PROTEOLYSIS IN REGULATION AND DISEASE

Organizers: Dennis Cunningham and Ralph Bradshaw

April 8-14, 1991

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Structure, Function and Evolution of Proteases and Protease Inhibitors

CH 001 STRUCTURE OF THE REACTIVE CENTRE LOOP OF SERPINS: IMPLICATIONS FOR HEPARIN-ANTITHROMBIN INTERACTIONS, Robin W. Carrell, Dyfed Evans, Paul Hopkins and Penelope Stein, Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, UK.

The inhibitory serpins, on cleavage of the reactive centre peptide bond, characteristically undergo a conformational change, the newly generated carboxy terminus moving some 70Å to the opposite pole of the molecule. The observation that this change does not occur with the non-inhibitory serpins, angiotensinogen or ovalbumin, has focused attention on the structure and role of the N-terminal portion of the reactive centre loop. We have now determined the structure of native ovalbumin to 1.95Å resolution and have found that the intact peptide loop that forms the homologue to the reactive centre of the inhibitory serpins takes the unexpected form of an isolated helix. This is formed by a 19 residue segment (P15-P'5) with the P1-P1' Ala-Ser exposed on the final turn of a 3-turn helix held in a wobbly conformation protruding from the molecule on two peptide stalks.

We present here experimental evidence based on natural and engineered mutants of the N-terminal stalk of this loop (P10-P14) which indicates that a requirement of inhibition is movement of the N-terminal segment of the loop to give partial re-entry of the strand into the A-sheet. This will enable the reactive centre loop to adopt the canonical structure present in other families of serine protease inhibitors. Thus the serpins have a mobile reactive centre capable of physiological modulation. We illustrate this with findings from the heparin-activated inhibitors protease nexin-1 and antithrombin. These show that antithrombin has two contiguous heparin binding sites formed by a row of positively charged residues extending across one face of the molecule: a high affinity pentasaccharide site on the D-helix and a low affinity site extending from it to the reactive centre. Binding to the pentasaccharide site is accompanied by a conformational change which affects the region of re-entry of the reactive centre loop and gives non-specific acceleration of inhibition in general as with plasmin and Factor Xa; binding to the larger low affinity site gives charge neutralisation and hence provides additional acceleration to the inhibition of cationic proteases such as thrombin.

CH 002 VIRAL PROTEASES AS TARGETS FOR THERAPEUTIC INTERVENTION: THE HIV PROTEASE AND ITS INHIBITORS. Paul L. Darke, Jill C. Heimbach, Joan A. Zugay, Nancy E. Kohl, Paula M. D. Fitzgerald, Joel R. Huff, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486.

The HIV-1 protease is currently accepted as a prime target for AIDS therapeutic agents and it has been demonstrated that HIV replication can be halted in tissue culture at low concentrations of protease inhibitors. The contributions to our understanding of important inhibitor/enzyme interactions from both crystallographic analysis of inhibitor complexes and mutational analysis of inhibitor binding and catalytic properties in solution will be discussed. In particular, changes in binding site residues Arg-8 and Asp-29 have profound effects upon inhibitor binding. In addition, change of Ala-28 to Ser or Thr (residues found in the pepsins at the homologous position) result in a lowering of the pH optimum for peptide hydrolysis, in agreement with some previous interpretations of the pepsin family crystal structures.

CH 003 POSITIVE DARWINIAN SELECTION IN THE EVOLUTION OF PROTEIN INHIBITORS OF SERINE PROTEINASES, M. Laskowski, Jr., Department of Chemistry, Purdue University, West Lafayette, IN 47907
Protein chemists widely believe that equivalent (orthologous) proteins from related species are functionally identical or nearly so. They also believe that in the evolution of these proteins structurally and functionally important amino acid residues are strongly conserved, while the other residues (those surface residues with little functional importance) vary a good deal. These are among the main tenets of the Kimura neutral mutation theory of molecular evolution. Somewhat paradoxically many protein chemists say that they are selectionists not neutralists even though they believe the neutralist conclusion.

The available observations on most proteins mainly support the neutralist conclusions especially so on the similarity of structure and on conservation of structurally important residues. The evidence for conservation of functional properties and of functional residues is far less impressive, but a reasonable case for conservation can probably still be made for most proteins.

Protein inhibitors of serine proteinases are striking a exception. Closely related natural inhibitors often inhibit quite different enzymes. Association equilibrium constants vary a great deal. This is not limited to protein engineering by natural evolutionary means. Since protein inhibitors of serine proteinases are very small and stable, variants have been made by various laboratories by semisynthesis, total synthesis, and by recombinant DNA technology. Many of the variants show predictable changes in switching the inhibition from one enzyme to another, e.g. P_1 Arg → Trp from trypsin to chymotrypsin, P_1 Arg → Val from trypsin to elastases, especially leukocyte elastase, P_1 Leu → Lys → Glu from chymotrypsin, elastase, subtilisin to trypsin to glutamic acid specific enzymes. More complex designs involving changes of as many as six amino acid residues were successful in changing an inhibitor with broad specificity to one with a narrow one.

Thus, part of the explanation for positive Darwinian selection in inhibitor evolution is at hand. In contrast to other proteins, inhibitors are tolerant of nonconservative changes of amino acid residues in their reactive sites. They respond by changing specificity not by loosing activity. Another aspect still remains a puzzle. Why do closely related organisms need to make the dramatic specificity changes in their inhibitors? It seems that we can show that the project is technologically feasible. We are less clear about whether and why it is desirable.
(Supported by NIH Grant GM 10831)

CH 004 EVOLUTION AND REGULATION OF THE RAT KALLIKREIN GENE FAMILY,
Raymond J. MacDonald, E. Michelle Southard, Debora R. Wines and Robert E. Hammer, Department of Biochemistry and the Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75235.
The rat kallikrein multigene family is a group of approximately twenty very similar and closely linked genes with disparate patterns of tissue-specific expression. Within the high sequence homology among family members wrought by recombination processes such as gene conversion and unequal crossing over, differences between genes provide for the altered substrate specificities of the encoded serine proteases and the diverse patterns of tissue-specific expression. Analysis of nucleotide sequence variants in nine kallikrein family genes shows that most variants are shared by multiple family members. The patterns of shared variants are complex and indicate the occurence of multiple, short gene conversions between family members and suggest an important role for gene conversion in the evolution of the functional diversity as well as sequence homogenization of the gene family. The sequence exchanges between family members have generated novel combinations of amino acid variants at key residues that may affect substrate specificity and thereby contribute to the diversity of enzyme activity. Small sequence exchanges also may play a role in generating the diverse patterns of tissue-specific expression of family members through the reassortment of gene regulatory elements in nearby gene flanking sequences. To identify tissue-specific transcriptional elements and to define the regulatory strategy of the kallikrein family locus that may be affected by these evolutionary processes, we analyzed the expression of several kallikrein-based transgenes in mice and rats. The results demonstrated that 5'-proximal gene flanking regions contain many of the regulatory elements for qualitatively correct tissue-specific expression of individual family members, but generally lack those necessary for normal, high expression in these tissues and for the salivary gland expression characteristic of the family. We propose that the diversity of expression of each member is determined principally by tissue-specific transcriptional control elements associated with that gene, whereas the high level and salivary gland expression is imposed on the family by a dominant enhancer (as yet unidentified) located somewhere in the kallikrein locus.

Protease Processing

CH 005 PROCESSING OF GROWTH FACTORS PRECURSORS, Ralph A. Bradshaw, Michael Blaber and Paul J. Isackson, Department of Biol. Chemistry, College of Medicine, University of California, Irvine, CA 92717.

Polypeptide growth factors generally function as soluble entities that are produced from larger precursors by limited proteolysis. These precursors themselves may vary in size and organization as a result of alternative splicing of corresponding transcripts and can occur in both soluble and membrane bound forms. Thus the presence or absence of appropriate proteases can control the nature and distribution of growth factor activities in various tissues and locales. Surprisingly, little information is available regarding these processing agents. Most cleavages are presumed to occur in the ER/Golgi continuum although some proteolytic modifications clearly can occur at extracellular sites. Some of the proteases that are involved may be themselves be membrane bound.

In the submaxillary gland of the adult male mouse, the precursors for nerve growth factor (NGF) and epidermal growth factor (EGF) appear to be processed, at least in part, by soluble kallikreins that remain associated with the mature growth factors in high molecular weight complexes. These interactions are highly specific and arise from a limited number of contacts between the precursor and the soluble enzyme. The binding sites have been defined using site directed mutagenesis followed by detailed analyses of the resulting derivatives. These studies also have revealed interesting features of serine proteases and the effects of individual residues on their catalytic properties. Supported by US Public Health Service Research Grant AG09735 and American Cancer Society Research Grant BE-41K.

CH 006 PROTEOLYTIC PROCESSING OF TUMOR NECROSIS FACTOR AND ITS' ROLE IN TUMOR REGRESSION AND INFLAMMATORY DISEASE, Michael Kriegler, Department of Molecular and Cellular Biology, Cytel Corporation, 11099 North Torrey Pines Road, La Jolla, California, 92037. Tumor necrosis factor is an inflammatory mediator that, in addition to its' potent anti-tumor activities, plays a key role in a variety of inflammatory disorders. These disorders include septic shock, cachexia, rheumatoid arthritis, diabetes and the induction of HIV expression in latently infected cells. Some of these pathologies are manifest locally in the body of an affected individual while others are manifest systemically. Our initial investigations were conducted in an attempt to determine how the TNF molecule could manifest such a complex biology. Initially thought to be a secretory molecule, we have shown that TNF exists as a cell-surface integral transmembrane precursor that is subsequently cleaved to form the "secretory" form[1]. We have also shown that the transmembrane form of TNF is biologically active[2]. The systemic physiologies of TNF may be associated with the secretory form and the localized physiologies may be associated with the cell-associated transmembrane precursor. Thus, the biodistribution of TNF in the body appears to be, at least in part, a function of a processing pathway and the regulation of this pathway may serve to explain the complex physiology of the molecule. It is clear from our studies that TNF release from activated inflammatory cells is mediated by a protease, a so-called TNF convertase. In an attempt to understand the molecular mechanism that controls TNF we have identified and characterized the TNF convertase. This work and its implications will be discussed.

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CH 007 PROCESSING OF PEPTIDE PRECURSORS AND OF ENZYME PRECURSORS. Richard E. Mains, Brian T. Bloomquist, E. Jean Husten, Richard C. Johnson, Hye-Young Yun, Gilles Noel, and Betty A. Eipper. Dept.Neuroscience, Johns Hopkins Univ. Med. School, Baltimore, MD 21205.
The production of bioactive α-amidated peptides from their glycine-extended precursors is a two step process involving the sequential action of two activities encoded by the bifunctional peptidylglycine α-amidating monooxygenase (PAM) precursor. The NH_2-terminal of the PAM precursor contains the first enzyme, peptidylglycine α-hydroxylating monooxygenase (PHM), a Cu^{++}, O_2 and ascorbate dependent enzyme; purification of this enzyme led to the initial cloning of the PAM precursor. The middle of the PAM precursor contains the second divalent metal-dependent enzyme, peptidyl-α-hydroxyglycine α-amidating lyase (PAL), necessary to complete the α-amidation reaction at the pH of secretory granules. The COOH-terminal of the PAM precursor encodes a transmembrane domain and cytoplasmic tail. Mammalian and bacterial expression vectors encoding the full PAM precursor or various domains of the precursor in the sense or antisense orientation were constructed. Cells transfected with PHM vectors secreted large amounts of PHM activity. Cells transfected with a PAL vector exhibited increased secretion of PAL activity, while mammalian cells transfected with another PAL vector including a transmembrane domain retained much of the PAL in a membrane-bound form. Stable mammalian cell lines with significantly elevated or diminished levels of PAM were generated by transfection of a mouse pituitary cell line, and a rate limiting role for PAM in peptide α-amidation was demonstrated. Overexpression of either the full-length PAM precursor or the PHM domain led to increased α-amidation of endogenous peptides. Overexpression of the full-length PAM led to a decrease in the endoproteolytic processing of endogenous prohormone; conversely, underexpression of PAM led to significantly enhanced endoproteolytic processing of endogenous prohormone. Mouse pro-ACTH/endorphin (POMC) contains in its sequence each of the 4 possible pairs of basic amino acids recognized as potential cleavage sites in higher molecular weight precursors: KR (Lysine-Arginine), RR, RK and KK. To examine the structural requirements for processing and routing in a cell line that already produces pro-ACTH/endorphin endogenously, a reporter mutation was introduced into the mouse pro-ACTH/endorphin cDNA. Since this mutation did not affect processing or secretion, additional mutations were introduced on the reporter background. The results show that the enzymatic machinery of AtT-20 cells fails to cleave efficiently at an Arg-Lys (RK) site even following elimination of possible structural hindrances by nearby O- or N-linked carbohydrate side chains. However, mutation of the RK sequence to RR allowed extensive cleavage to yield γ_3-MSH. Support: DK-32948,DK-32949,DA-00266,DA-00097,DA-00098.

CH 008 ROLE OF MAMMALIAN KEX2-LIKE ENDOPROTEASES IN PRECURSOR PROTEIN PROCESSING, Gary Thomas, Laurel Thomas, Patricia A. Bresnahan, Barbara A. Thorne, Richard Leduc, and Joel S. Hayflick, Vollum Institute, Oregon Health Sciences University, Portland, Oregon 97201.

Posttranslational proteolysis is a common mechanism required for the synthesis of biologically active proteins and peptides in all eukaryotes examined, including yeast, invertebrates, and mammalian cells. One of the early events in precursor protein maturation is endoproteolysis at the carboxyl side of pairs of basic amino acid sequences (especially -LysArg- and -ArgArg-). Several activities capable of cleaving at single or paired basic residues in vitro have been proposed as candidates for authentic mammalian precursor processing endoproteases. However, none of these candidate activities have been shown to be a bona fide precursor cleaving endoprotease in vivo. In contrast, genetic and biochemical studies unequivocally identified the gene required for excision of the peptide mating hormone in Saccharomyces cerevisiae. This locus, the KEX2 gene encodes a unique subtilisin-like membrane bound, calcium dependent, serine endoprotease (Kex2p) required for cleaving on the carboxyl side of pairs of basic residues. Recently, three mammalian cDNA sequences, fur [Roebroek et al. EMBO 5:2197 (1986)], PC2 [Smeekens et al. JBC 265:2997 (1990)] and PC1 (or PC3) [Seidah et al. DNA and Cell Biology 9:415(1990)], have been identified which share significant structural homology with the KEX2 gene sequence. The fur gene is expressed in a wide variety of tissues and cell lines whereas expression of PC2 and PC3 are restricted to endocrine and neural tissues. Expression of the fur gene product in BSC-40 cells reveals that this gene product, furin, encodes a Golgi compartment-localized, membrane-associated, calcium-dependent, serine protease capable of efficiently processing pro-β-nerve growth factor to 13 kDa β-NGF [Bresnahan et al. JCB 111:(December, 1990)]. Expression of the ACTH/β-endorphin precursor, POMC, together with PC3 in vivo reveals that this gene product can process POMC to several pituitary anterior lobe peptides including β-LPH whereas co-expression of PC3 and PC2 together with POMC results in production of peptides reminiscent of processing in the neurointermediate lobe including conversion of β-LPH to γ-LPH and β-endorphin. Taken together, these results identify furin, PC2 and PC3 as the initial members of a unique family of mammalian endoproteases capable of processing precursor proteins in the secretory pathway.

Proteases in Intracellular Degradation and Protein Turnover

CH 009 ROLE OF AMINOPEPTIDASES IN DEGRADATION AND TURNOVER OF PROTEINS IN *SACCHAROMYCES CEREVISIAE*, John A. Smith, Ulrich Teichert, and Yie-Hwa Chang, Departments of Molecular Biology & Pathology, Massachusetts General Hospital and Departments of Genetics & Pathology, Boston, MA 02114
The *in vivo* role of aminopeptidases in the synthesis and degradation of eukaryotic proteins is poorly understood at present. We are beginning to unravel their role by determining the number of soluble aminopeptidases, as well as their specificity, molecular properties, and intracellular distribution. Using Met-pNA and Met-Ala-Ser as substrates, we have found nine distinct aminopeptidases in *Saccharomyces cerevisiae*, including a methionine aminopeptidase (MAP) and an aminopeptidase-P (AP-P). MAP is a central enzyme in cotranslational protein processing, and AP-P is an important enzyme in peptide and protein degradation. Our purification of yeast MAP and AP-P, as well as the cloning and sequencing of their encoding genes provide the tools required to elucidate aspects of the *in vivo* function and control of NH_2-terminal methionine cleavage and aminopeptidase-mediated protein degradation in eukaryotic cells. Both MAP and AP-P are Co^{++}-dependent, and their functions can be inhibited by EDTA and Hg^{++} ions. MAP was purified to apparent homogeneity, and a cDNA encoding MAP was cloned and sequenced. The DNA sequence encodes a precursor protein containing 387 amino acid residues. The mature protein, whose NH_2-terminal sequence was confirmed by automated Edman degradation, consists of 377 amino acids. The function of the unique 10-residue presequence, which contains one serine and six threonine residues, remains to be established. The deduced amino acid sequence of yeast MAP shows 60% similarity and 42% identity to the amino acid sequence of the MAP from *Escherichia coli*. AP-P, which catalyzes the removal of amino acids from the NH_2-termini of peptides and proteins whose penultimate residues are prolines, was purified 1200-fold. The M_r of the native AP-P was estimated to be $140{,}000 \pm 5{,}000$ by gel filtration chromatography, and the M_r of the denatured protein was estimated to be $66{,}000 \pm 2{,}000$ by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The biological role of AP-P in protein degradation has been investigated by disrupting its gene.

CH 010 THE N-END RULE AND SELECTIVE PROTEIN DEGRADATION, Alexander Varshavsky, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

The N-end rule relates the metabolic stability of a protein to the identity of its amino-terminal residue (1). Similar but distinct versions of the N-end rule operate in all organisms examined, from mammals to bacteria. In eukaryotes such as yeast and mammals, the N-end rule-based degradation signal comprises a destabilizing amino-terminal residue and a specific internal lysine residue (2,3). The latter is the site of attachment of a multiubiquitin chain whose formation on a targeted protein is essential for the protein's subsequent degradation (4). The N-end rule is organized hierarchically (2). Specifically, amino-terminal Asp and Glu (and also Cys in mammalian reticulocytes) are *secondary* destabilizing residues in that they are destabilizing through their ability to be conjugated to Arg, one of the *primary* destabilizing residues. Amino-terminal Asn and Gln are *tertiary* destabilizing residues in that they are destabilizing through their ability to be converted, via selective deamidation, into the secondary destabilizing residues Asp and Glu (2).

I will discuss our recent studies of the N-end rule pathway that include the use of oligomeric protein substrates to address *cis-trans* recognition and subunit-specific protein degradation by this pathway, the discovery of the N-end rule pathway in bacteria, and molecular genetic analyses of both the components of the N-end rule pathway and its specific *in vivo* substrates.

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Proteolytic Processing During Infection by HIV and Other Viruses

CH 011 REGULATION OF PICORNAVIRUS PROTEIN PROCESSING

Bert L. Semler, Wade S. Blair, Mark A. Lawson, and Xiaoyu Li
Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717.

The structure/function relationships of picornavirus 3C proteinases have been analyzed by genetic manipulation of the viral 3C enzymes and polyprotein substrates. Chimeric 3C proteinases were generated between enzymes derived from poliovirus type 1 (PV1) and coxsackievirus B3 (CVB3). These suballelic replacement mutations were made possible by engineering new restriction endonuclease recognition sites into the 3C cDNAs that did not change the amino acids at such sites. Six different chimeric enzymes were tested for their *in vitro* cleavage activity on both PV1 and CVB3 polyprotein substrates. A comprehensive biochemical and immunological analysis of the protein products produced by the above enzymes was carried out. The results of this line of experimentation revealed that a specific order of events in processing viral polyprotein is employed by picornaviruses. In addition, the data suggested that, although structural elements throughout the 3C proteinase play a role in efficient substrate utilization, the carboxyl-terminal region of the enzyme contains amino acid sequence elements most important in species-specific substrate recognition. This latter conclusion has led us to alter putative active site amino acid residues in the poliovirus 3C proteinase. The rationale for such an approach is based upon evolutionary conservation of critical amino acids in this group of thiol proteinases and upon structural modeling studies which suggest that the 3C proteinases may bear structural resemblance to the well-characterized serine proteinases. Our preliminary results suggest that active site proteinase geometry may be more important in substrate recognition than the actual nucleophilic residue (i.e. cysteine versus serine) involved in catalysis. We have also analyzed the role of amino acid residues surrounding the proteolytic cleavage sites in the polyprotein recognized by the poliovirus 3C (or 3CD) proteinase. Such cleavage sites occur at glutamine-glycine (Q-G) pairs in poliovirus precursor polypeptides. To examine the role of amino acid sequences proximal to the VP3-VP1 cleavage site in the P1 capsid precursor, we have generated four or six amino acid insertions or single amino acid substitutions that disrupt the P4 residue proximal to the cleavage site. The effects of these alterations have been analyzed by an *in vitro* protein processing assay, by infectivity and growth assays using infectious cDNAs of the poliovirus genomic RNA, and by pulse-chase analysis of the *in vivo* processing phenotypes of any recovered mutant viruses. Our data provide *in vitro* and *in vivo* evidence that the alanine residue in the P4 position of the VP3-VP1 cleavage site is a required substrate determinant for the recognition and cleavage of that site by the 3CD proteinase, and that the P4 alanine residue may specifically interact with the enzyme itself.

CH 012 MOLECULAR ANALYSIS OF HIV PROTEIN PROCESSING, **Ronald Swanstrom, Andrew Kaplan, Marianne Manchester, Mark Moody, Steven Pettit, Lorraine Everitt, Daniel Loeb, Clyde Hutchison III, Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599**

Proteolytic processing of the viral Gag and Gag/Pol precursor proteins occurs as a late step in the human immunodeficiency virus life cycle and is required for the formation of infectious virus. Processing of the Gag precursor results in mature virion morphology with the formation of a condensed viral core. Processing of the Gag/Pol precursor is probably necessary to obtain full enzymatic activity of the proteins encoded within the *pol* gene, and occurs either during or immediately after budding of the virus from the cell membrane. It is likely that all cleavage events in these two precursor proteins are carried out by a virus-encoded protease, which, in the case of HIV-1, is found near the amino terminus of the Pol domain. Numerous studies have now shown that the retroviral protease is a member of the aspartic proteinase family of enzymes and that it must dimerize to form the nearly symmetric active site of the enzyme.

We have used a bacterial expression and processing system to carry out an extensive structure-function analysis of the HIV-1 protease. In this study we have examined the effects of over 400 independent amino acid substitutions on protease activity. There are three regions of the protein in which consecutive positions are sensitive to amino acid substitution. These regions correspond to the active site loop, the hydrophobic core, and a flap which covers the substrate during cleavage. One mutation in the flap reduces the efficiency of cleavage by the protease of one of the cleavage sites in Pol. This phenotype can be reverted by a second substitution at the cleavage site. Approximately 4% of the substitutions tested displayed a temperature sensitive phenotype. Eight such mutants have been identified thus far. They are well spaced over the 99 amino acid length of the protease from position 8 to 98.

Processing sites represent a heterogeneous collection of sequences. We have compared these sites to look for common sequence elements. Processing sites are characterized by restricted sequence heterogeneity and increased hydrophobicity from positions P4 through P3'. Furthermore, most sites can be classified into two sequence families depending on the identity of the amino acid at the P1' position.

In a effort to define the temporal relationship between virion formation and protein processing we have examined the fate of pulse-labelled Gag protein in infected cells. A fraction of the labelled Gag protein is processed after associating with the membrane during virion formation. The rest of the Gag protein remains in the cytoplasm but still undergoes processing. This observation suggests that the viral protease is inappropriately activated in the cytoplasm of infected cells. The viral protease may also cleave cellular proteins in the cytoplasm and thus contribute to cytotoxicity.

CH 013 COMPARISON OF THE STRUCTURES OF INHIBITOR COMPLEXES OF HIV-1 PROTEASE
Alexander Wlodawer, Alla Gustchina and Amy L. Swain, NCI-FCRDC, ABL-Basic Research Program, Frederick, MD 21702, USA.
The action of HIV-1 protease (PR) is essential for replication of infective virus. Its function is to free other viral proteins from a translated polyprotein into discrete components. PR is an attractive target for AIDS drug design because if it does not function properly, the virus is not infective. The structures of crystals which contain PR complexes with four different inhibitors were solved at 2-2.5 Å resolution. The enzyme used in the study of three of them was chemically synthesized, with the sequence corresponding to the SF2 isolate of HIV-1 PR, while the fourth inhibitor was cocrystallized with recombinant NY5 isolate of the protease. Data extending to 2 Å resolution were collected for a complex with MVT-101, a hexapeptide containing a reduced peptide bond. This structure has been refined to an R = 15.4% (1) with the inhibitor modeled in two orientations, indicating crystallographic disorder. Another inhibitor, JG-365, has a hydroxyethylamine group in place of the normal scissile bond. The structure of the complex has been refined to an R-factor of 14.8% at 2.4 Å resolution (2). A complex with an octapeptide inhibitor U-85548E, containing a hydroxyethylene non-scissile group, has been solved with 2.5 Å data and refined to an R = 13.8% (3). The latter two inhibitors appear to bind in a unique orientation. Most recently, we cocrystallized recombinant protease with acetylpepstatin in a space group different from that of the other complexes, as previously reported by Fitzgerald et al., (4). That structure is being refined and has a current R = 17.7% at 2.5 Å resolution. These four inhibitors span the range of K_i from micromolar to nanomolar and their comparison provides valuable information relating the observed structures with the inhibitor sequences length, and crystal packing. Such information is necessary if these inhibitors are to be used as lead compounds in designing drugs targeted against AIDS.

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Proteolysis in Cell Migration, Development and Cancer - I

CH 014 GENETIC AND BIOLOGICAL ANALYSIS OF THE CELL SURFACE CYCLE OF UROKINASE PLASMINOGEN ACTIVATOR. **Francesco Blasi, Lisbeth B. Møller, David Olson, Nina Pedersen, Jari Pollanen, Emilia Soravia.** University Institute of Microbiology, Øster Farimagsgade 2A, 1353 Copenhagen K, Denmark.

Cell surface urokinase-dependent plasminogen activator (uPA) is involved in a variety of normal and pathological cellular functions that result in cell migration, tissue remodeling and invasion. In order to establish the relativ contribution of the different components of the urokinase PA system (uPA, its receptor and inhibitors) in the invasion process, we have used a reverse genetic approach, transfecting a cell lacking uPA, receptors and inhibitors with a gene coding for only one of the component of the PA system. The so derived types of cells complement each other in a variety of in vitro invasive and tissue remodeling systems. The relative importance and mechanism of action of the different components of the PA system can be therefore assessed by co-cultivation experiments. These data, combinec with the results of biochemical and mutational analysis give us the following picture. The secreted single chain pro-enzyme (pro-uPA) binds to a specific surface receptor that is membrane associated via a glyco-lipid anchor. Receptor binding strongly stimulates pro-uPA activation to two chain active uPA, which exerts its plasminogen activating activity on the cell surface. This effect appears to be due to the close proximity between pro-uPA and plasmin, caused by the surface binding of both components. The plasminogen activator inhibitors, PAI-1 and PAI-2 control this process a: they can interfere with plasmin formation by blocking uPA activity via the formation of an SDS-resistant covalent complex. Once such a complex is formed on the cell surface, it is internalized and degraded.

