECIFICITY OF TRYPSIN, John J. Perona, Lizbeth Hedstrom, William J. Rutter, Charles S. Craik and Robert J. Fletterick, Departments of Pharmaceutical Chemistry and Biochemistry & Biophysics, and Hormone Research Institute, Univ. of California, San Francisco, CA 94143-0446

We employ an integrated approach designed to elucidate the structural basis for the substrate specificity of trypsin, and to re-engineer this specificity to produce variant proteases possessing altered discrimination properties. Through the use of a genetic selection and site-directed mutagenesis coupled to functional and structural analysis of variants, we have found that Lys and Arg substrate binding affinity to the S1 site is correlated with accessibility of the Asp189 negative charge to substrate, and not with the nature of the electrostatic contacts. Asp189 also plays a role in precise substrate positioning. Three surface loops have been identified which are critical to providing discrimination between Lys/Arg and hydrophobic (chymotryptic) substrates. Crystal structures of trypsin variants possessing chymotryptic specificity show that the conformation of Gly216, which forms hydrogen bonds with the P3 residue of the substrate, is a primary determinant of specificity. Comparative structural analysis suggests that this determinant is critical to elastase specificity also. We are using the knowledge gained from these analyses to guide us in the construction of large libraries which are used in genetic selections for trypsins of altered specificities.

S 342 MASS SPECTROMETRIC APPROACHES TO THE MOLECULAR CHARACTERIZATION OF PROTEOLYTIC PATHWAYS OF AMYLOID-β-POLYPEPTIDES RELATED TO ALZHEIMER'S DISEASE. Michael Przybylski*, Martina Jetschke*, Marianna Mák+, Martina Schuhmacher*, Gabor Toth+, Zoltan Szekely+, and Botond Penke+, *Faculty of Chemistry, University Konstanz, 78434 Konstanz, Germany; and +Department of Medical Chemistry, University of Szeged, and Hungarian Academy of Sciences, Hungary.

Proteolytic processing of the β-amyloid precursor protein (APP) has been shown to occur by a secretory non-amyloigenic pathway, and an alternate pathway leading to neurotoxic β-amyloid polypeptides (β-AB), but molecular details of these proteolytic pathways are not well understood. Recent developments of "soft" ionization methods have led to a breakthrough of mass spectrometry (MS) for the molecular characterization of polypeptides up to large proteins by plasma desorption (PDMS), laser desorption (LDMS), and particularly, electrospray (ESMS); these MS methods also provide direct identifications of proteolytic products even in complex mixtures (peptide mapping). A series of β-AB polypeptides, and specific site-mutation analogues encompassing N-terminal β-AB and C-terminal transmembrane sequences have been prepared by chemical synthesis, and their structures characterized by PDMS and ESMS. Initial proteolytic model studies were carried out with several serine- and cysteine-proteases; the complete identification of proteolytic products by MS-peptide mapping showed high structural dependence of proteolytic specificities. Furthermore, charge structures of macromolecular ions in ESMS indicate conformational differences of non-amyloigenic and β-AB peptides. These MS methods are currently applied to the molecular characterization of β-AB products from Alzheimer plaques, using a new isolation procedure from gel electrophoresis bands suitable for ESMS analysis.

S 343 DETERMINATION OF THE SOLUTION STRUCTURE AND DYNAMICS OF THE SUBTILISIN SAVINASE™ BY MULTINUCLEAR 3D-NMR METHODS, M.L. Remerowski[1], T. Domke[2], A. Groenewegen[2], H.A.M Pepermans[2], C.W. Hilbers[1], and F.J.M. van de Ven[1], [1]Department of Biophysical Chemistry, University of Nijmegen, and [2]Uni-Lever/Vlaardingen, the Netherlands

The subtilisin Savinase™ is used as a protein degrading additive in washing powders, but in general, subtilisins are an attractive model system for protein engineering because of their extensive data base and diverse applicability. They have been studied extensively by means of X-ray crystallograpy and many X-ray structures have been produced. Recent developments in NMR spectroscopy have brought proteins of up to 30kD within the realm of three-dimensional solution structure determination by NMR methods. This molecule is the largest monomeric protein to date for which an NMR solution structure is being attempted. So far, about 95% of the backbone atoms (^{15}N-$^{1}H_N$-^{13}C=O-$^{13}C\alpha$-$^{1}H\alpha$) have resonance assignments and the secondary structural elements have been determined. Additionally, the flexibility of each region of the molecule has been determined from amide proton-deuterium exchange data. At present we are working on a full three-dimensional solution structure.

This is the first crucial step for further investigations, in which the effects of environmental changes can be observed, as well as interactions with calcium ions, inhibitors, and detergent molecules under varied conditions. An additional goal is to develop methods of fast "screening" for mutants using NMR spectra, to quickly determine structural and dynamical differences in engineered variations of this molecule.

S 344 MUTATION OF Glu 80 → Lys RESULTS IN A PROTEIN C MUTANT THAT NO LONGER REQUIRES Ca^{2+} FOR RAPID ACTIVATION BY THE THROMBIN-THROMBOMODULIN COMPLEX, Alireza R. Rezaie, Timothy Mather, Fredy Sussman and Charles T. Esmon, Cardiovascular Biology Research, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104.

Binding Ca^{2+} to a high affinity site in protein C and Gla-domainless protein C (protein C lacking residues 1-44) results in a conformational change that is required for activation by the thrombin-thrombomodulin complex, the natural activator of protein C. Recent studies with factor VII and IX suggest that a Ca^{2+} binding site is present in the protease domain and involve Glu 70 and 80 (chymotrypsin numbering system). In thrombin, the Ca^{2+} binding site present in trypsin is replaced by an internal salt bridge between Lys 70 and Glu 80. Protein C has a single high affinity Ca^{2+} binding site outside the first EGF domain and, similar to factors VII and XI, contains Glu 70 and 80 in the protease domain. We constructed and expressed a Gla-domainless protein C mutant in which Glu 80 is replaced with Lys. Activation of GDPC E80K is accelerated to the same extent as wild type by TM, but unlike the wild type, Ca^{2+} is not required for this acceleration. Unlike wild type Gla-domainless protein C, Ca^{2+} no longer inhibits activation of the mutant by free thrombin, and Ca^{2+} stimulation of chromogenic activity is also absent. The characteristic Ca^{2+} dependent *quenching* of Gla-domainless protein C intrinsic fluorescence is also absent in the mutant. We conclude that the high affinity Ca^{2+} binding site in protein C critical for zymogen activation involves Glu 80. The Glu 80 to Lys mutation probably results in a salt bridge with Glu 70 that stabilizes protein C in a conformation similar to the Ca^{2+} stabilized conformation.

S 345 THE USE OF PYRIDOXYL DERIVATIVES FOR THE SYNTHESIS OF PROTEASE INHIBITORS
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We have developed a novel series of three-functional amino acid (lysine, aspartic acid, cysteine) pyridoxyl derivatives (PD) as the component of inhibitors of angiotensin converting enzyme (ACE). These compounds achieve tight binging to ACE due to the presence of a strong ligand for the active site zinc ion as well as features which mimic the binding of acyl tripeptides. Pyridoxyl and pyridoxamine analogues of thyrotropin releasing hormone (TRH) have breen prepared. These analogues and TRH itself have been shown to be inhibitors of metalloenzymes, that can explain releasing hormons mechanism of action. The most effective inhibitor of ACE has been tripeptide, containing S-(5-pyridoxyl)-cysteine. It has been proposed to use pyridoxal for preparation of inhibitors containing aldehydic group. Polyfunctional character of PD gave possible to synthesis multiplet peptides possessing a high activity as compared with monomeric peptide. High activity apears to be provides multiple binding with sites.

The multiplet forms chemotactic peptide, peptides of contact interaction of cells and antigen determinants have been obtained. We are explored the possibility of virus-cell interaction inhibition by the multiplet peptides and the antibodies induced these peptides.

S 346 IN VITRO PROTEIN SPLICING OF AN ARCHAEA INTERVENING PROTEIN SEQUENCE, Maurice W. Southworth, Ming-Qun Xu, Fana B. Mersha and Francine B. Perler. New England Biolabs, 32 Tozer Road, Beverly, MA 01915.

Examples of protein splicing have been described in representatives of three kingdoms. In each case, an internal peptide segment, which is an endonuclease, must be precisely removed and the external protein sequences must be joined to produce the active, mature protein. Splicing of intervening protein sequences (IVPSs) in heterlogous expression systems, suggests that protein splicing is self-catalyzed. Here we show that an archaeal IVPS, plus one downstream residue, contains sufficient information for protein splicing when inserted into three foreign proteins, a ß-galactosidase fusion and 2 maltose binding protein 3-part chimera's. In each case, protein splicing of the fusion protein is controllable by temperature, allowing purification of protein precursors and subsequent *in vitro* splicing of highly purified protein precursors. Residues found to be essential for self-catalytic protein splicing are His-Asn, and either Ser or Thr. Mutagenesis studies have resulted in fusion proteins that show 1. no splicing, 2. a reduced rate of splicing, 3. cleavage at only one of the two splice junctions and 4. excision of the internal peptide segment, but failure to join the external protein sequences. The data provides direct evidence for protein splicing yielding mechanistic insight into the process. Furthermore, controllable protein splicing opens up novel approaches to protein engineering.

S 347 UNIQUE STRUCTURAL FEATURES OF FACTOR D, A SERINE PROTEASE OF HUMAN COMPLEMENT, Sthanam V.L. Narayana, Sunghee Kim, Lawrence J. DeLucas and John E. Volanakis, Department of Optometry, University of Alabama at Birmingham, Birmingham, AL 35294

Recent x-ray crystallographic analysis of Factor D revealed unique differences from other serine proteases in the conformation of the catalytic residues His57 and Asp102. Furthermore, the two non-crystallographically related molecules in the triclinic unit cell, A and B, displayed distinctive active center conformations. Differences in residues known to form the primary and secondary substrate-binding sites were also noted. To investigate the contribution of these unique structural features of Factor D to its distinct functional properties, we constructed a series of Factor D mutants by site-directed mutagenesis. Some of these interesting mutants have been crystallized and structural studies are in progress. To further characterize Factor D, we have recently obtained crystals of the recombinant zymogen form of this enzyme. The determination of the structures of this proD and an inhibitor complex is in progress. Structural differences from the native structure of Factor D will be discussed.

S 348 USE OF PEPTIDE AND NONPEPTIDIC LIBRARIES FOR MAPPING OF ACTIVE SITE OF HUMAN THROMBIN

Peter Strop, Charlie Chen, Kevin Haney, Jim Spoonamore, Jim Ostrem, Alena Stirandova, Pavel Safar, Viktor Krchnak, Peter Kocis, Nikolai F. Sepetov, Dagmar Cabel, Farid-Abdul Latif and Michal Lebl.
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We used linear and cyclic peptide libraries with L and D amino acids, linear peptide-peptidomimetic libraries as well as nonpeptidic scaffold libraries to map the active site of human thrombin and also to verify different screening protocols for selection of thrombin inhibitors. Screening of primary libraries was done by enzyme-linked assay as described (1) using human thrombin labeled with biotin. Selected peptides were resynthesized and inhibition constants determined using chromogenic and fluorogenic substrates. Examples of structures of peptides of different chirality which were selected as inhibitors of human thrombin with on bead binding assays will be given. The potential of the Selectide process for lead compound optimization will be illustrated using the secondary libraries with known motif (active site inhibitor of thrombin d-Phe-Pro-Arg-Pro , Ki 20 uM). Five positions were randomized on each side of dPhe-Pro-Arg-Pro peptide and libraries containing 3 fold excess of 1.5 millions different sequences were screened using enzyme-linked assay. Peptide (Sel1172) with app. 1000 fold improvement of binding was selected.

References:

1) Lam, K.S. et al.(1991) Nature 354, 82-84

S 349 ACTIVATION OF HUMAN PLASMA PROCARBOXYPEPTIDASE B,

Anthony K. Tan and Dan L. Eaton, Department of Cardiovascular Research, Genentech, Inc., South San Francisco, CA 94080

A new basic carboxypeptidase (pCPB: plasma carboxypeptidase B) has been isolated from human plasma (Eaton, D.L., Malloy, B.E., Tsai, S.P., Henzel, W., and Drayna, D. (1991) J. Biol. Chem. 266, 21833-21838). The exact funciton of this newly discovered protein is still unclear. However, its affinity to plasminogen suggests a possible role in fibrinolysis.

The major obstacle in the characterization of pCPB is in the step of activation of the zymogen. A number of proteases has been examined and trypsin is found to be the best. Unlike carboxypeptidase A and B from pancreas, pCPB is extremely unstable to trypsin activation. Trypsin cleaves pCPB at two sites: Arg-92 and Arg-330. The active enzyme (M_r = 35,000) is released after the cleavage at Arg-92 and continuation of cleavage of the activated pCPB at Arg-330 yields a catalytically inactive enzyme (M_r = 25,000). We have designed a novel way to acitvate the proenzyme with very little unwanted cleavage. It was found that when pCPB was activated in the presence of a weak reversible inhibitor such as ε-aminocaproic acid (ε-ACA), the unwanted trypsinolysis could almost be eliminated. Further studies with other ε-ACA analogs suggest that there are two structural requirements for a successful protector and they are: a free carboxyl group and a basic side chain. The length of the methylene chain separating the free carboxyl group and the basic group influences the effectiveness of the protector. It was also found that both L-lysine and L-arginine were more effective than their corresponding D-isomer. All these compounds are active site directed inhibitor. The most likely mode of action is binding to the active site which induces a conformational change. The result of the conformational change is either restricting the mobility of the loop which Arg-330 is resided on or shielding the loop from the accessibility of the bulk solvent.

