CELLULAR PROTEASES AND CONTROL MECHANISMS

Organizer: Tony Hugli
February 21 - 26, 1988

Page

Plenary Sessions

Poster Sessions

Cellular Proteases and Control Mechanisms

Characterization of Cellular Proteases and Comparisons with the Plasma Enzymes

X 001 BIOSYNTHESIS OF LYSOSOMAL PROTEINASES, Ann H. Erickson, Department of Biochemistry, The University of North Carolina, Chapel Hill, N.C. 27599.

The basic proteolytic and glycolytic processing steps that occur during the biosynthesis of lysosomal proteinases are essentially the same for aspartic and cysteine proteinases. Using *in vitro* translation and pulse-chase analysis in tissue culture cells, we have compared the biosynthesis of porcine cathepsin D, a model aspartic proteinase, and rat cathepsin L, a model cysteine proteinase. Both undergo co-translational cleavage of an amino-terminal signal sequence which mediates transport across the ER membrane. Both enzymes are synthesized as inactive proenzymes possessing amino-terminal activation peptides, although the activation peptide is twice as long for cysteine proteinases as for aspartic proteinases. Both enzymes acquire high-mannose carbohydrate in the lumen of the ER, 2 chains for cathepsin D and 1 for cathepsin L. Removal of the pro group generates active single-chain enzyme. Whether the single-chain enzyme undergoes cleavage into a light and a heavy chain appears to be cell specific. Finally, late during biosynthesis both classes of enzymes undergo carboxyl-terminal processing, loosing a few amino acid residues. During biosynthesis both enzymes are also secreted to some extent. In most cells the secreted enzyme is the pro enzyme bearing some complex carbohydrate. One exception is the activated peritoneal macrophage, which secretes all biosynthetic forms except the last form having undergone carboxyl-terminal processing. Analysis of the biochemical nature of the various processing steps has enabled us to define the cellular pathway followed by newly synthesized proteinases targeted to the lysosome.

X 002 MATRIX METALLOPROTEINASES: MOLECULAR AND BIOCHEMICAL STUDIES. Gillian Murphy and *Andrew J.P. Docherty, Strangeways Laboratory, Cambridge, U.K. and *Celltech Ltd, Slough, U.K.

The resorption of the extracellular matrix of connective tissue can be effected by a group of metalloproteinases secreted by the indigenous cells. Three major matrix-degrading have been identified by biochemical purification and characterisation: collagenase, stromelysin and gelatinase. Further insights into the structure of collagenase and stromelysin are being obtained from the sequencing of cDNA clones and into their function by the analysis of recombinant proteins expressed in mammalian cells. Their similar basic properties are confirmed by the extensive homologies in their predicted amino acid sequences, including a putative Zn^{2+} binding site, an identifiable prosequence which is lost on activation and regions of homology with extracellular matrix proteins. In each case, central 'core' fragments of Mr 22-28K can be generated which retains varying degrees of proteinase activity, including the ability to bind to the specific tissue inhibitor of metalloproteinases, TIMP. The two types of metalloproteinase have different specificities for matrix macromolecules and the basis of this variability is a subject for investigation.

The metalloproteinases are initially secreted in a latent proform, requiring activation, which is thought to be an important regulatory point in vivo. The activation process can be mediated either by proteinases such as trypsin or plasmin, or by modification of the local environment of the enzyme (e.g. in the presence of organomercurials). Both treatments lead to an initial partial activation of the proteinases and self-cleavages subsequently generate the fully active enzyme. In the case of human stromelysin further self-cleavages occur, yielding a Mr 28K active form. Collagenase activation by proteinases or organomercurials is potentiated by active stromelysin, forming the basis for a possible activation cascade in vivo. Current data on these observations will be presented from studies using natural and recombinant proteinases.

X 003 NEUTROPHIL ELASTASE AND CATHEPSIN G: STRUCTURE, FUNCTION, AND BIOLOGICAL CONTROL, James Travis, Guy Salvesen, David Farley, Sukanto Sinha, and Wieslaw Watorek, Dept. of Biochemistry, University of Georgia, Athens, GA 30602.

Two of the major proteinases synthesized by phagocytic cells are elastase and cathepsin G, both of which are normally found exclusively in neutrophils. These enzymes are presumed to be involved in the turnover of both foreign and aged proteins during the process of phagocytosis. However, they have also been implicated in the development of connective tissue diseases due to their ability to degrade elastin, collagen, and proteoglycan as a result of cell leakage or cell death. The primary structure of both enzymes, which are glycoproteins, has been determined in this laboratory, and comparisons with similar enzymes from other species will be discussed. Since both enzymes are synthesized and secreted as two groups of isozymes we have performed studies to determine the differences between the various forms. Our results indicate that there are significant differences in carbohydrate side chain structure which would account for isozyme formation. However, there is no indication that this affects either the enzymatic activity of individual isozymes or the ability of each to interact with controlling proteinase inhibitors.

X 004 A FAMILY OF SERINE PROTEASES IN LYTIC GRANULES OF CYTOLYTIC T-LYMPHOCYTES, Jürg Tschopp, Danièle Masson and Dieter Jenne, Institute of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland

Proteases inhibitors are known to suppress CTL- and NK-cell induced target cell lysis. We show that cytoplasmic granules of CTL contain, in addition to the pore-forming, lytic protein perforin, a family of highly homologous serine esterases, designated granzymes A - H. The serine esterase affinity label diisopropyl fluorophosphate reacts only with granzymes A, B and D. While granzyme A is a disulfide-linked homodimer with trypsin-like specificity (cleaving best after arginine), no substrates have been found for the other granzymes. Granzymes D, E, F are highly glycosylated (up to five potential N-linked glycosylation sites accounting for 50 % of their molecular weight). Granzyme B seems to be restricted to CTL, whereas granzyme A is also expressed in non-cytolytic $CD4^+$ helper T-lymphocytes. Granzyme A is secreted by both T cell subsets in response to a specific antigenic stimulus of the target cell.