This systems allows the fusion of biochemical and biological data into a coherent physiological picture, and is therefore suitable in principle for detailed mechanistic analysis of the PA system in a variety of physiological processes.

CH 015 SERINE AND METALLOPROTEASE CASCADE SYSTEMS LINKED TO THE INVASIVE PHENOTYPE, James P. Quigley, Department of Pathology S.U.N.Y. at Stony Brook, NY 11794.

The serine protease, plasminogen activator (PA), functions in a protease cascade by generating the active protease plasmin which along with PA is responsible for hydrolyzing a number of glycoprotein components of the basement membrane and extracellular matrix (ECM). We have employed a highly specific anti-catalytic monoclonal antibody to PA to dissect the catalytic role of this enzyme in the degradation of ECM. It appears that the complete degradation of the ECM is not mediated by the PA/plasmin cascade alone but necessitates the additional involvement of a metalloprotease cascade comprising a number of specific zinc-requiring collagenases and gelatinases. We have purified and characterized a 70kDa metallo-collagenase/gelatinase whose expression is enhanced in Rous sarcoma virus transformed fibroblasts and along with PA and plasmin is catalytically responsible for most of the dissolution of fibroblast ECM. In addition we have recently purified and characterized a 92kDa collagenase/gelatinase from a number of human tumor cells. This metallo-enzyme is secreted in the inactive, zymogen form and its mode of activation and its catalytic role in cell invasion and migration are unknown. A highly specific, anti-catalytic monoclonal antibody has been raised against the 92kD enzyme by employing multiple, selective screenings of hybridoma cultures. The employment of this specific antibody in various migration, invasion and metastasis assay systems will be reported.

CH 016 TRANSLATIONAL CONTROL OF SERINE PROTEASE EXPRESSION: TISSUE PLASMINOGEN ACTIVATOR IN THE MOUSE OOCYTE, J.-D. Vassalli, J. Huarte, D. Belin, University of Geneva Medical School, Geneva, Switzerland, and M. L. O'Connell, W. G. Richards, S. Strickland, Department of Pharmacology, SUNY, Stony Brook, NY 11794

Primary mouse oocytes contain untranslated mRNA for tissue plasminogen activator (t-PA). During meiotic maturation, t-PA mRNA undergoes a 3' polyadenylation, is translated, and is degraded. Injections of maturing oocytes with different antisense RNAs directed against coding and non-coding portions of the t-PA mRNA blocked t-PA synthesis. Analysis of t-PA mRNA in matured oocytes after antisense injections suggested a cleavage of the RNA/RNA hybrid region. In primary oocytes, the 3' non-coding region was susceptible to antisense-catalyzed cleavage, while the other portions of the RNA are blocked from hybrid formation until maturation occurs. Antisense RNA complementary to the extreme 3' 103 nucleotides (nt) blocked elongation, translational activation, and destabilization.

Injected RNA fragments corresponding to part of the 3'-untranslated region (3'UTR) of this mRNA are also subject to regulated polyadenylation. Chimeric mRNAs containing part of this 3'UTR are polyadenylated and translated following resumption of meiosis. Polyadenylation and translation of chimeric mRNAs require both specific sequences in the 3'UTR and the canonical 3'-processing signal AAUAAA. Injection of 3'-blocked mRNAs and of *in vitro* polyadenylated mRNAs shows that the presence of a long poly(A) tract is necessary and sufficient for translation. These results establish a role for regulated polyadenylation in the post-transcriptional control of tissue plasminogen activator expression.

Based on these results, we are currently developing a new method for examining the role of this protease in mammalian oogenesis and early embryogenesis.

Proteolysis in Cell Migration, Development and Cancer - II

CH 017 ROLE OF PROTEASES IN NEURITE OUTGROWTH. Randall N. Pittman, Angela DiBenedetto, Jeff Ware, and Ann Marie Inglis, Department of Pharmacology-6084, Univ. of Pennsylvania School of Medicine, Philadelphia, PA 19104.

Rat sympathetic and sensory neurons in culture release urokinase plasminogen activator (uPA) and a metalloprotease. A large fraction of these proteases is released from the growth cones of growing neurites, suggesting that these proteases may be involved in growth cone functions. The metalloprotease degrades native and denatured Types I and IV collagen and fibronectin. Inhibition of the metalloprotease blocks neurite outgrowth in 3-dimensional collagen gels but does not affect outgrowth on 2-dimensional collagen substrates. These data are consistent with a role for the metalloprotease in degrading components of the extracellular matrix to create "channels" for neurite outgrowth in the peripheral nervous system.

Some of the uPA released by the neuron binds in discrete patches to the bottom surface of the neuron (next to the substrate). Inhibition of uPA activity with physiological or synthetic inhibitors or with antibodies increases neurite outgrowth about 2-fold. The effects of inhibiting uPA appear to be direct rather than indirect through inhibition of plasmin generation. Plaminogen is not present in our serum-free culture system and inhibitors of plasmin do not affect neurite outgrowth. Time lapse videomicroscopy of individual growth cones indicates that inhibition of uPA activity decreases lamellipodial activity on the sides and base of the growth cone and increases activity at the leading edge of the growth cone. The underlying molecular basis for the effects of uPA inhibitors on growth cone motility is being investigated. In addition, potential substrates for uPA in the extracellular matrix or on the neuronal surface are currently being examined.

CH 018 THE ROLE OF PROTEASES IN THE CONTROL OF ANGIOGENESIS, Rifkin, D.B., Department of Cell Biology, New York University Medical Center, New York, New York 10016.

Normal cell invasion, as exemplified by capillary growth during neovascularization, demonstrates a requirement for the elaboration of extracellular serine and metalloproteinases. Under normal conditions most endothelial cells are non-invasive when assayed in the amnion invasion assay. However, in the presence of the angiogenic factor bFGF, these cells become invasive. Coincident with this response there is an increase in the production of plasminogen activator (PA), collagenase, and the receptor for PA. This allows the cells to focus proteolysis in specific areas. It has also been shown that the release of soluble bFGF from a matrix-bound form may be mediated by plasmin. The inhibition of endothelial invasion may also rely on proteases as the angiostatic steroid, medroxyprogesterone, stimulates the production of high levels of an inhibitor of PA. Moreover, TGF-β, which is an inhibitor of endothelial cell invasion, is itself derived from a latent inactive precursor (LTGF-β) via the action of proteases. LTGF-β activation occurs on the cell surface and requires the action of PA plasmin. In addition,specific binding proteins and on the cell surface serve to regulate surface activation in a manner which may be similar to the activation of clotting factors. These aspects of the participation of proteases in the control of cell invasion will be discussed.

CH 019 PLASMINOGEN ACTIVATORS IN NEURAL DEVELOPMENT.
Nicholas W. Seeds, Glenn Friedman, Shahla Verrall and Paul McGuire. Neuroscience Program and Department of Biochem/Biophys/Genetics, University of Colorado Health Sciences Center. Denver, CO 80262.

Neural development in higher organisms requires that neurons must migrate from germinative zones to their proper place within the nervous system, and extend axons over very long distances to find their synaptic target. In the developing mouse cerebellum both of these processes, cell migration and axonal outgrowth, occur postnatally. Earlier studies have shown that the tissue levels of the extracellular protease plasminogen activator are elevated at the time of active cell migration. Using both a 360bp cDNA and an oligomeric DNA probe, we have found that tissue plasminogen activator (t-PA) mRNA levels are highest at this same time, and decrease markedly in the mature cerebellum. Cellular localization of t-PA activity has shown that it is concentrated at neuronal growth cones. Not only is t-PA is secreted by neurons, but migrating granule neurons of the developing cerebellum possess cell surface receptors, where t-PA is bound with high affinity (Kd=50pM) while retaining its proteolytic activity. t-PA binding is specific and appears to involve the EGF-like domain of t-PA. Protease activity appears to facilitate granule neuron migration, since serine protease inhibitors retard the migration both in vivo and in vitro. Plasminogen activator activity associated with axonal growth cones is more readily observed in the peripheral nervous system, where individual growth cones of sensory neurons are shown to possess urokinase-type plasminogen activator (u-PA). When grown on a fibronectin substratum these neurons rapidly extend axons, whose growth cones clear a path in the underlying matrix. This clearing occurs in the absence of plasminogen or in the presence of aprotinin, and leads to the release of ^{125}I-peptides of Mr = 220, 210 and 200,000 when the neurons are grown on a ^{125}-I-fibronectin matrix. This plasminogen-independent cleavage of fibronectin requires direct cell contact, and is insensitive to inhibitors of thrombin and metalloproteases. However, this activity is inhibited by protease nexin; suggesting that the plasminogen-independent limited cleavage of fibronectin by sensory neurons occurs by the direct action of u-PA on the matrix.

Proteolysis in Hemostasis, Inflammation and Wound Healing

CH 020 STRUCTURE AND BIOLOGY OF MAST CELL PROTEASES, George H. Caughey, Cardiovascular Research Institute and Department of Medicine, University of California, San Francisco, CA 94143-0911.

The principal mast cell serine proteinases, tryptase and chymase, are packaged with histamine in secretory granules and are released outside of the cell after mast cell activation. All human mast cells appear to contain tryptase. Chymase, however, is restricted to a subset of mast cells most heavily represented in tissues such as the dermis and the airway submucosa near glands. Molecular biologic studies suggest that the proteases are synthesized as inactive zymogens, which then must be processed to active forms prior to or during incorporation into secretory granules. Tryptase is active in the form of a heparin-associated tetramer of trypsin-like subunits, which are heterogeneous and appear to be the products of a multigene family. Chymase is a chymotrypsin-like monomer related to neutrophil cathepsin G and to lymphocyte granzymes. Following release, tryptase and chymase are resistant to inactivation by circulating inhibitors of serine proteases. In vitro experiments with dog and human enzymes suggest possible roles for tryptase in local anticoagulation and in the regulation of neuropeptide activity, bronchomotor tone, and collagenase activation. Recent evidence also suggests that tryptase is a potent mitogen and comitogen for cultured fibroblasts, a finding that could provide a molecular explanation for the observed association between mast cells and proliferating fibroblasts. Chymases may be involved in extravascular angiotensin conversion, regulation of submucosal gland secretion, inactivation of sensory neuropeptides released in neurogenic inflammation, and potentiation of histamine-induced vascular permeability in the skin. Thus, multiple lines of evidence suggest possible roles for mast cell tryptase and chymase in hemostasis, inflammation, wound healing and other biological processes.

CH 021 THE BIOCHEMISTRY AND PHYSIOLOGY OF THE PROTEIN C ANTICOAGULANT PATHWAY, Charles T. Esmon, Bernard Le Bonniec, Jia Ye and Arthur E. Johnson, Howard Hughes Medical Institute, Oklahoma Medical Research Foundation, Oklahoma City, and University of Oklahoma, Department of Chemistry and Biochemistry, Norman, OK.

Protein C (PC) is a major regulatory protein of the coagulation cascade. Defects in PC and accessory proteins contribute to thrombotic complications and appear to exacerbate inflammatory responses. The zymogen is converted to the vitamin K-dependent anticoagulant activated protein C by a complex of thrombin and thrombomodulin (TM), an endothelial cell surface protein. The specificity of thrombin is altered by TM such that thrombin activates PC 1,000x faster than free thrombin, but thrombin procoagulant functions are reduced. The mechanisms of acceleration of the PC activation are complex. Although activation by free thrombin is potently inhibited by Ca^{++}, activation of PC by the complex is Ca^{++} dependent. This is due to a conformational change in the protease domain in the region of the sessile bond upon binding of Ca^{++} to the first EGF domain of PC. This conformational change allows recognition by the T-TM complex. Studies with point mutants of PC and thrombin indicate TM may function to relieve an incompatibility between the P3 and/or P3' position (Asp) of PC (which is Gly or Phe in most thrombin substrates) and a Glu at position 192 in the primary specificity pocket of thrombin. This conclusion is based on the properties of a thrombin mutant in which Glu 192 is replaced with Gln (EQ192). Fluorescent and ESR studies of the thrombin catalytic center are consistent with alteration of the orientation of Glu 192 upon complex formation with TM. The Glu → Gln mutation is an example of alteration of enzyme catalytic efficiency without major changes in Km, a property shared by the protein-protein activation complexes of the coagulation system.

CH 022 PROTEOLYTIC CONTROL OF HUMORAL MEDIATORS Tony E. Hugli Department of Immunology, Research Inst. of Scripps Clinic, La Jolla, CA 92037

Various bioactive factors are generated from plasma proteins during activation of the coagulation and complement cascades. A group of potent inflammatory mediators known as anaphylatoxins are released from complement components during activation. These factors are called C3a, C4a and C5a and collectively induce smooth muscle contraction, granulocyte activation and migration, increase vascular permeability, and stimulate tissue macrophages and mast cells. These functions define a major role for the anaphylatoxins in inflammation. Mechanisms that regulate these factors physiologically include a serum carboxypeptidase (SCPN), proteases of the leukocytes and monocytes, and chymase and tryptase in the tissue mast cells. Removal of a C-terminal arginyl residue from these factors either diminishes or eliminates activity. Factor bound to monocyte or leukocyte receptors is internalized and then degraded by granulocytic proteases. Once the unbound anaphylatoxins escape the circulation, another mechanism is required to eliminate the buildup of these factors in the tissues. We have demonstrated that this mechanism may involve proteases from the tissue mast cells. In man the anaphylatoxins are able to activate the mast cells by both specific (i.e. receptor mediated) and non-specific (polycationic) mechanisms. Activation of mast cells in the peritoneum of a rat exposes surface chymase that in turn efficiently destroys the anaphylatoxin C3a when it is introduced in the peritoneal cavity. Radiolabelled C3a is degraded in the peritoneal cavity only after the mast cells have been stimulated. This mechanism of interstitial proteolytic degradation has major implications for tissue processing of bioactive humoral factors. The extravascular elimination of anaphylatoxins from injury sites contributes to the self regulation of hypersensitive and inflammatory responses in man.

CH 023 REGULATION OF TYPE 1 PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1) GENE EXPRESSION IN VITRO AND IN VIVO. D. J. Loskutoff, M. Keeton, S. Curriden and M. Sawdey. Committee for Vascular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037

PAI-1 is the primary physiologic inhibitor of tissue plasminogen activator (t-PA) and is a major biosynthetic product of cultured endothelial cells. It is an immediate early gene and its synthesis is stimulated by growth factors (TGFβ, PDGF, FGF, EGF), cytokines (IL-1, TNF), hormones (steroids, insulin) and other agents (PMA, endotoxin). PAI-1 levels are also regulated in vivo and abnormalities in this regulation are associated with vascular disease. We have been studying the mechanisms which regulate PAI-1 gene expression in vitro using cultured cells, and in vivo in mice. TGFβ, LPS, and TNFα each enhance the steady-state levels of endothelial cell PAI-1 mRNA by 30-fold or more. These increases are rapid, do not require de novo protein synthesis, and result from an increased rate of PAI-1 gene transcription. To further study PAI-1 gene expression, we have isolated the human PAI-1 gene and characterized its promoter. The gene itself is 12.2 kb in length and is organized into 8 introns and 9 exons. Comparative functional studies employing the firefly luciferase gene as a reporter gene show that fragments derived from the 5' flanking region contain the PAI-1 promoter, that it is expressed in a tissue-specific manner, and that it can be induced up to 40-fold by treating cells with TGFβ, PMA, or dexamethasone. Deletion mapping experiments indicate that each of these effects are mediated by two regions, a common "proximal" region located between -100 and +1 (10-fold induction), and distinct distal upstream regions for each agent. Endotoxin and TNF both stimulate PAI-1 gene expression in mice with the highest signal detected in heart, lung, adipose, kidney and liver. Analysis of these tissues by in situ hybridization reveals that the majority of PAI-1 mRNA is produced by the endothelial cells of the vessel wall. Evidence will be presented to suggest that elevated PAI-1 levels in the aorta and kidney may contribute to the thrombotic problems associated with atherosclerosis and glomerular nephritis, respectively.

Proteolysis in Disease/Therapy - I

CH 024 STUDIES ON THE PROTEOLYTIC DEGRADATION OF THE AMYLOID PRECURSOR PROTEIN IN ALZHEIMER'S DISEASE. Carmela R. Abraham, Gregory Papastoitsis and Bronwyn L. Razzaboni, The Arthritis Center, Department of Medicine, Boston University School of Medicine, Boston, MA 02118.

Alzheimer's disease (AD), Down's syndrome, and to a far lesser extent, normal aged brains exhibit abnormal extracellular deposits of amyloid. The major component of brain amyloid is the ß-protein, a 4Kd fragment of the larger ß-protein precursor (ßPP). The finding of the abnormally processed ß-protein and a protease inhibitor (α1-antichymotrypsin) in the amyloid deposits (1) prompted us to search for proteases which may generate the ß-protein from its precursor. We here report on the presence and partial purification of two such proteolytic activities from Alzheimer's brain. Normal physiologic C-terminal cleavage of the secreted form of ßPP occurs in the middle of the ß-protein suggesting that the ß-protein accumulates due to an alternative degradation pathway. We propose here that the protease activity we describe participates in this abnormal pathway.

AD brain homogenates were fractionated using centrifugation, ammonium sulfate fractionation and a DEAE column. Subsequent to the DEAE step, three proteolytic activities were detected by incubating the fractions with an iodinated peptide, P1 (P1=HSEVKMDAEF). This peptide was synthesised so as to flank the N-terminus of the ß-protein (aspartic acid); the histidine was added for the purpose of iodination. The cleaved radiolabeled products were separated by thin layer chromatography and exposed to X-ray film (2). One activity was further purified by size exclusion chromatography and the active fractions were characterized by: a) using a battery of protease inhibitors and activators and b) sequencing the P1 peptide after incubation with the protease fraction in order to identify the cleavage sites. Our first activity appears to be a calcium-activated serine protease which cleaves the peptide in three locations, one being between the methionine and aspartic acid. Thus, this protease fraction is able to generate the N-terminus of the ß-protein. Using the same procedure, a similar proteolytic activity was purified from perfused, fresh-frozen monkey brain, suggesting that this protease is present in the brain and not in the blood which may be entrapped in post-mortem human brain. Efforts are under way to characterize the other activities. Finding the protease(s) which generate the ß-protein can lead to the design of protease inhibitors aimed to arrest amyloid deposition which is believed to cause neuritic abnormalities, neuronal cell death and dementia.

1. Abraham et al., Cell **52**, 487-501 (1988)
2. Abraham et al., Peptide Research **3**, 211-215 (1990)

CH 025 PROTEASE NEXINS: REGULATION OF SERINE PROTEASES IN THE EXTRACELLULAR ENVIRONMENT. Dennis D. Cunningham, William E. Van Nostrand and Steven L. Wagner, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717

Protease nexin-1 (PN-1) and protease nexin-2 (PN-2) are protease inhibitors that are synthesized and secreted by a variety of cultured extravascular cells. They occur in several tissues and are abundant in brain. PN-1 and PN-2 form complexes with certain serine proteases; some of the complexes are stable in SDS. The complexes bind back to the cells and are rapidly internalized and degraded. This provides a mechanism for inhibiting and clearing certain serine proteases in the extracellular environment.

PN-1 is a 43 kDa protein that rapidly inhbits thrombin, urokinase and plasmin. PN-1 binds to the extracellular matrix; this accelerates its inactivation of thrombin and blocks its ability to inhibit urokinase or plasmin. PN-1 is identical to the glial-derived neurite promoting factor or glial-derived nexin. It stimulates neurite outgrowth in cultured neuroblastoma cells; this activity depends on thrombin inhibition. Thrombin brings about neurite retraction in these cells. PN-1 and thrombin also recriprocally regulation the stellation of cultured astrocytes. The activity of PN-1 was reduced about 7-fold in Alzheimer's disease (AD) postmortem brain compared to age-matched control cases with similar postmortem times. The AD samples contained increased PN-1-containing complexes that co-migrated with PN-1-thrombin complexes. The PN-1 mRNA levels for PN-1 were about equal in the AD and control cases tested. Together, these results suggest that increased thrombin or a thrombin-like protease in AD brain leads to the formation of PN-1-thrombin complexes and a decline in free PN-1. In normal human brain, much of the PN-1 occurs around blood vessels, suggesting a protective role against extravasated thrombin under conditions in which the blood brain barrier is compromised.

PN-2 is a 110 kDa protein that inhibits blood clotting factor XI_a, trypsin, chymotrypsin, the epidermal growth factor binding protein, and the γ-subunit of nerve growth factor, in order of decreasing effectiveness. PN-2 retains activity upon incubation at pH 1.5 or with SDS. PN-2 is contained in α-granules of platelets and is released upon platelet activation. This finding, along with its potent inactivation of factor XI_a, suggests that PN-2 may participate in the regulation of blood clotting at wound sites. PN-2 is identical to the secreted form of the amyloid β-protein precursor. The β-protein is the major component of neuritic plaques and cerebrovascular deposits that characterize AD. PN-2 contains at least a part of the β-protein judged by its cross reactivity with anti-β-protein antibodies. Studies by other investigators have indicated that the amyloid β-protein precursor is normally processed within the β-protein domain.

Proteolysis in Disease/Therapy - II

CH 026 MOLECULAR MODELING OF PARASITE PROTEASES AS A TOOL FOR INHIBITOR DESIGN AND FUNCTIONAL ANALYSIS, James H. McKerrow, Celeste Ray, Jacques Bouvier, Sharon Reed, Fred Cohen and Ann Eakin, Departments of Pathology and Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA 94143 and the San Francisco Veterans Administration Medical Center, San Francisco, CA 94121; Department of Pathology and Medicine, University of California, San Diego, San Diego, CA 92103.

Proteases play key roles in the pathogenesis of a number of parasitic diseases. Serine or metalloproteases, released by infectious helminth parasite larvae, function to degrade host tissue during invasion of skin by schistosomes (blood flukes) and migration through subcutaneous tissue by the nematode parasite *Strongyloides*. In both nematode and trematode parasites, cysteine proteases are secreted into the gut lumen for digestion of host hemoglobin. We have identified a homologous gut-specific cysteine protease in *Caenorhabditis elegans*. The gene coding for this protease is expressed in a temporal and gut-specific manner. It codes for a member of the papain superfamily. The gene has been localized to chromosome 5, and studies to identify regulatory elements that determine spatial and temporal expression will be presented. A related group of cysteine proteases are key metabolic enzymes in several parasitic protozoa. In the case of *Trypanosoma cruzi*, the causative agent of Chagas' disease, a fluoromethyl ketone-derivatized peptide inhibitor arrests intracellular invasion and replication of the parasite. In the case of *Entamoeba histolytica*, a unique cysteine protease gene is present only in pathogenic strains, and correlates with extracellular cysteine protease release that results in extraintestinal invasion and tissue destruction. Molecular modeling of the active sites of these enzymes, based upon their homology to other members of the papain superfamily, is being used to identify or modify inhibitors which might be lead compounds for new antiparasitic chemotherapy.

CH 027 BACTERIAL PROTEINASES IN PERIODONTAL DISEASE, James Travis, Zuxiong Chen, Antoni Polanowski, Maude Wikstrom, and Jan Potempa, Dept. of Biochemistry, University of Georgia, Athens, Ga. 30602. The degradation of connective tissue during the development of periodontitis is believed to be initially due to the release of bacterial proteinases into the dento-gingival crevice. These enzymes can degrade a host of structural proteins, including collagen, proteoglycan, fibrinogen, and fibronectin. In addition, inactivation of the neutrophil-derived bactericidal proteins cathepsin G and lysozyme, the plasma proteinase inhibitors alpha-1-PI and alpha-1-Achy, as well as the general degradation of all complement proteins and the release of C3a and C5a through conversion of C3 and C5 has also been observed. These data would support the hypothesis that neutrophils, recruited to the dento-gingival crevice to phagocytize invading bacteria, play a major role in the development of periodontitis through the release of active elastase. Thus, there would be gingival connective tissue degradation by neutrophil proteinases which are no longer susceptible to control by their target inhibitors as we-l as by those enzymes secreted by bacteria. In this laboratory we have studied the proteolytic enzymes present in and secreted by the organism _Porphyromonas gingivalis_, an anaerobe which has been associated with attachment loss during the development of periodontitis. We have isolated and characterized two trypsin-like enzymes from this organism and followed the degradation of a number of physiologically important proteins. In addition, we have found that both the amidase and proteinase activities in both crude extracts of this organism and purified enzymes are strongly enhanced by glycine-containing peptides. The data so far obtained supports the hypothesis that proteinases from _P. gingivalis_ both directly and indirectly cause the rapid degradation of connective tissue proteins during the course of progressive periodontitis. (Supported by grants from NIH and the Swedish Medical Research Council).

Proteolysis in Regulation and Disease

Proteases: Structure, Function and Evolution

CH 100 INHIBITION OF HUMAN SKIN INVASION BY CERCARIAE OF *SCHISTOSOMA MANSONI* WITH SYNTHETIC PROTEASE INHIBITORS CHOSEN BY COMPUTER MODELING, Payman Amiri, Fred E. Cohen, Lydia M. Gregoret, Ken Aldape, Johnny Railey and James H. McKerrow, Departments of Pathology, Pharmaceutical Chemistry, and Medicine, University of California, San Francisco, San Francisco, CA 94143.
Human schistosomiasis is a serious health problem, currently affecting twenty five percent of the world population. Infection of the human host is initiated by penetration of intact skin by the cercarial stage of the parasite. This penetration is mediated by a potent elastase which is released from cercariae upon contact with human skin. A computer model of the three-dimentional structure of the *S. mansoni* cercarial elastase was generated based on amino acid sequence data and structural information from other serine proteases with homologous sequences. The validity of the computer model of the cercarial protease was tested by performing kinetic analysis of a series of synthetic peptide substrates and inhibitors designed from predictions of the model. The results of kinetic studies showed that predictions of large hydrophobic S-1 and S-4 binding pockets were correct. Furthermore, tetrapeptide inhibitors with phenylalanine or leucine at the P-1 site inhibited invasion of human skin by live *S. mansoni* cercariae, whereas a corresponding inhibitor with alanine at P-1 site did not inhibit the invasion. Finally, the model predicted a tetrapeptide inhibitor with lysine at P-3 and phenylalanine at P-1, which increased the solubility four-fold (versus alanine at P-3), while minimally changing $^{k}3/K_i$.

CH 101 CHARACTERIZATION OF RECOMBINANT CHICKEN CYSTATIN VARIANTS, Ennes A. Auerswald[1], Gabi Genenger[1], Werner Machleidt[2], Hartmut Oschkinat[3] and Hans Fritz[1]; [1]Abteilung für Klinische Chemie und Klinische Biochemie, Universität München, W-8000 München 2, [2]Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, W-8000 München 2; [3]Max-Planck-Institut für Biochemie, W-8033 Martinsried, Germany

Chicken egg white cystatin is a tight-binding, reversible (competitive) protein inhibitor (116 aa) of cysteine proteinases. For detailed structural and functional analysis recombinant variants of chicken cystatin were constructed, via a synthetic master gene mutated, expressed with an E.coli secretion system and isolated from periplasm by Cm-papain and immunoaffinity chromatography. The amino acid substitutions were located particularly in and around the conserved 1st hairpin loop, the QLVSG region, which is supposed to be one of the three main interaction aereas for papain binding together with the N-terminus and the 2nd hairpin loop (W. Bode et al. 1988, EMBO J.7, 2593 - 2599). The biochemical analyses of the resulting variants include: electrophoresis, IEF, HPLC, peptide mapping, amino acid sequencing, estimation of binding behaviour, NMR spectroscopy and measurement of inhibition constants. Variants with single amino acid substitutions show different inhibition behaviour with the cognate enzymes papain, actinidin and cathepsin B. This demonstrates the importance of single amino acid interactions for inhibition strength.