S 350 MUTATIONAL ANALYSIS OF HUMAN RENIN: IDENTIFICATION OF A SURFACE LOOP INVOLVED IN BINDING OF HUMAN ANGIOTENSINOGEN,

Diane Thibeault, Louise Pilote, Ginette McKercher, Yvon Gaudette, Ivan Lessard and Daniel Lamarre, Dept. of Biochemistry, Bio-Méga/Boehringer Ingelheim Research Inc., Laval, Québec, H7S 2G5

Human renin, a key enzyme of the renin-angiotensin system, is a highly specific aspartyl protease with only one known natural substrate, angiotensinogen. Human angiotensinogen (hANG) is hydrolysed by renin at the Leu^{10}-Val^{11} bond with lower Km and kcat values than the synthetic human tetradecapeptide (hTDP) which corresponds to the amino-terminus of hANG (Asp^{1}-Asn^{14}). The Km difference could indicate that in addition to the interaction with the active site, hANG may have other sites of contact. In order to identify these sites, renin mutants were made by substituting segments (4 to 9 residues) of non conserved sequences between human and rat renin. Since human and rat renin have very similar three-dimensional structures, the substitutions should minimize major disruption of the native enzyme conformation. Ten chimeric human-rat prorenin enzymes have been transiently expressed in COS cells. Secreted prorenin mutants were converted into renin by cleavage with immobilized trypsin. Their ability to cleave hANG and hTDP has been assessed by quantitation of angiotensin I using a radioimmunoassay. The substitution mutant involving residues 59-66 (MR59-66) generated an enzyme able to cleave hTDP but with a greatly reduced ability to cleave hANG. MR59-66 has been purified by affinity chromatography using a competitive renin inhibitor as the ligand. Preliminary results have shown that the catalytic efficiency with hTDP is not significantly *different from the wild-type enzyme. However, the catalytic efficiency* with hANG is 20-fold lower due to a 6-fold increase in Km and a 3-fold decrease in kcat. The data suggests that human renin residues 59-66 may be involved in recognition of angiotensinogen.

S 351 THE STRUCTURAL BASIS OF LATENCY IN PAI-1,

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The active to latent conversion of plasminogen activator inhibitor type 1 (PAI-1) is accompanied by the movement of reactive center residues from their position within an arched loop on the surface of the protein and insertion of these residues as an antiparallel strand into the major β-sheet, sheet A. Using the known structure of latent PAI-1 as a rational guide, mutations were designed to prevent or slow the insertion of the reactive center residues into the A sheet. Three types of mutations have been constructed. In the first, we have attempted to block the insertion of β-strand 4A into the A sheet by altering the sequence of amino acid residues in the strand such that the residues inserted are energetically less favorable to the formation of a β-sheet. Such mutations include the substitution of glutamic acid for those normally found within strand 4A at the even-numbered positions from P14 to P4. Second, we have introduced specific changes into a region of the molecule that we call the "gate" in an effort to restructure this region to resemble α1-antitrypsin to prevent the active to latent transition. Third, using the model of antithrombin III as a guide, we have introduced a specific disulfide bond into the PAI-1 protein where none previously existed. This mutation is designed to stabilize the position of the gate region so as to block the active to latent transition. These various mutants have been engineered into a bacterial expression vector in which PAI-1 protein expression is driven from a T7 gene 10 promoter which in turn depends upon the IPTG-inducible expression of T7 RNA polymerase. A polyhistidine tag permits the affinity purification of the expressed protein on a nickel-agarose column and further purification of PAI-1 is accomplished on a heparin-Sepharose column. This combined expression/purification system permits the rapid expression and purification of milligram quantities of PAI-1 required for the analysis of "latency-resistant" mutants. Preliminary results suggest: 1) strand 4A is partially inserted into sheet A and this insertion is required for inhibitory activity of PAI-1; 2) changing the P8 and P6 residues to glutamate resulted in proteins with a 10X and 2.5X increase in half life at 42° C respectively compared to wild type; 3) the "gate" mutations resulted in a protein with a 2X increase in half life at 42° C compared to wild type; and 4) engineering a disulfide bridge into PAI-1 resulted in a protein with a 10X increase in half life at 42° C compared to wild type. In summary, we have determined that both the mobility of the gate region and the propensity of the reactive center loop to form a strand within the A sheet contribute to the active to latent transition.

S 352 REGULATION OF PROTEASE NEXIN-1 SYNTHESIS AND SECRETION IN CULTURED NEURAL CELLS BY INJURY RELATED FACTORS AND THE THROMBIN RECEPTOR. Patrick J. Vaughan and Dennis D. Cunningham, Department of Microbiology and Molecular Genetics, University of California, Irvine, California, USA.
Protease nexin-1 (PN-1) is a 43kDa protease inhibitor that can inactivate several serine proteases although its physiological target is probably thrombin. PN-1 is identical to the glial-derived neurite promoting factor or glial-derived nexin (GDN). This neurite promoting activity of PN-1 is mediated through it's ability to inhibit thrombin, a protease which can retract processes on both neurons and astrocytes. It is interesting in this respect that much of the PN-1 in human brain occurs around blood vessels where it may play a protective role against extravasted thrombin following injury within the brain. Other studies also indicate that PN-1 may play key roles after injury and may be important in certain pathological conditions. In the present studies we have utilised the neuroblastoma cell line SK-N-SH to examine the regulation of PN-1 synthesis and secretion by factors known to be produced after injury and in inflammatory processes. This cell line comprises two distinct cell types, one with the properties of glial cells and one with the properties of neuronal cells. Using cloned derivatives of each of these two types we have found that PN-1 is produced only by the glial cell type and that the secretion of PN-1 by these cells is stimulated by four factors ; interleukin-1, transforming growth factor-b, tumor necrosis factor a and platelet-derived growth factor. All of these factors have important roles in the wound repair process, thus supporting the hypothesis that following injury PN-1 plays an important protective role in the brain. In addition, activation of the thrombin receptor acted synergistically with IL-1 to further stimulate PN-1 secretion by the SK-N-SH cell line. However, this effect of thrombin.was mediated through the neuronal cell population. Thus, following exposure to thrombin, neuronal cells may release factors or induce signals which can stimulate PN-1 production by glial cells. Of further interest was the observation that the neuronal cell type secreted two thrombin inhibitors that were distinct from PN-1. We are currently investigating the nature and the regulation of these inhibitors.

S 353 ANATOMICAL DISTRIBUTION AND CELLULAR LOCALIZATION OF THE THROMBIN RECEPTOR IN RAT BRAIN, Jonathan R.Weinstein, Stephen J. Gold, Dennis D. Cunningham and Christine M. Gall, Department of Microbiology and Molecular Genetics, Department of Anatomy and Neurobiology, University of California, Irvine. Irvine, California , USA.
Recent findings have suggested that the coagulation serine protease thrombin may have a potential role in modulating neuronal plasticity in the central nervous system (CNS) as well as contributing to CNS pathophysiology following cerebrovascular injury. Thrombin can retract processes on neurons and astrocytes in culture at picomolar concentrations. Serum proteins equivalent in size to thrombin can extravasate across the blood-brain barrier following ischemia or trauma. Once in the CNS, thrombin may act as a trigger for reactive gliosis and contribute to the inflammatory response. In addition, prothrombin mRNA is expressed at early developmental stages in the rat CNS suggesting endogenous brain expression of the protease. The recently cloned thrombin receptor (TR), a proteolytically activated, seven transmembrane domain, G-protein coupled receptor mediates most of thrombin's cellular activation functions including those in the CNS. TR expression in the CNS has not been well characterized to date. In the present study we have mapped the anatomical distribution and cellular localization of TR mRNA in young adult rat brain by in situ hybridization and Northern blot analysis using a rat TR cRNA probe. TR mRNA expression occurs in both neuronal and glial cell populations. Specific regions exhibiting strong signal include olfactory bulb, neocortex, dentate gyrus, cerebellum and substantia nigra pars compacta. In the latter region TR mRNA is colocalized with immunoreactivity for tyrosine hydroxylase, a marker for dopaminergic neurons. These results demonstrate a broad CNS distribution of TR mRNA including expression by vulnerable neuronal groups and regions of high plasticity in adult brain. Work supported by NIA grant AG00538 to DDC and CMG.

S 354 MECHANISM OF PROTEIN SPLICING AND THE IDENTIFICATION OF A BRANCHED INTERMEDIATE, Ming-Qun Xu, Maurice W. Southworth, Henry P. Paulus#, Donald G. Comb, Fana B. Mersha, Linda J. Hornstra and Francine B. Perler. New England Biolabs, 32 Tozer Road, Beverly, MA 01915. #Boston Biomedical Research Institute, Boston, MA 02114.
Protein splicing is a post-translational processing event in which an internal protein region must be precisely removed from a protein precursor and the external domains must be joined together to form the mature protein. Alignment of splice junctions of known protein splicing elements reveals the presence of Ser, Thr or Cys at both splice junctions and His-Asn at the C-terminus. Direct examination of the mechanism of protein splicing was made possible by purification of protein precursor from two constructs in which a protein splicing element was inserted into β-galactosidase and MBP-paramyosin fusion protein. We demonstrate that protein splicing in vitro can be controlled by both temperature and pH. A splicing intermediate, observed during processing of the precursor, was found to contain two N-termini and showed rapid reversibility between itself and the precursor form, that was controllable by pH. These results agree with our postulated model that protein splicing is initiated by N-O acyl shifts at both splice junctions and involves a branched intermediate. Efficient in vitro splicing even in a foreign context implies that a protein splicing element contains the necessary information for appropriate folding to align the reacting groups in close proximity.

S 355 PHAGE DISPLAY OF TRYPSIN Yang, Q., Wang, C-I., and Craik, C.S. Departments of Pharmaceutical Chemistry and Biochemistry & Biophysics, University of California at San Francisco, San Francisco, CA 94143-0446
A goal of our protein engineering effort on trypsin is to understand the relationship between the three dimensional structure of the enzyme and its function and to use that information to redesign the enzyme. This work focuses on the development of an *in vitro* screening method, i.e., phage display, to investigate the substrate specificity of trypsin. Phage display is a novel technique based on the expression of proteins on the surface of filamentous bacteriophage for *in vitro* ligand binding. The gene III and VIII encoded proteins from bacteriophage M13 have been fused to the C-terminus of the trypsin gene. The fusion gene is inserted into M13mp18 and regulated by an inducible *tac* promoter. The fusion protein is secreted to the periplasmic space and assembled into trypsin M13 hybrid phage particles. Like wild-type trypsin, the trypsin-phage forms a non-covalent complex with an endogenous E.*coli* protease inhibitor, ecotin. This complex is disrupted at low pH and the fusion phage is separated from ecotin by dialysis against a 300 KDa cut-off membrane that also removes unbound trypsin or trypsin-coat protein. The trypsin-phage also display trypsin-like activity with the kinetic parameters (kcat and Km) that closely approximate that of the wild-type trypsin. The trypsin-phage can be selectively enriched from a background of wild-type M13 phage by panning with immobilized ecotin. A trypsin binding pocket library has been made on phage in which the amino acids that primarily determine the substrate specificity have been randomized. This library is being screened with various ligands and trypsin mutants are being characterized. Study of the amino acid combinations which afford new specificities will provide insights into the complex interplay of determinants which give the highly homologous trypsin family of proteases their diverse properties.
This work is supported by a grant to CSC from the National Science Foundation.

S 356 EXPRESSION OF HUMAN α1-ANTICHYMOTRYPSIN IN TRANSGENIC MOUSE BRAIN INDUCES THE FORMATION OF ALZHEIMER-LIKE LESIONS, Cho-Yau Yeung*, Rodrigo O. Kuljis§, Susan R.Ross† & Robert D. Beech*, Depts. of Genetics* and Biochemistry†, Univ. of Illinois College of Medicine, Chicago, Il 60612 and Dept. of Neurology§ The Univ. of Iowa & Vet. Affairs Med. Ctr., Iowa City, Iowa 52242.
Excessive amyloid plaques and associated neuritic abnormalities are two of the defining histopathological features of Alzheimer's disease (AD). The presence of the protease inhibitor α1-antichymotrypsin (ACT) in the amyloid plaques suggests that these plaques may result from altered proteolysis of the amyloid precursor protein (APP). Three independently derived strains of transgenic mice that constitutively expressed human ACT in the brain were generated. All offspring of these mice developed massive numbers of amyloid and ACT-containing plaques. In addition, analyses of their brain tissues under electron microscopy revealed cerebral amyloid deposits that were associated with ultrastructural abnormalities in the neuropil that include neuritic changes. These results demonstrate that a genetic alteration that elevates ACT expression in the brain is sufficient to induce several of the histopathological features associated with AD and suggests that mutations affecting ACT expression may underlie the familial AD locus identified on the long arm of human chromosome 14. Since ACT is a major acute phase response protein, these findings further suggest that various environmental factors capable of inducing an inflammatory response in the brain may also lead to the formation of AD-like lesions in the absence of any predisposing mutation in the APP gene.

S 357 STABILITY OF NATIVE α_1-ANTITRYPSIN AS PROBED BY SINGLE AMINO ACID SUBSTITUTIONS THAT CONFER ENHANCEMENT IN STABILITY, Myeong-Hee Yu, Kee Nyung Lee, Jeongho Kim, Ki-Sun Kwon, Protein Engineering Group, Genetic Engineering Research Institute, KIST, Taejon 305-333, Korea
α_1-antitrypsin, a member of serine protease inhibitor (serpin) family, is a major plasma inhibitor and abnormal production of which is responsible for human liver or lung disease. Unlike other globular proteins the native structure of inhibitory serpins is strained, which is important for regulation of activity. We isolated via mutagenesis and screening α_1-antitrypsin variants carrying single amino acid substitutions which showed prolonged activity at 60 ℃. Mutations did not affect inhibitory activity significantly, as revealed by normal association rate constants with porcine pancreatic elastase. Mutations, however, retarded aggregation rate during heat denaturation process, thus confering increased stability against heat-deactivation without altering inhibitory activity. The urea-induced unfolding transition probed by fluorescence intensity and circular dichroism signal, combined with analysis on urea gradient gel electrophoresis, indicated that the mutations also increased conformational stability. Pattern of amino acid substitutions and location of such mutations will be discussed with respect to a mechanism by which native α_1-antitrypsin folds and maintains strain. Suppression of a few genetic defects by these stable mutations is under investigation.