The N-terminal sequences of granzymes A and B are identical to those predicted from two cDNA clones previously isolated using T-cell specific libraries (1, 2). Polyclonal antibodies have been used to isolate cDNA clones coding for granzymes C, D, E, F. All granzymes are highly homologous to each other ($\geq$ 43 %) and contain a characteristic sequence of conserved amino acid between residues 9 - 16. Three other non-CTL proteases known to be stored in cytoplasmic granules, rat mast cell proteases 1 and 2, and cathepsin G of neutrophils, also belong to this growing protease family. Comparison of the cDNA-derived protein sequences with the amino-termini of the isolated proteins indicates that the granzymes (except granzyme A) contain an acidic dipeptide (GE or EE, propeptide) following the signal peptide (prepeptide). We predict that the propeptide is removed during packaging into their respective storage granules.

No direct role of the granzymes in CTL-mediated cytotoxicity has yet been demonstrated.

References

1) Gershenfeld & Weissmann, 1986, Science, 232: 854
2) Lobe et al., 1986, Science, 232: 858

Architecture of Cellular Protease Structure Analysis (X-Ray and NMR)

X 005 SITE DIRECTED MUTAGENESIS OF SUBTILISIN, Richard Bott*, Mark Ultsch*, John Burnier*, Paul Carter*, Dave Powers*, Jim Wells*, Scott Power#, Robin Adams#, Tom Graycar#, and Dave Estell#, *Genentech Inc., 460 Pt. San Bruno Blvd., So. San Francisco, CA 94080 and #Genencor Inc., 180 Kimball Way, So Sanfrancisco, CA 94080.
The sereine protease subtilisin is being studied as a model system to explore the feasibility of altering protein structure and function using site specific mutagenesis. At several positions, all nineteen possible variant enzymes have been expressed and characterized. Variants in which a single substitution has been introduced in the subtilisin polypeptide chain of 275 residues, manifest alterations of substrate specificity and increased activity toward different substrates. We have determined the three dimensional structures of a number of these variants. In all cases the substitutions have resulted in small, localized perturbations of the three dimensional structure. Comparison of these structures allow us to determine the role of different amino acid side chains in the active site.

X 006 INHIBITOR BINDING STUDIES ON THE ASPARTYL PROTEASE FROM PHIZOPUS CHINENSIS; IMPLICATIONS FOR GENERAL MECHANISM OF ACTION. David R. Davies, K. Suguna, and E.A. Padlan, Laboratory of Molecular Biology, NIDDK, NIH, Bethesda, MD 20892.
The three-dimensional structure of the aspartyl protease from Rhizopus chinensis has been determined by X-ray diffraction. The enzyme resembles the other aspartyl proteases such as pepsin, penicillopepsin and endothiapepsin. The structure consists of two domains between which runs a cleft containing the catalytic aspartic residues, the carboxylate groups of which are tightly held by a network of hydrogen bonds. Between them, approximately equidistant from the four carboxylate oxygens is a water molecule that has been proposed to be the nucleophile in the catalytic mechanism. A number of oligopeptide inhibitors including several pepstatin derivatives have been observed to bind in the cleft,thus providing a means of accounting for the extended subsite specificity of the enzyme. Another inhibitor studied has the sequence D-His-Pro-Phe-His-Phe[CH2-NH]-Phe Val-Tyr, with a reduced bond between the two adjacent phenylalanines. This binds in the cleft with the reduced bond in the active site making close contact with the two carboxyls. The active site water molecule is displaced by each of these inhibitors. Several mechanisms of action for this class of enzyme have been proposed and will be discussed.

X 007 STRUCTURAL BASIS OF THE ACTION OF THERMOLYSIN AND THE ZINC PROTEASES.
H.M. Holden, D.E. Tronrud, S. Roderick, L.H. Weaver and B.W. Matthews, Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403 U.S.A.

High resolution X-ray crystallography has been used to determine the modes of binding to thermolysin of a series of different inhibitors including dipeptides, mercaptans, hydroxamates, N-carboxymethyl peptides and phosphonamidates. The interactions displayed by such inhibitors illustrate interactions that are presumed to occur between the enzyme and its substrates during catalysis. The crystallographic analyses, together with model building, suggest a detailed stereochemical mechanism of action for thermolysin and, by analogy other zinc proteases such as carboxypeptidase A and the angiotensin converting enzyme (1,2). Recent analyses of a series of phosphonamidates, which are presumed to be transition-state analogues, has shown that chemically similar inhibitors can adopt dissimilar modes of binding. These different configurations provide a rationalization for large differences in the kinetics of binding that are observed for these inhibitors (3,4). Comparison of matched pairs of inhibitors allows the contribution of a single interaction (e.g. a hydrogen bond) to be determined (5,6).

1. Monzingo, A.F. and Matthews, B.W. (1984) Biochemistry 23, 5724-5729.
2. Hangauer, D.G., Monzingo, A.F. and Matthews, B.W. (1984) Biochemistry 23, 5730-5741.
3. Holden, H.M., Tronrud, D.E., Monzingo, A.F., Weaver, L.H. and Matthews, B.W. (1987) Biochemistry, in press.
4. Bartlett, P.A. and Marlowe, C.K. (1987) Biochemistry, in press.
5. Bartlett, P.A. and Marlowe, C.K. (1987) Science 235, 569-571.
6. Tronrud, D.E., Holden, H.M. and Matthews, B.W. (1987) Science 235, 571-574.