CH 102 EXPRESSION OF HUMAN RECOMBINANT TYPE IV COLLAGENASE (72KD) IN ESCHERICHIA COLI AND CHARACTERIZATION OF THE ACTIVE ENZYME. Robert E. Bird[1], Susan Kraus[1], Sylvia A. Crush-Stanton[1], Rafael Fridman[1,2], Michael Berman[1], and William G. Stetler-Stevenson[2]. [1]Molecular Oncology Inc., 19 Firstfield Road, Gaithersburg, MD 20878 and [2]Laboratory of Pathology, NCI, NIH, Bethesda, MD 20892.
The ability of tumor cells to metastasize to distant sites has been attributed to the secretion of a 72 kD metalloprotease capable of degrading collagen IV in the basement membrane. To investigate the biochemical properties of this enzyme, the human type IV collagenase (72kD) cDNA has been expressed in E. coli under the control of the lac promoter. The expressed protein has the sequence Met-Thr-Met-Ile-Thr-Asn-Ser-Mature procollagenase IV (630 residues) and is found in the insoluble fraction after lysis of induced cells. The insoluble fraction is solubilized in guanidine-HCl, diluted 10-fold, and dialyzed against buffer. The refolded enzyme was passed through a gelatin-Sepharose column and the bound enzyme was eluted with DMSO. Approximately five milligrams of homogeneous enzyme were obtained from 10 grams of induced cells. This enzyme has been characterized by gelatin zymography and in a soluble gelatinase assay. It is inactive as the proenzyme (or zymogen) but can be activated to the 62 kD form by treatment with p-aminophenylmercurial acetate (APMA). The active enzyme is inhibited by purified tissue inhibitor of metalloproteinase (TIMP-2) which is found in a non-covalent complex with native type IV collagenase (72 kD) secreted from human tumor cells. The enzyme does not autoactivate indicating that it is stable in the absence of the inhibitor TIMP-2. The purified recombinant enzyme is currently being assayed for activity against type IV collagen as substrate. This enzyme will be used in assays to monitor the activity of recombinant TIMP-2 during refolding and purification. Furthermore, this recombinant enzyme will be useful for studying type IV procollagenase/TIMP-2 interactions.

CH 103 MOLECULAR STRUCTURE OF LEUCINE AMINOPEPTIDASE AND ITS COMPLEX WITH BESTATIN.
S.K. Burley+, P.R. David*, R.M. Sweet%, A. Taylor#, & W.N. Lipscomb* +Laboratory of Molecular Biophysics, The Rockefeller University, New York, NY 10021; *Gibbs Chemical Laboratory, Harvard University, Cambridge, MA 02138; %Department of Biology, Brookhaven National Laboratory, Upton, NY 11973; #USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA 02111.
The three-dimensional structure of bovine lens leucine aminopeptidase (EC 3.4.11.1) complexed with bestatin, a slow-binding inhibitor, has been solved to 3.0Å resolution and refined at 2.25Å resolution. In addition, the structure of the isomorphous native enzyme has been refined at 2.3Å resolution. The enzyme is physiologically active as a hexamer, which has 32 symmetry and is triangular in shape with a triangle edge length of 115Å and maximal thickness of 90Å. The monomers are crystallographically equivalent and each is folded into two unequal α/β domains connected by an α-helix to give a comma-like shape with approximate maximal dimensions of 90x55x55Å^3. The secondary structural composition is 40% α-helix and 19% β-strand. The N-terminal domain (160 amino acids) mediates trimer-trimer interactions and does not appear to participate directly in catalysis. The C-terminal domain (327 amino acids) is responsible for catalysis and binds the two zinc ions, which are less than 3Å apart. The pair of metal ions is located near the edge of an 8-stranded, saddle-shaped β-sheet. One zinc ion is coordinated by carboxylate oxygen atoms of Asp-255, Asp-332 and Glu-334, and the carbonyl oxygen of Asp-332. The other zinc ion is coordinated by the carboxylate oxygen atoms of Asp-255, Asp-273 and Glu-334. The active site also contains two positively-charged residues, Lys-250 and Arg-336. The six active sites are themselves located in the interior of the hexamer, where they line a disk-shaped cavity of radius 15Å and thickness 10Å. Access to this cavity is provided by solvent channels that run along the two-fold symmetry axes. Active site conformational changes occur on bestatin binding, and the mechanism of bestatin inhibition will be discussed in detail.

CH 104 KALLIKREIN-BINDING PROTEIN IS A SERPIN: PURIFICATION, MOLECULAR CLONING AND EXPRESSION, Julie Chao, Karl X. Chai, Jian-Xing Ma and Lee Chao, Medical University of South Carolina, Charleston, South Carolina 29425-2211

Tissue kallikrein-binding protein (RKBP) was purified from rat serum and the cDNA encoding the protein was isolated. Sequence analysis indicated that RKBP belongs to the serpin superfamily and contains 416 amino acid residues. RKBP gene of 10 kilobases was cloned and it comprises of four introns and five exons. The organization of RKBP gene is homologous to those of human α1-antitrypsin and α1-antichymotrypsin. In the 5'-flanking region of RKBP gene, a variant TATA box sequence ATAAATA is found at 20 base pairs upstream from the transcription initiation site. This region also contains a putative steroid hormone responsive element. Northern and dot blot analyses using RKBP cDNA probe showed that the expression of RKBP gene in rat liver is reduced after acute phase inflammation and is induced by estrodiol and progesterone. Southern blotting revealed restriction fragment length polymorphisms between normotensive and spontaneously hypertensive rats at or near RKBP locus. Recombinant RKBP was expressed in *E. coli.* and purified as a 43 KDa protein which forms a 92 KDa SDS-and heat-stable complex with tissue kallikrein. N-terminal amino acid sequence of recombinant binding protein matches completely with that of RKBP purified from serum. The results open the way for studying the regulatory mechanisms of RKBP gene expression by hormones as well as the interactions of tissue kallikrein and the binding protein through protein engineering.

CH 105 **A 15 AMINOACID PROLINE-RICH REGION CONTROLS THE ACTIVATION AND SUBSTRATE SPECIFICITY OF RECOMBINANT HUMAN FACTOR XII,** Franca Citarella, Alessandro Aiuti, Claudia La Porta, Silvia Misiti and Antonio Fantoni, Dip. Biopatologia Umana, Universita' di Roma La Sapienza, Italy.
From FXII cDNA clones were prepared two recombinant constructs: (a) the full length FXII cDNA and (b) a partially deleted cDNA coding only for the leader peptide, 15 aminoacids af the proline-rich region and the catalytic region of FXII. The two constructs were introduced by homologous recombination into the vaccinia virus genome and the recombinant viruses were used to infect hepatoma HepG2 cells. Cells expressing full length FXII cDNA secrete a 80,000 dal protein reacting with a FXII specific mAb. The immunologically purified recombinant FXII is identical to native FXII for size, clotting activity, amidolytic activity and activation by negative charges. Cells expressing the partially deleted FXII cDNA secreted a 32,000 dal protein antigenically identified to FXII containing the catalytic region and 15 aminoacids of the proline-rich region, but lacking 319 aminoacids of the regulatory domains. This latter engineered FXII protein mantains the clotting activity of native FXII and is activated by negative charges in sharp contrast of FXII fragment, a naturally occurring degradation product of FXII comprising only the catalytic region, which is not activated by negative charges and is not active in coagulation. Thus, 15 aminoacids of the proline-rich region confer to this engineered FXII both the capacity to bind negative charges and the substrate specificity for initiating the coagulation cascade.

CH 106 STRUCTURE-BASED INHIBITION OF RECOMBINANT HIV1 PROTEASE USING NON-PEPTIDE COMPOUNDS, Dianne L. DeCamp, Lilia M. Babé, Paul R. Ortiz de Montellano, Paul S. Furth, Irwin D. Kuntz, and Charles S. Craik, Departments of Pharmaceutical Chemistry and Biochemistry/Biophysics, UCSF, San Francisco, CA 94143-0446

Recombinant HIV1 and 2 proteases have been expressed and purified from *E. coli* and *S. cerevisiae* , respectively (Babé *et al.*, (1990) Protein and Pharmaceutical Engineering, Wiley-Liss, Inc. 71-88; Pichuantes *et al.*, (1990) J. Biol. Chem. **265**, 13890-13898). Purified enzymes have been used to test the inhibitory effects of compounds found with the docking algorithm of Kuntz *et al.* (1982, J. Mol. Biol. **161**, 269). This molecular modeling program finds structures which will fit the shape of a macromolecular receptor site whose X-ray structure is known (DesJarlais *et al.*, (1988) J. Med. Chem **31**, 722-729). One of the compounds identified in the search was bromperidol. Haloperidol, a closely related compound and approved antipsychotic agent, was chosen for testing. Haloperidol inhibits the HIV1 and 2 proteases in a concentration-dependent fashion with a K_I of approximately 100 μM (DesJarlais *et al.*, (1990), PNAS **87**, 6644-6648). It is highly selective, having little inhibitory effect on pepsin activity and no effect on renin at concentrations as high as 5 mM. Both haloperidol and its hydroxy derivative have shown activity against maturation of viral polypeptides in bacterial and mammalian cell assay systems. Haloperidol is not useful as a treatment for AIDS but may be a useful lead compound for the development of an antiviral pharmaceutical. Analysis of the crystal structure of a peptide-enzyme complex shows that inhibitor binding enlarges a crevice in the vicinity of the carbonyl group of haloperidol when it is bound in its theoretically preferred orientation. Derivatives of haloperidol in which the carbonyl group has been replaced by groups that can interact with the crevice have been synthesized. The derivatives are all better inhibitors of the HIV1 protease than haloperidol, the IC_{50} values of the best inhibitors being approximately ten-fold smaller than that of haloperidol.

CH 107 INHIBITION OF AUTOPROTEOLYTIC ACTIVATION OF INTERSTITIAL PROCOLLAGENASE BY RECOMBINANT METALLOPROTEINASE INHIBITOR MI/TIMP-2, Yves A. DeClerck, Tsuey-Dawn Yean, Hsieng S. Lu, Jerry Ting and Keith Langley. Division of Hematology-Oncology, Children's Hospital of Los Angeles, Los Angeles, California 90027 and AMGEN, Inc., Thousand Oaks, CA 91320.

The purification and cloning of a novel metalloproteinase inhibitor (MI/TIMP-2) related to tissue inhibitor of metalloproteinases (TIMP) has been recently described by our laboratory (J. Biol. Chem. 264, 17445-17453, Proc. Natl. Acad. Sci. USA 87, 2800-2804). We have transfected Chinese hamster ovary cells with a vector containing human MI/TIMP-2 cDNA, and purified recombinant-derived MI/TIMP-2 (rMI/rTIMP-2) from the conditioned medium of such cells. We have investigated the inhibitory activity of rMI/rTIMP-2 toward rabbit fibroblast interstitial collagenase. The inhibition of activated collagenase by rMI/rTIMP-2 is stoichiometric and consistent with the formation of a 1:1 molar ratio complex. In addition to blocking the activated enzyme, rMI/rTIMP-2 inhibits the conversion of 52-kDa procollagenase to the 42-kDa active enzyme initiated by organomercurials. When plasmin is used as activator, rMI/rTIMP-2 does not inhibit the plasmin-mediated conversion of the 52-kDa proenzyme to the 46-kDa inactive intermediate but blocks further conversion of the 46-kDa intermediate to the 42-kDa active enzyme. The data indicate that rMI/rTIMP-2 blocks the autoproteolytic activation of procollagenase. Also, rMI/rTIMP-2 forms complexes with the 52-kDa procollagenase, the 46-kDa intermediate and with the 42-kDa activated enzyme which are stable to sodium dodecyl sulfate (SDS), such that the complexes can be visualized by SDS-polyacrylamide gel electrophoresis. It appears that the formation of a SDS-stable complex with procollagenase requires an initial conformational change of the procollagenase brought about by organomercurials or by plasmin cleavage. The data suggest that MI/TIMP-2 may be able to control the extracellular action of certain metalloproteinases not only at the level of the activated enzyme but also at the level of proenzyme activation.

CH 108 CHARATERISATION OF AN ALKALINE PEPTIDASE OF TRYPANOSOMATID ORGANISM, Helena Enahoro and Frank Ashall, Department of Pure and Applied Biology, Imperial College, London, SW7 2BB

An alkaline peptidase was detected in all the stages of the life cycle of *T.cruzi* and in other trypanosomatids, including African trypanosomes and *Leishmania*,using solution assays as well as direct detection of enzyme activity in gels, cleaves peptide substrates on the carboxyl side of arginine and lysine residues at alkaline pH. The enzyme has not been detected in any non-trypanosomatid protozoa or mammalian cells tested. We hypothesize that the alkaline peptidase is specific to organisms of the order, Kinetoplastida or to the family, Trypanosomatida and that it has a processing function *in vivo*. Subcellular localisation studies of the *C.fasciculata* and *T.cruzi* enzymes indicate that the alkaline peptidase is cytosolic. The enzyme was demonstrated to have a preference for cleavage of substrates with arginine or lysine at the P2 as well as P1 position of the peptide chain, although arginine and lysine are equally acceptable at P2, whereas arginine is preferred to lysine at P1. The enzyme was inhibited by DFP, TLCK, leupeptin and antipain,but was unaffected by PMSF, E-64, iodoacetic acid, pepstatin A or *o*-phenanthroline. All peptidylfluoromethanes, peptidyldiazomethanes and peptidylsulphonium salts containing arginine or lysine at P1 were also strong inhibitors. We hypothesize that the alkaline peptidase is a serine peptidase unusually sensitive to diazomethanes, or a cysteine peptidase unusually sensitive to E-64; or it maybe a novel type of peptidase.

CH 109 MOLECULAR STRUCTURE OF THE COVALENT INTER CHAIN CARBOHYDRATE CROSS-LINK IN PRE-α-INHIBITOR. Jan J. Enghild, Guy Salvesen, Stanley Hefta, Ida B. Thogersen, Shane Rutherfurd, Salvatore V. Pizzo. Pathology Department, Duke University Medical Center, Durham, N.C. 27710 and Beckman Research Instute of the City of Hope Duarte C.A. 91010.
Human blood contains two SDS stable proteinase inhibitors Pre-α-inhibitor and Inter-α-inhibitor. The two proteins are composed of one and two dictinct heavy chains respectivly. They contain the same light chain (bikunin) a Kunitz-type proteinase inhibitor of some serine proteinases. The chains of the two proteins are linked covalently by a chondroitin-4-sulfate that originates from Ser-10 of bikunin. Somewhere towards the non reducing end carbon-6 of an N-acetyl galactosamine is esterified, in the case of pre-α-inhibitor, to the C-terminal of α-carboxyl group of the heavy chain. The structure of this novel cross-link was analysed by carbohydrate and amino acid analysis, Edman degradation and FAB/MS.

CH 110 DISSOCIATION OF SPECTRIN PROTEOLYSIS AND PLATELET AGGREGATION: KUNITZ PROTEASE INHIBITOR DOMAIN OF APP/PN2 INHIBITS A23187 BUT NOT THROMBIN-INDUCED PLATELET AGGREGATION, Jill E. Foreman and David D. Eveleth, Cortex Pharmaceuticals,Inc., 15241 Barranca Parkway, Irvine, Ca.,92718

Calpain activity in platelets in situ, measured by analysis of spectrin proteolysis, was compared to aggregation and secretion in the presence of a variety of protease inhibitors. No correlation between inhibition of calpain and inhibition of aggregation or secretion was observed using either leupeptin (Ac-Leu-Leu-Arginal) or Calpain inhibitor 1 (Ac-Leu-Leu-Norleucinal). These compounds have similar effects on platelets stimulated with either thrombin or the calcium ionophore A23187. This suggests that calpain does not play an essential role in platelet activation. In contrast, aggregation induced by A23187 was inhibited by a synthetic amyloid precursor protein/protease nexin 2 (APP/PN2) fragment containing a Kunitz protease inhibitor domain. The APP/PN2 fragment failed to inhibit thrombin-induced aggregation or secretion induced by either agonist. These observations suggest that the APP/PN2 fragment inhibits expression of an aggregation system, possibly the proteolytic activation of GPIIa/IIIb fibrinogen receptor, induced by A23187. Since platelets secrete PN2 upon activation, this may represent a negative feedback loop influencing platelet aggregation.

CH 111 HUMAN AND DOG LUNG TRYPTASE: A COMPARISON OF SUBSTRATE SPECIFICITY AND INHIBITION, A.J. Ganzhorn, M-C. Chanal, S. Trumpp-Kallmeyer, B. Neises, D. Schirlin, J-M. Wihlm and G. Di Francesco, Marion Merrell Dow Research Institute, and Service de Chirurgie Thoracique, CHU, 67009 Strasbourg, France
Human lung tryptase may be involved in the pathology of asthma and other inflammatory diseases. The enzymes from human and dog lung were purified about 70-fold, starting with a high salt extract of whole tissue, and their substrate specificities compared. Both enzymes hydrolyze a series of peptide p-nitroanilides with similar turnover numbers, but dog tryptase has 3-50-fold higher catalytic efficiencies (V/K_m). Arginine in position P1 is preferred by a factor of about 2 over lysine by both enzymes. The inhibitor leupeptin binds better to the dog (K_i = 0.2 µM) as compared to the human (K_i = 1.4 µM) enzyme. A 3-dimensional model of tryptase was constructed based on the structure of the complex formed by bovine trypsin and bovine pancreatic trypsin inhibitor. Within the substrate binding site, Lys-192 and Asp-216 in the human enzyme are replaced by Gln and Gly in the dog enzyme. According to our model and kinetic data, residue 192 is the more likely candidate to account for the differences in substrate specificity and inhibition. Other changes in the two amino acid sequences do not appear to be close to the active site.

CH 112 THE SPECIFICITY OF VARIANT PROALBUMIN CLEAVAGE BY YEAST KEX2 PROTEASE AND THE HEPATIC PROALBUMIN CONVERTASE ARE IDENTICAL,
Peter M George, Stephen O Brennan, Robert J Peach, and Ian C Bathurst, Department of Biochemistry, Christchurch Hospital, Christchurch, New Zealand and Chiron Corporation, Emmeryville, CA94608
Yeast KEX2 protease was examined as a potential model for a human proalbumin convertase and, in all respects, mimicked the predicted properties of a proalbumin convertase. KEX2 rapidly cleaved the propeptide Arg-Gly-Val-Phe-Arg-Arg from the N-terminal of proalbumin but, unlike trypsin, did not cleave physiologically unprocessed human proalbumin variants. There was little or no cleavage of proalbumin Lille (-2 Arg-His) or Christchurch (-1 Arg-Gln), and there was negligible cleavage of proalbumin Blenheim (1 Asp-Val) which retains the dibasic processing site. Despite the absence of this dibasic sequence proalbumin Kaikora (-2 Arg-Cys), which is partially processed *in vivo*, was cleaved at about half the rate of normal proalbumin. Aminoethylation of the new cysteine increased the rate of cleavage to near that of normal proalbumin. The KEX2-catalysed cleavage of normal proalbumin was independant of pH between pH 6.0 and 8.0. Antitrypsin Pittsburgh (358 Met-Arg), an *in vivo* inhibitor of proalbumin cleavage, inhibited KEX2 in a reversible manner. KEX2 was also inhibited by a recombinant antitrypsin with a diarginyl reactive centre.

CH 113 PROTEOLYSIS OF HUMAN FIBRONECTIN AND APOLIPOPROTEIN AI BY NEUTRAL ENDOPEPTIDASE (24-11).
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Neutral metalloendopeptidases designated as elastase-type enzymes on the basis of their potential to degrade synthetic elastase substrates, were previously isolated from human skin fibroblasts and isolated HDL. Both enzymes cleaved fibronectin and Apo AI and were found to share high analogies with NEP (enkephalinase 24-11). We here demonstrated that purified NEP was able to degrade fibronectin (but not collagen types I, III, IV,V) similary as isolated enzymes from fibroblasts and HDL. NEP also hydrolyzed human skin elastin fibers (mainly elaunin and oxytalan fibers) as assessed by ex vivo studies on tissue sections and automated image analysis. NEP was previously described as a processing endopeptidase that cleaves biological active peptides; our studies demonstrating that it can also hydrolyse protein as fibronectin, raise the possibility it may modify cell-matrix interactions as an ectoenzyme. Increased levels of such enzyme, in the sera of patients with active sarcoïdosis associated with altered HDL cholesterol metabolism of these individuals could be linked to its apo AI degrading capacity. The acute enhancement of NEP activity from dermis fibroblasts during the phase III of senescence may also related to the parallel pericellular matrix disorganization.

CH 114 Expression of Biologically Active Human Antithrombin III in Sf9 Insect cells.
L. S. Gillespie, K. K. Hillesland and D. J. Knauer, Department of Developmental and Cell Biology, University of Calfiornia, Irvine, CA 92717
Antithrombin III (ATIII), a member of the SERPIN supergene family, is a plasma-borne thrombin inhbitor that plays a key regulatory role in hemostasis. Several members of this family, including ATIII, share the common property that their anti-protease activity is markedly accelerated by the sulfated glycosaminoglycan, heparin. In order to conduct a site-directed mutagenic analysis of the specific residues involved in the activation of ATIII by heparin, several of which are believed to be contained within the D helix, we have chosen to express ATIII in Sf 9 insect cells using recombinant baculovirus. In the present studies we demonstrate the successful construction and plaque purification of two different baculovirus recombinants that express the cDNA for human ATIII. Both recombinants express ATIII at a high level, approximately 10 to 35 $\mu g/10^6$ cells. The recombinant ATIII is indistinguishable from authentic human ATIII purified from plasma in terms of immunoreactivity, progressive thrombin inhibitory activity, selective recognition of heparin that is high affnity for authentic ATIII, and the kinetics and extent of activation by high affinity heparin.

CH 115 REGULATION AND EXPRESSION OF THE MULTIPLE α_1-PROTEASE INHIBITOR GENES OF *Mus Musculus*, Kenneth S. Krauter, Kate Montgomery, Jil Tardiff, and Frank Borriello, Department of Cell Biology, Albert Einstein College of Medicine, Bronx, N.Y. 10461

Murine α_1-Protease Inhibitor (α_1-PI), which is homologous to human α_1-Antitrypsin (α_1-AT), is encoded by a small multigene family located on chromosome 12. In order to establish a mouse model for a human α_1-AT deficiency disease, we have begun a detailed analysis of the regulation of expression of the mouse α_1-PI genes as well as a characterization of the properties of each member of the gene family. Using a combination of PCR and cloning techniques, we have shown that there are 5 α_1-PI genes which are greater than 95% identical in exon, intron and flanking regions. DNA sequence analysis of cDNA clones of all 5 mRNAs expressed in adult liver show that despite high overall sequence identity, the reactive sites of each gene have diverged from one another at an unexpectedly high rate. The pattern of changes is consistent with an evolutionary mechanism which selects for rapid diversification of the duplicated members in a functionally important region.

In other studies, we have shown that α_1-PI is expressed in a liver abundant manner and is transcriptionally active only in tissues in which the genes are expressed. Using a deletion-transfection approach, we have shown that the transcriptional regulation in liver is mediated, both positively and **negatively,** by a set of *cis*-acting DNA sequences extending as far as ~2500 bases upstream of the start site, which appear to bind to specific nuclear proteins. Unlike the situation in humans where significant macrophage expression of α_1-AT driven by an upstream promoter is seen, we have shown that expression of α_1-PI cannot be detected at significant levels in macrophage cells and that the extremely low levels of expression seen is exclusively from "leaky" transcription at the liver promoter.

CH 116 CHARACTERIZATION OF THE TWO MEMBRANE ASSOCIATED FORMS OF FURIN, Richard Leduc, Barbara A. Thorne, and G. Thomas, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201-3098.

Recently, three mammalian cDNA sequences, fur, PC2, and PC1 (or PC3) have been identified which share significant structural homology with the yeast KEX2 prohormone endoprotease gene sequence, a member of the subtilisin family of serine endoproteases which cleaves substrates at pairs of basic amino acids (-LysArg- or -ArgArg-). Structural analysis predicts that, like Kex2p, the fur gene product, furin, possesses a single membrane spanning domain whereas PC2 and PC3 apparently lack this structure. Expression of the fur cDNA in BSC-40 cells revealed that this gene product, furin, encodes a Golgi compartment-localized, membrane-associated, calcium-dependent, serine protease capable of efficiently processing pro-β-nerve growth factor to 13 kDa β-NGF [Bresnahan et al. JCB 111:(December, 1990)]. By using anti-furin antibodies, immunoblot analysis detected two furin translation products (90 and 96 kDa). Both products were tightly associated with membranes, presumably via the predicted membrane spanning domain, since each was resistent to alkaline carbonate extraction. Expression of a mutant furin lacking the predicted membrane spanning domain resulted in a soluble furin doublet suggesting that the predicted transmembrane spanning domain is essential for membrane localization and that the size heterogeneity does not result from a modification of the C-terminus of the protein. Examination of the amino terminal domain of furin reveals several potential paired basic amino acid cleavage sites. Endoproteolysis at these sites would generate a protein approximately 6 kDa smaller than the initial translation product suggesting that the furin doublet may represent precursor and processed forms of the enzyme.

CH 117 USE OF PROTEINASE RADIOZYMOGRAPHY TO DISTINGUISH CATALYTIC CLASSES OF SECRETORY GRANULE PROTEINASES. Iris Lindberg and Steven F. Roberts, Dept. of Biochemistry and Molecular Biology, Louisiana State University School of Medicine, New Orleans, LA 70112.

The radiozymographic method of Irvine et al (Analyt. Biochem. 190:141, 1990) was used to investigate the presence of various types of proteinases within chromaffin granule subfractions. This method involves the *in situ* cleavage of recombinant copolymerized [35S]- proenkephalin by proteinases separated by SDS-PAGE. By incubating gels with reversible inhibitors at 4 C prior to incubation of the gel at 37 C, this technique can be extended to the biochemical characterization (with respect to activators and inhibitors) of individual enzymes within a mixture. Three distinct enzyme bands, at 30, 66 and 76 kDa, were found in both the soluble and membrane-bound portion of chromaffin granules; all of these proteinases were found to be preferentially associated with the membranes. The 76 and 30 kDa enzymes were shown to be serine proteinases based on complete inhibition by soybean trypsin inhibitor (STI); inhibition was not observed using o-phenanthroline, PCMS, EDTA, or pepstatin. The 66 kDa enzyme was the only one which showed much greater activity in the presence of 5 mM Ca+2; this enzyme was inhibited by EDTA but not DFP, STI, PCMS, o-phenanthroline, or pepstatin.

CH 118 CONTRIBUTION OF THE GLUTAMINE 19 SIDE CHAIN TO TRANSITION-STATE STABILIZATION IN THE OXYANION HOLE OF PAPAIN, Robert Ménard, Julie Carrière, Henri E. Khouri, Céline Plouffe, Pierre Laflamme, Thierry Vernet, Daniel C. Tessier, David Y. Thomas and Andrew C. Storer, Biotechnology Research Institute, National Research Council of Canada, Montréal, Québec, H4P 2R2, Canada.
To investigate the relevance of an oxyanion hole to the mechanism of papain, mutants aimed at modifying one of the hydrogen bond donors (Gln19) thought to be involved in this oxyanion hole have been produced. Mutagenesis of residue 19 to an alanine results in a significant decrease in k_{cat}/K_M from which a value of 2.5 kcal/mol can be calculated for the binding energy of the H-bond linking the glutamine side chain to the substrate's carbonyl oxygen in the transition-state. Gln19 in papain has also been replaced by Ser, Glu and His. Introduction of an amino acid with an ionizable side chain in the oxyanion hole lead to enzyme variants Gln19Glu and Gln19His that are still active against the substrate CBZ-Phe-Arg-MCA. However, the activity is modulated by deprotonation of the glutamic acid side chain in the Gln19Glu mutant and possibly also of the imidazole side chain in the Gln19His mutant. Interaction of the Gln19Ala mutant with several inhibitors (chloromethyl ketones, aldehydes, nitriles) has also been investigated. The results obtained with these mutants are providing valuable mechanistic information for both enzyme-substrate and enzyme-inhibitor interactions.