Posters Relevant to Oral Sessions 7, 10 and 12

S 400 MUTATIONAL ANALYSIS OF THE MASON-PFIZER MONKEY VIRUS PROTEINASE, Martin Andreansky and Eric Hunter, The University of Alabama at Birmingham, Department of Microbiology, Birmingham, AL 35294.
We have previously reported on the expression of a Mason-Pfizer monkey virus (M-PMV) proteinase precursor in *E. coli*. During isolation and purification the 26kDa precursor undergoes self processing, yielding three active forms of the recombinant enzyme (17kD and 12kD homodimers and a 17/12kD heterodimer), all of which have identical N-termini (NH_2-W-V-Q-P-I-T-), similar to that determined previously for the MMTV proteinase. During preparation and storage, the active 17kD species is processed at the C-terminus to the 12kD form, thereby enabling formation of the heterodimer. The slower cleavage occurs 45 amino acid residues upstream from the C-terminus at a scissile I-M-M-C-S><P-N-D-I-V peptide bond. Preliminary substrate specificity and inhibition studies suggest that the substrate specificity of the M-PMV proteinase is closely related to that of MAV proteinase.
In order to further characterize this enzyme and its role in the assembly of mature M-PMV particles we have designed several mutations within the proteinase reading frame. In addition, we have developed an expression/mutagenesis system, that allows for both efficient in vitro mutagenesis and tightly regulated expression of proteins in *E. coli* from a single construct.
Expressing the mutated precursors in bacteria, we were able to identify mutations that alter the rate of precursor self-processing into mature enzyme. Mutation of T to S in the substrate binding cleft has no effect on the C-terminal processing, yet appears to completely block the N-terminal cleavage. Since all the other mutations that we have made are at the P1 Y of the N-terminal and at the P2' N of the C-terminal cleavage site, none of the latter mutations change the structure of the mature enzyme itself. While F and M at the N-terminal P1 have no effect on the rate of self-processing, S, L and A retard the processing and V and T inhibit the cleavage at this site. Most of the mutations at the P2' of the C-terminal cleavage site (S, M, T, K, and V) do not change the rate of cleavage, however, substitutions of D, E, I and L slow down the processing. So far, we have not identified any mutations that would completely inhibit the cleavage at the C-terminal cleavage site.
We are currently cloning the mutations that alter the rate of proteinase self-processing into a proviral construct, to establish their effect on the in vivo polyprotein processing and viral viability.

S 401 EFFECTS OF HIV-1 PROTEASE MUTATIONS ON THE EFFICIENCY OF POLYPROTEIN PROCESSING AND INFECTIVITY OF VIRAL PARTICLES.
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The effect of single amino acid changes within the viral protease gene have been analyzed in a system in which viral polyproteins are processed and assembled into mature virions. The parent vector, HIV-gpt, contains all the HXB2 genes except for *env*, which was replaced by the E. coli *gpt* gene for selection purposes. Infectious particles can be produced by co-transfection of these HIV-gpt vectors and a plasmid encoding *env* in COS-7 cells. The titer of these particles may be quantitated on CD4+HeLa cells. Through this system, it is possible to identify the intermediates and the end products of HIV gag and gag/pol processing, and compare to the infectivity of the particles containing the mutated proteases. We have characterized mutants which produce lowered activity in the HIV protease to assess their effect on infectious particle formation. One such mutant, Thr26Ser, shows only 4-5 fold decrease in k_{cat} when measured using recombinant protease and a synthetic peptide substrate. In the virus, this mutation causes a significant reduction in the rate of processing of the gag substrate, particularly the C-terminal cleavage of p25 to the mature p24 protein. The infectivity of mutant-bearing viral particles is dramatically reduced during the first 24 hours of virus production. Over time the viral titers obtained are comparable to wild type, indicating that slow maturation of the gag polyproteins can yield viable infectious particles. This system can probe the effect of varying protease activity levels on the maturation process.

S 402 INTERNAL CLEAVAGE SITES OF THE UL80 PROTEASE OF HUMAN CYTOMEGALOVIRUS: IMPLICATIONS FOR PROTEASE ACTIVITY AND TURNOVER.
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In human cytomegalovirus (HCMV) and in other herpesviruses, proteolytic processing of the assembly protein is necessary for the packaging of DNA into the virion. Proteolysis is accomplished by a site-specific protease encoded by the N-terminal domain of the HCMV UL80 gene, which also encodes the assembly protein itself, by translation of separate mRNAs which are 3' coterminal (Liu and Roizman, J. Virol. 65, 5149-5156 (1991); Welch et al., PNAS 88, 10792-10796 (1991). We have recently demonstrated that the ~85-kDa protease precursor is cleaved internally at three sites: (i) at the C-terminus of the assembly protein domain, (ii) between the 30- and 57-kDa proteins, and (iii) within the 30-kDa protease itself, which yields the 16- and 13-kDa proteins and may be a mechanism to inactivate the protease (Baum et al., J. Virol. 67, 497-506 (1993). Pulse-chase radiolabelling of HCMV-infected cells indicates that cleavage occurs first at the C-terminus of the 85-kDa polyprotein, followed by release of the 30-kDa protease from the N-terminus, and finally by cleavage within the 30-kDa protease. Mutations at each of the three cleavage sites demonstrated that none of these cleavages is necessary to activate the protease, either in *E. coli* or in transfected cells. However, the observation that the unprocessed 85-kDa protease precursor, and its assembly protein substrate, initially share identical C-terminal regions has implications for compartmentalized proteolysis.

S 403 ASPARTIC PROTEINASES FROM THE HUMAN MALARIAL PARASITE *PLASMODIUM FALCIPARUM*,
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Over 40% of the world's population live in regions where malaria is endemic. In 1989, there were an estimated 110 million clinical cases of malaria worldwide. Increasing resistance of parasites to currently available anti-malarial agents and vector mosquitoes to insecticides makes the search for novel methods of control a high priority. During the intraerythrocytic stages of malarial infection, an aspartic proteinase plays the key role at the rate limiting step of haemoglobin degradation by the parasite to yield amino acids for its metabolism. The gene encoding an aspartic proteinase (PFAPD) has been characterised. The parasite enzyme shows >30% identity and >50% homology to *human* Cathepsin D, Cathepsin E and Renin.
A 3-dimensional model of PFAPD has been constructed using rule based procedures showing that the primary sequence may be folded into a structure closely resembling those of other known aspartic proteinases. PFAPD has been expressed in *E. coli* and purified to homogeneity. The recombinant proteinase is active against a range of synthetic peptide substrates and haemoglobin with an optimum pH of 4.7. Its activity is inhibited by 100 nM pepstatin. The genes encoding further related proteins are also being characterised and expressed.

S 404 CHARACTERIZATION OF METALLOPROTEINASES IN *TRYPANOSOMA CRUZI*.
Myrna C. Bonaldo, Jussara M. Salles, Luiz N. d'Escoffier & Samuel Goldenberg. Fiocruz, Dept. Biochem.Molec.Biol., Av. Brasil 4365, Rio de Janeiro, RJ, 21045-900, Brasil

Two major metalloproteinase activities of 52 kDa and 65 kDa have been described during the metacyclogenesis of *T.cruzi* clone Dm28c. The 52 kDa enzyme is constitutively expressed, whereas the 65 kDa metalloproteinase is specifically expressed by the infective metacyclic trypomastigotes (Exp. Parasitol.,73: 44, 1991). These proteinases partition into the detergent phase following TX-114 extraction, indicating their association with membranes of the insect stages of the parasite. Analysis of zymograms obtained from extracts of different strains of *T.cruzi* indicate that these metalloproteinases are highly polymorphic. Polyclonal antisera against the major *Leishmania sp.* metalloproteinase (GP63), that cross reacts with different genera of trypanosomatids, did not cross-react with the *T.cruzi* enzymes. Two-dimensional SDS-PAGE of the 52kDa enzyme showed that it displays isoelectric points ranging from 4.6 to 5.4. Interestingly, two distinct 52kDa metalloproteinases were observed, with optimal pH activities at 5.5 and 10.0, respectively.

Support TDR-WHO, PADCT, CNPq, PAPES-FIOCRUZ

S 405 **ACTIVATION OF THE HIV-1 PROTEASE IN PRECUROR FORMS OF THE ENZYME**
C.A. Carter and G. Zybarth, Dept. of Microbiology, S.U.N.Y, Stony Brook, N. Y. 11794.

Retroviral proteases (PR) are synthesized as part of polyprotein precursors. They are released ansd function as small homodimeric enzymes. A critical factor in the assembly of infectious virions is the activation of this virally-encoded PR and its maturation from the precursor. To examine the role of domains in the precursor on the autoprocessing activity of PR, PR precursors with N-terminal extensions of increasing length were constructed and the efficiency of PR maturation *in vitro* was determined by measuring the appearance of mature 11 kDa PR. Large differences in the efficiency of maturation among the various extended forms were observed. Experiments using a genetic assay to test for dimerization capability of mature and extended forms of PR suggested that domains in the extended PR influenced the ability of PR to dimerize and would thereby influence autoprocessing activity. The results indicate that Gag and Pol domains in processing intermediates play an important role in PR maturation and in addition, suggest that the location of the initial cleavage event may be an important determinant of the efficiency of viral maturation and infectivity.

S 406 BIOCHEMICAL CHARACTERIZATION OF HIV-1 PROTEASE MUTANTS RESISTANT TO A-77003, A-80987,

Chih-Ming Chen, Ayda Saldivar, Mary Turon, Norman E. Wideburg, Terry Robins, Sudthida Vasavanonda, Kent Stewart, Chang Park, Xiangpeng Kong, Dale J. Kempf, David Ho[1], John Erickson[2] And Daniel W. Norbeck, Pharmaceutical Products Division, Abbott Laboratories, Abbott Park, IL 60064; [1]Aaron Diamond AIDS Research Center, N.Y10016; and [2]Structural Biochemistry Program, Frederick Biomedical Supercomputing Center, National Cancer Institute, Frederick, MD 21702

Studies of mutant proteins have played an important role in elucidating the function of biological systems. In the case of HIV-1 protease, site specific mutation of the catalytic aspartate residue demonstrates its essential role in the maturation of HIV virus and as the target of anti viral intervention of AIDS through the design of specific inhibitors. On the other hand, study of the enzyme-inhibitor interaction at molecular level , especially of inhibitor-selected mutants, may help one delineate the importance of various functions and guide future improvement of inhibitor design to overcome the potential problem of drug resistance.

Here we report the characterization of HIV-1 protease mutations selected by its inhibitors A77003 and A80987. HIV-1 variants with decrease sensitivity to A77003 or A80987 have been generated in tissue culture systems and shown to contain mutations at arginine 8, valine 32, methionine 46, isoleucine 47, and valine 82. Recombinant proteins containing various single as well as double mutations were generated by standard techniques. Kinetic parameters of purified enzymes were determined using an artificial, fluorogenic substrate-based assay. Determination of binding constants (K_i) for each enzyme against these inhibitors and other related compounds showed potency reductions ranging from 1 to 93 fold compared to wild type. The recombinant protease containing methionine 46 mutation, although found in all three HIV-1 resistant viruses, showed no significant difference in both enzymatic activity and its inhibitor potency. The possible role of each residue in inhibitor binding will be discussed based on X-ray crystal structures of protease/inhibitor complexes.

S 407 STRUCTURE-BASED INHIBITOR SCREENING AND DESIGN FOR PARASITIC PROTEASES,

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A major requirement in the application of structure-based drug design and discovery is the availability of a high resolution structure of the target protein [Kuntz (1992) *Science* 257, 1078]. One approach to overcome this limitation is to obtain such a structure by computer modeling based on the known structures of homologous proteins. We have successfully tested this approach. Potential inhibitors against important proteases in the schistosome and malaria were identifed computationally, based on our model-built structure, and were comfirmed experimentally for their high affinity for targeted proteases ($K_i < 10$ μM for three best inhibitors) [Ring, et al. (1993) *Proc. Natl. Acad. Sci.* 90, 3583]. Moreover, given the low resolution of current search algorithms, it is encourging that such an approach does show the ability to distinguish the subtle structural difference between two homologous proteins. Further work is under way to improve upon the identified lead compounds for better parasitic protease inhibition.

S 408 MUTATIONS IN HIV-1 PROTEASE RESIDUES AFFECTING SUSCEPTIBILITY TO L-735,524.

Jon H. Condra*, Olga M. Blahy, Bruce L. Bush† ‡, Chris Culberson†, Leah Gotlib, Donald J. Graham, Pia L. Graham, M. Katharine Holloway†, Robert L. LaFemina, Julio C. Quintero, Audrey Rhodes, Elizabeth Roth, Vinod V. Sardana, Christine L. Schneider, Abner J. Schlabach, William A. Schleif, Donna L. Titus, Bohdan S. Wolanski, Jill A. Wolfgang and Emilio A. Emini. Departments of Antiviral Research and † Molecular Systems, Merck Research Laboratories, West Point, PA 19486 and ‡Rahway, NJ 07065.

The aspartyl protease of HIV-1 catalyzes the cleavage of the viral polyprotein, is essential for viral infectivity and is, therefore, an attractive target for antiviral intervention. L-735,524 is a potent and selective competitive inhibitor of the enzyme and is currently undergoing clinical evaluation. However, the long-term efficacy of antiviral therapies is often compromised by the emergence of resistant viral variants.

In an effort to assess the potential for clinical resistance to L-735,524, we have attempted to derive resistant mutant enzymes and viruses *in vitro*. Guided by molecular modeling, amino acid substitutions were introduced at specific residues in and around the active site of the protease.

As assessed by a bacterial colony color screen and direct Ki measurements of purified bacterially expressed mutant enzymes, several L-735,524-resistant mutants were identified. While Ki values for L-735,524 were increased in these mutants, Km values for their peptide substrate were also elevated. However, introduction of the responsible mutations into proviral clones yielded virus variants that were fully susceptible to L-735,524, although some exhibited resistance to other inhibitors. Attempts at direct cell culture selection for virus variants resistant to L-735,524 have also been unsuccessful. Hence, mutations conferring resistance to this inhibitor do not appear readily *in vitro*.

S 409 CATHEPSIN L2 SECRETED BY THE PARASITIC TREMATODE *Fasciola hepatica* CLEAVES FIBRINOGEN IN A NOVEL MANNER THAT PRODUCES A FIBRIN CLOT.