X 008 ISOTOPE DIRECTED NMR ANALYSIS OF INHIBITOR/PROTEASE COMPLEXES, Paul G. Schmidt[a,b], Mitsuo Oka[a], Douglas Kalvin[b], Juergen Maibaum[b], Theo Hofmann[c] and Daniel H. Rich[b].
[a]Vestar, Inc., 939 E. Walnut St. Pasadena CA 91106; [b]School of Pharmacy, University of Wisconsin-Madison, Madison WI 53706; [c]Department of Biochemistry, University of Toronto, Toronto, Canada.

Tight binding enzyme inhibitors are being used increasingly as lead compounds for the development of new drugs and for characterizing enzyme-ligand interactions and catalytic mechanisms. We have explored NMR methods for measuring structural features of synthetic peptides bound to acid protease active sites. The most useful peptides contain ^{13}C incorporated in specific loci, on the one hand for direct observation of the isotope-enriched carbon signal and, on the other hand, to allow us to select individual proton signals from otherwise overwhelming muddle of the protein-peptide NMR spectrum. The latter method works by taking the difference of proton NMR spectra with and without decoupling the ^{13}C signal. Protons not attached to the enriched ^{13}C (about 2000 atoms) cancel out, leaving the signal from the single enriched site - usually a doublet split by a singlet of opposite sign. The most important variation on this method incorporates presaturation of the singlet proton resonance before each collection pulse. Transfer of this saturation can occur to protons of protein active site amino acids which lie within about 4 A of the labeled inhibitor group. The difference spectrum then reveals these nearby peaks. Systematically employed, such experiments can be used to "map" the active site from the viewpoint of peptide inhibitors. We have obtained data from several ^{13}C labeled acid protease inhibitors which illustrate the method and provide orientation of some peptide side chain groups near aromatic residues of porcine pepsin.

Molecular Biology of Cellular Proteases and Regulators

X 009 **DESIGNS ON TRYPSIN-ENGINEERING AN ENZYME.** C.S. Craik, J. Higaki, H. Guo, M. Mc Grath, L. Evnin, J. Vasquez and R. Fletterick, Departments of Pharmaceutical Chemistry and Biochemistry/Biophysics, University of California, San Francisco, CA 94143.

Three aspects of enzyme structure/function are being studied using trypsin: substrate specificity, catalytic mechanism and folding. We have developed a system for the production, purification and characterization of natural or variant enzymes. Correctly folded trypsinogens or trypsins are expressed in bacteria and purified to levels of one milligram per liter. Yields of purified protein vary by 50 fold depending on the mutant. The variation in yield may depend on the stability and/or solubility of the variant enzyme in the bacteria. The expressed protein is directed to the periplasm of the bacterial host permitting correct disulfide bond formation and purification to crystalline quality. Approximately half of the expressed enzyme is isolated as a complex with the *E. coli* protease inhibitor, ecotin. A rapid procedure for the creation of single or multiple DNA modifications has been devised. Using oligonucleotides and single-stranded DNA templates containing uracil, single or multiple point substitutions, insertions or deletions can be constructed, isolated and sequenced directly. A single plasmid permits protein expression or DNA mutagenesis as required. An assay has been developed that permits rapid screening of the recombinant trypsins. Periplasmic fractions of recombinant *E. coli* that express the variant trypsins are fractionated with polyacrylamide gel electrophoresis. The enzyme activity is monitored directly by overlaying an activity gel impregnated with the appropriate enzyme substrate on the separation gel. This assay has been used to screen variant enzymes that have been altered in active site residues, substrate binding pocket residues and surface loops. Select variants have been chosen for detailed kinetic and structural investigations. The function of the side chains of His57, Asp102 and Ser195 have been tested by replacing them with alternate residues to produce the variants His57Ala, Asp102Asn, Ala, Ser, Glu, Ser195Cys and Asp102Asn + Ser195Cys. Kinetic studies show that the variant enzymes have reduced activities (k_{cat}) but virtually unmodified binding properties as reflected in K_M for ester or peptide substrates. The Asp at 102 is required for catalysis in physiological conditions since it is essential for positioning and maintaining the correct tautomeric form of the side chain of His57. Similarly the loss of activity following the replacement of Ser195 with Cys underscores the requirement for the oxygen atom as a nucleophile within the framework of a serine protease active site. The similarity of the positions of the three catalytic residues to Asn, His and Cys in the sulfhydryl proteases suggests that the 50-fold less activity (relative to papain) found for Ser195Cys trypsin is due either to steric incompatibilities among the catalytic triad or altered electronic charge distribution. The complete inactivity of Asp102Asn + Ser195Cys trypsin is likely due to the incorrect tautomer for His that we expect to be in the active site. The side chains of Gly216, Gly226, Asp189 and Ser190 are primary determinants of specificity in the P1 site. Kinetic analyses of Gly216Ala, Gly226Ala and Asp189Glu show that the Arg>Lys specificity of trypsin can be affected. The changes in specificity are observed in k_{cat} suggesting that correct positioning of the scissile bond is essential for catalytic efficiency. The inversion of the charge at the base of the specificity pocket in Asp189Lys surprisingly leads to an enzyme with only chymotryptic activity. The positive charge is presumed to be buried in a hydrophilic hole outside the substrate binding pocket. Surface loops on proteins, though variable in length and amino acid sequence, are presumed to be necessary for structural integrity and to provide flexibility for folding. Two loops in trypsin that show variable length and sequence in the family of serine proteases are the autolysis loop 145-149 and the loop at 71-79. Preliminary results suggest that deletion of amino acids 71-79 or 145-149 permits the proteins to survive in the host and be secreted to the periplasm. These enzymes and the double-deletion variant are stable *in vitro* though the activity of the enzyme is 100-1000 fold lower in k_{cat} for all three variants; the K_M's are normal. In contrast, deletion of the last three amino acids dramatically reduces enzyme activity. These data suggest that loop deletions may be used to study the structural and functional properties of selected surface loops in the native protein.