CH 119 DEGRADATION OF BASEMENT MEMBRANES BY MATRIX METALLOPROTEINASES: SYNERGISTIC EFFECTS OF MATRIX METALLOPROTEASES 3 (STROMELYSIN) AND MATRIX METALLOPROTEASE 9 (92-KDA GELATINASE/TYPE IV COLLAGENASE), Yutaka Ogata, Sripad Gunwar, Ko Suzuki, Milton E. Noelken and Hideaki Nagase, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66103
Degradation of basement membranes (BMs) occurs in many pathological conditions such as glomerular nephritis, tissue ulceration, and tumor cell invasion and metastasis. We previously reported that MMP-3 effectively degraded collagen IV and laminin in intact BMs from bovine glomeruli (GBM) and bovine anterior lens capsules (LBM). Here we report degradation of BMs by two other MMPs, MMP-2 (72-kDa gelatinase/type IV collagenase) and MMP-9 (92-kDa gelatinase/type IV collagenase) and compare their abilities with that of MMP-3. Degradation products were measured by the amounts of protein and hydroxyproline released from BMs. Both MMP-2 and MMP-9 degraded GBM and LBM but their abilities were about 1/10 and 1/4 of that of MMP-3. However, when MMP-9 and MMP-3 were combined, the extent of BM degradation exceeded the sum of the products released by each enzyme, while such synergistic effects were not seen with a mixture of MMP-2 and MMP-3. Degradation products were also analyzed by SDS/PAGE and immunoblottings to identify the origin of fragments, i.e., collagen IV or laminin. The fragments of collagen IV were further mapped using specific antibodies against the N-terminal (7S) domain, the C-terminal (NC-1) domain or the major triple-helical region. Degradation of collagen IV was extensive by these three MMPs, and fragments in the M_r range 25,000-380,000 were detected. These results suggest that the three MMPs play an important role in the catabolism of BMs, especially in the combination of MMP-3 and MMP-9. (Supported by NIH Grant AR39189 and DK18381).

CH 120 EXPRESSION OF RECOMBINANT HUMAN FACTOR X IN CHINESE HAMSTER OVARY CELLS Debra D. Pittman, Kathleen Thomkinson, Terri L. Messier, William R. Church and Randal J. Kaufman Genetics Institute, Cambridge, MA and University of Vermont, Burlington VT.
Factor X, a vitamin K-dependent protease, is synthesized as a single chain polypeptide and undergoes extensive post-translational modifications. These modifications include cleavage of the propeptide, γ-carboxylation and β-hydroxylation. Prior to secretion, factor X is cleaved to a two chain molecule consisting of a heavy chain of 55 kDa and a light chain of 17 kDa. A 1.5 kb cDNA encoding full length human factor X was isolated from a human fetal liver library, inserted into a mammalian expression vector and transfected into dihydrolfolate reductase deficient Chinese hamster ovary cells. Factor X expression was assayed using a chromogenic assay and an Elisa. Cloned cell lines were isolated which efficiently secrete high levels of factor X. The expression of biologically active factor X was dependent on the presence of vitamin K in the culture medium. Less than 20 % of the secreted protein was biological active. Addition of warfarin, an antagonist of vitamin K, abolished activity. Immunoprecipitaion of [^{35}S]-cysteine labeled conditioned medium indicated that the factor X was secreted as a 55 kDa heavy chain and a heterogenous light chain of 17 to 19 kDa. In addition, a band of 75 kDa was observed, suggesting that the precursor polypeptide was not efficiently processed.

CH 121 LIMITED PROTEOLYSIS OF THE α-MACROGLOBULIN RAT α_1-INHIBITOR-3: IMPLICATIONS FOR A DOMAIN STRUCTURE, David S. Rubenstein, Jan J. Enghild and Salvatore V. Pizzo, Department of Pathology, Duke University Medical Center, Durham, NC 27710.
Rat α_1-inhibitor-3 is a 180 kDa monomeric proteinase inhibitor found in high concentration in rat plasma. By several criteria it has been shown to be a member of the family of α-macroglobulin proteinase inhibitors often exemplified by the tetrameric human α_2-macroglobulin. We have used limited proteolysis of rat α_1-inhibitor-3 to probe the domain structure of this family of proteins. Proteinases of different specificities, including trypsin, chymotrypsin, thermolysin, and *Staphylococcus aureus* V8 proteinase, were employed and a common fragmentation pattern was observed when the reaction products were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. These fragments were electrotransferred to polyvinylidene difluoride membranes and subjected to N-terminal amino acid sequence analysis in order to position them within the context of the primary structure. The fragmentation pattern may define the domain structure of α_1-inhibitor-3 and serve as a model for the domain organization of the α-macroglobulin family of proteins.

CH 122 EVALUATION OF SERPIN CONFORMATIONAL STABILITIES, Guy Salvesen, Alan Mast, Salvatore Pizzo and Jan Enghild, Pathology Department, Duke University Medical Center, Durham, N.C. 27710.
Many serpins, particularly those that inhibit proteinases, have a tendency to unfold at quite low urea concentrations (2-4 M). Proteolytic cleavage within the reactive site loop (RSL), a region that is thought to form intermolecular contacts to proteinases, enhances conformational stability of many serpins so that they now fail to unfold in 8 M urea. This stabilization is likely due to increased favorable interactions within the dominant structural feature of serpins, the A-β-sheet (Loebermann et al., J. Mol. Biol. 177, 531-556, 1984). We have examined the conformational stabilities of native, proteinase-complexed, and RSL-cleaved serpins. We find that proteinase-complexed serpins are closer to RSL-cleaved serpins than their native counterparts, in terms of stability. In some cases, we find that the position of cleavage within the RSL effects the conformational stability and other properties of the cleaved form. Moreover, incorporation of synthetic peptides corresponding to RSL regions alters the structure and inhibitory properties of native serpins. These data are discussed in terms of the proteinase-inhibitory mechanism of serpins.

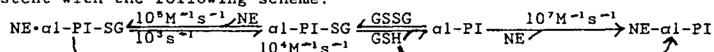
[Serpins used: α_1-proteinase inhibitor, α_1-antichymotrypsin, antithrombin III, ovalbumin and angiotensinogen.]

CH 123 STRUCTURAL REQUIREMENTS FOR THE EXPRESSION OF COLLAGENOLYTIC AND PROTEOLYTIC ACTIVITIES OF MATRIX METALLOPROTEINASE I (TISSUE COLLAGENASE), Ko Suzuki and Hideaki Nagase, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS 66103
Matrix metalloproteinase 1 (MMP-1/tissue collagenase) is a zinc enzyme secreted from connective tissue cells into extracellular matrix in zymogen form. When activated it digests triple helical regions of interstitial collagens I, II and III into 3/4 and 1/4 fragments. It has been also reported that MMP-1 can digest substrates other than interstitial collagens. To understand the relationship of collagenolytic and general proteolytic activities of MMP-1 we have investigated the expression of the two activities using collagen I and reduced, carboxymethylated transferrin as substrates following activation of the precursor (proMMP-1) with 4-aminophenylmercuric acetate (APMA). ProMMP-1 was first converted to the 43-kDa and then to the 41-kDa MMP-1, and both collagenolytic and proteolytic activities were detected simultaneously. However, after a longer incubation, the 41-kDa MMP-1 was gradually processed into the 22-kDa and the 27-kDa fragments. This process was accompanied by a loss of collagenolytic activity, while the proteolytic activity of MMP-1 was virtually unchanged. NH_2-terminal sequence analyses of the two fragments indicated that the Pro^{250}-Ile^{251} bond was cleaved by autolysis. The 22-kDa fragment was derived from the zinc-containing catalytic domain of MMP-1. Reduction and alkylation of the only disulfide bond of the 41-kDa MMP-1 located in the 27-kDa COOH-terminal domain also destroyed the collagenolytic activity of MMP-1, but this treatment did not alter the proteolytic activity. These results suggest that the 27-kDa COOH-terminal domain is required for MMP-1 to recognize the triple helices of interstitial collagens while the proteolytic activity of MMP-1 resides solely in the catalytic domain of 22-kDa. (Supported by NIH Grant AR39189).

CH 124 CHARACTERIZATION OF A MOLUSC (OCTOPUS VULGARIS) α–MACROGLOBULIN. Ida B. Thogersen, Frederic H. Brucato, Salvatore Pizzo and Jan J. Enghild, Pathology Department, Duke University Medical Center, Durham, N.C. 27710.
α–Macroglobulins have been isolated and charactered from severel mammalian species as broad specificity proteinase inhibitors. Thiol ester containing dimeric proteins (360 kDa) have also been purified from three invertebrate species; crayfish (Pacifastacus leniusculus), lobster (Homarus americanus) and horseshoe crab (Limulus polyphemus). These proteins are believed to be evolutionary related to the mammalian α–macroglobulins and have been charactered mainly by their ability to inhibit proteinases. The invertebrate α-macroglobulins mentioned above are all contained within the Arthropod phylum. We found that the Octopus (Octopus vulgaris), a representative of the Mollusc phylum, also posesses a dimeric thiol ester containing proteinase inhibitor. This is probably the most primitive animal currently known to posess an α–macroglobulin.

CH 125 BIOCHEMICAL AND MOLECULAR GENETIC STUDIES OF *LEISHMANIA PIFANOI* AMASTIGOTE SPECIFIC CYSTEINE PROTEINASES: PURIFICATION, ANALYSIS OF PROCESSING, cDNA AMPLIFICATION, AND SEQUENCE ANALYSIS, S. Monroe Duboise, Yara Traub-Cseko, Luis Rivas, Alfred A. Pan, and Diane McMahon-Pratt, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06510
Leishmania amastigote thiol proteinase(s) are essential for parasite survival. Axenic cultivation of an amastigote-like form of *Leishmania pifanoi* facilitates purification of an abundant developmentally regulated cysteine proteinase using either thiol interchange chromatography or immunoaffinity chromatography. Pulse-labeling experiments reveal a 40-kDa precursor that yields a 27-kDa proteinase. Binding of concanavalin A to both molecules indicates presence of a mannose carbohydrate moiety. Precursor enrichment was achieved by inhibition of processing. Amino terminal sequences of 40-kDa and 27-kDa forms are identical and have extensive homology with papain and cathepsin L. Two amplified gene segments, *Lpcys1* and *Lpcys2* encoding regions between cysteine and asparagine active sites were sequenced. Deduced amino acid sequences of probable active sites were particularly conserved with respect to corresponding sequences from other trypanosomatids. Existence of multiple *Lpcys2* gene copies and distinct chromosomal locations of the two genes were demonstrated. Both genes were shown to be preferentially transcribed in amastigotes with *Lpcys2* being more highly expressed than *Lpcys1*. Nucleic acid sequences of the two proteinases were as dissimilar to each other as to cysteine proteinase genes of *Trypanosoma brucei* and *T. cruzi*, suggesting an ancient divergence. Amino acid sequence analysis of internal fragments of the immunoaffinity-purified 27-kDa proteinase suggests that this protein is an *Lpcys2* gene product.

CH 126 DISULFIDE EXCHANGE REGULATES INTERACTION OF THIOL-MODIFIED α1-PROTEASE INHIBITOR WITH NEUTROPHIL ELASTASE, Suresh C. Tyagi and Sanford R. Simon, Departments of Biochemistry and Cell Biology, and Pathology, SUNY at Stony Brook, Stony Brook NY 11794
We have isolated a disulfide-bridged conjugate of α1-protease inhibitor and glutathione (α1-PI-SG) with one -SG is incorporated per α1-PI molecule. α1-PI-SG is a reversible inhibitor of amidolytic activity of neutrophil elastase (NE); all the NE•α1-PI-SG complex is completely dissociated after SDS-PAGE. Addition of one molar equivalent of reduced glutathione (GSH) regenerates free α1-PI which inhibits NE irreversibly and forms a nondissociable NE-α1-PI complex as seen on SDS-PAGE. We have estimated the rate of regeneration of α1-PI from α1-PI-SG after reduction with equimolar GSH in the presence of NE by monitoring the rate of appearance of the nondissociable NE-α1-PI complex on SDS-PAGE, and have calculated a second order rate constant in the range of $10^4 M^{-1} sec^{-1}$. The proposed mechanism for reduction of α1-PI-SG and formation of the nondissociable NE-α1-PI complex is consistent with the following scheme:



We have also recorded the intrinsic fluorescence of α1-PI after reaction with GSSG as a measure of conformational changes in the vicinity of the tryptophans. The Trp fluorescence was red shifted in the α1-PI-SG conjugate, but returned to that of native α1-PI after reduction with GSH. These results show that thiol modification of α1-PI alters both its inhibition of NE and its conformation. (Supported by NIH [HL-14262] & Cortech, Inc.)

CH 127 PURIFICATION AND PROPERTIES OF THE ADENOVIRUS PROTEASE, Joseph M. Weber and Karoly Tihanyi, Department of Microbiology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Québec, Canada, J1H 5N4

We have cloned and expressed the human adenovirus type 2 protease gene in E. coli (Houde and Weber, Gene 88: 269-273, 1990). The expressed protease was isolated from inclusion bodies by a four step chromatographic procedure including a DEAE-Sephacell, hydroxyapatite, Phenyl-Sepharose column chromatography and a gel filtration step on Sephacryl S-200. The final preparation showed a single protein by silver staining on one dimensional SDS-PAGE or on 2D (isoelectric focusing-SDS-PAGE) slab gels. The pure enzyme consisted of one subunit with a molecular weight of 27,000 and it had a pI of 10.2. The purified protease was stable for at least two months at -20°C. It cleaved the viral substrate protein pVII to VII. Enzyme activity was inhibited by p-Chloromercuribenzoate, N-ethylmaleimide, dithiodipyridin and Iodoacetamide. Soya bean trypsin inhibitor, E64 did not inhibit the enzyme. Labelled Iodoacetic acid bound the pure enzyme under nonreducing conditions while labelled disopropyl-fluorophosphate did not. These data suggest that the human adenovirus type 2 protease is a cystein rather than a serine protease.

CH 128 URSOLIC ACID AND ITS TRITERPENOID ANALOGS ARE NATURAL SLOWLY BINDING INHIBITORS OF PLASMIN, Qi-Long Ying and Sanford R. Simon, Departments of Pathology, and Biochemistry and Cell Biology, SUNY at Stony Brook, Stony Brook, NY 11794-8691.

Ursolic acid is a pentacyclic triterpenoid acid which is present in high concentrations in the cuticles of common fruits. This compound and its triterpenoid analogs are potent competitive inhibitors of the amidolytic activity of human plasmin. The mechanism of inhibition is consistent with a rapid pre-equlibrium binding of the triterpenes, involving a fast bimolecular association, followed by a slow unimolecular relaxation step to form a relatively stable enzyme-inhibitor complex. The apparent equilibrium constant for the initial binding of ursolic acid to plasmin is 1830 nM. The subsequent relaxation step generates a stable complex with a half life of 5.2 min; slow dissociation of the triterpene from plasmin restores amidolytic activity. The overall apparent inhibition constant for ursolic acid is 65 nM. Other triterpenoid analogs of ursolic acid, such as oleanolic acid, uvaol, and erythrodiol, are also effective slowly binding inhibitors of plasmin. Hederagenin, the triterpenoid aglycone of α-hederin, is even more potent than these analogs, but the intact saponin, hederin, is totally inactive. Albumin at concentrations found in plasma effectively binds the triterpenes as they dissociate from plasmin, facilitating reversal of inhibition of the amidolytic activity. This binding capacity of albumin may serve a physiological protective role, preventing inhibition of plasmin by triterpenes absorbed from ingested fruits. Albumin may also transport triterpenes effectively, preventing their clearance from the circulation and facilitating uptake by tissues. (Supported by NIH [HL-14262], NYS Science & Technology Office, and Cortech, Inc.)

Proteolytic Processing and Protein Degradation

CH 200 UBIQUITIN-SPECIFIC PROCESSING PROTEASES OF THE YEAST *SACCHAROMYCES CEREVISIAE,* Rohan T. Baker, John W. Tobias and Alexander Varshavsky, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Ubiquitin (Ub), a highly conserved 76-residue protein, is present in eukaryotes either free or covalently joined, via its C-terminal Gly residue, to a great variety of proteins. A major function of Ub is to mark proteins destined for selective elimination. Unlike the posttranslationally produced, branched Ub-protein conjugates, linear Ub-protein adducts are formed as the translation products of either natural or engineered Ub gene fusions. In the yeast *S. cerevisiae* and in other eukaryotes, Ub is generated exclusively by proteolytic processing of precursors in which Ub is joined either to itself to yield a linear polyubiquitin, or to the unrelated amino acid sequences of two ribosomal proteins. Miller et al. (*Bio/Technology* 7, 698, 1989) have cloned a *S. cerevisiae* gene, named *YUH1*, for a Ub-specific protease that cleaves Ub off relatively short C-terminal fusions but is nearly inactive with larger fusions such as Ub-β-galactosidase. We now report the isolation of three genes, named *UBP1-UBP3*, encoding three other yeast Ub-specific proteases. These proteases cleave Ub off its C-terminal extensions irrespective of their size. The UBP1, UBP2 and UBP3 proteases lack statistically significant sequence similarities to YUH1. They are also largely dissimilar to each other, except for two short Cys- and His-containing regions encompassing their putative active sites. We are characterizing the substrate specificities and *in vivo* functions of UBP1-UBP3. Deubiquitination of Ub-protein fusions by these proteases allows the *in vivo* or *in vitro* generation of proteins bearing predetermined amino-terminal residues, a method with applications in both basic research and biotechnology.

CH 201 PURIFICATION AND MOLECULAR CLONING OF THE IL-1β PROCESSING ENZYME, Roy A. Black, Shirley R. Kronheim, Bruce Mosley, Carl Kozlosky, Nicole Nelson, Kirk Van Ness, Teresa Greenstreet, David Gearing, Carl J. March and Douglas Pat Cerretti, Immunex Corp., 51 University St., Seattle, WA 98101

Interleukin-1β (IL-1β) is a key hormone of the immune system, with roles in hematopoiesis, inflammation, and wound healing. Mature IL-1β is generated by proteolytic cleavage of an inactive precursor between Asp-116 and Ala-117. We have reported previously that lysates of the human monocytic cell line, THP-1, carry out this cleavage. We report here that the processing enzyme (termed IL-1β protease) has been purified to homogeneity and the amino terminal 23 amino acids determined. Full length IL-1β protease cDNAs were then isolated in a three-stage process. In the first stage, fully degenerate PCR primers based on amino acid residues 1-6 and 11-16 were used to amplify DNA from cDNA prepared from THP-1 poly-A$^+$ mRNA. The amplified cDNA, after subcloning and sequencing, was found to encode amino acids 1-16 of the IL-1β protease. In the second stage, nucleotides 1-17 were used in a 3'-anchored PCR amplification to generate a cDNA encoding IL-1β protease from the amino-terminal amino acid to the poly-A tail. Finally, in the last stage, full length cDNAs were isolated from a human neutrophil library using the anchored PCR product as a probe. To demonstrate that these cDNAs encode the IL-1β protease, we inserted them into the mammalian expression vector, pDC303, and co-transfected these constructs into COS-7 cells with a second mammalian expression plasmid encoding precursor IL-1β. The transfected COS-7 cells were metabolically labeled with ^{35}S-Met and ^{35}S-Cys, and IL-1β was immunoprecipitated from lysates of the cells. Analysis of autoradiograms after SDS-PAGE of the immunoprecipitates showed that the precursor was specifically processed to mature IL-1β in the cells which received the IL-1β protease cDNA.

CH 202 PURIFICATION, AMINO-TERMINAL SEQUENCE AND KINETIC CHARACTERIZATION OF A SECRETED FORM OF THE YEAST PROHORMONE-PROCESSING KEX2 PROTEASE, Charles Brenner and Robert S. Fuller, Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305.

The Kex2 protease of the yeast *Saccharomyces cerevisiae* is a membrane-bound, Ca^{2+}-dependent serine protease that cleaves precursors of secreted peptides at the carboxyl side of pairs of basic residues. In order to characterize the properties of this molecule in detail, we have purified to homogeneity a soluble, secreted form of the enzyme produced by deletion of C-terminal sequences that include a single membrane-spanning anchor. The predicted primary sequence suggests initial synthesis of Kex2 as a pre-pro-enzyme with a pro-segment of approximately 100 residues preceding a domain homologous to subtilisin. Amino-terminal sequence of the purified protein demonstrates that Kex2 undergoes N-terminal proteolytic cleavage at the second of two Lys-Arg sites prior to the subtilisin domain, and suggests that cleavage of the pro-segment is autoproteolytic. Steady state kinetics of hydrolysis of a variety of synthetic substrates were examined with the purified enzyme. Substrates containing Lys-Arg and Arg-Arg as the P2-P1 residues were cleaved with similar kinetics. Substitution of residues other than Lys or Arg at P2 resulted in increases in K_M, whereas substitution of Lys for Arg at P1 resulted in changes in both k_{cat} and K_M such that k_{cat}/K_M was decreased by nearly 10,000-fold.

CH 203 MOLECULAR CLONING AND SEQUENCING OF A cDNA ENCODING METHIONINE AMINOPEPTIDASE FROM *SACCHAROMYCES CEREVISIAE*. Yie-Hwa Chang, Ulrich Teichert, John A. Smith, Departments of Molecular Biology and Pathology, Massachusetts General Hospital and the Departments of Genetics and Pathology, Harvard Medical School, Boston, Massachusetts 02114.
Methionine aminopeptidase (MAP), which catalyzes the removal of NH_2-terminal methionine from proteins, was purified to apparent homogeneity from *Saccharomyces cerevisiae*. A yeast cDNA encoding MAP was cloned and sequenced. The DNA sequence encodes a precusor protein containing 387 amino acid residues. The mature protein, whose NH_2-terminal sequence was confirmed by automated Edman degradation, consists of 377 amino acids. The function of the 10-residue presequence, which contains one serine and six threonines, remains to be established. The deduced amino acid sequence of yeast MAP shows 59.6% similarity and 42% identity to the amino acid sequence of the MAP from *E.coli*. Further, the yeast MAP gene was determined by DNA blot analyses to be a single copy gene located on chromosome VII.

CH 204 STRUCTURE OF A UBIQUITIN-DEPENDENT DEGRADATION SUBSTRATE: A 3-DISULFIDE FORM OF LYSOZYME, Robert E. Cohen, Christopher P. Hill and David Eisenberg, Department of Chemistry & Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, CA 90024-1570
Both ubiquitin conjugation and ubiquitin-dependent degradation of chicken egg white lysozyme in a reticulocyte lysate depend upon the reduction of one of the four disulfide bonds (Dunten *et al.*, J. Biol. Chem. 266, in press). Cleavage of the Cys6-Cys127 disulfide is required to convert lysozyme into a substrate for the ubiquitin-protein ligase, E3. Reduction and carboxymethylation of Cys6 and Cys127 yields a derivative (6,127-rcm-lysozyme) that is ubiquitinated and degraded faster than the unmodified protein in a reaction that no longer requires a reducing agent. Thus, the 6,127-rcm derivative appears to mimic an intermediate in the ubiquitin-dependent degradation of lysozyme. Presumably, determinant(s) critical for the binding to E3 are exposed upon reduction of the Cys6-Cys127 disulfide. To address this issue, the x-ray crystal structure of 6,127-rcm lysozyme is being compared with that of native lysozyme. Crystals of the 6,127-rcm derivative were obtained that belong to the orthorhombic space group $P2_12_12_1$ with cell dimensions $a = 77.7$ Å, $b = 81.1$ Å, $c = 37.9$ Å and two molecules per asymmetric unit. The crystals diffract well to better than 2.0 Å. The structure, solved by molecular replacement, will be discussed in the context of its recognition by the ubiquitin system.

CH 205 FUNCTIONAL STUDIES ON HIV-1 AND OTHER RETROVIRAL PROTEASES, Christine Debouck, Dept of Molecular Genetics, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406.
HIV-1 protease (PR) cleaves the HIV-1 *gag-pol* precursor at eight sites which differ in their primary sequence except for three of them which match the consensus sequence Ser/Thr.Xaa.Yaa.Phe/Tyr*Pro. One of our goals has been to analyze the efficiency of cleavage at these various sites by the HIV-1 and other aspartyl proteases in order to better understand the cleavage specificity of these proteases. To this end, we have engineered the eight HIV-1 PR cleavage sites in the middle of the *E. coli* galactokinase gene. The efficiency of processing at these sites was then examined using a double plasmid co-expression system followed by immunoblot analysis with a galactokinase-specific antibody. These studies revealed that all sites are cleaved well by the HIV-1 protease with the best sites being Tyr*Pro (p17-p24gag junction), Met*Met (between the end of p24 and p7gag), and Phe*Pro (flanking the protease). The other sites are being cleaved 2-3 times less efficiently. Similar analyses were carried out with the simian immunodeficiency virus (SIV_{MAC}) protease, SIV PR. This enzyme was found to cleave all sites in HIV-1 *gag-pol* with the exception of the p51/p15 bond (Phe*Tyr) in HIV-1 reverse transcriptase using the galactokinase co-expression system or with synthetic peptides. To further examine the resemblance between HIV-1 and SIV PR, we are currently testing their sensitivity to rationally designed mechanism-based inhibitors. Preliminary results indicate that these two enzymes are equally sensitive to a handful of inhibitors tested so far. This suggests that SIV infection in macaques should be a useful model for the preclinical evaluation of AIDS therapeutic agent targeted toward the virally encoded HIV-1 protease.

CH 206 CYSTATINS INHIBIT ATP-DEPENDENT PROTEOLYSIS IN RETICULOCYTE LYSATE, Julie M. Fagan and Lloyd Waxman, Department of Animal Sciences, Rutgers University, New Brunswick, NJ 08903

A major non-lysosomal pathway for protein breakdown in mammalian cells requires energy. The best studied ATP-dependent proteolytic system involves the participation of several enzymes to covalently attach ubiquitin to potential substrates. These ubiquitin-protein conjugates are by a large (26S) multienzyme complex, one component of which may be the 20S multicatalytic proteinase (MCP), or proteasome. Although much is known about the enzymes which conjugate ubiquitin to proteins, the proteinases that hydrolyze these conjugates are not well characterized. We have investigated the effects of the cystatins, a group of thiol proteinase inhibitors, on ATP-dependent proteolysis in reticulocyte lysates. Cystatin C, chicken egg white cystatin, and the high and low molecular weight kininogens were found all to inhibit the breakdown of ^{125}I-BSA with an IC_{50} ~1 µM, although cystatin C was generally the most effective. The same inhibitors also blocked the breakdown of endogenous proteins in lysates. Cystatins A and B showed little inhibition, even at 2.5 µM. Interestingly, the low molecular weight thiol proteinase inhibitors leupeptin, antipain, E-64, E-64-c, and E-64-d had no effect on proteolysis in lysates. Since the MCP is thought to play a role in the ATP-dependent proteolytic pathway, we investigated the effect of cystatins on the different hydrolytic activities of this enzyme. Similar to its effect in lysates, cystatin (2.5 µM) inhibited the degradation of ^{125}I-BSA by 95%. The MCP also has multiple active sites which are able to hydrolyze fluorogenic peptides. Cystatin C (2.5 µM) inhibited by >90% the hydrolysis of Succinyl-Leu-Leu-Val-Tyr-aminomethyl coumarin (AMC) and CBZ-Leu-Leu-Glu-naphthylamide, while having little effect on the cleavage of CBZ-Ala-Arg-Arg-AMC or CBZ-Gly-Gly-Leu-methoxynaphthylamide (all at 100 µM). Since recent evidence has suggested that the active sites of the MCP are of the serine type, the mechanism of inhibition by the cystatins is unclear and will require more detailed kinetic analysis. Supported by NIH, USDA, and the American Heart Association.