John P. Dalton, Sharon McGonigle and Andrew J. Dowd, School of Biological Sciences, Dublin City University, Republic of Ireland.

Fasciolia hepatica (liver fluke) is a parasitic trematode of mammals. The disease is primarily one of agricultural animals such as sheep and cattle but in certain regions it is also a important human pathogen. The parasite, following ingestion by the host, burrows through the gut wall and migrates to the liver where it causes extensive damage before moving into the bile ducts. The parasite is a blood feeder, even in the bile ducts, where it can scour the duct walls to gain access to the circulation.

We have purified two cysteine proteinases from medium in which mature liver fluke parasites were cultured. Although both enzymes have been characterised as cathepsin L-like proteinases (CL1 and CL2) they can be distinguished by their substrate specificites. For example, the substrates Boc-val-pro-arg-AMC, Tos-gly-pro-arg-AMC and Tos-gly-pro-lys-AMC are cleaved only by cathepsin L2.

Whilst searching for macromolecules that are cleaved by these two enzymes we discovered that when cathepsin L2 was mixed with a solution of fibrinogen a clot was formed. Several data indicate that the cathepsin L2-produced clot is formed in a manner that differs to that of a thrombin-produced clot. These data include **a**. the clotting abilty of cathepsin L2 is not inhibited by hirudin and is not prevented by the anti-polymerant H-gly-pro-arg-pro-OH. **b**. electroporetic analysis of the cathepsin L- and thrombin-produced clots under reducing conditions reveal a different profile; the profile obtained for the cathepsin L-produced clot revealed the appearance of polypeptides of high molecular size (125 & 100 kDa) with a concurrent disappearance of the fibrinogen α and β-chains. This observation may suggest a covalent crosslinking. **c**. cathepsin L can cause the clotting of fibrinogen that has been cleaved by thrombin to remove the fibrinopeptides but prevented from clotting by the addition of H-gly-pro-arg-pro-OH.

S 410 INACTIVATION OF A HYBRID TRANSACTIVATOR (GAL4::PR::VP16) BY THE FUSED HIV-1 PROTEINASE: A SIMPLE MAMMALIAN CELL BASED ASSAY FOR INHIBITORS OF THE VIRAL ENZYME ACTIVITY.

Bimalendu Das Mahapatra, Michael G. Murray and Jerome Schwartz

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The human immunodeficiency virus type 1 (HIV-1) proteinase (PR) and its flanking sequences have been fused in frame between the DNA-binding domain of the GAL4 protein of yeast and the transcription-activation domain of the VP16 of Herpes virus (GAL4::PR::VP16). The autocatalytic activity of PR in the hybrid protein physically separates the DNA binding domain from the transactivating domain, abolishing transcription of a reporter gene, however specific inhibition of the PR activity allows the GAL4::PR::VP16 fusion protein to activate transcription of the reporter gene. This provides a simple assay to monitor the HIV-1 PR activity within mammalian cells. A dose-dependent effect of a potent PR-specific inhibitor is demonstrated in this system and illustrates the sensitivity of the assay. The assay is used as a primary, high throughput screen to identify novel inhibitors of the viral PR. In order to measure inhibition of PR activity in this assay the macromolecular synthetic processes of transcription, translation, and secretion of the reporter gene product must be functional, therefore, inhibitors of these processes score negative in this screen. Moreover, the method can be used to generate and analyze mutants and revertants of this important clinical target.

S 411 IDENTIFICATION OF THE SPECIFIC SERINE RESIDUE AT THE ACTIVE SITE OF THE HERPES SIMPLEX VIRUS TYPE 1 PROTEASE, C.L. DiIanni,*+ J.T. Stevens+, M. Bolgar^, D.R. O'Boyle II+, S.P. Weinheimer+ and R.J. Colonno,+ +Virology Department, ^Analytical Research and Development, Bristol-Myers Squibb PRI, Princeton, NJ 08543
Herpes simplex virus type 1 (HSV-1) protease is responsible for proteolytic processing of itself and of the nucleocapsid associated protein, ICP35 (infected cell protein 35) [Liu and Roizman (1991) *J. Virol.* **65**, 5149-5156]. A temperature sensitive mutation in the gene encoding the protease affects processing of ICP35 and results in failure of nucleocapsids to package DNA. Inhibitor studies indicated that the HSV-1 protease is sensitive to the serine protease inactivator, diisopropyl fluorophosphate (DFP), and therefore, was investigated further. The irreversible inactivation by DFP is dependent on time and concentration of DFP. Saturating amounts of a peptide substrate protected the HSV-1 protease from the inactivation by DFP whereas a peptide which is not a substrate did not protect against the inactivation indicating that the modification occurs at the active site. Loss of activity correlates linearly with the incorporation of $[^3H]$ DFP and extrapolation to zero activity indicated that 0.7 $[^3H]$ DFP molecules reacted per active site. Analysis of inactivated protease by mass spectrometry indicated a stoichiometry of 1 DFP per molecule protease. In order to identify the specific residue modified by DFP, the protease was labeled with $[^3H]$ DFP and subsequently digested with chymotrypsin. The resulting peptides were separated by reverse phase HPLC and 80 % of the radioactivity was contained in one peptide. Sequencing analysis by mass spectrometry identified the active site serine as the residue modified by DFP. This residue and the region in which it is found is highly conserved among the herpes viral proteases. This data demonstrates that the HSV-1 protease is a serine protease.

S 412 RATIONAL DESIGN OF POTENT, BIOAVAILABLE NONPEPTIDE CYCLIC UREAS AS HIV PROTEASE INHIBITORS. Susan Erickson-Viitanen, Patrick Y.-S. Lam, Prabhakar K. Jadhav, Charles J. Eyermann, C. Nick Hodge, Yu Ru, Lee T. Bacheler, James L. Meek, Michael J. Otto, Marlene M. Rayner, Y. Nancy Wong, Chong-Hwan Chang, Patricia C. Weber, David A. Jackson and Thomas R. Sharpe. Departments of Virology Research and Chemical and Physical Sciences, The DuPont Merck Pharmaceutical Company, Wilmington, Delaware, 19880-0400.
The HIV protease represents an attractive target for the utilization of computer-aided drug design to discover therapeutics for treatment of HIV diseases. A novel series of cyclic urea compounds have been designed which are potent inhibitors of the HIV protease and of HIV replication using published HIV-1 protease-inhibitor X-ray crystal structures, 3D database searching, and structure-based design methods. A fundamental design feature of these inhibitors is that the cyclic urea's carbonyl oxygen mimics the hydrogen-bonding features of a key structural water molecule observed in published X-ray crystal structures. The success of the design was confirmed by x-ray crystallographic studies which clearly show these cyclic inhibitors link the protein active site aspartic acid carboxylates to the flexible protease flaps via a hydrogen bond network that displaces the water molecule. The unique features of the design permit synthesis of high affinity and selective inhibitors of relatively low molecular weight and, as a result, improved oral bioavailability.

S 413 ISOLATION OF CYSTEINE PROTEINASE GENES FROM NEMATODES, **Stephen A. Harrop[1,2], Paul Prociv[2], and Paul J. Brindley[1]**. [1]Queensland Institute of Medical Research, Brisbane, Queensland 4029, and [2]Department of Parasitology, University of Queensland, St. Lucia, 4072 Australia.

In order to characterize the proteinase genes of parasitic nematodes, Sakanari *et al.* 1989 (*Proc. Natl. Acad. Sci. USA* 86, 4863) employed a series of consensus oligonucleotide primers based on conserved amino acid sequence domains of eukaryotic serine and cysteinyl protease genes in the polymerase chain reaction (PCR) with a variety of nematode genomic DNAs as templates. Notwithstanding the success obtained with these kinds of primers with parasitic pathogens in a number of laboratories, the primers are exceedingly degenerate (greater than 4000-fold) which has resulted in amplification of spurious target sequences in many PCR experiments.

Recently, we derived a set of primers exhibiting marked reduction in degeneracy (only 8 and 32-fold) to more readily isolate nematode cysteine proteinase genes. The primers were designed to anneal to the active site coding regions, as were the Sakanari *et al.* primers, but we biased our primer design toward the nucleotide sequence and codon usage of published cysteine proteinases of *Caenorhabditis elegans*, *Haemonchus contortus* and *Ostertagia ostertagi*. Using these primers and genomic DNA or cDNA as templates, we amplified PCR products from the parasitic nematodes *Strongyloides stercoralis* and *Ancylostoma caninum*, and from *C. elegans* (GenBank accession numbers L22306, U02611, and L22447 respectively).

Cloned sequences of the PCR products show that we have isolated a 350 bp fragment from *S. stercoralis* which bears the deduced amino acid active site residues cysteine and asparagine and amino acid transition motifs predicted for cysteine proteinases. This may be the first reported protein-encoding sequence from this important human pathogen. A 593 bp cDNA *A. caninum* fragment contains the cysteine protease catalytic triad, and has ≈60% homology to numerous cathepsin Bs. In addition, we have cloned a 583 bp fragment from *C. elegans* which contains an intron and all three active site residues for cysteine proteinases (C,H,N), and which exhibits 61% homology to a recently described *C. elegans* gut-specific cathepsin B, *gcp*-1 (Celeste & McKerrow 1992 *Mol. Biochem. Parasitol.* 52, 239). This gene may encode a protease previously shown to decline with age in *C. elegans* (Sarkis *et al.* 1988 *Arch. Biochem. Biophys.* 21,80).

Our primers should be of value in isolating other cathepsin and protease genes from nematodes, and may find utility with isolating proteinase genes of non-nematode parasitic helminths, including the human schistosomes.

S 414 CHARACTERIZATION OF RECOMBINANT PROTEASE FROM HUMAN CYTOMEGALOVIRUS,
Barry Holwerda, Art Wittwer, Linda Carr, Roger Wiegand, Mihaly Toth, Christine Smith, Kevin Duffin and Martin Bryant, Searle Infectious Diseases Research and Monsanto Corporate Research, St. Louis, MO 63198
The UL80 open reading frame of human cytomegalovirus (HCMV) encodes a protease and the capsid assembly protein. The products of this complex open reading frame have been studied during natural virus infection and by expression in *E. coli* and baculovirus-infected insect cells. The 80-kD initial translation product was autocatalytically processed to yield a mixture of 34-kD, 20-kD and 14-kD fragments from the N-terminal protease domain and 55-kD and 39-kD fragments from the C-terminal capsid assembly protein domain in the recombinant systems. Sequence analysis of these products indicated that HCMV protease recognized and cleaved the sequences VxA/S and VxA/A. The 34-kD protease was the result of proteolytic release from the 80-kD polyprotein whereas the 20-kD and 14-kD fragments resulted from internal processing of the 34-kD protease. The 55-kD and 39-kD fragments were the products remaining after release of the protease from the 80-kD precursor. The two-chain (20+14 kD) and single-chain (34 kD) forms of the enzyme retained hydrolytic activity against purified capsid assembly protein and peptides possessing the protease recognition sequence, VxA/S. The two fragments that comprise the two-chain form of the enzyme are not associated by interchain sulfhydryl bonds and both forms of the enzyme are inhibited by the sulfhydryl reagent p-hydroxymercuribenzoate. Pulse-labelling experiments showed that autoproteolytic processing occurs rapidly in the recombinant systems in comparison to markedly slower release and internal processing in HCMV-infected fibroblasts.

S 415 IN VITRO TRANSLATION OF CATHEPSINE D-LIKE PROTEASE OF PLASMODIUM FALCIPARUM,
Ginette Jaureguiberry, Nipa Rujithamkul, Isabelle Hatin, INSERM U13 190 Bld McDonald 75944 Paris Cedex 19.France.
Native electrophoresis followed by imprint digest method using human hemoglobin as substrate allowed the detection of a strong parasite hemoglobinase activity at acidic pH (3.9 to 5). This protease, with a 55 kDa molecular weight, closely ressembles Cathepsin D in terms of its substrate specificity, pH dependance and inhibitor sensitivity.
The transcription of *Plasmodium falciparum* gene encoding for the protein Cathepsin D like has been studied in the parasite's asexual growth stage. For this purpose poly(A+) RNA was isolated from in vitro culture of *P.falciparum*. The presence of mRNA encoding cathepsin D was tested by rabbit reticulocyte in vitro translation and subsequent analysis of the translation products by immunoblotting assay with a polyclonal antiserum raised in rabbit against bovine cathepsin D. The results indicated efficient translation of *P.falciparum* mRNA indicating that the mRNA preparation contained the gene encoding for the cathepsin D as translation product. Two bands were detected by immunoblotting, 83 kDa and 68 kDa which could be two isozymes of *P.falciparum* Cathepsin D. In addition the Northern hybridization analysis with a probe obtained by PCR using a DNA Vectorette library and Cathepsin D primer showed two transcripts of 2.8kb and 1.9kb compatible to be encoding for this two cathepsin D isozymes.

S 416 X-RAY CRYSTALLOGRAPHIC STUDIES OF HIV-1 PROTEASE COMPLEXED WITH A NOVEL SERIES OF PENICILLIN-DERIVED INHIBITORS,
H.Jhoti, A. Mistry, O. Singh and A. Wonacott. Protein Structure Group, Glaxo Group Research, Greenford Rd, Greenford, Middlx UB6 0HE, UK

The inactivation of the HIV-1 protease has been identified as a mechanism of intervening in the formation of infectious progeny virus. Thus, the HIV-1 protease is regarded as a target in the development of drugs for the treatment of AIDS. Using a high-throughput enzyme-based screen a novel penicillin derived C2-symmetric compound was discovered which led to a series of potent and selective inhibitors of HIV-1 protease. X-ray studies were initiated to experimentally determine the mode of binding of these inhibitors to HIV-1 protease.

The structures of HIV-1 protease complexed with 6 different penicillin derived inhibitors, 3 symmetric (GR116624, GR122505,GR127370) and 3 asymmetric, (GR123976,GR126045,GR137615) were solved using molecular replacement and difference Fourier techniques. Each complex was refined using X-PLOR and PROLSQ and the active site analysed. A comparison of the structures showed how the active site was able to accomodate different groups in the P and P' pockets and, thus, the molecular basis of recognition by HIV-1 protease of this novel series of non-peptidic inhibitors was established.