Acknowledgements: Support by National Sceince Foundation grant DMB-8608086 (CSC) is gratefully acknowledged.

X 010 Establishment of the dorsal-ventral axis in Drosophila requires the function of a gene, snake.

Robert Delotto, Max-Planck-Institut für Entwicklungsbiolgie, 7400 Tübingen, West Germany

During the few hours of embryonic development in Drosophila melanogaster, the functions provided by a set of twelve maternal effect genes are necessary for the proper differentiation of cells along the dorsal-ventral body axis of the embryo. Loss of function mutations at many of these loci can result in "dorsalized" embryos, in which cells at defined positions along this axis develop like cells normally found at more dorsal positions in the wild type animal. Other mutations may "ventralize" the embryo, deleting dorsal tissues at the expense of ventral ones. In extremely "dorsalized" embryos cells along the entire dorsal-ventral axis develop like cells normally found at the extreme dorsal position of the wild type embryo. The activities for many of these genes can be found in transplantable factors in the cytoplasm of the fertilized egg. Mutations at most of the loci can be rescued by the injections of either wild type cytoplasm or polyA+ RNA into mutant embryos. The snake gene is represented by four strongly "dorsalized" alleles and mutations at this locus respond most dramatically to rescue in cytoplasmic injection experiments. The snake gene has been cloned (Delotto and Spierer, 1986, Nature 323: 688) and a molecular characterization of the locus suggests that it encodes a protein of 430 amino acids which appears to be a member of the serine protease superfamily and exhibits structural similarities to some of the blood clotting enzymes. A synthetic mRNA has been made from a cDNA utilizing an SP6 promotor and it can be used to rescue the mutant phenotype by injection into mutant embryos. An immunological characterization of the snake protein is presently underway in order to test the hypothesis that snake may function in a series of cascaded protealytic activities which will play an essential role in the generation of positional informations along the dorsal-ventral axis during morphogenesis.

X 011 GENES, ZYMOGENS AND ACTIVATION CASCADES OF YEAST VACUOLAR PROTEASES. E.W. Jones, C.A. Woolford, C.M. Moehle, J.N. Van Arsdell, M.A. Innis, Dept. of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213 and Cetus Corporation, Emeryville, CA 94608.

Several of the hydrolases found in the lysosome-like vacuole of the yeast Saccharomyces cerevisiae are synthesized as inactive precursors that must be proteolytically processed to yield mature enzymes. Two proteases, A and B, appear to catalyze these specific cleavages. The PEP4 gene encodes the preproPrA precursor form of protease A, an aspartyl protease with marked homology to pepsin (1,2). Entry of proPrA into the acidic vacuole is thought to trigger autoactivation of proPrA to active protease A, by analogy to pepsinogen. DNA sequence analysis of one mutant allele, pep4-7, indicates that replacement of Ser by the bulky Phe in the second half-active site results in accumulation and failure of activation of proPRA, although the mutant proPRA can still be activated in trans, apparently by mature protease A. The DNA-derived amino acid sequence of PRB1, the structural gene of the subtilisin-like serine protease, protease B, is a 635 codon ORF. The N-terminus of mature protease B is amino acid 281(3). Studies of mutants indicate that the mature N- and C-termini of protease B are both generated by proteolytic processing. The cleavage(s) that generate the mature N-terminus are completed prior to transit from the endoplasmic reticulum. Protease A activity is required for generation of the mature C-terminus, presumably in the vacuole itself. Supported by NIH AM18090 and GM29713 and Cetus Corp.

1) Woolford, C.A., et al. (1986) Mol. Cell. Biol. 6: 2500-2510.
2) Ammerer, G., et al. (1986) Mol. Cell. Biol. 6: 2490-2499.
3) Moehle, C.M., et al. (1987) Mol. Cell. Biol. 7: in press.

X 012 PROTEASES INVOLVED IN PARASITE INVASION, James H. McKerrow, Johnny F. Railey, George Newport, Margaret Brown, and William E. Keene, Department of Pathology and School of Pharmacy, University of California, San Francisco, San Francisco, CA 94143.

Initiation of infection of the human host by a variety of helminth and protozoan parasites may be mediated by proteases expressed during the infectious stage of the parasite life cycle. Studies on a number of nematode, trematode and protozoan parasites have shown that the proteases used to facilitate tissue invasion are of three types. The first type, represented by the trematode parasite *Schistosoma mansoni*, is a serine protease with elastase activity that allows the parasite to invade the elastin-rich extracellular matrix of dermis. Structural and kinetic studies of this enzyme show it has primary sequence similarity to eukaryotic serine proteases including the pancreatic elastases and a substrate specificity similar to pancreatic elastase II. The enzyme is expressed only in the invasive cercarial stage of the parasite. Regulation is both transcriptional and post-translational. The second group of enzymes is represented by proteases of nematode parasites such as *Strongyloides* and hookworm. These are also elastases but of the metalloprotease class, and they are elaborated by the L3 larvae of nematode parasites which are skin invasive. The third category of enzymes are thiol proteases expressed by trophozoites of *E. histolytica* and metacercariae of *Paragonimus*. In contrast to the skin-invasive larvae of helminth parasites, these organisms invade the gastrointestinal tract. They have no elastase activity, but can degrade collagen and fibronectin. The expression of the *E. histolytica* thiol protease correlates with the relative virulence of different strains of the organism. Its substrate specificity appears similar to that of cathepsin B, but rather than being internalized in lysosomes, it is released by the organism into culture medium. Structural and genetic analysis of parasite proteases is providing important new information not only on the pathogenesis of the diseases they produce, but also the molecular evolution of thiol, metallo, and serine proteases.