CH 207 DIFFERENTIATION-DEPENDENT CHANGE OF PROTEIN STABILITY IN THE HUMAN COLON CANCER CELL LINE HT-29. Germain Trugnan[1], Eric Ogier-Denis[2], Dalila Darmoul[1], Catherine Sapin[1], Laurent Baricault[1], Chantal Bauvy[2], and Patrice Codogno[2]. (1)INSERM U178, 16, avenue Paul Vaillant Couturier, Villejuif , and (2)INSERM U180, 45 rue des Saint Pères, Paris, France

When the human colon cancer HT-29 cells undergo an enterocytic differentiation, they correctly process their N-glycans, whereas their undifferentiated counterpart are unable to process Man_{9-8}-$GlcNac_2$ species. The mechanisms for such an observation have been studied. In the presence of 1-deoxymannojirimycin, a specific inhibitor of mannosidase I, differentiated HT-29 cells, as expected, accumulate Man_{9-8}- $GlcNac_2$ species, whereas in undifferentiated HT-29 cells these compounds continue to be rapidly degraded. In contrast, the use of leupeptin, a specific inhibitor of thiol and serine proteases, leads to the accumulation of these oligosaccharides in undifferentiated HT-29 cells. We therefore suggest that a direct pathway should exist between the rough endoplasmic reticulum and a leupeptin-sensitive degradative compartment in undifferentiated HT-29 cells. The emergence of this new pathway could explain why protein stability and N-glycan processing may vary as a function of the state of cell differentiation.

CH 208 THE YEAST PROHORMONE-PROCESSING KEX2 PROTEASE, A MEMBER OF THE SUBTILISIN SUPERFAMILY, CONTAINS A NOVEL DOMAIN ESSENTIAL FOR PROTEOLYTIC ACTIVITY.

Pablo Gluschankof and Robert S. Fuller, Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, CA 94305.

The Kex2 protein of the yeast *Saccharomyces cerevisiae* is a highly specific, Ca^{2+}-dependent serine protease required for post-translational maturation of pro-α-mating factor by cleavage at the carboxyl side of paired basic residues (Lys-Arg). Kex2 protease is anchored to the membrane of a late Golgi compartment via a single C-terminal transmembrane domain (TMD). Deletion of the TMD results in secretion of an active, soluble form of the enzyme. N-terminal to the TMD is a domain of 295 residues that is homologous to the subtilisin family of serine proteases. In order to establish the C-terminal boundary of sequences necessary for forming an active species, random, progressive C-terminal deletions were constructed, expressed in yeast and assayed both for *in vivo* function and for secreted proteolytic activity. Remarkably, the endpoint for production of active protease was not at the end of the subtilisin domain, but at a point 150 residues downstream that corresponds precisely to the endpoint of homology between Kex2 protein and a newly discovered family of homologous mammalian proteases. Disruption of this novel domain ("P-domain", for precursor-processing) blocks an early step in the maturation of pre-pro-Kex2 protease, the removal of an N-terminal pro-domain by cleavage at a Lys-Arg site.

CH 209 THE REGULATED PROTEOLYSIS OF THE BACTERIOPHAGE LAMBDA cII PROTEIN, Martín Gonzalez and Harrison Echols, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

The cellular concentration of the cII protein of phage lambda plays a key role in the lysis-lysogeny decision. Past work has determined that the relative activity of cII is controlled primarily through the stability of the cII protein, for which degradation is facilitated by the *Escherichia coli* HflA and HflB functions. By creating a carboxy-terminal deletion of the cII protein, designated cII-205, we have identified a region of the cII protein which is important for HflA mediated proteolysis. Additional experiments suggest that this region is not essential for HflB action, supporting the concept that HflA and HflB comprise elements of distinct degradative pathways for the cII protein.

CH 210 THE TWO MITOCHONDRIAL MATRIX PROCESSING PEPTIDASES (MPP AND MIP): STRUCTURAL AND FUNCTIONAL CHARACTERIZATION, Grazia Isaya, Frantisek Kalousek, Jörg Kleiber, Victor Saavedra and Leon E. Rosenberg, Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510

Upon import of nuclear-encoded precursor proteins into mitochondria, their amino-terminal leader peptides are cleaved by one (MPP, mitochondrial processing peptidase) or two (MPP and MIP, mitochondrial intermediate peptidase) peptidases in the mitochondrial matrix. We separated the rat liver mitochondrial MPP and MIP and purified MPP to homogeneity. The final MPP preparation consists of two polypeptides of 55 and 52 kDa. The 55 kDa subunit contains a 32 amino acid leader peptide and a mature protein which shows 35% identity with MPP of *Neurospora* and yeast. A conserved negatively-charged α-helical segment in the amino-terminal half of the subunit might be involved in the recognition of positively-charged mitochondrial leader peptides. *In vitro* mutagenesis in the region surrounding the MPP and MIP cleavage sites of once- and twice-cleaved precursors, indicates that the two peptidases recognize a higher order structure rather than specific amino acids at the junctions between the leader peptides and the mature amino termini. Cleavage by MPP requires a compatible mature protein on the carboxy-terminal half of the cleavage site. Those precursors whose mature amino terminus is not compatible with MPP cleavage evolved intermediate octapeptides which supply the structural requirements for MPP cleavage. They share the motif PheXX(Ser/Thr/Gly)XXXX and are specifically cleaved by MIP. A synthetic octapeptide is a potent inhibitor of the MIP processing activity. Inhibition is abolished when the amino-terminal Phe is deleted, whereas it is reduced by 10-fold when the Phe is substituted with a non-hydrophobic amino acid such as Tyr.

CH 211 ALPHA-CRYSTALLIN: ATP-DEPENDENT FORMATION OF HIGH MOLECULAR WEIGHT AGGREGATES AND DEGRADATION, J. Jahngen-Hodge, R.D. Lipman, A. Dean, L.L. Huang, and A. Taylor, Lab. for Nutrition and Vision Research, USDA Human Nutrition Research Center on Aging, Tufts University, 711 Washington St., Boston, MA 02111

Alpha-crystallin is the major protein component of lens, a tissue with little protein turnover. It accumulates damage over time and is known to form aggregates in-vivo.

One proteolytic pathway for disposal of damaged or obsolete proteins involves ubiquitin as a biological marker to initiate this ATP-dependent process. Ubiquitin is conjugated to proteins, forming aggregates of various molecular weights. Conjugates of high molecular weight are often degraded in an ATP-dependent fashion. Previously, we have demonstrated that the lens has an active ubiquitin conjugation system. Age influenced the quantity and molecular weight range of ubiquitin conjugates found in lens. Many conjugates were of high molecular weight.

Using ^{125}I-alpha-crystallin as substrate and enzymes of a reticulocyte lysate, we demonstrate that at 37°C, ATP-dependent and ATP-independent degradation occur. Simultaneously, there is ATP-dependent formation of high molecular weight aggregates which include ^{125}I-alpha-crystallin and ubiquitin. This suggests that ^{125}I-alpha-crystallin-ubiquitin conjugates are formed.

Supported by grants from USDA/ARS Contract No. 53-3K06-0-1 and Fight for Sight, Inc.

Proteolysis in Regulation and Disease

CH 212 EXPRESSION OF A FUNCTIONAL HUMAN PROPROTEIN PROCESSING ENZYME IN MAMMALIAN CELLS. Randal J. Kaufman+, Alnawaz Rehemtula+, Anthony J. Brake*, Philip J. Barr*, and Robert J. Wise^Δ. +Genetics Institute, Cambridge MA, ^Δ Brigham and Women's Hospital, Harvard Medical School, Boston, MA, and * Chiron Corp, Emeryville, CA 94608.
Intracellular proteolytic processing is an essential step in the maturation of many secretory proteins including plasma proteins, hormones, neuropeptides, growth factor, and viral glycoproteins. Frequently propeptide cleavage occurs after paired basic amino acid residues. To date, no mammalian propeptide processing enzyme with such specificity has been cloned and molecularly characterized. A cDNA clone encoding a human propeptide processing enzyme was isolated from a human liver cell line cDNA library which has structural homology to the well-characterized subtilisin-like protease Kex2 from yeast. The expressed product from the cDNA, called PACE (paired basic amino acid cleaving enzyme), was shown to mediate correct cleavage of the von Willebrand Factor precursor when coexpressed in either COS-1 monkey kidney cells or Chinese hamster ovary cells. Data will be presented on further characterization of the substrate specificity of the PACE protein for cleavage after paired basic amino acid residues and on its ability to cleave HIV-1 envelope gp160.

CH 213 FUNCTIONAL EXPRESSION OF A FAMILY OF GENES ENCODING PROHORMONE PROCESSING ENZYMES, Judith Korner, Jay Chun, David Harter, Laura O'Bryan and Richard Axel, Department of Biochemistry and Molecular Biophysics and Howard Hughes Medical Institute, Columbia University, New York, NY 10032
All cells are likely to express proteolytic cleavage enzymes capable of cleaving precursor proteins, such as the insulin receptor, at specific pairs of basic amino acids. In contrast to this ubiquitous cleavage activity, secretory cells are thought to express processing enzymes which recognize additional target sequences containing paired basic residues. We have combined gene cloning with a sensitive assay for protein processing in *Xenopus* oocytes to examine the function of a set of genes encoding a family of proteases. The *Xenopus* oocyte expresses an endogenous processing activity that cleaves precursor proteins after the sequence R-X-K/R-R, but does not cleave at paired basic residues within other contexts. A mutation that generates this sequence renders a precursor, such as the yeast α-factor pheromone, susceptible to cleavage by this endogenous protease activity. We have cloned a human furin cDNA that is ubiquitously expressed and may be the enzyme responsible for this cleavage activity. In addition, we have cloned a homologue of furin, FUR 97, whose expression is restricted to specialized secretory cells. Expression of FUR 97 RNA in *Xenopus* oocytes results in the proteolytic processing of prohormones expressed in these cell types. These studies suggest that organisms have evolved a ubiquitous processing activity and a set of more highly regulated cleavage activities which results in the differential processing of precursors in different tissues.

CH 214 CHLOROQUINE ADMINISTRATION TO RATS: A MODEL OF LYSOSOMAL STORAGE DISEASE? Tatjana A.Korolenko, Elena V.Rukavishnikova and Alfija F.Safina, Department of Cellular Biochemistry and Physiology, Institute of Physiology, Siberian Branch of the Academy of Medical Sciences of USSR, Novosibirsk, 630117, USSR
Suppression of intralysosomal proteolysis by chloroquine in isolated hepatocytes,macrophages,cell cultures includes pH-elevating and inhibition of lysosomal proteinases activity. Earlier we have found inhibition of purified cathepsins H, B, L by chloroquine. The single and repeated chloroquine administration to the rats was followed however by the increased activity of cystein proteinases and especially cathepsin D activity in liver. The amount and kinetics of chloroquine accumulation in liver were similar in both cases, as well as swelling of lysosomes. It suggests that increase of lysosomal proteinases activity reflects rather secondary changes of the particles (intralysosomal lipid storage) and disturbances of processing and excretion of lysosomal enzymes. We try to increase chloroquine accumulation in liver using model of extrahepatic cholestasis and chloroquine administration. In normal conditions chloroquine excretion is known to occur via bile.Cholestasis (on the 9th day) did not influence significantly on the amount of chloroquine accumulated. Chloroquine administration in vivo can be considered as a model of lysosomal storage disease, especially in repeated or chronic modes of drug administration.

CH 215 PURIFICATION AND CHARACTERIZATION OF A NOVEL THIOL PROTEASE INVOLVED IN ENKEPHALIN PRECURSOR PROCESSING. Timothy J. Krieger and Vivian Y.H. Hook, Dept. of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, MD.

We have isolated a novel thiol protease from bovine chromaffin granules that meets several requirements expected of a proenkephalin processing enzyme. Firstly, the thiol protease is present within highly purified secretory vesicles (chromaffin granules), the site of neuropeptide precursor processing. Secondly, the enzyme cleaves the model enkephalin precursor ^{35}S-(Met)-preproenkephalin (^{35}S-(Met)-PPE) optimally at pH 5.5 which is consistent with the acidic pH 5.5-5.8 intravesicular environment. Thirdly, thiol proteolytic processing of ^{35}S-(Met)-PPE resulted in high molecular weight intermediates (8-25 kDa) possessing the NH_2-terminal fragment of the precursor that lacks (Met)enkephalin squences; proenkephalin in vivo is also processed in this manner. Furthermore, authentic (Met)enkephalin was generated from peptide F by cleavage at Lys-Arg and Lys-Lys dibasic amino acid sites. Of particular interest, was the finding that this thiol protease demonstrated precursor selectivity, since it prefered the enkephalin over a tachykinin precursor as substrate. Biochemical characterization showed that the enzyme's binding to concanavalin A, molecular weight of 33 kDa, pI of 6.0, and pH optimum differ from cathepsin B, H, and other thiol proteases. These results suggest that a novel thiol protease may be involved in proenkephalin processing for the synthesis of active opiate peptides and possibly other neuropeptides.

CH 216 CHANGES IN BRAIN PROTEASE LEVELS UNDER VARIOUS CONDITIONS, Lajtha, A., Banay-Schwartz, M. and Kenessey, A., Center for Neurochemistry, The N.S. Kline Inst. for Psychiatric Research, Orangeburg, NY 10962
Although we understand a great deal about the mechanism and function of protein synthesis, much less is known about protein breakdown. Brain protein turnover is unexpectedly rapid and extensive, requiring high catabolic activity in vivo. The brain contains a number of exo and endopeptidases that have roles in the formation and inactivation of neuropeptides and in the metabolism of specific brain proteins. The enzymes are in general at high levels, considerably higher than required for physiological activity, and their activity is regulated by sequestration and or by endogenous inhibitors and activators. Endogenous inhibitors are at high level. Enzyme content and in vivo enzyme activity undergo a number of changes. We observed a significant decrease during development in brain protein turnover (in vivo protease activity), with a smaller change in content, and an increase in content with a smaller change of turnover, in the aging brain. Activity also changes under various stress conditions. The changes in malnutrition seem to be specific for brain, in that in tissues such as muscle protein, synthesis decreases while breakdown increases, leading to net protein loss, whereas in brain the decrease in synthesis is balanced by a decrease in breakdown. This indicates specific control mechanisms in brain. The changes of protease activity in the brain will be reviewed.

CH 217 Purification of Human Rhinovirus 14 protease 3C by fusion to glutathione-S-transferase.
Louis E.C. Leong and Alan G. Porter, Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511.

Human Rhinovirus 14 (HRV-14) is a picornavirus whose genome is expressed as a large polyprotein.This polyprotein is processed to mature viral proteins by two viral encoded proteases; 2A and 3C.The protease 3C has been expressed and purified from *E. Coli* by R.J. Colonno et.al.,though retaining protease 3C activity the protease has an unnatural methionine residue in its N-terminus. We have recently purified the HRV-14 3C protease by fusion to glutathione-S-transferase(GST).On cleaving the protease from the GST moiety we have generated protease 3C of greater than 90% purity which possesses its natural N-terminus.Immunoprecipitation and N-terminus sequencing confirms the protein purified as HRV-14 protease 3C.We are currently determining 3C activity with its native substrates and with the GST fusion expression system we have sufficiently high yields of the 3C protease to crystalize the protein.

CH 218 PROTEIN DEGRADATION IN THE YEAST EXOCYTIC PATHWAY

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A recently described protein degradation process appears to operate in an early compartment of the mammalian cell exocytic pathway. Our studies indicate that such a degradative pathway also exists in the yeast. To describe this process, we have studied the expression of alpha-1-proteinase inhibitor (A1Pi) in yeast cells transformed with human A1Pi cDNA genes. A1Pi is an appropriate choice for a reporter protein to study degradation in the yeast exocytic pathway because the Z variant (A1PiZ) is degraded while the wild type (A1PiM) protein is not. Transformed cells expressing A1PiM secrete about 20-30% of the A1Pi into the periplasmic space. A1PiZ transformed cells secrete little A1Pi and appear to degrade this protein, as the intracellular pool of A1PiZ is only 15-30% of that of A1PiM. Dot blot of whole cell RNA and northern analyses of poly-A RNA, indicate that the level of transcription of the A1PiZ and A1PiM genes is similar in the transformed cells. Studies of A1Pi in yeast strains deficient in vacuolar proteases suggest that degradation is non-vacuolar. A1Pi expression in yeast strains deficient in ubiquitin-mediated proteolysis, and pulse-chase protein radiolabelling experiments to determine the rates of degradation are in progress. (Supported by NIH grant HL 37128)

CH 219 ACTIVATION OF PROTEASE B OF THE YEAST, *S. CEREVISIAE*, BY AUTOCATALYSIS AND BY AN INTERNAL SEQUENCE, Vicki L. Nebes and Elizabeth W. Jones, Dept. of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213

Protease B is a serine protease found in the lysosome-like vacuole of yeast. The subtilisin-like protease is synthesized as a 76 kDa precursor that traverses the secretory pathway where it is extensively processed and modified before active enzyme is formed. Both O-linked and N-linked sugars are added in the endoplasmic reticulum, where, after signal peptide cleavage, an autocatalytic, intramolecular cleavage separates a 39 kDa precursor (aa 281-635) from the *pro* region, which is rapidly degraded. Sugar addition in the Golgi converts the 39 kDa precursor to a 40 kDa intermediate. In the late Golgi or vacuole, the vacuolar aspartyl protease, protease A, cleaves a C-terminal fragment to form a 37 kDa precursor that is rapidly processed, apparently autocatalytically, to form the 31 kDa mature enzyme. The *pro* region is required if processing of the 40 kDa precursor to the 37 kDa species by protease A is to occur. Normally the requirement is met intramolecularly but supplying the *pro* region as a separate molecule also suffices.

CH 220 COINCIDENT EXPRESSION OF THE PROCESSING PROTEINASE FOR AN INSECT VITELLIN WITH ACIDIFICATION OF YOLK GRANULES, J.H.Nordin, E.L. Beaudoin and X. Liu, Department of Biochemistry, Univ. of Massachusetts, Amherst, MA 0100.

Vitellin (Vt), the major nutrient protein for insect embryo development, is endocytosed by the oocyte and concentrated in yolk granules (YGs). Utilization is coordinated with embryo development, but what regulate the process is unknown. *Blattella germanica* (Bg) eggs store Vt unchanged through day 3 postovulation. Degradation is initiated at day 4 by the proteolytic processing of Vt's 3 subunits to specific peptides, which are consumed by the embryo. A cathepsin B-like yolk proteinase (assayed *in vitro* with Z-LNE and Z-AMC), first expressed at day 3, showed an 18-fold increase in Sp. Act. through day 6. Two other observations support its role in processing: (a) it processed Vt subunits *in vitro* and (b) mutant eggs, deficient in Z-LNE esterase, did not degrade Vt *in vivo* and their embryos failed to develop. Microscopy of YGs revealed that they decrease abruptly in size coincident with Vt processing at day 4. YGs at days 4-6, treated with 50 uM acridine orange (AO), fluoresced red-orange. YGs from yolk of earlier eggs had a non-specific, green or yellow-green fluorescence. Red-orange fluorescence was abolished by 4 uM CCCP, 8 uM monensin, 50 mM NH_4Cl and 0.05% Triton X-100. Quenching of AO fluorescence by granules was also reversed by these agents. Day 6 YGs have a pH of 5.9. Thus, these YGs apparently lower their internal pH by a proton pump. Vt processing was also inducible "prematurely" *in vitro* by acidification of yolk. Our results suggest that in Bg, Vt consumption is activated by a processing proteinase, whose expression is linked to developmentally regulated YG acidification. [NSF DCB 88-19371.]

CH 221 CLEAVAGE OF THE MEMBRANE PRECURSOR FOR TGF-α IS A REGULATED PROCESS.
Atanasio Pandiella and Joan Massagué.
Cell Biology and Genetics Program and Howard Hughes Medical Institute
Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Transforming growth factor-α (TGF-α) is generated by cleavage of a membrane-anchored precursor protein, proTGF-α. ProTGF-α is cleaved at a slow rate and accumulates on the cell surface thereby mediating cell-cell adhesion and mitogenic stimulation. We show here that cleavage of membrane proTGF-α by an elastase-like enzyme constitutes an important regulatory step in the generation of soluble TGF-α. Different activators of various intracellular signaling mechanisms were tested for their ability to stimulate proTGF-α cleavage in Chinese hamster ovary cells transfected with a proTGF-α cDNA. Cleavage is activated in response to serum factors, tumor-promoting phorbol esters, and calcium ionophores, leading to depletion of cell-surface proTGF-α which disperses as soluble factor. Activators of protein kinase A and protein kinase G did not stimulate proTGF-α cleavage. The effect of the calcium ionophore A23187 on proTGF-α cleavage was dependent on extracellular calcium influx, since it could be prevented by an excess EGTA or EDTA in the culture media, and was largely independent of protein kinase C activation. In contrast, phorbol 12-myristate 13-acetate (PMA) stimulated proTGF-α cleavage via protein kinase C independently of extracellular calcium. Activation of proTGF-α cleavage by serum was largely independent of both protein kinase C and extracellular calcium influx. These results demonstrate the existence of mechanisms that control the switch of TGF-α from a juxtacrine to a paracrine growth factor, and suggest that regulation of proTGF-α cleavage is a complex process that can be controled by a number of agents via at least three distinct signal transduction pathways.

CH 222 STUDY ON THE EFFECT OF A MUTATION IN THE SIGNAL PEPTIDE OF HUMAN FACTOR X ON SIGNAL PEPTIDASE PROCESSING *IN VITRO*, M. Racchi[1], H.H. Watzke[2], K.A. High[2] and M.O. Lively[1], [1]Biochemistry Dept., Bowman Gray Sch. of Medicine of Wake Forest University, Winston Salem, NC 27103; [2]Div. Hematology, University of North Carolina, Chapel Hill, NC 27599
The intrinsic and extrinsic blood coagulation pathways converge in the common pathway with the activation of Factor X (FX), a vitamin K-dependent coagulation protein. A single point mutation in the FX gene of a patient with severe FX deficiency has been described [Watzke,H.H. et al., Blood 76:442A (1990)] which results in the substitution of an Arg for a Gly at the -3 position of the signal peptide, three residues on the N-terminal side of the putative site of cleavage by signal peptidase (SP). This position in secretory signal peptides plays an important role in the recognition of the site of cleavage by SP [von Heijne, G., J. Mol. Biol. 184:99 (1985)] and this mutation is predicted to block cleavage of the nascent protein by SP. The wild type and mutant FX cDNAs have been subcloned into the pGEM vector (Promega) for transcription and translation *in vitro*. Post-translational and co-translational assays with the mRNAs for both mutant and wild type proteins have been used to study the effect of the mutation on translocation, segregation, and proteolytic processing. We observed that the mutant protein is not a substrate for SP *in vitro*. This result suggests that the absence of circulating FX in this patient is due to failure of SP to cleave the nascent precursor protein. [Supported by NIH GM32861 (M.O.L.) and HL06350 (K.A.H.)]

CH 223 EXPRESSION, PROCESSING, AND CELLULAR EFFECTS OF VIRAL PROTEASE/B-LACTAMASE GENE FUSIONS. Christopher J. Rizzo and Bruce D. Korant, DuPont Company, Wilmington, DE 19880.
Plasmid constructions in which HIV-1 protease was fused in frame to the 5' end of the Bacillus licheniformis B-lactamase gene were expressed in E. coli. The proteins were expressed to high levels under control of the T7 promoter. In constructs containing wild type protease the fusion protein is processed by the active HIV enzyme yielding mature B-lactamase and the 99 amino acid HIV-1 protease. An Asp_{25} > Gly mutation in the protease rendered the enzyme inactive and resulted in accumulation of the full length fusion protein as 60% of the total in the cell. The protease/lactamase fusion reduced the cytotoxic effects seen in clones expressing HIV-1 protease alone. In addition, the 99 amino acid protease cleaved from the fusion protein has increased solubility facilitating further studies of the enzyme. The B-lactamase enzyme in these constructions retains its ability to hydrolyze penicillin conferring an ampicillin resistant phenotype upon transformed E. coli.

CH 224 **A HIGH THROUGHPUT ASSAY FOR INHIBITORS OF HIV-1 PROTEASE: SCREENING OF MICROBIAL METABOLITES.**

Edoardo Sarubbi, M. Luisa Nolli, Franca Andronico*, Sergio Stella, Gerard Saddler, Enrico Selva, Antonio Siccardi* and Maurizio Denaro.
Lepetit Research Center, MMDRI, Gerenzano VA, Italy and *Dipartimento di Biologia e Genetica, Università di Milano, Italy.

A novel method for discovery of HIV-1 protease inhibitors in complex biological samples has been developed. The assay is based on two specific reagents: a recombinant protein constituted by a portion of the HIV-1 Gag polyprotein comprising the p17-p24 cleavage site, fused to E. coli ß-galactosidase, and a monoclonal antibody which binds the fusion protein in the Gag region. Binding occurs only if the fusion protein has not been cleaved by the HIV-1 protease. The assay has been adapted for the screening of large numbers of samples in standard 96-well microtiter plates. Using this method about 12,000 microbial fermentation broths have been tested and several HIV-1 protease inhibitory activities have been detected. One of these has been studied in detail.

CH 225 **mPC1 AND mPC2 ARE DISTINCT PRO-HORMONE PROCESSING PROTEINASES,**

Nabil G. Seidah, Suzanne Benjannet, Robert Day and Michel Chrétien,
Laboratories of Biochemical and Molecular Neuroendocrinology, Clinical Research Institute of Montréal, Montréal, Qué, Canada H2W 1R7.

Proteinases within neuroendocrine secreting cells are responsible for the processing of most pro-hormones at either single or pairs of basic residues. Recently, we reported the full cDNA structures of two new mouse subtilisin-like serine proteinases which display a 47% amino acid sequence similarity to the catalytic domain of the yeast KEX2 α-mating factor processing enzyme. These putative enzymes, called mPC1 and mPC2 were found to be selectively distributed and exclusively synthesized within neuronal and endocrine cells. The genes coding for PC1 and PC2 are located on two different chromosomes in both mouse and human. Co-expression of pro-opiomelanocortin (POMC) and either mPC1 or mPC2 was performed in three cell lines, namely within the constitutively secreting BSC-40 cells and within the regulated PC12 and AtT-20 cells. In all three cells, POMC is cleaved by mPC1 into ACTH and βLPH, whereas mPC2 cleaves POMC into αMSH and βEndorphin. This result agrees with the tissue distribution of PC1 and PC2 and emphasizes the narrower cleavage specificity of PC1 as compared to PC2 which cleaved the 5 pairs of basic residues analysed. A systematic catalogue of various cell lines for the presence of PC1 and PC2 transcripts, showed that one or both of these enzymes are expressed in AtT-20, βT3 and GH3 cells, but not in PC12, Ltk- nor BSC-40 cells. In order to further support the hypothesis of the physiological participation of PC1 and PC2 in pro-hormone processing in vivo, data will be presented regarding the co-regulation of these mRNAs with those of POMC in the pituitary gland.