These crystallographic studies, together with molecular modelling and NMR studies, provided information for on-going synthetic chemistry to optimise potency of this series of inhibitors and resulted in compounds with IC_{50} against HIV1- protease in the nanomolar range. Studies are in progress to assess the potential of these novel compounds as chemotherapeutic agents for the treatment of AIDS.

S 417 CLEAVAGE OF HUMAN SERPINS BY HIV-1 PROTEINASE,
Alexei F. Kisselev, Reinhardt Mentele, Andreas Schulze and Klaus von der Helm, Max-von-Pettenkofer-Institut & Max-Planck-Institut für Biochemie, Munich, Germany
Purified recombinant proteinase of HIV-1 cleaves the human serpins, $alpha_1$-proteinase inhibitor (a_1PI) and antithrombin III (ATIII). Other serpins, such as $alpha_1$-antichymotrypsin and chicken ovalbumin, are not cleaved. Wild type a_1PI is cleaved at the Met351-Phe352 and Phe352-Leu353 residues (part of the reactive site loop), resulting in the complete inactivation of the inhibitor function. The [M351-E, M358-R] mutant of a_1PI is cleaved only at the corresponding Phe-Leu residues. ATIII is cleaved at the Phe37-Tyr38, Phe60-Ala61 and Phe337-Leu338 (a_1PI numdering) residues, which are not part of the reactive site loop.
A possible biological meaning of these processes will be discussed.
The cleavage of these serpins by other aspartic proteinases such as pepsin A and HTLV-1 proteinase is under investigation.

S 418 THE USE OF CYSTEINE PROTEINASE INHIBITORS AS A POSSIBLE ANTISCHISTOSOMAL APPROACH,

Mo Klinkert, Livia Pica-Mattocia, Donato Cioli and Elliott Shaw, Institute of Cell Biology, Consiglio Nazionale delle Ricerche, Rome, Italy and Friedrich-Miescher Institute, Basel, Switzerland.

A "hemoglobin protease" from the human parasite *Schistosoma mansoni* is a cysteine proteinase with substrate and inhibitor specificities similar to cathepsin B. The amino acid sequence of the proteinase was deduced from the nucleotide sequence of a full-length cDNA clone and found to exhibit a high degree of homology to lysosomal cathepsin B of mouse, rat and man. The enzyme is located in the gut of the schistosome and has been postulated to participate in the digestion of host hemoglobin. One way to test the hypothesis that the protease is essential for nutrition is to examine whether inhibition of enzyme activity would deprive the parasite of its source of nutrients and thus have a lethal effect. Based on biochemical data obtained from assays of human cysteine proteinases, we therefore tested a panel of peptidyldiazomethanes against live *S. mansoni* in culture. We found that the presence of inhibitors in the medium caused morphological changes and reduced motility and finally led to death of the parasites. As diazomethanes inactivate not only cathepsin B but also other cysteine proteinases such as cathepsin L and calpain, it is at this stage of our studies not possible to determine whether parasite death is due to inhibition of cathepsin B or another cysteine proteinase or if the effect is synergistic. However, the gut location of cathepsin B favours it as a digestive enzyme. Future studies will be directed at elucidating the precise mechanism of hemoglobin degradation by cysteine proteinase(s) and understanding enzyme-inhibitor interactions, which could lead to the construction of novel agents against schistosomiasis.

S 419 A GENETIC SCREEN FOR PROTEIN/PROTEIN INTERACTION WITHIN THE PERIPLASMIC SPACE OF *E. COLI*

Harald Kolmar, Frank Hennecke, Christian Frisch, Kerstin Bründl and Hans-Joachim Fritz, Institut für Molekulare Genetik der Georg-August-Universität, Grisebachstraße 8, D-37077 Göttingen, F.R.G.

The function of numerous proteins, most notable that of many cell surface receptors or proteases depends on protein/protein contacts mediated by the binding of a ligand or a substrate. We try to mimick such type of interactions with the aim to develop generally applicable schemes of genetic screening and selection for protein/protein and protein/ligand interaction occuring in the *E. coli* periplasmic space. Our recent work has concentrated on the study of homodimer formation and folding stability of the immunoglobulin light chain REI_V[1] located in the *E. coli* periplasm.

We were able to derive a transcriptional signal from the folding state of REI_V by replacing the periplasmic domain of the ToxR receptor protein from *Vibrio cholerae*[2] by REI_V. This fusion protein (ToxR'/REI_V) is anchored in the cytoplasmic membrane by a single transmembrane helix. The cytoplasmic ToxR' domain is an activator of the *V. cholerae ctx* transcription control region, its function being dependent on dimerization mediated by dimer formation of the respective periplasmic domain. REI_V, itself being a homodimer, is able to mediate transcription activation when fused to ToxR'. As a convenient monitor of transcriptional activity, we placed *lacZ* under *ctx* control.

When fusing REI_V derivatives with different conformational stabilities to ToxR', we found different levels of β-galactosidase activity in crude cell extracts which positively correlated with the free energy of dimer formation of the respective REI_V derivatives. Since large numbers of clones can be investigated in parallel, using an ELISA reader, this method already constitutes a genetic screen for protein-protein interaction.

Obviously, the procedure lends itself to generalization and possibly to expansion towards genetic selection. Further work will also concentrate on attempts to derive transcriptional signals from the binding of defined or randomly generated peptide inhibitors to various proteins (*e.g.* proteases, receptor domains) linked to ToxR'.

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S 420 ANALYSIS OF TEMPERATURE-SENSITIVE MUTANTS OF HIV-1 PROTEINASE.

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Morphogenesis of retroviruses involves assembly of the structural Gag and Gag-Pol polyproteins followed by the release of immature non-infectious viral particles from the plasma membrane. Subsequent conformational change of the viral core which is triggered by proteolytic cleavage of the structural polyproteins by the virus encoded, virion associated proteinase (PR) generates the fully infectious, mature virion. Inactivation of PR abolishes viral infectivity whereas premature activation prevents particle assembly and induces rapid cell death suggesting that tight control of PR activation is critical for viral replication.

To study the requirements for PR activation and analyze the cause of PR induced cytotoxic effects, we generated temperature-sensitive mutants of HIV-1 PR. Expression of truncated viral polyproteins in Escherichia coli yielded complete and correct cleavage at the permisive temperature and mostly stable precursor proteins at the non-permissive temperature. The relative activities of PR mutants in terms of autocatalytic cleavage from a precursor and/or cleavage of specific peptide substrates will be discussed. The mutant PR domains were also cloned into a full length proviral plasmid and their effect on polyprotein processing, viral replication and infectivity was analyzed.. One PR mutant showed a ts-effect on viral infectivity with non-infectious virus at the non-permissive temperature and infectious virus, albeit at a reduced titer, at the permissive temperature. This phenotype correlated with a temperature-dependent difference in proteolytic processing of viral polyproteins both in transfected cells and in extracellular particles.

S 421 PROTEASES AND PATHOGENESIS IN MALARIA PARASITES.

Langsley, G. Fricaud, A-C., Barale, J-C., Morales-Betoule, M-E., Lensen, T. and Braun-Breton, C. Unit of Experimental Parasitology, Dep. of Immunology, Institut Pasteur, Paris France.

We have identified two different stage specific serine proteases in malaria parasites. The first is a merozoite specific GPI-anchored protease whose activity depends on its solubilisation by a parasite specific GPI-PLC and whose function appears to be involved in red blood cell invasion (1, 2, 3, 4). Inhibition of the merozoite protease affects only entry and not attachment to erythrocytes, and the potential substrate on the red blood cell surface has been identified as the anion transporter band 3 (2). The second serine protease activity resembles that of urokinase and is specific for gametocytes and gametes. The possible role of a parasite specific plasminogen activator in the development of sexual stage parasites will be discussed as will our current progress in the characterisation of the merozoite protease crucial for erythrocyte invasion.

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S 422 PROTEASES AND COLLAGENASE ACTIVITY DETECTED IN ELECTRON-DENSE GRANULES FROM *Entamoeba histolytica*, Gloria León-Avila, Rosalinda Tovar and María de Lourdes Muñoz, Department of Genetics and Molecular Biology, Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional. Ap. postal 14-740, 07300, México, D.F., MEXICO.

"In vitro" interaction of *Entamoeba histolytica* with collagen type I induces intracellular formation and release of electron-dense granules (EDGs) to the extracellular milium. In order to asses the role of EDGs in the pathogenicity we made a partial characterization. We determined by SDS-PAGE that EDGs contains approximately 13 major polypeptides among them there are proteolytic and collagenase activities. We purified this collagenase from EDGs by FPLC and determined a molecular weight of 102kDa. To define the pattern of the gelatinase activities we incubated the trophozoites and collagen type I for 1, 2, 3, 5, 7 and 10h. Total extracts from trophozoites, EDGs and supernatants showed 10 proteolytic activities by the zymogram technic. Among them a major band of 40kDa was identified in supernatants EDGs and total extracts. In addition EDGs showed a principal band of 62kDa. EDGs treated with agents such acetic acid, Triton X-100 or a mix of this compounds didn't show any modification of these cysteine proteinases. These results indicate that the proteolytic activities contained in the EDGs secreted by *E. histolytica* may participate in the pathogenicity of the parasite.

S 423 PURIFICATION AND CHARACTERIZATION OF A VIRAL ENHANCING PROTEASE, Leslie S. Lepore and Robert R. Granados, Boyce Thompson Institute for Plant Research at Cornell University, Ithaca, NY 14853

Much effort is presently being focused on the use of insect viruses as biopesticides. The primary obstacle to the successful use of these viruses is their slow rate of kill. We are studying a viral enhancing protein ("enhancin") found in a granulosis virus (GV) pathogenic to the lepidopteran, *Trichoplusia ni* (cabbage looper). This protein can increase the susceptibility of larvae to nuclear polyhedrosis virus (NPV) infections and increase the rate of kill (Derksen and Granados (1988) *Virology* **167**, 242-250.; Gallo et al. (1991) *J. Invertebr. Path.* **58**, 203-210.). We believe the primary mode of enhancement to be the proteolysis of the larval peritrophic membrane (PM), a chitin and protein matrix which lines the digestive tract of many insects. Though several viral enhancing proteins have now been identified, many questions remain about the biochemistry and mode of action of these proteins.

We have purified the viral enhancing factor from T. ni GV and correlated the ability to enhance infections with a 104 kD protein. When *T. ni* neonates are co-fed enhancin and *Autographa californica* NPV, only 50 pg of enhancin per larva is necessary to increase the larval mortality from ~ 25% to 100%. Though contaminating proteases have been identified in the enhancin preparation, *in vitro* studies with inhibitors demonstrate that the PM degradation is caused by a metalloprotease while the contaminating proteases are primarily serine proteases. Successive removal of the contaminating proteases did not diminish enhancin's ability to increase larval susceptibility to an NPV infection. The purified enhancin also retained the ability to degrade PM's *in vitro*. Chitinolytic activity has also been identified in the purified enhancin preparation. This activity is not inhibited by typical metalloprotease inhibitors and thus may not be involved in the viral enhancing effect. Future studies are focused on production of enhancin in tissue culture using recombinant baculoviruses and investigation of the possibilty that the active enhancing protein is derived from the 104 kD enhancing protein.

S 424 CRYSTAL STRUCTURE OF HUMAN RHINOVIRUS TYPE 14 3C PROTEASE, D. Matthews, W.W. Smith, R.A. Ferre, B. Condon, G.Budahazi, W. Sisson, J.E. Villafranca, C. Janson, H. McElroy, C. Gribskov and S. Worland, Agouron Pharmaceuticals, San Diego, CA 92121

Human rhinoviruses(HRVs) are members of the picornavirus family of small single-stranded RNA viruses that translate genomic RNA directly into a precursor polyprotein which is co- and post- translationally processed to yield functional viral proteins. Proteolytic cleavages between glutamine and glycine residues necessary to generate capsid and non-structural proteins are carried out by the viral 3C and 3CD proteases. Although picornavirus 3C proteases are thio proteases, recent evidence suggests they may be related structurally to the trypsin-like family of serine proteases. We have crystallized HRV type 14 3C protease and determined its structure using x-ray diffraction methods. The crystal form used in these studies is triclinic with eight HRV molecules in the asymmetric unit arranged with approximate 422 point group symmetry. Each HRV 3C protease molecule consists of 182 residues folded into two topologically equivalent six-stranded beta barrels with the substrate binding site located between the two domains. The overall polypeptide fold is similar to that of trypsin-like serine proteases although the juxtaposition of beta barrels is somewhat different. There are also variations in the lengths of individual beta strands and significant differences in the orientation of loops connecting elements of secondary structure. In contrast to the Ser-His-Asp triad found at the active site of trypsin-like serine proteases, the corresponding catalytically important residues in HRV 3C protease are Cys-146, His-40, and Glu-71.

S 425 CHARACTERIZATION OF A KERATINOLYTIC PROTEASE FROM A STRAIN OF *Bacillus licheniformis*, Eric S. Miller[1], Xiang Lin[2] and Jason C. H. Shih[2], Departments of Microbiology[1] and Poultry Science[2], North Carolina State University, Raleigh, NC 27695

Keratin is generally recalcitrant to proteolysis by trypsin, chymotrypsin, pepsin and common microbial proteases. *Bacillus licheniformis* PWD-1 was isolated from a farm poultry waste digester. We have identified and characterized a secreted protease from *B. licheniformis* PWD-1 that hydrolyzes feather keratin. The purified enzyme is monomeric (33 kDa), and has temperature and pH optima of 50°C and 7.5, respectively. It is more active in keratinolysis than the other proteases examined, and is more hydrolytic for elastin and collagen than some elastases and collagenases. Keratinolysis does not require a reducing agent and is not coupled with the production of sulfhydryls. The enzyme is sensitive to phenylmethylsulfonyl fluoride, suggesting that it is a serine protease. Studies of the *B. licheniformis* PWD-1 "keratinase", and identification of the gene in a genomic library, are in progress. Supported by a grant from the U.S. Department of Agriculture.

S 426 The Serine Protease of the Tissue Penetrating Larval Stage of the Parasitic Nematode *Anisakis simplex*. Stephen R. Morris and Judy A. Sakanari, Department of Pathology, University of California at San Francisco, VAMC 113B, 4150 Clement St., San Francisco, CA 94121.