Processing Mechanisms of Protein Synthesis

X 013 CARBOXYPEPTIDASE E AND PROTEIN PROCESSING, Lloyd D. Fricker, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx NY 10461. Many peptide hormones and neurotransmitters are initially produced as large precursors which must be enzymatically processed into the bioactive peptide. The processing sites are usually pairs of basic amino acids, and the sequential action of a trypsin-like endopeptidase and a carboxypeptidase B-like exopeptidase would produce the bioactive peptides. Carboxypeptidase E ('CPE'; EC 3.4.17.10, also designated enkephalin convertase and carboxypeptidase H) appears to be the carboxypeptidase B-like enzyme associated with the biosynthesis of numerous peptide hormones and neurotransmitters. CPE is present in many tissues where peptide biosynthesis is thought to occur, such as brain, pituitary, and adrenal medulla. Within the pituitary and adrenal medulla, CPE activity is associated with the peptide-containing secretory granules, which is the proposed site of peptide processing. CPE is maximally active at pH 5.6, the intragranular pH of most secretory granules. Purified CPE is very specific for substrates with C-terminal lysines and arginines, and is able to remove these amino acids from a wide range of bioactive peptide precursors without further degrading the peptide. This suggests that CPE may be involved with the biosynthesis of many different peptide hormones and neurotransmitters.

Recently, a cDNA clone encoding CPE has been isolated and sequenced. This analysis reveals that CPE is initially produced as a precursor containing an additional 42 amino acids on the N-terminus of the active form of the enzyme. The first 25 amino acids of this peptide resemble a signal peptide and are presumably removed by a signal peptidase. The remaining 17 amino acids are very hydrophilic. This peptide could be removed by a trypsin-like endopeptidase; there are five adjacent arginines immediately preceding the N-terminus of the active form of CPE. Within the 434 amino acids of the active form of CPE there are four pairs of basic amino acids. Two of the pairs of basic amino acids are near the C-terminus, and it is possible that cleavage at these sites produces the different protein forms of enzymatically active CPE that have been observed. The enzymatic activity of CPE presumably resides in the first 340 amino acids of the active form (residues 43-383), based on homology with carboxypeptidases A and B. While the overall homology between bovine CPE and either bovine CPA (20%) or bovine CPB (17%) is low, all of the amino acids thought to be involved with catalytic activity in CPA and CPB are present in comparable positions in CPE. This suggests that CPE, CPA, and CPB have evolved from a common ancestral carboxypeptidase.

X 014 **HEN OVIDUCT SIGNAL PEPTIDASE**, Mark O. Lively, Bowman Gray School of Medicine, Wake Forest University Medical Center, Winston-Salem, NC 27103. Signal peptidases are responsible for one of the earliest acts of limited proteolysis required for the production of most secretory proteins, certain organellar proteins and many membrane proteins. We have purified a microsomal signal peptidase present in the endoplasmic reticulum of tubular gland cells of the oviduct of laying hens. Hen oviduct signal peptidase (HOSP) is an integral membrane protein that requires detergent to remain soluble in aqueous solution. We have purified the enzyme [1] by the following series of steps: solubilization of carbonate-treated microsomes with Triton X-100; anion exchange chromatography on DEAE cellulose at pH 8.2; cation exchange chromatography on CM-cellulose at pH 5.8; hydroxylapatite chromatography; and affinity chromatography on concanavalin A-Sepharose. Purified HOSP requires only two proteins for limited proteolysis of secretory precursor proteins in vitro. It is composed of a 19 kDa protein and *an electrophoretically heterogenous 23 kDa glycoprotein which migrates with distinct* forms at 22, 23, and 24 kDa. Structural analysis of the individually purified forms of the glycoprotein has shown that they are differentially glycosylated forms of the same polypeptide backbone. Peptide maps of tryptic fragments separated by high performance liquid chromatography are the same for each glycoprotein. Amino acid sequence analysis of the purified tryptic peptides has revealed the same amino acid sequences for all tryptic fragments of the three forms which have been sequenced. In contrast, the tryptic map of the 19 kDa protein is distinct from that of the glycoprotein and no amino acid sequences common to both proteins have been found. Approximately one third of the amino acid sequence of the 23 kDa glycoprotein has been determined in nonoverlapping fragments. Comparison of these sequence data with the amino acid sequence database has not revealed any similarities with other known proteins. From these limited data, we conclude that HOSP is apparently not related to either of the known E. coli signal peptidases whose sequences have been determined. HOSP is also apparently not related to any of the known classes of proteolytic enzymes. The catalytic activity of HOSP has been resistant to inhibition by a wide range of reagents known to inactivate other classes of proteolytic enzymes. The data presently available suggest that signal peptidase may be a representative of a new mechanistic class of proteolytic enzymes.

[1] Baker, R.K. and Lively, M.O. (1987) Biochemistry 26, in press.