CH 226 PRECURSOR PROCESSING BY PC2 AND PC3,

S.P. Smeekens, C. Albiges-Rizo, L.A. Phillips, A. Montag*, M. Schwin+, H. Swift+, W. Chutkow, M. Benig, and D. F. Steiner, Howard Hughes Med. Inst. and the Depts. of *Path., and +Molecul. Genetics and Cell Biol., Univ. of Chicago, Chicago, IL 60637

PC2 and furin (or PACE) are members of a novel class of mammalian proteins homologous to the yeast precursor processing endoprotease kex2 and the related bacterial subtilisins. We have recently identified and cloned a cDNA, designated PC3, from the mouse AtT20 anterior pituitary cell line that encodes an additional member of this family. Both PC2 and PC3 carry out endoproteolytic cleavages at dibasic residues within proopiomelanocortin, indicting that they have the specificities expected for precursor processing. We are also studying the activities of these proteases on proinsulin and other substrates and have expressed both PC2 and PC3 in a variety of systems. Northern blot analysis of a variety of tissues and cultured cell lines indicates that both PC2 and PC3 are expressed in neuroendocrine cells but are absent or very low in liver, kidney, muscle, and spleen. Immunohistochemical analysis indicates that PC2 is expressed within the islets of Langerhans, while electron microscopic analysis shows PC2 to be localized to the secretory vesicles of the β-cells. In Western blots of human insulinoma granule fraction extracts PC2 is detected as a 67 kDa protein, whereas analysis of the protein synthesized in Xenopus oocytes following injection of in vitro transcribed PC2 RNA reveals a 69 kDa glycoprotein that is processed at its N-terminus, suggesting that in vivo, PC2 itself is synthesized as a proprotein and may require proteolytic processing for activation. Taken together, these results suggest that PC2 and PC3 are endoproteolytic precursor processing proteases of the regulated secretory pathway. (Supported by HHMI and NIH Grants DK13914 and DK20595.)

CH 227 ISOLATION OF BOVINE KIDNEY LEUCINE AMINOPEPTIDASE cDNA: COMPARISON WITH THE LENS ENZYME AND TISSUE-SPECIFIC EXPRESSION OF TWO mRNAs,
Allen Taylor and Barbara P. Wallner, USDA-HNRCA at Tufts, Boston, MA 02111
Bovine lens leucine aminopeptidase (LAP) is the prototypical aminopeptidase. Immunological studies indicate that bovine lens and kidney LAPs are indistinguishable and that LAP is a very conserved protein (Taylor, A.et al. [1984] Exp. Eye Res. 38, 217-229). The cDNA sequence indicates that bovine kidney LAP is synthesized as a 513 amino acid protein containing a precursor with a 26 amino acid prosequence. In the C-terminal region an octapeptide is present in kidney LAP, which was not noted in the lens LAP sequence. Sequence homology between bovine lens LAP and *E. coli* xerB gene product had been noted, and we postulate that these--and several other--enzymes may belong to a new class of zinc metallopeptidases with common mechanistic and physical parameters. While only one (2.4 kb) LAP transcript was observed in lens tissue, two transcripts (2.0 and 2.4 kb) were observed in kidney tissue and cultured lens cells. As lens cells are progressively passaged, LAP mRNA concentrations are regulated in a manner consistent with the transient increase in LAP activity in these cells. However, the relative amounts of the 2.0 and 2.4 kb LAP messages are independently regulated. Northern blot analysis indicate that the two transcripts arise by differential splicing of a common precursor RNA.
Funded at least in part from USDA/ARS Contract 53-3K06-0-1 and the Guggenheim Foundation.

CH 228 THE N-END RULE OPERATES IN BACTERIA, John W. Tobias, Thomas E. Shrader, Gabrielle L. Rocap and Alexander Varshavsky, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

The N-end rule relates the metabolic stability of a protein to the identity of its amino-terminal residue (1-4). In eukaryotes, the presence of ubiquitin-specific processing proteases makes possible efficient deubiquitination of either natural or engineered linear ubiquitin fusions such as Ub-X-β-galactosidase, yielding an X-βgal test protein that bears a predetermined residue X at the amino terminus. Bacteria such as *Escherichia coli* apparently lack ubiquitin and ubiquitin-specific enzymes. This precluded the generation of N-end rule test substrates in bacteria. By expressing in *E. coli* the recently cloned yeast gene (*UBP1*) for a ubiquitin-specific processing protease (5), we bypassed the above difficulty, and have found, using X-βgals as test proteins, that a version of the N-end rule operates in bacteria as well. Specifically, amino-terminal Arg, Lys, Phe, Leu, Trp and Tyr conferred short half-lives (~2 min at 36°C) on the corresponding X-βgal proteins, whereas the X-βgals bearing any of the other amino-terminal residues were long-lived in *E. coli* ($t_{1/2}$ > 10 hr). A variety of *E. coli* mutants known to perturb proteolysis or stress response are being tested for their influence on the N-end rule; in a converse approach, a genetic screen is also being used to isolate the relevant mutants directly.

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CH 229 PROTEOLYTIC PROCESSING OF ANTIGENS INSIDE MACROPHAGE ENDOSOMES
Johannes M. van Noort[1], Jacqueline Boon[1], Alfons C.M. van der Drift[1], Josée P.A. Hilbers[2] and Claire J.P. Boog[2]. [1]TNO Medical Biological Laboratory, P.O. Box 45, 2280 AA Rijswijk, The Netherlands and [2]Dept. Immunology, State University of Utrecht, The Netherlands.
T cells usually respond to complexes between MHC proteins and antigen-derived peptides. Such peptides are liberated from exogenous antigens by limited proteolysis inside the endosomes of antigen presenting cells such as macrophages and B cells. By comparing the degradation of a model antigen, viz. myoglobin, after uptake by macrophages with the result of in vitro digestion with endosomal proteases, processing sites in myoglobin were mapped and could be attributed to different enzymes.
The endosomal aspartyl protease cathepsin D was found to account for most of the initial cleavages in myoglobin while the thiol protease cathepsin B appeared active in selectively trimming the C-termini of some cathepsin D-released peptides. Subsequently, we demonstrated that the predominant cathepsin D-released fragments of myoglobin were efficiently recognized by myoglobin-specific T cells. Each known T-cell epitope of the protein was located on a different cleavage product, consistently at the very N-terminus. We hypothesize that this N-terminal location relates to the role of cathepsin D as a pivotal processing enzyme. With different protein substrates, it was found that cathepsin D preferentially cleaves within a motif of about seven amino acid residues, five of which form the N-terminus of the newly generated fragment. We postulate that MHC proteins often focus on this structure -presumably common among many different processing products- for binding and presentation of peptides to T cells. As a result, T-cell epitopes of antigens may frequently be found at the N-terminus of cathepsin D-released fragments. We are currently testing the general validity of this hypothesis.

CH 230 CHARACTERIZATION OF MULTIPLE ENDOPEPTIDASES IN BOVINE ADRENAL CHROMAFFIN VESICLES,David C.C.Wan, Emanuel J. Diliberto, Jr., Lester Taylor, Barbara Merrill, Robert L. Johnson, William G. Chestnut and O. Humberto Viveros, Division of Pharmacology, and Division of Organic Chemistry, Burroughs Wellcome Co., RTP, NC 27709, USA

Putative neuropeptide processing endopeptidases were isolated from consecutive chromatographic fractionations of dialysed soluble fractions of purified bovine chromaffin vesicles on p-chloromercuribenzoate-agarose (PCMB-agarose), p-aminobenzamidine-agarose (p-ABA-agarose) and soybean trypsin inhibitor-agarose (STI-agarose) affinity columns. Proenkephalin precursor peptides (BAM12P, BAM22P and amidorphin) were used as substrates. Degradation peptide fragments were separated by RP-HPLC and identified by FAB mass spectrometry, amino acid composition and sequencing. Fractions eluted from PCMB-agarose affinity chromatography hydrolyzed the Arg-Arg sequence of BAM12P, resulting in the generation of Met-enkephalin and Met-enkephalin-Arg at pH 5.7. This activity was inhibited by PCMB and E64, indicating that a thiol protease is involved. The eluates from the p-ABA-agarose affinity column contained enzyme activity which cleaved at the Lys-Arg of BAM22P and at the Lys-Lys of amidorphin at pH 7.4. This activity was not inhibited by STI indicative of a non-trypsin-like endopeptidase. The eluates from STI-agarose affinity column had an enzyme activity capable of hydrolyzing amidorphin at the carboxy side of Lys-Lys. This activity was completely inhibited by STI indicative of a trypsin-like endopeptidase. This study demonstrates that a variety of different endopeptidase activities are found in soluble lysates of adrenal medulla chromaffin vesicles which may be involved in the synthesis of neuropeptides in the adrenal medulla.

CH 231 PROTEOLYTIC PROCESSING OF THE CYTOMEGALOVIRUS ASSEMBLY PROTEIN: IS A VIRAL GENE RESPONSIBLE? Anthony R. Welch and Wade Gibson, Virology Laboratories, Department of Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD 21205

Assembly and maturation of the herpesvirus capsid appears to involve the phosphorylation, proteolytic cleavage and ultimate elimination of an abundant "procapsid" constituent referred to as the assembly protein. Although the chronology of these modifications is not yet established, there is evidence that the cleavage event may be required for DNA packaging and therefore essential to virus replication and a potential target for antivirals.

Our studies of the cytomegalovirus assembly protein have demonstrated that its 40-kDa precursor is synthesized at late times after infection, moves slowly into the "NP-40 nuclear fraction", and undergoes cleavage(s) which eliminates its carboxyl end and produces the mature procapsid assembly protein. In order to pursue structural and functional studies of the assembly protein at the molecular level, we identified, cloned, and sequenced the genomic region encoding it.

Results of this work and subsequent studies established that the assembly protein is one of four in-frame, overlapping, carboxy-coterminal proteins that are independently transcribed and translated from a gene near the middle of the viral genome. These studies also revealed an eight amino acid candidate cleavage site that is perfectly conserved near the carboxyl end of the assembly proteins of human and simian cytomegaloviruses, and partially conserved in the counterpart protein of herpes simplex virus type 1. The sequences encoding the precursor of the largest of the overlapping proteins and of the assembly protein have been cloned, transcribed, and translated *in vitro*, and the resulting [^{35}S]methionine-labeled products are being used as substrates to detect the maturational protease. Progress in identifying this enzyme and defining its cleavage site will be presented.

Proteases in Cell Migration, Development and Cancer

CH 300 MECHANISM-BASED SERINE PROTEASE INHIBITORS AS POTENTIAL ANTITUMOR AGENTS: CELLULAR TARGETS, M.M. Ames, *D.H. Kinder and M.J. Wick, Department of Oncology, Mayo Clinic and Foundation, Rochester, MN 55905; and *College of Pharmacy, Washington State University, Pullman, WA 99169

Our laboratories have designed a series of novel di- and tripeptide boronic acid substrate analogs of the serine proteases pancreatic elastase and α-chymotrypsin. The roles of proteases and protease inhibitors in cancer prompted our interest in these compounds as potential antitumor agents. These analogs, which are potent, selective inhibitors of the target enzymes, are also effective growth inhibitors of murine and human tumor cells in culture (Wick et al., The Pharmacologist 30:157, 1990). The compounds do not exert their effects through broadly based poisoning of proteases, or through mechanisms commonly associated with antitumor agents (e.g., macromolecular synthesis inhibition, DNA damage), suggesting that the mechanism of growth inhibition is related to protease inhibition. We wish to focus on characterizing and isolating potential targets of the inhibitors in tumor cells. Preliminary studies using chromogenic and fluorogenic substrates showed chymotrypsin-like and elastase-like activity in whole cell lysates as well as subcellular fractions of A375 melanoma cells. These activities were substantially inhibited by appropriate boronic acid analogs. Recently, we have begun to utilize affinity chromatography to further characterize target proteins. Tripeptide inhibitors were coupled to CNBr activated sepharose. Solubilized, radiolabeled proteins from tumor cell lysates were applied to the column. Proteins bound to the column were eluted with acid. SDS-PAGE followed by autoradiography verified that proteins had bound specifically to the column. Work is currently underway to further characterize these proteins.

CH 301 PROTEASE INHIBITOR BLOCKS OSTEOBLAST MIGRATION INDUCED BY MAST CELL LYSATE (MCL), Kresimir Banovac, Department of Orthopaedics and Rehabilitation, University of Miami, School of Medicine, Miami, FL 33101

Numerous mast cells (MCs) were found in bone of patients with osteoporosis, renal osteodystrophy and in healing fracture callus. The role of MCs in bone remodeling remains uncertain. Our previous studies indicated that MCL alters interaction of bone cells and extracellular matrix suggesting its possible role in control of cell motility. The present study evaluates further the effect of MCL on bone cell migration. MCs were obtained after lavage of rat peritoneal and pleural cavities and were lysed by repetitive freezing to prepare MCL. Osteoblast-like cells were isolated from fetal rat calvaria by collagenase digestion. Cell migration was analyzed on glass coverslips coated with colloidal gold. The moving cells cleared the gold particles and their tracks were graded on a semiquantitative scale. Osteoblasts were incubated in BGJa medium with 10% fetal bovine serum at 37° in a CO_2 incubator using 12-well culture dishes containing glass coverslips. Osteoblasts incubated with MCL (17 ug protein/ml) for 24 h showed a significantly higher migration than controls. The effect of MCL on bone cells was also studied on collagenase digested bone fragments and on intact endosteum of fetal rat parietal bone. Cell morphology was analyzed by scanning electron (SEM) and light microscopies. The addition of MCL (50-75 ug protein/ml) induced in 1 h cellular morphology of typical "moving" cells (e.g. pseudopodia, trailing processes and gradual detachment from substratum). The effect of MCL on osteoblast migration and morphology was abolished by neutral protease inhibitor-phenylmethane-sulfonyl fluoride. These data suggest that mast cell protease(s) may have a role in bone remodeling through their effect on cell motility.

CH 302 CHARACTERIZATION OF THE COLLAGEN AND TIMP BINDING DOMAINS IN COLLAGENASE USING TRUNCATED ENZYMES AND A CHIMERIC MATRIX METALLOPROTEINASE, J.D. Becherer, A. Howe, I. Patel, B. Wisely, H. LeVine, and G. McGeehan, Glaxo Research Laboratories, Dept. of Biochemistry, 5 Moore Dr., Research Triangle Park, N.C. 27709

Human skin fibroblast collagenase (MMP1) cleaves native interstitial collagens at a single locus 3/4 from the N-terminus between a Gly-Leu or Gly-Ile bond. In order to understand the structural features of collagenase which are responsible for its collagen specificity, several recombinant collagenase enzymes have been generated in *E. coli*. Successive deletions from the C-terminus of the full length collagenase (43 kDa) have produced truncated collagenase enzymes of 37 kDa, 31 kDa, 25 kDa, and 19 kDa. All of the truncated collagenases demonstrate similar enzyme kinetics toward the synthetic thioester substrate, Ac-Pro-Leu-Gly-thioester-Leu-Leu-Gly-OEt. Km values range from 2.0 - 2.3 mM while k_{cat}/Km values for the truncated collagenases ranged from 0.7 - 1.1 relative to the full length enzyme. In addition, the truncated enzymes are able to digest casein or gelatin but only the full length collagenase degrades native collagen. Alkylation of the cysteine residues in the full length collagenase molecule abolishes only its ability to cleave native collagen. A chimeric metalloproteinase constructed from the N-terminal catalytic fragment of stromelysin (MMP3) and the C-terminal fragment of collagenase also cleaves the thioester substrate but fails to cleave collagen. These results suggest that the integrity of the 24 kDa C-terminal end of the collagenase molecule is essential for the enzyme's ability to recognize and cleave collagen but not for its activity against gelatin, casein, and a variety of synthetic substrates. Furthermore, TIMP inhibits the ability of all of these enzymes to cleave the thioester substrate, casein, gelatin, and collagen. The ability of TIMP to inhibit the truncated collagenases demonstrates that the TIMP binding site is localized within a 19 kDa N-terminal fragment and is distinct from the collagen binding site.

CH 303 THE LIGAND-BINDING DOMAIN OF THE CELLULAR RECEPTOR FOR UROKINASE PLASMINOGEN ACTIVATOR: IDENTIFICATION AND DEMONSTRATION OF FUNCTION IN PROTEOLYSIS ON THE CELL SURFACE, Niels Behrendt, Ebbe Rønne, Vincent Ellis, Michael Ploug and Keld Danø, Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark.

By chemical cross-linking experiments and protein sequencing, the amino acid sequence 1-87 of the human receptor for urokinase was shown to constitute the ligand-binding domain of this membrane protein. A monoclonal antibody, specifically directed against the ligand-binding domain, hindered access of the ligand and abolished an enhancing effect of cell surfaces on urokinase-dependent plasminogen activation. While cell surface plasminogen activation is fascilitated by the urokinase receptor on intact cells, no increase in the activity of the ligand was found in receptor-binding experiments using the purified reagents. The identification and isolation of the ligand-binding domain in this receptor provides a tool to study the role of the surface-dependent, urokinase-mediated plasminogen activation cascade in a number of processes including cellular invasion.

CH 304 ROLE OF THE UROKINASE RECEPTOR IN FACILITATING EXTRACELLULAR MATRIX INVASION BY CULTURED COLON CANCER[1], Douglas Boyd, William Hollas and Francesco Blasi[2], Department of Pharmacology, Baylor College of Medicine, Houston, TX 77030

This study was undertaken to discriminate between soluble and cell surface bound urokinase (UK) as a potential mediator of in vitro invasion by cultured colon cancer. Extracellular matrix invasion by a colon cancer cell line GEO, characterized as being a poor secretor of UK and having few receptors ($<10^4$ /cell) was not augmented when these cells were made to secrete up to 8 times as much UK, in response to an exogenous UK gene driven by the RSV LTR promoter. In contrast, the cell line HCT 116 equipped with 10 times as many binding sites, ($>10^5$/cell), the majority of which, are occupied with endogenous ligand was an efficient invader of the extracellular matrix. Inhibition of UK binding to the cell surface receptors using an antibody to the A chain of the plasminogen activator, reduced invasion by 65 %. The cell line RKO is equipped with 3 X 10^5 receptors/cell, 15 % of which are tagged with endogenous UK. Pretreatment of these cells with UK led to a 5 fold increase in extracellular matrix invasion. Together, these data suggest that for cultured colon cancer, at least, invasion is a function of the amount of cell surface receptor bound UK.

[1] This work was supported by an NIH grant CA 51539-02.

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CH 305 THE DETECTION AND CHARACTERIZATION OF CATHEPSIN B-LIKE ENZYMES FROM BREAST TUMOUR CELLS IN CULTURE USING NOVEL BIOTINYLATED PROBES, Breda M. Cullen, John Nelson, Margaret McGivern, Gillian Kay and Brian Walker, Division of Biochemistry, School of Biology and Biochemistry, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, N. Ireland.

Cathepsin B-like activity has been detected in a number of human breast tumour cell lines. An active form of the enzyme was found associated with the plasma membrane whereas a latent, higher molecular weight form was secreted into the medium. Both these forms have been visualised using specific biotinylated probes. This novel method has allowed the molecular weights of the enzymes to be determined as 31kDa. for the active species and 43kDa. for the latent form. Activation of the latter results in a molecular weight decrease to 31kDa., which is in agreement with earlier published work. E.G.F. stimulation of both these forms of cathepsin B-like activity has also been demonstrated.

CH 306 EXPRESSION AND CHARACTERIZATION OF RECOMBINANT TYPE IV COLLAGENASE (72 KD) AND TIMP-2 IN MAMMALIAN CELLS USING A VACCINIA VIRUS/T7 RNA POLYMERASE SYSTEM.

Rafael Fridman[1,2], Robert E. Bird[2], Matti Hoyhtya[2], Michael Berman[2], William G. Stetler-Stevenson[1], and Thomas R. Fuerst[3]. [1]Laboratory of Pathology, NCI, NIH, Bethesda, MD 20892, [2] Molecular Oncology Inc. and [3]MedImmune Inc., 19 Firstfield Rd., Gaithersburg, MD 20878.

The 72 kD type IV collagenase, a metalloprotease capable of degrading basement membrane collagen IV, is thought to play a critical role during tumor cell invasion, angiogenesis and wound healing. Type IV collagenase is secreted in a latent form which specifically associates with the tissue inhibitor of metalloproteases-2 (TIMP-2) in a non-covalent complex. Exposure of the complex to organomercurial compounds releases 80 aminoacids of the NH_2-termini of the zymogen resulting in enhanced proteolytic activity which can be completely inhibited by addition of exogenous TIMP-2. However, due to the difficulty in isolating type IV collagenase free of TIMP-2, the nature of the molecular interactions between TIMP-2 and type IV collagenase has not yet been addressed. In this study we report the use of a novel expression system based on a recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase in the cytoplasm of infected cells and plasmid vectors containing the cDNA of either TIMP-2 or the 72 kD enzyme flanked by T7 promoter and termination sequences to produce analytical amounts of recombinant TIMP-2 and type IV collagenase to investigate TIMP-2-type IV collagenase interactions. Transfections of these T7 promoter-controlled genes in epithelial cells resulted in high levels of expression of either TIMP-2 or type IV collagenase which are secreted into the culture media. The recombinant type IV collagenase was found to be free of endogenous TIMP-2 and to maintain its enzymatic activity as well as its ability to bind gelatin. Likewise, recombinant TIMP-2 retains its inhibitory activity when compared to native TIMP-2. Also, vaccinia recombinant TIMP-2 was affinity purified with E. coli recombinant type IV collagenase bound to Sepharose beads. Experiments using either supernatants of cells co-transfected with TIMP-2 and type IV collagenase genes or a mixture of isolated recombinant proteins followed by immunoprecipitation with specific antibodies revealed that TIMP-2 and type IV collagenase can form a complex in vitro. The production of recombinant vaccinia viruses containing the gene of either TIMP-2 or the 72 kD enzyme is also reported.

CH 307 THE ROLE OF FIBRINOGEN AND FIBRINOLYSIS IN T CELL PROLIFERATION,

Gladstone, P., Engardt, S.E., Oncogen/Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121

Previously, we have shown that our monoclonal antibodies which inhibit urokinase (uPA) inhibit monocyte-dependent T cell proliferation in serum. The present work shows that (1) monocyte uPA digests Fibrinogen (Fgen) into T cell stimulatory fragments and (2) the importance of uPA is underestimated in serum. Proliferation is inhibited 25-70% by anti-uPA when the usual serum-based medium is used. Although anti-uPA altered the levels of IL-1, TNF and γ IFN., none of these accounted for the inhibition. The classic PA/plasmin substrates, Fgen and/or fibrin, despite their nominal absence from serum, might be carried in by the fresh cells and might be the uPA targets. Thus, experiments were repeated in 10% plasma vs. serum. Inhibition by anti-uPA was higher(>90%) in plasma versus 25-45% in serum-based medium. Purified Fgen at a concentration equal to 10% plasma inhibits T cell proliferation in serum by 79% in the absence of monocytes. If monocytes are present, Fgen inhibition is only 21%. Fgen digested briefly with PA+plasminogen loses its suppressive effect on purified T cells and becomes stimulatory (+18-85%), while still retaining major fragments D(94K) and E(120K). The Fgen digest has been analysed and fractionated on an HPLC column. Apparently, monocytes can digest Fgen; T cells alone can't. Using 2-color FACS staining; prior to stimulation, monocytes bear much more Fgen than CD4+and CD8+ T cells.

CH 308 CHARACTERIZATION OF A PROTEASE ACTIVITY ATTRIBUTABLE TO HUMAN RECOMBINANT BONE MORPHOGENETIC PROTEIN-1 (hrBMP-1),

Tomoko Harada, David Israel, Kelvin Kerns, Elizabeth Wang, Genetics Institute, 87 CambridgePark Drive, Cambridge, MA 02140-2387.

BMP-1 has a domain homologous to *Astacus* protease, a metallo-endoprotease discovered in the digestive tract of a freshwater crayfish. Protease activity implicated by this homology has been studied in hrBMP-1. Human BMP-1 in CHO cells is expressed as a 93kD full-length and other lower MW forms. Using digestion of ^{125}I-calcitonin as an assay, we have found protease activity in partially purified CHO hrBMP-1. Preliminary data indicate inhibition of the hrBMP-1 protease activity by a BMP-1-specific affinity purified polyclonal antibody. Additional characterization of the protease activity is underway. BMP-1 was originally purified with other Bone Morphogenetic Proteins from bovine bone. BMP-1 to date has greatest homology to *tolloid*, a *Drosophila* gene implicated in development.

CH 309 MATERNAL TRANSCRIPTS FROM THE *DROSOPHILA MELANOGASTER* FURIN PROTEASE GENE ARE DEPOSITED IN DEVELOPING OOCYTES, Joel S. Hayflick, William Wolfgang, Michael Forte and Gary Thomas, Vollum Institute, Oregon Health Sciences University, Portland, OR 97201-3098
A family of mammalian proteases has recently been discovered that shares sequence homology with the subtilisin-like peptide precursor endoprotease from yeast, Kex2p. To study the roles that these proteases have in vivo by a genetic approach, we chose *Drosophila melanogaster* as our model system. To determine whether *D. melanogaster* has a member(s) of this family, degenerate oligonucleotide PCR primers where designed using highly conserved sequences surrounding the active site amino acids (asparagine, histidine and serine). These primers were used to amplify a sequence from a *Drosophila* head specific cDNA library. The PCR product was characterized by DNA sequencing and found to contain significant homology to Kex2p. This fragment was used to isolate a cDNA insert of 3.5 kb from the library. The cDNA encodes an amino acid sequence with 55% identity to the human furin protease. In adult *Drosophila* bodies, the single gene is transcribed into mRNA species of 4.0, 4.5 and 6.8 kb, whereas the heads have an additional 8.4 kb transcript. In situ hybridization shows specific signals in adult female nurse cells and developing oocytes, as well as cell soma of the brain. We hypothesize that the highly conserved protein, dFurin, functions in the oocytes during early embryogenesis to convert inactive precursors to active molecules, including morphogens.

CH 310 QUANTITATION OF THE UROKINASE AND TISSUE PLASMINOGEN ACTIVATORS ACTIVITY OF THE MALIGNANTLY TRANSFORMED AND NORMAL HUMAN FIBROBLASTS, Jerzy Jankun[1], Veronica M. Maher, and J. Justin McCormick, Carcinogenesis Laboratory, Michigan State University, East Lansing, MI 48823,

The presence of zymogens secreted into tissue culture medium by cells or released from cell surface can be detected by electrophoresis of the sample, overlaying the electrophoresis gel with an appropriate indicator gel, and examining it for zones of lysis (zymography). We have made this method quantitative for detection of urokinase plasminogen activator (u-PA) and tissue plasminogen activator (t-PA) by scanning the zones of lysis with computerized image analysis system and determining that a correlation exists between their enzymatic activity and square root of the lysed area. The method simultaneously detects plasminogen activators (PAs) of different molecular weight types. Using the method, we analyzed the PAs bound to the receptors on the cell surface and secreted into the medium of normal and malignantly transformed human fibroblasts. We found that the receptor-bound u-PA activity was significantly higher in malignantly transformed fibroblasts than in the non-transformed cells. Supported by DOE grant DEFG02-87-ER60524.

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CH 311 BACILLUS CALMETTE-GUERIN ABROGATES IN VITRO INVASION OF HUMAN BLADDER TUMOR CELLS BY PROTEASE PROTECTION OF FIBRONECTIN. Brian C.S. Liu, Richard J. Garden, Robert E. Weiss, S. Mark Redwood, and Michael J. Droller. Dept. of Urology, Mt. Sinai School of Medicine, New York, NY 10029.