The nematode *Anisakis simplex* causes anisakiasis by accidental ingestion of the larval stage in raw or undercooked seafood. The pathology is caused by worm penetration of the stomach or intestine followed by visceral larval migration. In the host parasite relationship, proteases have been implicated in tissue penetration, particularly with migratory helminth parasites of the digestive tract. A serine protease which has an apparent molecular weight of 28 kDa has been purified from the larvae of this parasite. Of several substrates tested, the best was the peptide Z-Gly-Pro-Arg-pNA, with a K_m of 70 μM. However, the peptides Z-Phe-Arg and Z-Arg-Arg were not cleaved by the enzyme. This enzyme is irreversibly inhibited by peptide-chloromethyl ketones and PMSF. It is reversibly inhibited by the protein inhibitors soy bean trypsin inhibitor and ecotin, the latter being 1000-fold better than SBTI. It is not inhibited by peptide fluoromethyl ketones, E-64, or 1,10 phenanthroline. An endogenous serine protease inhibitor has also been purified which has an apparent M_r of 21 kDa. It inhibits trypsin activity but not that of chymotrypsin or elastase. Amino acid sequence information obtained from this inhibitor is 80% identical with soy bean trypsin inhibitor.

S 427 Determination of Kinetic Rate Constants for HIV-1 Protease: Binding of Inhibitors and Monomer/Dimer Interconversion, Christopher A. Pargellis[#], Maurice M. Morelock[#], Edward T. Graham[‡], Peter Kinkade[#], Susan Pav[#], Klaus Lubbe[†], Daniel Lamarre[¶], Paul Anderson[§]
Department of [#]Biochemistry, [‡]MIS and [†]Molecular Biology, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road., P. O. Box 368, Ridgefield, CT 06877. Departments of [§]Medicinal Chemistry and [¶]Biochemistry, BioMega / Boehringer Ingelheim Research, Inc., 2100 rue Cunard, Laval, PQ, Canada H7S 2GS.

Transition state analogues of HIV-1 protease were evaluated kinetically. Rate constants for association and dissociaton were determined by a novel method employing paired rate equations. This method is termed the paired progress method and will be presented in detail. During the course of the analysis nonlinearity was observed in control reactions containing enzyme and substrate only. This was subsequently shown to be due to a reversible inactivation process resulting from enzyme dilution. Integrated rate equations were developed based on dissociation of dimeric enzyme during dilution and a reassociation of dilute monomers following the addition of substrate. The equations were modelled to the data generated for this process yielding a dissociation constant of 6.82×10^{-3} sec^{-1} and an association constant of 2.59×10^{5} $M^{-1}sec^{-1}$ corresponding to a K_d of 4.4×10^{-9} M.

S 428 HEMOLYSIS AND HEMAGGLUTINATION IS MEDIATED BY HIGH MOLECULAR MASS CYSTEINE PROTEINASES IN *PORPHYROMONAS GINGIVALIS*, Robert Pike, Jan Potempa, Takahisa Imamura and James Travis, Biochemistry Department, University of Georgia, Athens, GA. 30605.

Porphyromonas gingivalis has been implicated as one of the major pathogenic bacteria involved in the progression of destructive adult periodontitis. It secretes a number of virulence factors, amongst them attachment factors such as the hemagglutinin/s and the "trypsin-like" cysteine proteinases, which are actually a population of arginine and lysine-specific proteinases. The high molecular mass forms of the lysine and arginine-specific proteinases (hereafter KGP and RGP, respectively) are synthesized in a form which includes a domain which is at least partially responsible for hemagglutination. *P. gingivalis* has an absolute requirement for iron, which it usually obtains in the form of heme groups sequestered from erythrocytes hemolysed by the bacteria. The factors mediating the hemolysis of the erythrocytes are unknown, although it has been reported that a cysteine proteinase isolated from the culture medium was able to lyse erythrocytes. In this work it was shown that the hemolysis of erythrocytes by *P. gingivalis* takes place via a process of attachment and then gradual lysis of the cell, possibly induced by proteinase-mediated breakdown of the erythrocyte membrane proteins. The attachment to the erythrocyte was most dependent on the KGP enzyme, although RGP does function in this regard to a certain extent. Lysis of the erythrocytes then appeared to proceed in a manner which could generally be mimicked by purified KGP, although other mechanisms may also function in this regard. This may represent an important mechanism whereby *P. gingivalis* could obtain heme from the erythrocytes in a limited manner, without causing complete lysis, thus conferring a competitive advantage on the organism.

S 429 COMPARISON OF TWO ARGININE-SPECIFIC CYSTEINE PROTEINASES FROM *PORPHYROMONAS GINGIVALIS*, Jan Potempa, Robert Pike and James Travis, Institute of Molecular Biology, Jagiellonian University, Cracow, POLAND, and Department of Biochemistry, University of Georgia, Athens, GA 30602

Proteolytic enzymes produced by the periopathogenic bacterium, *Porophyromonas gingivalis*, have been implicated as important virulence factors in the initiation and progression of periodontal disease. Previous work in our laboratories resulted in the purification of low and high molecular weight forms of an arginine-specific cysteine proteinase, referred to as Arg-gingipain-1 (RGP-1) and high molecular mass Arg-gingipain (HRGP), respectively. In this study, the major variant of the low molecular weight form of Arg-gingipain (RGP-1A) was isolated from *P.gingivalis* (strain H66) culture fluid. Although the enzyme differs from RGP-1 and HRGP in the N-terminal amino acid sequence and the degree of the stimulation of the amidolytic activity by glycyl-glycine, it shares the unusual features of Arg-gingipain as regards a narrow specificity limited to peptide bonds containing arginine residues and resistance to inhibition by known protein proteinase inhibitors. To compare the destructive potential of the different forms of Arg-gingipain, HRGP and RGP-1A were active site titrated and extensively characterized in terms of their enzymatic activity on chromogenic peptide substrates. Their susceptibility to inhibition by synthetic inhibitors of proteolytic enzymes and ability to inactivate and/or degrade some physiologically important proteins e.g. human plasma proteinase inhibitors (α-1-proteinase inhibitor, α-1-antichymotrypsin, antithrombin III, α-2-antiplasmin, α-2-macroglobulin), plasminogen, fibrinogen, cathepsin G, lysozyme and immunoglobulins were also tested.

S 430 AUTOPROTEOLYTIC ACTIVITY OF THE *ENTAMOEBA HISTOLYTICA* 122 KDA ADHESIN,

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The 122 kDa adhesin is a glycoprotein playing a role in the pathogenicity of *Entamoeba histolytica*. Different chromatography methods were performed in order to purify it. The first step was always affinity purification on Agarose con A column. The second step was either ion exchange (DEAE-Sepharose), or selective adsorption (hydroxyapatite), or immunoaffinity (Sepharose binding Monoclonal antibody 122 kDa) column. Two proteins of 65 and 55 kDa were co-eluted with the 122 kDa adhesin.
These same peptids (65 and 55 kDa) were also observed after incubation of 122 kDa gel fragment in diethylamin buffer. The 122 kDa adhesin was found to have protease activity evidenced by its ability to degrade gelatin.
In addition antibodies raised in mice against these two products do recognize the 122 kDa adhesin.
Our interpretation to these results is that the 65 and 55 kDa fraction could likely be degradation of the 122 kDa adhesin.

KEY WORDS: *Entamoeba histolytica*, 122 kDa adhesin, chromatography, autoproteolysis.

S 431 Biosynthesis, Purification and Biochemical Characterization of a Histidine-linked MoMLV Protease;

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The replication of Moloney murine leukemia virus (MoMLV) relies, like that of other retroviruses, on the correct processing of two viral precursor proteins by a virally encoded protease. Until today this protease was only available in small amounts, purified from viral particles. Therefore, we constructed the gene encoding the MoMLV-protease by synthesizing six overlapping oligonucleotides which were inserted in a bacterial expression vector adding three histidine codons to the 5'- and 3'-end of the protease sequence. The expressed histidine-linked MoMLV protease could be purified from bacterial lysates by convenient one-step affinity chromatography on a metal chelate column under denaturing conditions, yielding high amounts of his-MoMLV-protease. The denatured protein was refolded by dialysis and displayed proteolytic activity. Using a peptide substrate the specific activity of the his-MoMLV protease was determined to be 334 nmol/min/mg. A narrow pH optimum was determined at pH 6. The specificity of the enzyme was demonstrated by the cleavage of two peptide substrates mimicking the cleavage sites in the MoMLV precursor protein $Pr65^{gag}$. The substrate specificity of the enzyme differed from that of a histidine-linked HIV-1 protease expressed and purified under the same conditions. Substantial differences between these two proteases were also observed in respect of specific activity and the pH dependence. These data demonstrate for the first time the bacterial expression of a MoMLV protease and the biochemical characteristics of the purified enzyme.

S 432 SYNTHESIS AND ENZYMATIC EVALUATION OF N-PROTECTED PHENYLALANINE ANALOGS AS INHIBITORS OF THE HIV-1 PROTEASE.

Gilles Sauvé, Abdallah Ezzitouni, Jocelyn Yelle, Dorin Dolejan, Benoit Ochietti, and Jeannot Lettre, Institut Armand-Frappier, 531 boul. des Prairies, Laval, P.Q. Canada H7N 4Z3

The HIV-1 protease plays a crucial role in the replicative cycle of the virus. Inhibition of this viral enzyme leads to the production of immature, uninfectious viral particles, thus limiting progression of the infection. Several inhibitors have been described in the literature however their size (2-4 amino acids) and their peptidic nature represent major drawbacks responsible for their low bioavailability and their instability *in vivo*. In order to obtain small molecules devoid of peptidic bonds, we have synthesized N-protected phenylalanine analogs carrying in C-terminal position different hydrophilic functions (e.g. diol, diketone, hydroxyketone, epoxide, etc.) adjacent to a hydrophobic group (e.g. pentafluorophenyl). Synthesis of the analogs and evaluation of their activity will be presented.

S 433 MAPPING THE S' SUBSITE SPECIFICITY OF α-LYTIC PROTEASE AND CERCARIAL PROTEASE FROM SCHISTOSOMA MANSONI REVEALS S'-P' CONTACTS BEYOND THE S'_3 SUBSITE,

Volker Schellenberger, Chris W. Turck and William J. Rutter, Hormone Research Institute, Box 0534, University of California, San Francisco, CA 94143

The S' subsite specificity of α-lytic protease and cercarial protease was studied using a large number of peptide-derived nucleophiles of the structure H-Xaa-Ala-Ala-Ala-Ala-NH_2, H-Ala-Xaa-Ala-Ala-Ala-NH_2, and H-Ala-Ala-Xaa-Ala-Ala-NH_2 where Xaa is D-Ala, Cit, and all natural amino acids except Cys. The variable residues of these nucleophiles occupy the P'_1-, P'_2-, and P'_3-positions during acyl transfer. Mixtures of the nucleophiles were analyzed in the acyl transfer reactions and second-order rate constants were calculated based on the theory of multiple substrate kinetics (Schellenberger, V., Turck, C., Hedstrom, L., & Rutter, W. (1993) Biochemistry 32, 4349-4353). The P'_1 and P'_2 subsites of α-lytic protease and cercarial protease exhibited a significant degree of specificity, and variations in the measured second-order rate constants by up to two orders of magnitude were observed. Both enzymes exhibited very little specificity towards the P'_3 side chains, but the efficiency of nucleophiles with P'_3 D-Ala or Gly was significantly reduced. This suggests the existence of enzyme-nucleophile contacts beyond the S'_3 subsite. In order to study the number of functional S' subsites we analyzed the efficiency of nucleophiles with the structure H-Ala_n-NH_2 and H-Ala_n-OH with n between 1 and 5. The resulting data indicate two S' subsites for chymotrypsin, four S' subsites for α-lytic protease and at least five S' subsites for cercarial protease.

S 434 TRACKING DOWN THE SITE OF THE HEPATITIS A VIRUS PRIMARY CLEAVAGE WITH A RECOMBINANT 3C PROTEINASE

Tina Schultheiß*, Yuri Kusov+ and Verena Gauss-Müller*, Institute for Medical Molecularbiology, Medical University of Lübeck, 2400 Lübeck, Germany,+Pres. address: Institute for Clinical Microbiology and Immunology, St.Gallen, Switzerland

Hepatitis A virus (HAV) is distinguished from other members of the picornavirus family by an extremely protracted course of infection without cytopathic effect on most cells and extremely high thermic stability of the mature virus particle. From the alignment of the HAV amino acid sequence with those of other picornaviruses it can be inferred that proteolytic cleavage of the primary translation product is controlled in a different way as only 3C but not 2A is a proteinase.
Our work is concerned with the role of hepatitis A virus proteinase 3C which was expressed in bacteria as a mature enzyme and purified by ion exchange chromatography. Intermolecular cleavage activity was demonstrated on substrates generated by DNA-directed *in vitro* translation (TNT) and compared to intramolecular cleavage. The identification of cleavage products by immunoprecipitation suggests that cleavages at the P1/P2 and P2/P3 junctions are catalyzed by 3C and occur instantly as well as almost simultaneously. The data also suggest that the extent of processing at various sites within the polyprotein is dependent on the concentration of proteinase 3C. Cleavage at the P1-P2 site seems to be regulated by the context of the site.
cis and *trans* cleavage yied the same products.
These data support the notion that HAV 3C has an activity profile similar to that of cardiovirus.

S 435 EXPRESSION OF A CYTOTOXIC PICORNAVIRAL PROTEINASE IN EUKARYOTIC CELLS,

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Proteinase 2A of entero- and rhinoviruses (both members of picornaviridae) is responsible for the essential primary cleavage of the nascent viral polyprotein by separating structural from nonstructural protein precursors. Further processing of the polyprotein is carried out by a second virally encoded proteinase 3C or 3CD respectively. 2A is also involved in the shutoff of host cell protein synthesis by cleaving indirectly or directly the eukaryotic translation initiation factor eIF-4γ (p220). As a result, cap-dependent translation is turned off in the infected cell; picornaviral RNAs initiate internally and are unaffected. In order to characterize the role of 2A during host cell shutoff, to identify cellular targets of proteinase 2A and to distinguish between 2A and 3C cleavage events a transient and a permanent expression system for proteinase 2A was established. Proteinase 2A of human rhinovirus serotype 2 (HRV2 2A) was expressed transiently by "transferinfection" utilizing a variety of expression vectors. According to the strength of the promoter used a gradual cytotoxic effect was observed. As an approach for permanent expression a tetracyclin inducible system was employed. Based on the selective expression of 2A effects on the host cell can be studied in the absence of 3C or any other viral protein.