X 015 THE YEAST PRO-HORMONE CLEAVING ENZYME, KEX2, Robert S. Fuller[1] and Jeremy Thorner[2], [1]Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305 and [2]Department of Biochemistry, University of California, Berkeley, CA 94720.
In baker's yeast (Saccharomyces cerevisiae), maturation of the precursor to a secreted peptide hormone (α-factor) requires cleavage at the carboxyl side of -LysArg- sites. We have demonstrated previously that this specific proteolytic processing event is catalyzed by the endoprotease encoded by the yeast KEX2 gene, and have cloned and sequenced this DNA. Expression vectors were used to overproduce the enzyme for biochemical studies. The enzyme is a membrane-bound, Ca^{2+}-dependent neutral protease. Activity is inhibited by moderate concentrations of some thiol-directed reagents, but is resistant to high concentrations of trans-epoxysuccinate derivatives (which are potent inhibitors of known thiol proteases). Conversely, activity is resistant to several serine protease inhibitors, yet can be inhibited by diisopropylfluorophosphate (a reagent diagnostic for serine proteases). These findings suggest that the KEX2 enzyme represents a novel class of serine protease. Correspondingly, the predicted amino acid sequence deduced from the nucleotide sequence has an ∿300-residue segment that has significant homology to the subtilisin family of bacterial serine proteases and to a related protease of a prokaryotic actinomycete, thermitase. When optimally aligned, the KEX2 enzyme shares 36% identity to a consensus sequence for subtilisin/thermitase; and, homology is even greater (up to ∿60%) if conservative amino acid replacements are allowed. Further, residues of the so-called "charge relay system" (Asp-His-Ser) are present in nearly the same relative positions in the yeast and bacterial enzymes; and, the stretches of strongest homology between the two proteins are clustered around these and other residues known to be essential for catalysis and substrate binding in subtilisin. This catalytic domain of KEX2 contains 7 Cys residues, which may account for the sensitivity of the enzyme to SH-directed compounds. Other aspects of the predicted amino acid sequence fit well with the known enzymic and topological features of the enzyme in vivo. Deletion analysis of the KEX2 gene shows that a single transmembrane domain (TMD) near the C-terminus is responsible for tethering the enzyme in the secretory system. Only a few of the available -Asn-X-Thr/Ser- sites in the protein carry N-linked oligosaccharide chains; in contrast, a Ser- & Thr-rich region just proximal to the TMD is heavily decorated with O-linked carbohydrate. Subcellular fractionation indicates that the enzyme is in an internal compartment distinct from secretory vesicles. The yeast enzyme, when expressed from a vaccinia virus vector, functions in a variety of animal cell lines to carry out proper processing of prepro-opiomelanocortin to yield a spectrum of correct products, including β-endorphin$_{1-31}$.

Protease Control Mechanisms: Natural and Synthetic Regulators of Proteases and Inhibitors

X 016 PROTEASE NEXINS: CONTROL OF EXTRACELLULAR PROTEINASES, Dennis D. Cunningham, Steven L. Wagner, David H. Farrell and William E. Van Nostrand, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717

The protease nexins (PNs) are protein proteinase inhibitors that are synthesized and secreted by a variety of cultured cells and form denaturation-resistant complexes with certain serine proteinases. The PN:proteinase complexes bind to the cells which secrete the PN and are rapidly internalized and degraded. Although the PNs resemble certain plasma serine proteinase inhibitors (serpins), they are present in only minute concentrations in plasma.

PN-1 rapidly inhibits thrombin, urokinase, plasmin, and trypsin. It possesses a heparin binding site and heparin markedly accelerates the inactivation of thrombin by PN-1. In cultures of human fibroblasts, PN-1 occurs in the medium and also bound to the extracellular matrix (ECM) where it colocalizes with fibronectin. Although the ECM component(s) responsible for the binding of PN-1 have not yet been identified, our studies have shown that this binding significantly affects PN-1 activity. First, the ECM accelerates the inactivation of thrombin by PN-1; this is mostly due to ECM heparan sulfate. Second, binding of PN-1 to the cell surface blocks its ability to form complexes with urokinase. These results lead to the suggestion that PN-1 is primarily a thrombin inhibitor in vivo where the amount of extracellular fluid is small compared to cell culture conditions, and where much of the PN-1 is probably bound to the cell surface and ECM.

Less is known about PN-2 because it was purified only recently. PN-2 forms denaturation-resistant complexes with three serine proteinases: the epidermal growth factor binding protein, the gamma subunit of nerve growth factor and trypsin. PN-2 also inactivates chymotrypsin, but by a reversible mechanism that does not lead to the formation of denaturation-resistant complexes. Like PN-1, PN-2 has a heparin binding site and binds to the cell surface/ECM.

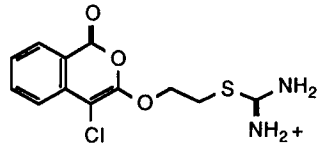
X 017 TYPE 1 PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1), David J. Loskutoff and Jun Mimuro, Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

Plasminogen activation provides an important source of localized proteolytic activity during fibrinolysis, ovulation, cell migration, epithelial cell differentiation, and tumor cell invasion. Precise regulation of plasminogen activator (PA) activity thus constitutes a critical feature of many biological processes. This control is achieved in large part through the action of PAI-1, an efficient, endothelial cell (EC) derived inhibitor of both urokinase and tissue-type PA (tPA; K_d=1.3x10^{-13}M; K_{assoc}=3.9x10^{7}M^{-1}s^{-1}). PAI-1 accounts for as much as 12% of the total protein released by ECs, and is also a major component of the extracellular matrix (ECM) of ECs. It has an M_r of 50,000, an isoelectric point of 4.5-5.0, and is relatively stable to conditions which inactivate most protease inhibitors (acid pH, SDS). However, it is extremely sensitive to inactivation by oxidants, possibly because it has a methionine at the P_1 position of it's reactive center. The molecular cloning of a cDNA for PAI-1 revealed that the mature human protein is 379 amino acids long, lacks cysteines and is a member of the serine proteinase inhibitor (serpin) family. The PAI-1 gene itself is 12.2 kilobase pairs in length and is organized into nine exons and eight introns. The production of PAI-1 by ECs is stimulated by endotoxin, interleukin-1, tumor necrosis factor, and transforming growth factor β (TGFβ). ECs are extremely sensitive to TGFβ with maximal effects (100-fold stimulation) observed with 1-2 ng/ml. These changes are relatively specific for PAI-1, and can be detected at both the protein and the RNA level. Interestingly, TGFβ also stimulates the amount of PAI-1 present in the ECM of ECs. PAI-1 exists in blood and in various cellular samples in both an active and an inactive (latent) form. The latent form can be converted into the active one by treatment with denaturants like SDS or guanidine-HCl. Although the majority of cell-associated PAI-1 is active, it rapidly decays ($t_{½}$=3 h) into the latent form once it is released from the cells. In contrast, ECM associated PAI-1 has a $T_{½}$> 24 h. These data suggest that PAI-1 is produced by ECs in an active form, and is then either released into the medium where it is rapidly inactivated, or released into the subendothelium where it binds to ECM. The specific binding of PAI-1 to ECM protects it from this inactivation.