Intravesical Bacillus Calmette-Guerin (BCG) has been shown to be an effective treatment for superficial transitional cell carcinoma of the bladder (TCC). The mechanisms by which BCG limits tumor cell activity have so far been unclear. We previously demonstrated that invasive human TCC cell line EJ has the ability to degrade basement membrane components, and that the degradation of extracellular matrix was due to a redistribution of activated cysteine protease cathepsin B to the plasma membrane of the invasive EJ cells (J Urol 144:798-804, 1990).

Using a modified Boyden chamber and an artificial basement membrane, we observed that BCG inhibited the invasion of EJ cells through the membrane by limiting tumor cell motility. Attachment and proliferation of tumor cells were not affected by BCG. The effects of BCG in tumor cell migration were mediated by fibronectin (FN), a basement membrane component. Using purified FN and purified activated protease cathepsin B from the EJ cells, we now demonstrate that BCG protects a specific sequence of the FN molecule from protease degradation. Furthermore purified peptides of the FN molecule containing the specific sequence stimulate motility of the EJ cells, whereas fragments lacking such sequence do not act as chemoattractants.

These findings suggest that BCG functions as a potent inhibitor of tumor cell invasion by protease protection of specific FN domain, and that the sequence contained in the domain may act as motility factor.

CH 312 MODULATION OF TUMOR CELL PROTEOLYTIC ACTIVITY BY EXTRACELLULAR MATRIX COMPONENTS, Andrew R. Mackay and Unnur P. Thorgeirsson, Division of Cancer Etiology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

In this study we have assessed the ability of various extracellular matrix components to alter the pattern of proteolytic enzyme expression in tumor cells. Laminin, fibronectin and type IV collagen did not effect the level of tumor cell 65kDa and 92kDa gelatinase or urokinase activity. Limited laminin-mediated stimulation of caseinolytic activity was noted in certain tumor cell lines. However, a type IV collagen preparation induced the secretion of an additional proteolytic enzyme in several tumor cell lines. Preliminary evidence suggests that this enzyme is an EDTA inhibitable gelatinolytic metalloproteinase not inhibited by either leupeptin, soybean trypsin inhibitor or PMSF. Interestingly, this enzyme did not undergo a reduction in molecular mass when incubated with the metalloproteinase activator APMA. Further characterization of this protease will be presented.

CH 313 EXPRESSION OF THE MATRIX METALLOPROTEINASE STROMELYSIN INCREASES INVASION OF MURINE PAPILLOMA-DERIVED KERATINOCYTES. Susan McDonnell*, Mary J.C. Hendrix[#], Elisabeth A. Seftor[#], Marc Navre[&], and Lynn M. Matrisian*, *Dept. of Cell Biology, Vanderbilt University School of Medicine., Nashville, TN, [#]Dept. of Anatomy, Univ. of Arizona, Tucson, AZ, and [&]Syntex Research, Palo Alto, CA.

The expression of the ECM-degrading metalloproteinase stromelysin correlates with the progression of chemically-induced murine carcinomas; stromelysin mRNA is not detectable in normal epidermis, is expressed in approximately 10% of benign papillomas, 75% of malignant carcinomas, and 100% of carcinomas induced by a chemical treatment which results in an increased incidence of metastastic tumors. To determine if stromelysin expression can play a causal role in tumor invasion, an established line of keratinocytes derived from chemically-induced papillomas was stably transfected with an SV40 promoter-controlled expression vector producing stromelysin. The stromelysin cDNA contained a single-base pair mutation so that the enzyme was secreted in an active form and did not require exogenous activation. Two clones of cells expressing stromelysin protein were selected and tested in an in vitro invasion assay through a matrigel-coated filter. The stromelysin-expressing clones demonstrated a 1.5 and 2.3 -fold increase in the ability to traverse the matrigel barrier compared to their parental controls. These results suggest that the expression of stromelysin contributes to tumor cell invasion and may play a causal role in one step of tumor progression.

CH 314 MATRIX METALLOPROTEINASE 9 (92KDA GELATINASE / TYPE IV COLLAGENASE) FROM HUMAN FIBROSARCOMA CELLS (HT-1080): PURIFICATION, PROPERTIES AND ACTIVATION MECHANISMS. Okada Y., Gonoji Y., Naka K., Nakanishi I., [*]Iwata K., [*]Yamashita K., and [+]Hayakawa T., Dep. of Pathol., Sch. of Med., Kanazawa Univ., [*]Fuji Chem. Industry, [+]Dep. of Biochem., Sch.of Dentistry, Aichi-Gakuin Univ., Japan

We have purified the zymogen of matrix metalloproteinase 9 (proMMP-9) from the culture medium of HT-1080 cells treated with TNFα by five chromatographic steps. Purified proMMP-9 was free of tissue inhibitor of metalloproteinases (TIMP) and homogeneous on SDS-PAGE with Mr=92,000. The enzyme degraded type I gelatin and type III, IV, V collagens. $\alpha 2$ (I) chain was also cleaved probably at an NH_2-terminal portion. The activity was inhibited in 1:1 stoichiometry by TIMP. ProMMP-9 was activated maximally by the incubation with 1mM aminophenylmercuric acetate (APMA) for 24h at 37°C and the activity was gradually decreased after the activation. During the activation proMMP-9 was converted to an intermediate species of Mr=83,000 and to an active form with Mr=67,000. Addition of purified TIMP in the activation processes inhibited the conversion of the intermediate species to the active one, suggesting that TIMP regulates the activity by inhibiting both activation and activity of the enzyme. Among endopeptidases examined, neutrophil cathepsin G and trypsin activated the zymogen to its full activity. HOCl also activated proMMP-9 in 30 min up to ~35% of the activity. These data suggest that MMP-9 secreted by cancer cells and activated by the interaction with neutrophils may play a significant role in pathologic destruction of extracellular matrix components in cancer invasion and metastases.

CH 315 EXPRESSION OF A MATRIX METALLOPROTEINASE (MMP-7) mRNA IN THE PROSTATE CELL LINE DU-145 INCREASES ITS INVASIVENESS *IN VITRO*. William C. Powell*, Elisabeth A. Seftor#, Mary J.C. Hendrix#, Raymond B. Nagle‡, G. Tim Bowden*. *Dept. of Radiation Oncology, ‡Dept. of Pathology, #Dept. of Anatomy, Univ. of Arizona Health Sciences Center, Tucson, AZ 85724.

Matrix metalloproteinases have been shown to be involved in tissue remodeling and cellular invasion. We have previously shown that with increasing grade of prostate carcinoma there is a corresponding decrease in the amount of basement membrane in the prostate (Mod. Path. 1989, Vol.2 2:105-111). We have also shown that MMP-7 is differentially expressed in prostate carcinomas when compared to normal prostates (Cancer Res. Clin. Onc., In Press). To further study the significance of this observation, we have stably transfected a human β-actin promoter expression construct containing the full length MMP-7 cDNA and a neomycin selectable marker into the tumorigenic (but nonmetastatic) cell line DU-145, which does not express detectable amounts of MMP-7. Forty-nine selected colonies were cloned and screened by northern analysis, and 15 were found to express a range of steady-state levels of the MMP-7 message. A clone (DUC-26) expressing increased amounts of MMP-7 was compared to the parental DU-145 and control transfected cells (vector only) in an *in vitro* invasion assay using a reconstituted basement membrane (Matrigel). In the absence of serum DUC-26 was >2x more invasive than DU-145, and >3x more invasive than the control transfected clone. These data suggest that expression of MMP-7 in prostate carcinoma may lead to a more invasive phenotype. This work was supported by an ACS grant (PDT-388) and an NIH grant (CA-40584).

CH 316 BIOCHEMICAL CHARACTERIZATION AND TISSUE DISTRIBUTION OF THE FIRST COMPONENT OF HAMSTER COMPLEMENT C1S, Hisako Sakiyama, Kiichiro Yamaguchi, Hiroshi Ohtsu, and Shigeru Sakiyama, Division of Physiology and Pathology, National Institute of Radiological Sciences, Anagawa, Chiba 260, Division of Biochemistry, Chiba Cancer Center Research Institute Nitona, Chiba 260, Japan.

We have purified a calcium-dependent serine proteinase (CASP) from the conditioned medium of hamster embryo fibroblasts, Nil2C2 cells. Nucleotide sequence of the cDNA of CASP was found to have high homology with that of human C1s. CASP cleaved human C4 and C2 to form C3 comvertase. Monoclonal antibodies against human C1s recognized CASP and the converse was the case. These evidences suggest that CASP is hamster C1s. Hamster C1s was shown to degrade type I and type IV collagen. Human complement, C1s, was also shown to cleave type I and type II collagen and gelatin (FEBS Lett. 268, 206, 1990). The proteolytic activity was inhibited by a serine protease inhibitor, DFP. An immunohistochemical examination revealed a widespread yet specific staining of hamster C1s in various tissues such as in chondrocytes of hyaline cartridge, surface epithelium of the stomach, muscle and so on. The synthesis of hamster C1s in these organs was confirmed by RNA blot hybridization. (J. Immunol. in press). Based on the biochemical and immunological evidences, C1s is supposed to have physiological functions in addition to the role in the classical complement pathway.

CH 317 ROLE OF 92 kDa TYPE IV COLLAGENASE IN METASTASIS OF TUMOUR CELLS, Hiroshi Sato[1], Yuri Kida[1], Masayoshi Mai[1], Yoshio Endo[1], Takuma Sasaki[1], Yasunori Okada[2] and Motoharu Seiki[1], [1] Cancer Research Institute, [2]School of Medicine, Kanazawa University, Kanazawa 920 Japan. Human tumour cell lines were examined for their mRNA transcript levels of genes whose products were expected to be associated with the degradation of type IV collagen and for their metastatic abilities in embryonated chicks. All 10 tumour cell lines originated from mesenchymal cells expressed higher levels of mRNA for 72 kDa type IV collagenase, however, this message was detected in only one out of 16 cell lines of epitherial origin. Expression of 92 kDa type IV collagenase mRNA was detected in only a few tumour cell lines but was independent of their cell types. TIMP and TIMP-2 mRNA transcripts were detected in most of cells examined. Thus, mRNA transcript levels of 92 kDa, 72 kDa type IV collagenases, TIMP and TIMP-2 were differentially regulated from each other in cell culture. Metastases were observed in livers and lungs of embryonated chicks with human tumour cells expressing high levels of 92 kDa type IV collagenase mRNA or low levels of TIMP mRNA. Synthesis of 92 kDa type IV collagenase was detected by immunostaining in human tumour cells infiltrating into livers of embryonated chicks suggesting that 92 kDa type IV collagenase may play an important role in metastasis of tumour cells.

CH 318 MODULATION OF PLASMINOGEN ACTIVATION AND TYPE IV COLLAGENASE ACTIVITY BY A LAMININ-DERIVED SYNTHETIC PEPTIDE, M.S. Stack, R.D. Gray and S.V. Pizzo, Dept. of Pathology, Duke University, Durham, NC 27710 and Dept. of Biochemistry, Univ. of Louisville, Louisville, KY, 40292.
Laminins constitute a family of multidomain glycoproteins with diverse biological activities including stimulation of neurite outgrowth, enhancement of tumor metastasis and promotion of cell growth, adhesion, and differentiation. A synthetic peptide derived from the E8 fragment of the laminin A chain (CSRARKQAASIKVAVSADR-NH_2) was identified which promotes metastasis and stimulates collagenase IV activity in the culture medium of melanoma cells (Kanemoto et al. (1990) PNAS 87, 2279). We report that this peptide, designated $LamA^{2091-2108}$, is a potent stimulator of tissue plasminogen activator-catalyzed plasminogen activation, resulting in a 22-fold increase in the k_{cat}/K_m of activation. The activity of purified types I and IV collagenase was inhibited by $LamA^{2091-2108}$ with IC_{50} values of 3 µM and 43 µM, respectively. These data suggest an alternative mechanism for observed increases in collagenase activity in the culture medium of melanoma cells, namely that the peptide stimulates plasmin formation followed by plasmin enhancement of procollagenase activation.

CH 319 INTRAMOLECULAR PROCESSING OF PAPAIN PRECURSOR, Thierry Vernet, Henry E. Khouri, Daniel C. Tessier, Pierre Laflamme, Andrew C. Storer and David Y. Thomas, Biotechnology Research Institute, National Research Council of Canada, Montréal, Québec, H4P 2R2, Canada
The 39-kDa papain precursor (propapain) has been secreted from insect cells infected with a recombinant baculovirus (Vernet et al., J. Biol. Chem. (1990), **265**, 16661). The protein was stabilized by mercury and purified to homogeneity using lectin-based affinity chromatography, gel filtration and ion exchange chromatography. The zymogen was processed *in vitro* at pH 4 and under reducing conditions into a 24-kDa active mature papain Processing of the precursor is prevented by inhibitors that specifically inhibit the mature papain enzyme. Substitution of the Cys25 of the active site the precursor by a Ser by site-directed mutagenesis also impairs processing. The initial rate of conversion of the precursor into papain is linear with time and is directly proportional to the initial concentration of the precursor. These data indicate that cleavage of the precursor's pro region during processing is an intramolecular event that requires the functional thiol active site of the mature domain of the precursor.

Proteases in Disease/Therapy

CH 400 AUTOANTIBODIES AGAINST THE MULTICATALYTIC PROTEINASE IN PATIENTS WITH SYSTEMYC LUPUS ERYTHEMATOSUS. Joaquín Arribas*, Maria Luz Rodríguez*, Rita Alvarez-Do Forno+ and José G. Castaño*. *Dpto. de Bioquímica e Ins. de Inves. Biomédicas CSIC. Fac. de Medicina UAM and + Servicio de Inmunologia C. S. La Paz. 28029 Madrid. Spain.
Multicatalytic proteinase (MCP), also known as proteasome or prosome, is a high molecular weight proteinase composed of several polypeptides ranging in size between 20 and 35 kdal and shown to be identical to a previously described 19S particle present in all eukaryotes. We have found that sera from patients with systemic lupus erythematosus (SLE) contain specific autoantibodies directed against different polypeptides of the MCP. These human autoantibodies, in contrast with polyclonal antibodies obtained in rabbits, recognize highly conserved epitopes of the MCP polypeptides from yeast to human. Two main implications from these findings are: 1) Circulating immune complexes are likely to occur in these patients and as the autoantibodies do not inhibit the peptidase activity of the MCP, they may be involved, directly or indirectly, in attacking membrane proteins producing cell damage being part of the complex response that eventually produce the pathological lesions observed in different tissues of autoimmune patients. 2) Some of the autoantibodies identified against the MCP polypeptides are likely to recognize the E. coli protease (Ti or C1p) that resembles the eukaryotic MCP. This would be an indication that immunological memory of bacterial or fungal infection may lead in certain individuals to the autoimmune response. In this sense MCP from yeast and their corresponding bacterial homologues would play a role, in certain patients with SLE, similar to mycobacterial hsp60 in patients with rheumatoid arthritis.

CH 401 PEPTIDASE ACTIVITIES IN THE EXCRETORY/SECRETORY (E/S) OF *TRICHURIS MURIS*, Lesley Drake, Donald Bundy and Frank Ashall, Department of Biology, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2BB, UK.
When excretory/secretory material of adult *T.muris* worms was electrophoresed in gelatin-containing polyacrylamide gels, two peptidases of Mr 120 kD and 97 kD were detected. Control experiments showed that these peptidases were indeed produced by worms and were not contaminants from host tissue or intestinal contents. These peptidases do not readily cleave haemoglobin, casein or bovine serum albumin (BSA) incorporated into polyacrylamide gels. The peptidases are not present in adult worm extracts in an active form, but appear to become activated during or following secretion from the worm. Both enzymes are strongly inhibited by diisopropylfluorophosphate and other serine peptidase inhibitors and have a pH optimum of about 6.5 for gelatin cleavage. The 120 kD and 97 kD peptidases of *T.muris* E/S material have the same pH optima, identical inhibitor profiles and both are present in E/S material but not in adult worms. Therefore we hypothesise that these peptidases are related to each other. The possibility exists that surface and secreted peptidases may be useful diagnostic tools for worm infections and we are investigating the corresponding peptidases of the human parasite, *T.trichiura* In addition, monoclonal antibodies to the peptidases are being produced, in order that further biochemical characterisation and histochemical localisation studies can be carried out.

CH 402 BIOCHEMICAL AND MOLECULAR GENETIC STUDIES OF *LEISHMANIA PIFANOI* AMASTIGOTE SPECIFIC CYSTEINE PROTEINASES: PURIFICATION, ANALYSIS OF PROCESSING, cDNA AMPLIFICATION, AND SEQUENCE ANALYSIS, S. Monroe Duboise, Yara Traub-Cseko, Luis Rivas, Alfred A. Pan, and Diane McMahon-Pratt, Department of Epidemiology and Public Health, Yale University School of Medicine, New Haven, CT 06510
Leishmania amastigote thiol proteinase(s) are essential for parasite survival. Axenic cultivation of an amastigote-like form of *Leishmania pifanoi* facilitates purification of an abundant developmentally regulated cysteine proteinase using either thiol interchange chromatography or immunoaffinity chromatography. Pulse-labeling experiments reveal a 40-kDa precursor that yields a 27-kDa proteinase. Binding of concanavalin A to both molecules indicates presence of a mannose carbohydrate moiety. Precursor enrichment was achieved by inhibition of processing. Amino terminal sequences of 40-kDa and 27-kDa forms are identical and have extensive homology with papain and cathepsin L. Two amplified gene segments, *Lpcys1* and *Lpcys2*, encoding regions between cysteine and asparagine active sites were sequenced. Deduced amino acid sequences of probable active sites were particularly conserved with respect to corresponding sequences from other trypanosomatids. Existence of multiple *Lpcys2* gene copies and distinct chromosomal locations of the two genes were demonstrated. Both genes were shown to be preferentially transcribed in amastigotes with *Lpcys2* being more highly expressed than *Lpcys1*. Nucleic acid sequences of the two proteinases were as dissimilar to each other as to cysteine proteinase genes of *Trypanosoma brucei* and *T. cruzi*, suggesting an ancient divergence. Amino acid sequence analysis of internal fragments of the immunoaffinity-purified 27-kDa proteinase suggests that this protein is an *Lpcys2* gene product.

CH 403 THE DETECTION OF CATHEPSIN B-LIKE ACTIVITY IN RHEUMATOID AND OSTEOARTHRITIC SYNOVIAL FLUIDS, Sean Duffy*, Brian Walker** and R.A.B. Mollan, * Department of Orthopaedic Surgery, Musgrave Park Hospital, Belfast BT9 7JB, ** Division of Biochemistry, School of Biology and Biochemistry, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, N. Ireland.

The role of proteolytic enzymes in the pathogenesis of arthritic disease is not well understood. Because of their destructive nature these enzymes can have a potentially damaging effect within the joint cavity. They are not only capable of degradation of the articular cartilage but also of the synovial fluid network. Cathepsin B, a cysteine protease, is one such enzyme that may have a role in the pathogenesis of arthritic disease.
In this study elevated levels of cathepsin B-like activity have been detected in rheumatoid synovial fluid when compared to the levels in osteoarthritic fluid. This was achieved by the use of a steady state fluorimetric assay and visualization of the enzyme was achieved using biotinylated active site directed inhibitors. A latent pro-form of cathepsin B has also been detected in synovial fluid from both disease states by the above methods.

CH 404 CALPAIN INHIBITOR REDUCES BRAIN SPECTRIN BREAKDOWN FOLLOWING KAINATE TOXICITY, David D. Eveleth, Juan Estrada, David Lutz, Reginald Dean, Steven Mennerick and Raymond T. Bartus, Cortex Pharmaceuticals, Inc., Irvine, CA 92718

Excitotoxicity is associated with many neuropathologies, including ischemia, stroke, and epilepsy. Elevations of intracellular calcium and activation of the calcium dependent protease calpain are associated with and may be causally linked to cell death in excitotoxicity. Systemic administration of the excitotoxic glutamate analog kainate to rats produces an acute phase of convulsions and seizures followed by extensive cell death in the hippocampus and other brain regions. The breakdown of the cytoskeletal protein spectrin correlates well with the extent of cell death observed histologically. Administration of Ac-Leu-Leu-Nle-H, a potent inhibitor of calpain, reduced the breakdown of spectrin measured 4 days after administation of kainate. The inhibitor is effective when given intraperitoneally at the time of kainate administration. Ac-Leu-Leu-Nle-H also reduced the convulsions induced by kainate. These observations suggest that this inhibitor can reach brain cells after peripheral administration in this model and that calpain is activated following peripheral adminstration of kainate. Further, activation of this protease plays an important role in the spectrin breakdown in nerve cells which accompanies the acute phase of kainate toxicity.

CH 405 Cell-mediated proteolytic cleavage of Pseudomonas exotoxin is an important rate-limiting step in toxin action. D. J. FitzGerald, M. Ogata, V. K. Chaudhary, C. M. Fryling and I. Pastan. Lab of Molecular Biology, NCI. Bethesda, MD 20892.

Pseudomonas exotoxin and its derivatives have been used to make a variety of chemical conjugates and recombinant gene fusion proteins. To improve the cytoxic activity of PE conjugates, we have studied rate-limiting steps in toxin action. After binding and internalization, PE is cleaved by a cell-associated protease to produce an N-terminal fragment of 28 kDa and a C-terminal fragment of 37 kDa. The 37 kDa fragment, containing ADPribosylating activity, translocates to the cytosol where it inhibits protein synthesis. Generation of the 37 kDa fragment is essential for toxicity since mutant proteins which cannot be cleaved are nontoxic. Only 8-10% of cell associated toxin molecules are cleaved to produce active 37 kDa fragments. This is an important rate-limiting step in toxin action, since toxin that remains intact cannot be translocated and toxin that is extensively degraded is inactive. Characterization of the protease responsible for cleavage has revealed that it is membrane-associated, that it cleaves PE with a pH optimum of 5.5 and that it requires divalent cations for activity. The site of cleavage has been located to arginine at position 279. Specific sequences at both the N and C-termini of the 37 kDa fragment are required for translocation to the cytosol.

CH 406 TETRACYCLINE (TC) INHIBITION OF METALLOPROTEINASES (MP) IN ARTHRITIC AND DIABETIC RATS. R. A. Greenwald, L. M. Golub. Long Island Jewish Medical Center, New Hyde Park, 11042 NY and SUNY Stony Brook, 11794.
Extensive studies (8 different laboratories, over 30 tissues) have confirmed that TCs, including a chemically modified non-antimicrobial analog (CMT), can inhibit a variety of interstitial MPs as well as collagen breakdown in tissues. Additional in vivo studies now extend these observations to diabetic rat skin, arthritic rat joint, and human cancer cells (pancreas, lung), from which both gelatinase (GELase) and/or Type IV COLLase can be inhibited by TCs, CMT, TIMP, and EDTA, but not by eriochrome black T, non-TC antibiotics, or serine or thiol proteinase inhibitors. In diabetic rats, daily oral gavage with a TC or CMT substantially suppressed the marked excess activity of GElase and IV/COLLase in glomerular extracts. In adjuvant arthritic rats, GELase & Type I COLLase activity were detected directly in extracts of inflamed paw tissue. CMT reduced GELase by 40% and the effect was independent of the severity of the inflammation. TC treatment also suppressed COLLase activity on SDS-PAGE. Combined treatment with TC and an NSAID obliterated all MP activity and mitigated erosive bone destruction. Pathologically excessive MP activity in diabetes, arthritis, and tumor cells may be amenable to blockade with TC analogues which could ameliorate some of the long term consequences of those processes.

CH 407 METAL ION-DEPENDENT INHIBITION OF ACTIVATED PROTEIN C BY α_2-MACROGLOBULIN (α_2M) AND ANTIPLASMIN (α_2AP) IN HUMAN BLOOD, Griffin JH, Gruber A, Heeb, MJ, Dept. Molecular & Exp. Med., Scripps Clinic & Research Fdn., La Jolla, CA 92037
The half-life of activated protein C (APC) activity in whole blood was 18 min whereas it was 31 min in citrated blood. To study the effect of divalent metal ions on the reaction of APC with protease inhibitors, incubation mixtures of blood and APC were analyzed using immunoblotting. Two APC-inhibitor complexes, previously identified on blots as APC-protein C inhibitor (APC-PCI) and APC-α_1-antitrypsin (APC-α_1AT), were observed in citrated blood. In whole blood two other bands containing APC were observed. Formation of one band was stimulated by Ca^{++} and another by Mg^{++}. The calcium-dependent band of APC was removed by an anti-α_2M antibody. Purified α_2M inhibited APC activity in a calcium-dependent reaction and formed stable complexes with APC. The magnesium-dependent band containing APC antigen was not identified. However, complexes of APC-α_2AP were identified in incubation mixtures of APC and blood or of APC and purified α_2AP, and the immunoblot band of these complexes comigrated with the band for APC-PCI complexes. The second order association rate constants (k_2) for inhibition of APC by α_2M and α_2AP were 99 and 100 $M^{-1}s^{-1}$, respectively, in the presence of calcium ions. These studies and an analysis of the half-lives for APC inhibition, calculated from the k_2 values for the four identified inhibitors and their concentrations in blood, suggest that PCI and α_1AT are the most effective APC inhibitors, that inhibition is significantly affected by calcium and magnesium ions, and that α_2M and α_2AP may be significant auxilliary inhibitors of APC.

CH 408 CONSTRUCTION OF SINGLE-CHAIN-Fv FRAGMENTS OF ANTIBODIES INHIBITING THE NIa PROTEASE OF PLANT POTYVIRUSES,
Gottfried Himmler, Irina Korschineck, Johann Kohl, Regina Sagl, Florian Rüker and Hermann W.D. Katinger, Institute of Applied Microbiology, University of Agriculture, Wien, Austria; William G. Dougherty, Dept. of Microbiology, Univ. Oregon, Corvallis; Robert Johnston, Dept. of Immunology, Univ. North Carolina, Chapel Hill

The monoclonal antibodies 3D6 and MC4911 have been characterized to bind to the NIa-proteins of Tobacco Etch Virus (TEV) and Potato Virus Y, respectively. The NIa is a cystein protease involved in the posttranslational processing of potyviruses and can be inhibited in vitro by MC4911. We have cloned and sequenced the variable regions of the heavy and the light chain of the immunoglobulin genes of the 3D6 and MC4911 cell lines, respectively.
These genes were cloned into an E.coli expression vector that provides a synthetic linker gene to give a fusion protein of the variable light and heavy chain. Experiments were undertaken to show if the binding characteristics of these constructs remain the same as that of the complete antibodies. The cloning of such single-chain-Fv fragments in transgenic tobacco is also shown.

CH 409 HEPARIN DERIVATIVES CAN MODULATE IN VITRO AND IN VIVO ELASTOLYSIS CATALYZED BY HUMAN LEUKOCYTE ELASTASE. *Lafuma C., Frisdal E., *Hornebeck W.
*Laboratoire de Biologie du Tissu Conjonctif, Faculté de Médecine, Créteil, France.
U. 296 INSERM, Faculté de Médecine, Créteil, France.

The inhibition of human leukocyte elastase (HLE) by heparin derivatives of known Mw and degree of sulphation can be classified as hyperbolic non competitive reaction. Ki (40nM-100µM) were found to decrease with the increasing degree of the sulphation of the compounds. They were inversely correlated with the chain lenght of oligosaccharides. Heparin fragments of defined Mr and degree sulphation were tested for their ability to prevent experimental emphysema in mice induced by intratracheal instillation of HLE. When administrated subcutaneously each day (2.5 mg/kg body weight) for 12 weeks (4wks before HLE instillation and 8 wks following enzyme treatment) heparin derivatives nearly totally abolished increased lung MLI induced by enzyme treatment.
Heparin derivatives, used in humans for many years with only few side reactions and allergic manifestations could be also a useful drug in HLE-mediated lung injury.