S 436 NOVEL CHYMOTRPYSIN-LIKE PROTEASES OF *STREPTOMYCES GRISEUS*,

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This report describes the discovery of two novel prokaryotic members of the chymotrypsin superfamily of serine proteases which are, despite similarity in sequence and activity to other family members, remarkably distinct in aspects of structure. One novel enzyme, *Streptomyces griseus* Protease C (SGPC) has acquired a carboxy-terminal chitin-binding domain implicating it in the degradation of structures associated with chitin. The second enzyme *Streptomyces griseus* Protease D (SGPD) has acquired an amino-terminal leader which is atypical of prokaryotic secretion signals and has homology to mitochondrial import signals. The mature form of recombinantly expressed SGPD is a stable dimer that demonstrates an inability to cross cell membranes post-translationally. These observations suggest that SGPD is not secreted, rather it is targeted to a subcellular location or vesicle.

S 437 PURIFICATION AND KINETIC CHARACTERIZATION OF HUMAN CYTOMEGALOVIRUS ASSEMBLIN CONTAINING A CHELATING PEPTIDE PURIFICATION HANDLE,

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We report the expression and facile preparation of pure HCMV assemblin with the aid of a chelating peptide purification handle. Chelating peptide-immobilized metal ion affinity chromatography (CP-IMAC) uses an engineered metal binding site, or chelating peptide (CP) at either the N-terminus or C-terminus of a recombinant protein for a one-step affinity purification using IMAC. These CP sequences are easily incorporated into the protein with recombinant DNA cloning techniques. The kinetic characterization of CP-assemblin was carried out using an HPLC assay to measure the hydrolysis of a peptide that mimics the maturational cleavage site of the assembly protein precursor (pAP). A non-linear regression analysis was used to determine the K_m and V_{max}. These data and the purified enzyme were used to develop a high throughput assay to screen for inhibitors.

S 438 THE INHIBITORS AND ACTIVATORS OF ENZYMES FROM STR.RESIFENSIS VAR.LYTICUS 2435,
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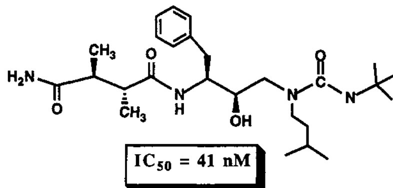
The lytic enzymes from Str.resifensis var.lyticus 2435 strain are able to produce substances which regulate enzyme activity.
The method of purification for inhibitors and activators was as following : aceton precipitation, ion-exchange chromatography on the column with CM-sephadex, affinity chromatography on the BrCN-activated sepharose column (trypsin was the ligand).
Four secured fractions have reduced commercial trypsin activity by 80-100%. The inhibitor activity was completely suppressed by three chloral acetic acid within 10 min. So we made a suggestion about protein structure of inhibitors. 0.1 N HCL and 0.1 N NaOH slightly depressed effect of inhibition.
Two other fractions were combined with ligands and they increased trypsin activity in 2.4-7.5 times. 5% three chloral acetic acid, 0.1 N HCL, 0.1 N NaOH and urea hasn't any influence on the activators activity. The inhibitors and activators possess high thermostability.
Probably these fractions could play an important role in the producer cell cycle.Trypsin inhibitor was collected in the cultural supernatant of Str.resifensis var.lyticus 2435 during the first hour of production.
We suppose,that inhibitors could defence young cells. One may notice the accumulation of activators in the stable state of the producer.

S 439 2A PROTEINASES OF COXSACKIE- AND RHINOVIRUS CLEAVE PEPTIDES DERIVED FROM eIF-4γ VIA A COMMON RECOGNITION MOTIF, W. Sommergruber, H. Ahorn, H. Klump, J. Seipelt, D. Blaas, E. Kuechler, H.-D. Liebig and T. Skern, Department of Cell Biology, BENDER+CO Ges mbH; Ernst Boehringer Institut fuer Arzneimittelforschung, Dr. Boehringergasse 5-11, A-1120 Vienna, AUSTRIA.
The 2A proteinases of coxsackieviruses (some serotypes of which are implicated in heart disease), poliovirus, and human rhinovirus (the main causative agents of the common cold) all belonging to the picornaviridae specifically cleave eukaryotic translation initiation factor eIF-4γ (p220) during viral life cycle. As a result, the infected cell is unable to translate capped mRNAs; picornaviral RNAs initiate internally and are unaffected. This cleavage was proposed to involve a quiescent cellular proteinase activated by 2A proteinases; however, evidence using purified components argues for a direct interaction. An essential prerequisite for such an interaction is the cleavage of an oligopeptide derived from the eIF-4γ cleavage site by each of the viral 2A proteinases. The cleavage specificities of the 2A proteinases from coxsackievirus B4 (CVB4) and human rhinovirus 2 (HRV2) on oligopeptide substrates have been determined. Comparison of the specificity of CVB4 2A proteinase with that of HRV2 2A proteinase allowed cleavable peptides to be designed using the common motif Ile/Leu-X-Thr-X * Gly. The data also allowed the prediction of three possible cleavage sites for 2A proteinases on eIF-4γ ; two peptides derived from these sequences were cleaved by both 2A proteinases. One of these peptides corresponds to the cleavage site for 2A proteinases mapped on eIF-4γ. This supports the hypothesis that cleavage of eIF-4γ by picornaviral 2A proteinases occurs directly.

S 440 **STRUCTURE-BASED DESIGN OF A NOVEL DIMETHYL SUCCINAMIDE HIV PROTEASE INHIBITOR WITH ANTIVIRAL ACTIVITY.** **William C. Stallings*, Huey-Sheng Shieh*, Roderick A. Stegeman*, Deborah E. Bertenshaw*, Gary A. DeCrescenzo*, Daniel P. Getman*, Robert M. Heintz*. John J. Talley*, Mark E. Gustafson*, Kurt D. Junger*, Martin L. Bryant#, Michael Clare#, Kathryn A. Houseman# Richard A. Mueller#. *Monsanto Corporate Research/Searle, 700 Chesterfield Village Parkway North, St. Louis, Missouri 63198 U.S.A. & #Searle Discovery Research, 4901 Searle Parkway, Skokie, Illinois 60077 U.S.A.**

Human immunodeficiency virus-1 (HIV-1), the causative agent of AIDS, encodes in its RNA genome the sequence of a unique aspartyl protease which is critical to the life cycle of the virus. Inhibition of this enzyme could arrest the replication of the virus in an infected individual with AIDS. The structure of the complex of HIV-1 protease with a representative of a new class of succinamide inhibitors has been determined by x-ray diffraction at 1.7 Å resolution.



The crystals were grown under conditions closely related to those described by Miller *et al.*[1], and protein coordinates from their structure provided the starting point for refinement. The P1' and P2' moieties of this compound are constructed from the R-hydroxyethylene urea isostere and the P2 moiety contains a dimethyl succinamide group; both of these components bind to the target protease in a manner which would have been difficult to model without determination of the crystal structure. The contributions of an earlier structure[2] in the rational design of the present molecule will be presented.

1. Miller M., Schneider J., Sathyanarayana B. K. , Toth M. V., Marshall G. R., Clawson L., Selk L., Kent S. B. H. & Wlodower A. (1989) *Science* 246, 1149-1152.

2. Getman D. P., DeCrescenzo G. A., Heintz R. M., Reed K. L., Talley J. J., Bryant M. L., Clare M., Houseman K. A., Marr J. J., Mueller R. A., Vazquez, M. L., Shieh H. -S., Stallings W. C. & Stegeman R. A. (1993) *J. Med. Chem.* 36, 288-291.

S 441 **SUBSTRATE RECOGNITION OF HIV-1 PROTEASES MUTANTS**
Jeffrey W. Stebbins and Christine Debouck, Department of Molecular Genetics; SmithKline Beecham Pharmaceuticals King of Prussia, PA 19406

The retroviral aspartyl proteases cleave immature viral polyproteins at discrete sites. These specific sites are predominantly hydrophobic in character, but vary widely in amino acid composition. Unraveling, the determinants of enzyme-substrate recognition is important for understanding both enzyme inhibition as well as possible viral resistance to protease inhibitors. The HIV-1 aspartyl protease is a homodimer (MW=21,500) consisting of two 99 residue-long monomers. The three dimensional structure of this enzyme has been determined to bond resolution in the presence and absence of inhibitors. In order to further understand the mechanism of HIV-1 protease-substrate recognition, protein engineering has be used to investigate the interaction of the peptide substrate backbone with the "flap" region of the enzyme.

Analysis of the three dimensional structures of the HIV-1 protease and pepsin from Endothia parasitica, a non-viral aspartyl protease, in the presence of acetyl-pepstatin and pepstatin, respectively, reveals beta sheet like contacts between the peptide backbone of the substrate and the enzyme. These contacts are made with a loop structure known as the "flap" of the enzymes. In the case of HIV-1 protease, two flaps, one from each monomer, interact with the substrate, whereas in the monomeric Endothia pepsin, the single flap interacts with the substrate. In order to investigate the role of the flaps in HIV-1 protease, a mutant version of this enzyme has been generated by cassette mutagenesis using a tethered dimer form of the enzyme. Specifically, in this mutant version of HIV-1 protease, one native flap has been replaced by an endothia pepsin like flap and the other flap has been truncated. Analysis of this mutant version of the enzyme may give new insights into the role of the flaps in substrate specificity.

S 442 CRYSTAL STRUCTURES OF TWO RATIONALLY SELECTED, STRUCTURALLY NOVEL HIV-1 PROTEASE INHIBITORS: COMPARISONS WITH MODELING,

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The structures of two crystal complexes of recombinant human immunodeficiency virus type 1 (HIV-1) protease with structurally novel, potent peptide inhibitors have been determined. The acquired knowledge of key molecular interactions occurring between inhibitors and aspartyl proteases, as well as the structural similarities between HIV-1 protease and human renin have been used to rationally select two analogs. These 'dipeptides' are Mor-Phe-Bac-Hch-Ψ[CHOHCH$_2$]-Mva-Nle (PD134922) and Mor-Phe-Ptg-Hch-Ψ[CHOHCH$_2$]-Mva-Nle (PD135390), and their K_i for inhibition of the HIV-1 protease are 15nM and 2nM, respectively (Mor = morpholinylsulfonyl, Phe = phenylalanine, Hch = hydroxy-modified form of cyclohexyl-alanine, Bac = butylaminocarbonyl, Ptg = 2-propenylthio Gly, Mva = a methyl-modified form of valine, Nle = norleucine). The structure of the PD134922 complex has been refined to a crystallographic R factor of 0.142 at 2.3 Å resolution, and the bound inhibitor diastereomer has the S configuration at the chiral carbon of Bac. The PD135390 complex structure has been refined to an R factor of 0.138 at 2.5 Å resolution. The chiral center at the latter P_2 site (Ptg) is racemic, and both diastereomers could be refined, although only the R-form is presented here. The hydroxyl groups in both structures make optimal hydrogen bonds with both the active site aspartates, such that three of the total four interactions average 2.4-2.9 Å, while the fourth interaction is longer (3.2 Å). Comparison of these X-ray structures with model-built structures of the inhibitors reveal similar positioning only at the hydroxyethylene isostere at the active site, and of the hydrophobic groups, Hch and Mva, which occupy the S_1 and S_1' pockets, respectively.

Research sponsored in part by the National Cancer Institute, DHHS, under contract no. N01-CO-74101 with ABL.

S 443 COMPARISON OF AN ELASTASE-LIKE PROTEINASE TO PSEUDOMONAS ELASTASE.

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Pseudomonas aeruginosa proteinases are involved in the pathology of *Pseudomonas* keratitis. Zymography of the exoproducts from various strains of *P. aeruginosa* revealed additional bands of proteolytic activity other than those corresponding to the known proteinases, alkaline proteinase and elastase. One of these proteinases (P115) was isolated from an overnight Mueller-Hinton conditioned broth by chromatography on DEAE-MemSep and Superose 12. P115, like elastase, cleaves multiple substrates such as casein, gelatin, albumin and hemoglobin and is inhibited by the chelating agents, EDTA and 1, 10 phenanthroline. It has a pH maximum of 8.5 for FITC-casein cleavage. On reducing gels, both elastase and P115 have a molecular weight of 33 kDa. However, when elastase and P115 are electrophoresed on non-reducing casein or gelatin SDS-polyacrylamide gels, they aggregate to form proteolytically active complexes of approximately 90 kD and 60 kD, respectively. The isoelectric point for P115 is 6.2 in contrast to 7.2 for elastase. P115 is closely related to elastase in that it reacts with antibodies to elastase. These results show that *P. aeruginosa* synthesize two related enzymes with different in some properties.

S 444 PROTEOLYTIC ACTIVITY OF THE HTLV PROTEINASE

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The proteinase encoded by the genome of the human T-cell leukemia/lymphoma virus, HTLV-I, has been produced in E.coli by recombinant technique. Although the majority of the expressed enzyme was in inclusion bodies, a significant part of the proteinase became (self)-activated in E.coli. After purification by the acid-method (1) the enzyme was active in trans processing the recombinantly processed HTLV gag precursor. Related proteins such as the HIV gag-precursor were also cleaved by the HTLV-proteinase at sites different from but nearby to the identical processing sites. The a.a. sequences of the cleaving sites of cytosceletal and extracelluar proteins, found to be cleaved by the HIV-proteinase and also cleaved by the HTLV proteinase will be discussed. The aim of this study is to compare the specificity of the HTLV proteinase in comparison to the HIV encoded counterpart towards cellular protein substrates to reveal whether or not these proteinases might be involved in the cytopathic host destruction occurring upon virus infection.