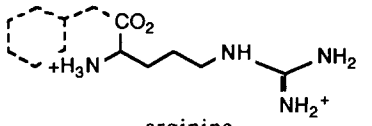
X 018 MECHANISM-BASED ISOCOUMARIN INHIBITORS FOR SERINE PROTEASES

James C. Powers and Chih-Min Kam, School of Chemistry, Georgia Institute of Technology, Atlanta, GA 30332.

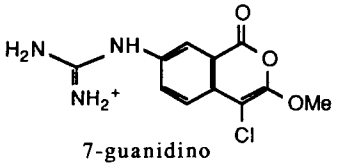
Isocoumarins are potent irreversible inhibitors for a variety of serine proteases. Most serine proteases are inhibited by isocoumarins such as 3,4-dichloroisocoumarin, while isocoumarins containing hydrophobic 7-acylamino groups are potent inhibitors for human leukocyte elastase. Isocoumarins containing basic side chains which resemble arginine (shown below) have been constructed and are potent inhibitors for trypsin-like enzymes. The 7-guanidino isocoumarin is a potent inhibitor of bovine thrombin, human factor Xa, human factor XIa, human factor XIIa, human plasma kallikrein, porcine pancreatic kallikrein and bovine trypsin. The 3-(2-isothiureidoethoxy)isocoumarin is less reactive toward many of the same enzymes, but is an extremely potent inhibitor of human plasma kallikrein. Several guanidinoisocoumarin have been tested as anticoagulants in human plasma and are effective at prolonging the prothrombin time.



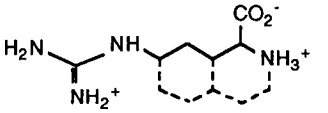
3-(2-isothiureidoethoxy)



arginine



7-guanidino



The mechanism of inhibition involves formation of an acyl enzyme by reaction of the active site serine with the isocoumarin carbonyl group. Isocoumarins with 7-amino or 7-guanidino groups will then decompose further to quinone imine methide intermediates which react further with an active site residue (probably His-57) to form stable inhibited enzyme derivatives.

X 019 HUMAN THROMBOMODULIN GENE IS INTRON DEPLETED: NUCLEIC ACID SEQUENCES OF THE cDNA AND GENE PREDICT PROTEIN STRUCTURE AND SUGGEST SITES OF REGULATORY CONTROL. Jackman, Robert W.; Beeler, David L.; Fritze, Linda; Soff, Gerald; and Rosenberg, Robert D.
Massachusetts Institute of Technology, Cambridge, MA 02139
ABSTRACT: We have isolated a human thrombomodulin cDNA, and a human genomic clone containing the putative promoter domain, as well as the translated and untranslated regions of the endothelial cell receptor. The nucleotide sequence of the thrombomodulin cDNA allows us to provide a complete picture of the structure of this endothelial cell receptor, and to confirm its homology to the human LDL receptor. The nucleotide sequence of the thrombomodulin gene suggests areas within the putative promoter domain which may be critical for regulating expression of the human endothelial cell receptor, indicates a potential signal peptide, and shows that no introns are present within the coding region. The overall organization of the human thrombomodulin gene is surprising because it represents a unique example of a gene that contains EGF type B repeats and a membrane spanning region which are not isolated within discrete exons.

Therapeutic Application of Proteases and Inhibitors

X 020 TREATMENT OF PULMONARY EMBOLISM WITH TISSUE PLASMINOGEN ACTIVATOR, Samuel Z. Goldhaber, M.D. on behalf of the Participating Investigators, Brigham and Women's Hospital (Harvard Medical School) Boston, MA 02115 and Other Institutions.
We assessed the efficacy and safety of peripheral intravenous recombinant human tissue-type plasminogen activator (rt-PA) in 47 patients with angiographically documented pulmonary embolism (PE). We administered 50 mg/2 hours and, if necessary, an additional 40 mg/4 hours (1,2). By 6 hours, 94% of the patients had angiographic evidence of clot lysis that was slight in 5, moderate in 12, and marked in 27 patients. Among the 34 patients with pulmonary hypertension prior to treatment, the pulmonary artery pressure decreased from 43/17 ($\overline{27}$) to 31/13 ($\overline{19}$) mm Hg ($p<0.0001$). The lung scan perfusion defect decreased from 35% before therapy to 14% ($P<0.01$) after therapy among the 19 patients who had pre and post treatment lung scans (3). Of 7 patients with pre and post treatment Doppler echocardiograms, hypokinetic right ventricular wall movement (mild in 1, moderate in 2, and severe in 4) normalized in 5 and improved to mild hypokinesis in 2. Right ventricular diameter decreased from 3.9 ± 1.0 to 2.0 ± 0.5 cm ($P < 0.005$) (4). Fibrinogen decreased 33% from baseline at 2 hours and 42% from baseline at 6 hours. However, patients with the greatest degree of angiographic clot lysis at 2 hours had a preponderance of fibrinogenolysis over fibrinolysis, as assessed by the ratio of cross-linked fibrin degradation products to fibrin(ogen) degradation (0.14 ± 0.09 vs. 0.54 ± 0.82) ($P<0.04$) products (5). Among selected patients, peripheral intravenous rt-PA can rapidly lyse PE, improve pulmonary perfusion, and improve right ventricular function.