CH 410 MOLECULAR POLYMORPHISM AND EPIDEMIOLOGY OF NEISSERIA MENINGITIDIS IgA1 PROTEASES, Hans Lomholt*,Knud Poulsen*,Dominique A. Cougant# and Mogens Kilian*.
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The principal immunoglobulin at human mucosal surfaces, IgA1, is specifically cleaved by extracellullar proteases produced by several bacterial pathogens, including the three leading causes of bacterial meningitis, N.meningitidis, S.pneumoniae, and H.influenzae. These IgA1 proteases are thought to be important virulence factors and may have potential usage as vaccines. We examined the IgA1 proteases and iga-gene regions of 133 isolates of N.meningitidis representing major epidemics and carrier isolates from 19 countries. Two isoenzyme types of meningococcal IgA1 proteases, based upon the exact cleavage site in the IgA1 hinge region, have previously been described. Isolates from epidemic clones were found to produce primarily type 1 enzyme. Antigenic variation was detected using enzyme neutralising antisera and this identified only five different inhibition types. An antiserum raised against the IgA1 protease of a single strain was found to inhibit the activity of all other strains in the material, a potentially important finding regarding possible future attempts to base a vaccine on meningococcal IgA1 proteases. Restriction endonuclease digestion of the iga-gene region revealed more than twenty fragment patterns, and even among closely related strains assigned to a single multilocus enzyme electrophoretic type, we found a high degree of polymorphism in the iga-gene. This indicates that frequent interstrain recombination processes occur in vivo. Our results are consistent with the mosaic-like structure of the iga-gene observed both in N.gonorrhoeae and H.influenzae.

CH 411 CHARACTERISATION OF THE PROTEASES OF TRYPANOSOMA CONGOLENSE AND T. BRUCEI, John D. Lonsdale-Eccles, George, W.N. Mpimbaza, Zeres R. Mbawa and Judith Kornblatt.
International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya.
African trypanosomes are haematozoan protozoan parasites that can cause high morbidity and mortality in infected animals. They possess endopeptidase activity that can be released into the surrounding milieu. Both Trypanosoma congolense and T. brucei have lysosomal cysteine proteases which are inhibitable by E-64 and cytosolic serine-like endopeptidase activities which are inhibitable by diisopropylfluorophosphate. We have isolated these enzymes by a combination of ion exchange, gel filtration and affinity chromatography. The specificity of the purified enzymes has been determined using a variety of protease inhibitors and a range of fluorogenic peptidyl 7-amido-4-methylcoumarin substrates. Both the lysosomal and cytosolic activities differ in several respects from their mammalian counterparts. For example, while the substrate specificity of the lysosomal enzyme from these trypanosomes is similar to that of mammalian cathepsin L, it is unusual in that its activity can be enhanced in vitro by a protein (of approximate Mr 70 kDa) from serum. The trypanosomal cytosolic activity from T. brucei is trypsin-like but is unusual in that it preferentially cleaves after two basic residues rather than one, exhibits strong substrate inhibition with particular substrates, and it can be inhibited by peptidyl diazomethyl ketones which are putative cysteine protease-specific inhibitors. We conclude that African trypanosomes contain several unusual proteases of the cysteine and serine classes whose characteristics may predispose them to chemotherapeutic intervention.

CH 412 STAGE-SPECIFIC EXPRESSION OF ENDOPEPTIDOLYTIC ACTIVITY IN AFRICAN TRYPANOSOMES,
Zeres R. Mbawa, Ian D. Gumm, Wallace, R. Fish and John D. Lonsdale-Eccles. International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya.
The African trypanosomes, Trypanosoma congolense, T. vivax and T. brucei have a complex cycle that alternates between mammalian host and an insect vector. We have analysed each of the different life cycle stages of T. congolense and T. vivax as well as selected stages of T. brucei for alterations in the levels of their endopeptidase activity. With the exception of T. vivax, the protein substrates were hydrolysed best by bloodstream forms of the parasites. In T. vivax proteolytic activity was highest in epimastigote forms. Z-Phe-Arg-NHMec hydrolytic activity was highest in forms that possessed a variable surface glycoprotein coat (i.e. metacyclic and bloodstream forms of the parasites). This activity was observed at both acidic and alkaline pH. In pleomorphic forms of T. brucei, the short stumpy forms had higher levels of Z-Phe-Arg-NHMec hydrolytic activity than did the long slender forms. Hydrolysis of Z-Arg-Arg-NHMec and Z-Gly-Gly-Arg-NHMec was observed predominantly at alkaline pH, and occurred in lysates of both insect and mammalian infective forms of T. brucei and T. congolense. Compared to their other life cycle stages, procyclic forms of T. brucei and epimastigotes forms of T. congolense exhibited enhanced hydrolysis of Z-Arg-Arg-NHMec and Z-Gly-Gly-Arg-NHMec. Low levels of hydrolysis of Z-Arg-Arg-NHMec were observed in the bloodstream and epimastigote forms of T. vivax. The hydrolysis of Z-Gly-Gly-Arg-NHMec in each of the life cycle stages of T. vivax was generally below detectable levels. We conclude that there is an induction of lysosomal proteolytic activity in the mammalian infective forms of these parasites.

CH 413 ISOLATION OF BACULOVIRUS DERIVED SECRETED AND FULL-LENGTH β- AMYLOID PRECURSOR PROTEIN,
Lisa McConlogue, Kelly Johnson-Wood, Dale B. Schenk, Sukanto Sinha, Ivan Lieberburg and Jeroen Knops, Athena Neurosciences, Inc., 800F Gateway Blvd., So. San Francisco, CA 94080.
We have expressed two forms of the Alzheimer's β-amyloid precursor protein (βAPP), the 695 amino acid form (695βAPP), and the 751 amino acid form (751βAPP) in a baculovirus system. Both forms were expressed as full-length precursor, and were subsequently processed in vivo to release extracellular secreted proteins. The secreted forms were cleaved from the full-length βAPP in a manner analogous to the cleavage of βAPP during constitutive secretion in mammalian cells. High levels of expression of 20 to 50 mg/L were achieved. Both full-length and secreted forms were purified using a combination of ion-exchange and immunoaffinity chromatography using a monoclonal antibody directed against βAPP. The 751βAPP derived full-length and secreted forms, which contain the Kunitz protease inhibitor domain, were shown to be as active in the inhibition of trypsin as is mammalian derived secreted βAPP. The availability of purified full-length βAPP from the baculovirus system will be valuable for biochemical analyses that elucidate the mechanism of the inappropriate proteolytic processing that leads to β-amyloid formation in Alzheimer's disease.

CH 414 THE APPLICATION OF NOVEL, ACTIVE-SITE DIRECTED AFFINITY LABELS FOR THE CHARACTERIZATION OF PARASITIC PROTEASES.
Ann McGinty*, Melanie Moore*, David W. Halton** and Brian Walker*.Divisions of Biochemistry* and Cell and Experimental Biology**, School of Biology and Biochemistry, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn road,Belfast BT9 7BL, N. Ireland.

A number of novel, irreversible diazomethylketone inhibitors based on peptidyl sequences previously shown to produce very effective inhibition of the lysosomal cysteine proteinases Cathepsin B and Cathepsin L have been synthesised. These reagents incorporate biotin or coumarin amino-terminal reporter groups and have been employed to successfully label the actively-secreted cysteine proteinases of juvenile and mature Fasciola hepatica maintained in vitro. Using these probes, it has proved possible to localize labelled proteolytic activity following SDS-PAGE and Western-blotting, and thus determine molecular weights. In addition, further characterization of the sub-site specificities of these proteinases has been achieved by blocking this labelling using various, non-labelled, active-site directed dipeptidyl inhibitors.

CH 415 A MUTANT OF Entamoeba histolytica WITH COLLAGENASE SECRETION AND VIRULENCE DIMINISHED, M. Lourdes Muñoz, Jesús Serrano, Rosalinda Tovar, Miguel Moreno, Edgar Rangel, Victor Tsutsumi and Mireya de la Garza, Centro de Investigación y de Estudios Avanzados del I.P.N. Apdo. 14- 740, México D.F. MEXICO.

Trophozoites of *Entamoeba histolytica* are able to attach and destroy host tissues. This property has been attributed to activities such as hydrolytic and proteolytic enzymes. Since collagen is a major component of the extracellular matrix, in previous studies we have focused our attention on the *E. histolytica* collagenase. The levels of the enzyme correlate with the virulence of different *E. histolytica* strains. This enzymatic activity was detected in electrondense granules (EDG) secreted by *E. histolytica* trophozoites incubated with collagen type I. We have characterized a mutant (BG-3) selected from the virulent strain HM1: IMSS by its resistance to cytochalasin D. Eritrophagocytosis and cytopathic effect were diminished in this mutant. Our studies showed that BG-3 had --- virulence, adherence to collagen type I, collagenase activity and EDG secretion diminished compared to the parental strain HM1. In addition, a monoclonal antibody anti- EDG had low recognition of the BG-3 plasma membrane.
In conclusion our results showed a correlation between collagenase activity and virulence.

CH 416 PROTEOLYTIC ACTIVITIES IN ALZHEIMER'S DISEASE BRAIN. Gregory Papastoitsis and Carmela R. Abraham. The Arthritis Center, Department of Medicine, Boston University School of Medicine, Boston, MA 02118.

The abnormal proteolytic processing of the ß-amyloid precursor protein in Alzheimer's disease (AD) brain results in the accumulation of ß-protein in the brain plaques and blood vessels. The purification and characterization of the enzymes involved in the abnormal processing is necessary for understanding the etiology of the disease. We have begun to identify, purify, and characterize proteolytic activities from AD brain through the use of a number of ion-exchange, gel-filtration and affinity resins. One proteolytic activity obtained is a cysteine metalloprotease that has been purified to near homogeneity. This protease acts as an endopeptidase cleaving after a methionine residue in a 10 amino acid synthetic substrate made according to the sequence flanking the N-terminus of the ß-protein. The enzyme requires the presence of a reducing agent e.g. dithiothreitol, for its activity. The enzyme is strongly inhibited by hydroxymercurybenzoate, E-64, N-ethylmaleimide, 1-10 phenanthroline, EGTA and EDTA. An apparent molecular weight of 57,000 was found by gel filtration and 55,000 by SDS-PAGE.

A second proteolytic activity has been identified from the same AD brain. This enzyme appears to cleave the 10 amino acid synthetic substrate internally, after a lysine residue. The enzyme's activity is strongly inhibited by 1,10 phenanthroline, EGTA, and bestatin, while PMSF had no effect on this proteolytic activity. Efforts are under way to further purify and characterize this second enzyme.

CH 417 ANTIBACTERIAL ACTIVITY OF AN INTERNAL PEPTIDE DERIVED FROM HUMAN LEUKOCYTE CATHEPSIN G, Jan Pohl[1], William M. Shafer[1,2], Victor J. Onunka[2], and James Travis[3], Emory University[1], V.A.Medical Center[2], Atlanta, GA 30322 and University of Georgia[3], Athens, GA 30602
Cathepsin G (CG) exerts potent in vitro antimicrobial action which is independent of its serine protease activity. Previously, we showed that both the isolated and the synthetic peptide of CG, HPQYNQR (CG residues 77-83), possesses broad spectrum antibacterial action in vitro. Using synthetic peptides containing single alanine replacements at each position we found that His-1 and Tyr-4 are critical for the antibacterial action. We now report that the antibacterial capacity of HPQYNQR is dependent on the stereochemical features imposed by L-amino acids and on the linear nature of its peptide chain. By using an agarose diffusion assay to quantitate antibacterial activity we found that HPQYNQR was as active as certain clinically useful antibiotics. When tested against *Staphylococcus aureus* strains resistent to methicillin, the antibacterial activity of the peptide was reduced, suggesting that HPQYNQR acts at the level of peptidoglycan synthesis. This may explain its broad spectrum antibacterial activity.

CH 418 HUMAN α_1-PROTEINASE INHIBITOR BINDS TO EXTRACELLULAR MATRIX *in vitro* WITH RETENTION OF INHIBITORY ACTIVITY, Alex R. Rinehart and Sanford R. Simon, Dept. of Biochemistry, SUNY at Stony Brook, Stony Brook, NY 11794

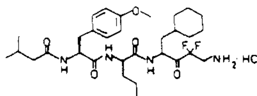
α_1-proteinase inhibitor (α_1-PI) is the major endogenous inhibitor of human leukocyte elastase (HLE). We have employed an ELISA to quantitate the binding of α_1-PI to extracellular matrix (ECM), composed of 51% glycoproteins and proteoglycans, 37% types I and III collagen and 12% elastin derived from rat aortic smooth muscle cells. α_1-PI is bound to ECM in a saturable manner which is unaffected by prior removal glycoproteins with trypsin. Binding to ECM is not decreased in the presence of high salt but is decreased at low pH. A 40X excess of unlabeled α_1-PI can displace only 50% of ^{125}I-α_1-PI pre-bound to ECM. A 30% decrease in binding to ECM occurs after washes with DTT, suggesting that a fraction of bound α_1-PI is covalently linked to ECM through disulfide bond formation. To demonstrate covalent linkage, the free SH of α_1-PI was modified with dithionitrobenzoic acid (DTNB). Upon reaction of TNB-α_1-PI with ECM, TNB$^-$ ion was released from 50% of the modified protein, consistent with disulfide bond formation between α_1-PI and some component(s) of the ECM. Results using a cross-linking reagent also suggest that elastin may be one of the ECM components involved in non-covalent binding of α_1-PI. 71% of total active ECM-bound α_1-PI and 48% of active α_1-PI bound non-covalently to ECM retains the ability to inhibit HLE-mediated ECM proteolysis. α_1-PI from plasma or serum can also be bound to ECM with retention of inhibitory activity. ECM-bound α_1-PI should be considered as a previously unrecognized defense against HLE-mediated tissue destruction. (Supported by NIH [HL-14262], SUSB Biotechnology Center, and Cortech, Inc.)

CH 419 A CATHEPSIN L-LIKE ENZYME FOUND IN *TRYPANOSOMA CRUZI* EPIMASTIGOTES, Helen Roberts, Elliott Shaw and Frank Ashall, Department of Biology, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2BB, UK.

Trypanosma cruzi (Y strain) epimastigotes were shown to have hydrolytic activity for the substrate Z-phenylalanyl-argininyl-aminomethylcoumarin (Z-Phe-Arg-MCA) at pH 4.0 which was activated by 2-mercaptoethanol. This activity is inhibited by 20µM E-64, 10µM tosyllysyl chloromethylketone, 1mM iodoacetamide and 20µM leupeptin. The peptidyl diazomethane, Z-Phe-AlaCHN$_2$ strongly inhibited the enzyme at a concentration of 12.5mM, whereas Z-Ala-PheCHN$_2$ only weakly inhibited the activity. Likewise the fluoromethane Z-Phe-AlaCH$_2$F, but not Z-Ala-PheCH$_2$F, strongly inhibited the Z-Phe-Arg-MCA hydrolase at pH 4.0. The activity was shown to have a digitonin-solubilisation profile similar to that of - mannosidase, a marker enzyme for lysosomes. *Crithidia fasciculata* choanomastigotes and *Leishmania donovani* amastigotes show simiolar characteristics for the hydrolysis of Z-Phe-Arg-MCA. The acidic Z-Phe-Arg-MCA hydrolase of *T.cruzi* appears not to readily cleave Z-Arg-Arg-MCA. This together with its pH profile, apparently lysosomal location and inhibitor characteristics led us to propose that the enzyme is a cysteine peptidase equivalent to cathepsin L of higher eukaryotes.

CH 420 ß-AMINO-α,α-DIFLUOROKETONES, NOVEL INHIBITORS OF HUMAN RENIN. STRUCTURE-ACTIVITY RELATIONSHIP, SPECIES AND ASPARTYL PROTEASES SELECTIVITIES, D. Schirlin, C. Tarnus, S. Baltzer and J.M. Rémy, Departments of Discovery Chemistry and Enzymology, Marion Merrell Dow Research Institute, Strasbourg, 67000, France.

The combination of an amine function and a difluoromethyleneketone has led to a new class of potent small and soluble inhibitors of human renin. These structures are inhibitors of primate renin but are much less active against non primate renins as well as other aspartyl proteases such as pepsin, cathepsin D or HIV protease.
MDL 74147 has an IC_{50} = 16 nM when tested against human plasma renin and IC_{50} = 22 nM versus monkey plasma renin. MDL 74147 is much less effective against dog renin (IC_{50} = 3 µM) or rat renin (IC_{50} = 100 µM).
A structure-activity relationship study will be presented and the putative mechanism of inhibition will be discussed.

MDL 74147 

CH 421 TETRACYCLINES (TCs) INHIBIT MUSCLE PROTEIN DEGRADATION IN VITRO AND IN VIVO. B. S. Schneider, R. A. Greenwald, L. M. Golub, J. Maimon. Long Island Jewish Medical Center, New Hyde Park, NY 11042

TCs, widely used as antibiotics, have a recently discovered novel action: inhibition of extracellular metalloproteinase activity, especially collagenase and gelatinase. This property, confirmed in 8 different laboratories using >30 tissue sources, includes natural and synthetic TCs as well as chemically modified TCs (CMT) devoid of antimicrobial activity. We have used 14C-Tyr biosynthetically labelled intracellular proteins in fetal myoblast culture as a test system to assess intracellular proteolysis. Starvation induces proteolysis which can be suppressed by various agents. Insulin, glyburide, minocycline, doxycycline, and CMT all reverse intracellular protein degradation in a dose dependent and synergistic manner. CMT also reverses proteolysis caused by tumor-cell conditioned medium (a model for cancer cachexia). In vivo studies show that CMT administered to diabetic rats partially reversed the usual loss of wet weight and diameter of the gastrocnemius muscles, consistent with the observation that TCs ameliorate cachexia in these animals. A CMT stripped of its metal chelation site lost its antiproteolytic effect. These findings suggest novel strategies for conditions characterized by muscle protein degradation and wasting.

CH 422 THE CLUSTER OF ACIDIC AMINO ACIDS AT THE TAILS OF PKA AND RECEPTOR KINASES IS SPECIFICALLY RECOGNIZED BY A MEMBRANAL PROTEINASE. S. Shaltiel, R. Seger, R. Riven-Kreitman, E. Mozes, and M. Fridkin The Weizmann Institute of Science, Rehovot, Israel.

The C-terminal tails of protein kinase A (PKA) and the EGF and insulin receptor kinases contain a cluster of acidic amino acid residues (positions 327-337, 979-991 and 1268-1286, respectively). These tails are clipped off by a kinase-splitting membranal proteinase (KSMP) in a conformation-dependent manner, i. e., no proteolysis occurs if these substrates are pre-denatured (Alhanaty et al., PNAS 78, 3492-3495 (1981); Seger et al., J.B.C 263, 3496-3500 (1988), EMBO J. 8 435-440 (1989)). This negatively charged cluster of amino acids is now shown to be a key biorecognition element for KSMP since: (1) monoclonal antibodies (mAb-103) against a branched polyamino acid ((T,G)-A-L) containing clusters of D and Y cross react with the C subunit of PKA but not with its KSMP cleavage product (C'), in which the C-terminal tail is clipped off; (2) mAb-C9, a monoclonal antiidiotype of mAb-103, specifically binds to the active site of KSMP and inhibits its activity; (3) Synthetic peptides containing a DDYEEEE stretch are competitive inhibitors of KSMP (K_i=24-46 μM); (4) The peptide FDDYEEEEI inhibits the cleavage of all three substrates and prevents the binding of mAb-103 to KSMP. In view of the important role played by this cluster of acidic amino acids in the endocytosis of the EGF receptor (W.S. Chen et al., Cell 59, 33-43 (1989)) KSMP provides a useful tool for studying the ligand induced internalization of growth factor and hormone receptors, while the monoclonal antibodies and the peptide inhibitors may be used to elucidate the physiological role of KSMP.

CH 423 STUDIES ON NATURAL CALPAIN INHIBITOR IN SPONTANEOUSLY HYPERTENSIVE RAT STRAINS. *Laura Soldati, Fulvio Serra*, Isabella Molinari*, Barry R. Barber*, Marco Ruggiero* and Giuseppe Bianchi.* Milan Univ., via Olgettina 60, 20132 Milano; and *Prassis Research Inst., 20019 Settimo Milanese, Italy.

Calpain is a cytosolic calcium-activated neutral protease involved in the control of critical passages of cell activation. Its activity is regulated by an endogenous protein inhibitor termed calpastatin. It has been previously found that calpastatin level is decreased in erythrocytes and renal cortex of Milan hypertensive rats (MHS) as compared with the normotensive control strain (MNS). In order to further assess the relationship between hypertension and calpastatin level, we measured calpastatin in erythrocytes and renal cortex of MHS, MNS, in F1 and F2 hybrids, and in two inbred strains derived from F2, one hypertensive (MHNE) and the other normotensive (MHNA). We found that calpastatin level is not correlated with hypertension: hypertensive MHNE had normal calpastatin level, and F2 hybrids showed no correlation between the two parameters. Our characterization of a hypertensive strain with normal calpastatin level (MHNE) will prove particularly useful in studying the role of calpastatin alteration in different pathological situations.

CH 424 STAPHYLOCOCCAL EXFOLIATIVE TOXIN INDUCES CASEINOLYTIC ACTIVITY

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It is well known that intraepidermal splitting in staphylococcal scalded skin syndrome (SSSS) is caused by the action of staphylococcal exfoliative toxin (ET); however, ET itself has no proteolytic activity, and the cells in the skin lisions do not show any cytolysis. The mechanisms of the action of ET remains as enigma. We eill present data showing an incease in caseinolytic activity in the supernatant of the epidermis from new-born mice after incubation with ET. This activity was inhibited by adding α_2 macroglobulin. Our findings suggest the exfoliation in SSSS is induced by proteinases caused by ET.

Casein-hydrolyzing enzyme(s) induced by ET were partially purified by several column chromatographies. However, on SDS-PAGE, the partially purified fractions exhibited several protein bands. When the partially purified enzyme(s) was preincubated with EGTA or EDTA, substantial inhibition of the activity was observed; however, no recovery of the activity was detected after the addition of $CaCl_2$.

CH 425 INACTIVATION OF CATHEPSIN B BY N-CHLOROACETYL DIPEPTIDE ANALOGUES: EXPLOITATION OF CARBOXY DIPEPTIDYL ACTIVITY, Brian Walker, Carvel Williams and Julia Nunn. Division of Biochemistry, School of Biology and Biochemistry, The Queen's University of Belfast. Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, N. Ireland.

We have synthesised a number of N-chloroacetyl dipeptide sequences and tested them as inactivators of bovine cathepsin B with the hope of exploiting the known exopeptidase activity of the enzyme. Of the sequences tested, N-chloroacetyl.F.M.OH (I) was found to be the most potent inactivator, bringing about complete inactivation of cathepsin B within 2 min (37 oC) at an inhibitor concentration of 20µM. In contrast to this, N-chloroacetyl.G.G.OH (II) was devoid of any inhibitory activity even at concentrations as high as I mM, thus demonstrating the preference of the enzyme for hydrophobic residues. We believe that compounds of this type could form the basis for the design of reagents that could selectively inactivate cathepsin B in the presence of cathepsin L, since the latter has never been demonstrated to possess carboxy dipeptidase activity.

CH 426 BIOCHEMICAL AND KINETIC CHARACTERIZATION OF TICK ANTICOAGULANT PEPTIDE (TAP): A NOVEL, HIGHLY SELECTIVE INHIBITOR OF BLOOD COAGULATION FACTOR Xa, Lloyd Waxman, Michael Neeper, Susan Jordan, Donna Smith, Assunta Ng, Denise Ramjit, Mohinder Sardana and George P. Vlasuk, Merck Sharp & Dohme Research Laboratories, West Point, PA 19486

Tick anticoagulant peptide (TAP), which was isolated from the tick Ornithodoros moubata, is a novel, highly specific inhibitor of Factor Xa (fXa). TAP is a polypeptide of 60 amino acids with 3 disulfide bonds. Although the molecule has limited homology to the Kunitz-type inhibitors, including the pairing of its disulfide bonds, it has no effect on 11 other serine proteases tested, including trypsin. Recombinant TAP (rTAP) was synthesized in S. cerevisiae as a secreted protein using a synthetic gene segment encoding TAP linked to the alpha-mating factor precursor. A detailed kinetic analysis was performed on the interaction of purified rTAP and fXa. rTAP was determined to be a reversible, slow, tight-binding competitive inhibitor of fXa. The overall inhibition constant for rTAP with fXa is 0.19 nM with a calculated association rate constant of 2.51 x 10^6 $M^{-1}sec^{-1}$. Direct binding experiments indicate that the active site of fXa is required for rTAP inhibition. rTAP blocked the incorporation of Dansyl-Glu-Gly-Arg-chloromethylketone into fXa, and binding of ^{35}S-rTAP was only observed in the presence of fXa and not DFP-treated fXa or the zymogen fX. Chemical modification of rTAP suggests that arginine residues as well as acidic amino acids are required for inhibition. When site-directed mutagenesis of each of the 5 arginine residues of rTAP was carried out, only Arg^3 was important in the inhibition of fXa. Blocking the alpha and epsilon amino groups or the single histidine residue had no effect on activity. Removal of the N-terminal tyrosine also eliminated TAP's ability to inhibit fXa. In addition to its in vitro anticoagulant properties, rTAP has been shown to prevent thrombus formation in vivo using a rabbit model of venous thrombosis. TAP's exquisite specificity and size suggest that it may have therapeutic value as an anticoagulant.

CH 427 PROTEOLYSIS OF HEMOGLOBIN α CHAINS IN β THALASSEMIA RETICULOCYTES,

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Beta thalassemia is an inherited disorder charactered by the inbalance synthesis of the α and β polypeptide chains. Evidence showed that proteolytic system could degrate exccess hemoglobin chains.
We herein reported deta of two thalassemia patients. Purified reticulocytes from patients and normal donors were incubated with ^{3}H leucine and pyromycin, which stopped further protein synthesis. At defferent time interval the samples were washed and reticulocytes were lysed, then the radioactivity of the total hemolysate was counted. The deta showed that the radioactivity was reduced more significantly in patients samples(25-35%) then those of normal donors(< 5%) after 4-6 hour incubation. This indicated arapid degradation of α chains by proteolysis. While adding N-ethylmaleimide to the samples, the proteolytic effect was prevented.

Late Abstract

INVESTIGATION OF MEMBRANE-ASSOCIATED PEPTIDASES OF TRYPANOSOMATIDS

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We examined the peptidases associated with detergent phases of Triton X-114 extracts of Trypanosoma cruzi, Crithidia fasciculata and Leishmania donovani, using polyacrylamide gel electrophoresis in which gelatin was used as substrate. T.cruzi and C.fasciculata expressed a major membrane-associated peptidase that was inhibited by o-phenanthroline but not by inhibitors of serine, cysteine or aspartic peptidases. The peptidase of both organisms was optimally active at alkaline pH and had an Mr of about 60 kD. L.donovani expressed two membrane-associated peptidases, both of which were inhibited by o-phenanthroline and had alkaline pH optima: one of these (Mr 68 kD) was thought to be the previously described gp63 promastigote surface peptidase. All enzymes detected were strongly inhibited by Zn^{2+} and Cd^{2+} ions. We are investigating the biochemical and biological relationships between these trypanosomatid membrane peptidases.