(1) Nitschko, Seelmeir, Kisselev, von der Helm, Meth. in Enzymol. (in press)

S 445 Peptidyl fluoromethyl ketones inhibit cysteine protease activity in *Schistosoma mansoni*.

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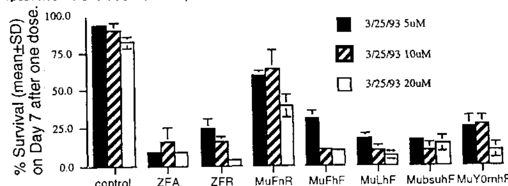
Schistosomiasis is a parasitic disease affecting over 250 million people in Africa, South America and Asia. It is hypothesized that a cysteine protease of the blood-feeding stages of *Schistosoma mansoni* degrades hemoglobin, thus providing essential nutrients to the schistosome. Compounds which specifically inhibit this cysteine protease may be effective against the disease of schistosomiasis. Peptidyl fluoromethyl ketones (FMK) inhibited the cysteine protease activity by enzyme assay(Table 1). These peptidyl fluoromethyl ketones reduced the survival of schistosomula in culture (Figure 1).

Table 1. Kinetic constants of peptidyl fluoromethyl ketones versus the cysteine protease of *Schistosoma mansoni*.

Compound	k3 (sec-1)	Ki (M)	k3/Ki(M-sec-1)
Z-F-A-FMK	4.0×10^{-3}	2.7×10^{-8}	1.5×10^{5}
Z-F-R-FMK	2.0×10^{-3}	9.6×10^{-9}	2.1×10^{5}
Mu-F-nR-FMK	2.9×10^{-2}	1.7×10^{-7}	1.7×10^{5}
Mu-F-hF-FMK	2.2×10^{-3}	2.8×10^{-9}	7.9×10^{5}
Mu-L-hF-FMK	3.3×10^{-3}	9.8×10^{-9}	3.4×10^{5}
Mu-bsu-hF-FMK	6.2×10^{-4}	1.7×10^{-9}	3.6×10^{5}
Mu-Y-Om-hF-FMK	5.7×10^{-4}	3.0×10^{-10}	1.9×10^{6}

The K_m of the cysteine protease of *S. mansoni* is 14 uM. n=nitro; Mu=morpholine; Z=3-N-benzyloxycarbonyl; bsu=benzylsuccinyl; m=methyl.

Figure1 Survival of schistosomula treated with peptidyl fluoromethyl ketones. Schistosomula were given the peptidyl fluoromethyl ketone in triplicate at 3 doses on day 1.



S 446 IDENTIFICATION OF ACTIVE SITE RESIDUES OF THE ADENOVIRUS ENDOPEPTIDASE, Joseph M. Weber, Claudine Rancourt, Karoly Tihanyi, Hossein Keyvani and Martin Bourbonniere, Department of Microbiology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec, Canada, J1H 5N4
Adenoviruses, like many complex viruses, encode an endopeptidase which is required for virion maturation and infectivity (Tihanyi, et al., JBC 268:1780). The enzyme appears to be a thiol endopeptidase which requires substrate-mediated activation (Webster, et al., Cell 72:97; Mangel et al., Nature 361:274). Multiple sequence alignment of the twelve adenovirus endopeptidases known to date identified a number of conserved residues which might be important for enzyme activity. Eleven mutants were created in the cloned gene by site directed mutagenesis to identify the active site of this thiol endopeptidase. The analysis of the proteolytic activity in a crude system using viral precursor proteins as well as in a purified system with activated proteinases using a new, chromophoric octapeptide substrate, yielded results consistent with cys104 and his54 being two members of the active site. This result was confirmed by the carboxymethylation of the reactive cys104, and its prevention by the active-thiol specific agent E64. Although cys122 and cys126 were also reactive cysteines, mutation of these residues did not affect enzyme activity. Replacement of the active site cys104 by ser104 converted the enzyme into a serine-like proteinase, sensitive to serine proteinase inhibitors. The absence of homology to other proteinases, particularly at the active site cysteine, coupled with the requirement for activation by a substrate cleavage fragment, indicate that the adenovirus endoproteinase may represent a new subclass of cysteine proteinases.

S 447 STRUCTURAL STUDIES OF PENICILLIN-DERIVED HIV-PROTEASE INHIBITORS
As reported at last year's meeting, ring-opened β-lactam dimers are potent and selective HIV-1 protease inhibitors (Humber et al, J. Med. Chem. 1992, 35, 3080-3081) which bind symmetrically to the enzyme. This dimeric series' potency derives from good hydrophobic interactions, with poor electrostatic interactions to the catalytic Asps. In order to improve potency and decrease size, a monomeric penicillin derivative with weak (micromolar) activity, GR123976, was elaborated based on molecular modelling to form good interactions with the Asps. Crystal structures of this series of compounds (GR123976, GR126045 and GR137615) complexed with HIV-1 protease show the basis for sequential improvements in affinity, resulting in nanomolar-potency compounds with good activity in antiviral assays, although the pharmacokinetics is still poor in spite of their reduced size.

S 448 THREE PREDICTED PROTEASES ENCODED IN ORF 1a OF MOUSE HEPATITIS CORONAVIRUS. Susan R. Weiss, Pedro J. Bonilla, Scott A. Hughes, Josefina Pinon, Mikhail Rozanov, Julie D. Turner. Department of Microbiology, University of Pennsylvania, Philadelphia, PA. 19104-6076.
Mouse hepatitis virus is a positive stranded RNA virus that causes demyelinating disease in rodents. Sequence analysis of the gene 1, the 21 kb putative RNA replicase gene has predicted the presence of three protease domains , two distantly related to papain like cellular proteases and one chymotrypsin like protease related to picornavirus 3C-like proteases. It is presumed that these protease activities play an important role in the processing of viral replicase polypeptides. Previous work has shown that the more amino terminal papain like protease acts *in cis* to cleave a 28 kd polypeptide which is encoded upstream of the protease activity. We have preliminary evidence suggesting that this cleavage occurs at gly/val site. This papain-like protease may also cleave at another site downstream of p28. We are currently carrying out studies designed to determine whether we can demonstrate activity for the second predicted papain-like activity. Recent *in vitro* translation studies suggest that a protease is indeed present in the carboxyterminal portion of ORF 1a, the region predicted to encode the picornavirus 3C-like protease. Current work is directed at demonstrating the cleavage sites for this activity and also the catalytic residues.

S 449 APROTININ INHIBITS INFLUENZA VIRUSES O.P.Zhirnov, P.B.Golyando, A.V.Ovcharenko; The D.I.Ivanovsky Virology Institute; Federal Research Center of Chemical-Biology & Ecology Problems, Moscow 123098, Russia
Chicken embryo proteinases, one of which is a blood clotting factor Xa-like proteinase, are known to specifically cleave the haemagglutinin (HA) of Influenza viruses to permit their activation in chicken embryonated eggs. We have found that injection of the serine proteinase inhibitor, aprotinin, into the allantoic cavity of eggs infected with Influenza viruses suppresses the viral HA cleavage and reduces the virus proteolytic activation and replication. Effective inhibition dose was determined as 0.1 uM concentartion. Aprotinin injections did not cause side toxic effects in embryos. However heparin, which is known to be a direct inhibitor of the Factor Xa, was not able to suppress Influenza virus hemagglutinin cleavage and replication in chicken embryo system. These data suggest that (i) exogenous aprotinin fails to inhibit directly the mature FXa enzyme but may prevent its activation via the blockage of the blood clotting IXa and VIIa-like proteinases; (ii) a second proteinase(s) besides Xa-like factor may be involved in the activation cleavage of the virus HA protein in chicken embryos. These findings extend a medical significance of aprotinin as antiviral.

S 450 SEPARATE EXPRESSION OF THE TWO SUBUNITS OF THE *THERMOPLASMA* PROTEASOME

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The proteasome is a high molecular weight (approx. 700 kDa), non-lysosomal multicatalytic proteinase ubiquitous in eukaryotic cells. Recently we have isolated proteasomes from the thermoacidophilic archaebacterium *Thermoplasma acidophilum* (1), however we could not detect proteasomes in eubacteria. Unlike eukaryotic proteasomes which are composed of at least 14 different though related subunits, all in the molecular weight range from 20 to 35 kDa, the *T. acidophilum* proteasome is made of multiple copies of two subunits only, α and β, with molecular weights of 25.9 and 22.3 kDa respectively. All the amino acid sequences of proteasomal subunits from eukaryotes available to date can be related to either the α-subunit or the β-subunit of the *T. acidophilum* 'Urproteasome' (2). Also the basic molecular architecture is conserved from *Thermoplasma* to higher eukaryotes; four seven-subunit rings collectively form a cylinder- or barrel-shaped structure.

Expression of the two genes encoding the constituent subunits of the *T. acidophilum* proteasome in *E. coli* yielded fully assembled and proteolytically active recombinant proteasomes (3). After separate expression of the two genes, the α-protein assembled into seven-subunit rings, whereas the β-protein was only found as monomeric subunit. The purified α-rings and β-subunits showed no proteolytic activity. At the moment site directed mutagenesis is performed to identify essential structural or functional amino acid residues.

References:
(1) Dahlmann *et al.*, (1989), FEBS Lett. 241, 239-245.
(2) Zwickl *et al.*, (1992), Biochemistry 31, 964-972.
(3) Zwickl *et al.*, (1992), FEBS Lett. 312, 157-160.

Late Abstracts

THE EFFECTS OF SUBSTITUTING THE REACTIVE SITE LOOP OF α_1-ANTICHYMOTRYPSIN WITH THOSE OF ANTITHROMBIN-III AND PROTEASE NEXIN INHIBITOR, Marylyn Z. Djie & Stuart R. Stone

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α_1-Antichymotrypsin (ACT), antithrombin III (ATIII) and protease nexin inhibitor (PNI) all belong to the serpin family of protease inhibitors. The mechanism of inhibition involves several steps and although it appears essentially irreversible and first order in excess of serpin, the initial binding seems to be reversible at low inhibitor concentrations. A characteristic equimolar SDS stable complex between serpin and its cognate enzyme usually forms, but the association between serpin and enzyme can also result in rapid cleavage of the inhibitor.

ACT inhibits chymotrypsin and mast cell chymase in addition to its target protease neutrophil cathepsin G, but does not efficiently inhibit trypsin, thrombin or urokinase. ATIII and PNI inhibit trypsin, thrombin and urokinase with association rate constants in excess of 10^4 $M^{-1}s^{-1}$. Substitution of the P1 leucine residue for arginine in ACT results in the P1/R mutant being able to inhibit thrombin with a association rate constant 10-fold higher than ATIII (in the absence of heparin). To investigate other possible determinants involved in the specificity of ACT, chimeric mutants, where the entire reactive site loop was exchanged for that of another serpin, were constructed and expressed in *E. coli*.

Replacing residues P8 to P'9 of ACT with the corresponding reactive site loop sequence of ATIII, results in a mutant (ACT-ATIII) which inhibits thrombin with an association rate constant comparable to that of ATIII. However, with the PNI reactive site loop on the ACT backbone, although the resulting variant (ACT-PNI) now acquired inhibitory properties towards thrombin, the association rate constant was 1000-fold lower than that of PNI. In addition little to no inhibition of urokinase, could be detected by ACT-PNI. Although initial inhibition could be detected between trypsin and all the chimeric mutants analysed, rapid cleavage was observed.

While this study confirms the importance of residues within the reactive loop of serpins, it also suggests that other determinants in the inhibitor and/or the protease contribute to the serpin specificity. In particular the length of the reactive loop may be essential to the conformation, stability and function of these inhibitors.

STRUCTURAL ANALYSIS OF THE CATALYTIC DOMAIN OF HUMAN FIBROBLAST COLLAGENASE, Brett Lovejoy, Anne Cleasby, Anne M. Hassell, Michael A. Luther, Debra Weigl, Gerard McGeehan, Millard H. Lambert and Steven R. Jordan,

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The crystal structures of the catalytic domain (residues 102-270) of human fibroblast collagenase in a native form (CF1, *R*-factor 20.2%, 1.9 Å resolution) and as a complex with a carboxyalkylamino-based inhibitor (CPLX, *R*-factor 18.6%, 2.4 Å resolution) provide new direction for the design of compounds that selectively inhibit individual members of the matrix metalloproteinase family. Despite scant sequence homology, collagenase shares structural homology with the 'N' domain of crayfish astacin and the 'N' domain of bacterial thermolysin. The catalytic domain consists of a twisted five-stranded beta sheet and three alpha helices. The distinct collagenase active site cleft contains a catalytic zinc ion that is ligated by His 218, His 222 and His 228. In addition, the enzyme contains a second zinc ion and a calcium ion that stabilize a long, highly exposed loop between two beta strands. In the complex structure, the carboxylate group of the inhibitor is bound to the catalytic zinc while the inhibitor P1' Leu side chain occupies a hydrophobic pocket (P1' pocket) that is adjacent to the catalytic zinc. Eight hydrogen bonds between collagenase and the inhibitor also contribute to the tight binding constant (K_i =135nM). In particular, Glu 219 and Ala 182 form hydrogen bonds with the α-amino group of P1' Leu. These bonds possibly mimic interactions that occur between the enzyme and substrate during hydrolysis of the scissile bond. A water molecule is observed at the base of the P1' pocket, where it is hydrogen bonded to Arg 214, a residue that is unique to collagenase. Even though the side chain of the inhibitor P1' leucine is completely buried in the P1' pocket, the closest approach between this leucine and the guanidino group of Arg 214 is 5 Å. Oδ1 and Nδ2 of Asn 180 form hydrogen bonds with the P1 substituent (benzyloxycarbonylamine). Like Arg 214, Asn 180 is not present in other matrix metalloproteinases. In the native structure (CF1), residues Leu 102 to Gly 105 occupy the active site cleft of a crystallographic symmetry related collagenase molecule. In CF1, both the nitrogen (N-terminus) and carbonyl oxygen of Leu 102 ligate the symmetry related catalytic zinc. Thr 103 is buried in the symmetry related P1' pocket and Thr Oγ1 forms a hydrogen bond with the symmetry related Glu 219 Oε1.