1. Goldhaber SZ, Vaughan DE, Markis JE, et al. Acute pulmonary embolism treated with tissue plasminogen activator. Lancet 1986; 2:886-889.
2. Goldhaber SZ, Markis JE, Kessler CM, et al. Perspectives on treatment of acute pulmonary embolism with tissue plasminogen activator. Seminars in Thrombosis and Hemostasis 1987; 13:221-227.
3. Parker JA, Markis JE, Palla A, et al. Early improvement in pulmonary perfusion after recombinant human tissue-type plasminogen activator therapy for acute pulmonary embolism: Segmental perfusion scan analysis. Radiology 1988; in press.
4. Come PC, Kim D, Parker JA, et al. Early reversal of right ventricular dysfunction in patients with acute pulmonary embolism after treatment with intravenous tissue plasminogen activator. JACC 1987; in press.
5. Vaughan DE, Goldhaber SZ, Kim J, et al. Recombinant tissue plasminogen activator in patients with pulmonary embolism: Correlation of fibrinolytic specificity and efficacy. Circulation 1987; 75:1200-1203.

Alterations of Cellular Proteases in Disease Processes

X 021 ADIPSIN: A POTENTIAL REGULATOR OF ADIPOCYTE METABOLISM AND OBESITY,
Bruce M. Spiegelman[t], Kathleen S. Cook[t] and Jeffrey S. Flier[*t], Dana-Farber Cancer Institute and Harvard Medical School[t], Boston, MA 02115 and Beth Israel Hospital, Boston, MA 02115[*].

When mouse adipocytes differentiate in culture, they express many new gene products. By cDNA cloning and sequence analysis, we have shown that one newly expressed mRNA encodes a novel serine protease homologue termed adipsin. Adipsin has approximately 30% amino acid homology to most members of the serine protease family and a closer homology (60%) with human Complement D. Adipsin has a charge-relay system typical of serine proteases and a potential site for activation of the zymogen. Using antipeptide antibodies to predicted adipsin peptides, we have shown that this protein is secreted from fat cells into the peripheral blood. It appears as a heavily glycosylated protein with a molecular mass of 37-41 Kd, far larger than the predicted size of 26 Kd.

Adipsin mRNA is regulated by the nutritional status of rodents. Initiation of a catabolic, lipolytic state via fasting, streptozotocin-induced diabetes or a partial pancreatectomy causes a 3-5 fold increase in adipsin mRNA in rat adipose tissue. Stimulation of an anabolic, lipogenic state through direct glucose infusion or refeeding fasted animals suppresses adipsin mRNA levels. Because the above data suggest an important systemic role for this fat-derived protein, we have examined adipsin synthesis in a disorder of systemic energy balance, i.e. obesity. Adipsin mRNA and circulating protein are greatly reduced (25-100 fold) in three experimental models of obesity having a genetic or metabolic basis: the ob/ob mouse, the db/db mouse and obesity induced by injection of monosodium glutamate (MSG) into neonatal mice. In contrast, normal rats made obese through purely dietary means (cafeteria diet) show no impairment in adipsin mRNA levels. These data raise several intriguing possibilities: (1) adipsin levels may distinguish between animals rendered obese through genetic or metabolic lesions versus obesity arising from pure overfeeding. (2) Since adipsin may participate in adipose catabolism, the reduction in this protein could play an important role in the development of certain obese syndromes. (3) Understanding the defect in adipsin regulation may improve our understanding of neuroendocrine defects which lead to aberrant gene regulation and lipogenesis in the fat of obese animals. The catalytic activity and substrate specificity of adipsin protein produced with baculovirus and mammalian virus vectors are currently being explored.

X 022 METALLOPROTEINASE GENE EXPRESSION IN DEVELOPMENT, DIFFERENTIATION, TRANSFORMATION AND DISEASE, Zena Werb, Richard A. Adler, Ole Behrendtsen, Carol A. Brenner, Jennie R. Chin, Elizabeth J. Clark, Steven M. Frisch, Daniel A. Rappolee, Patrice M. Tremble, and Elaine N. Unemori, Laboratory of Radiobiology and Environmental Health, University of California, San Francisco, CA 94143-0750.

The metalloproteinases are a multigene family of enzymes that degrade components of the extracellular matrix in the pericellular environment. The best studied examples of these proteinases are collagenase (a type I collagen-degrading proteinase), stromelysin (a proteoglycan, fibronectin and laminin-degrading enzyme), and two gelatinases (that also degrade collagen type IV and V), which are secreted by cells as proenzymes that must be activated for expression of their proteolytic potential. Their activities are controlled in part by endogenous inhibitors, also produced locally by cells. The best studied of these inhibitors is the tissue inhibitor of metalloproteinases (TIMP). We have studied the regulation of expression of these proteinases and TIMP in early mouse development, and in response to SV-40 transformation of fibroblasts, and have determined the cellular mechanisms regulating expression of these genes and activities of their gene products. During preimplantation mouse embryogenesis mRNA transcripts for stromelysin, collagenase, and TIMP are first seen as maternal transcripts in unfertilized eggs. These transcripts increase with development through the blastocyst stage. Metalloproteinases secreted by embryos in culture are detected as early as cleavage stages, and increase with development to blastocyst outgrowth stage, which is equivalent to implantation. In the embryonal carcinoma cell lines F9 and PSA-1, the transcripts for proteinases and TIMP and their biological activities are also regulated with endodermal differentiation. In fibroblasts in culture, the expression of metalloproteinase genes are up-regulated by phorbol diesters, cAMP, growth factors, interleukin 1, calcium ionophores, agents remodeling the actin cytoskeleton, and transformation and down-regulated by glucocorticoids and retinoids. Although collagenase and stromelysin are usually regulated coordinately during SV-40 transformation, collagenase is induced to a much greater extent than stromelysin, and regulation of collagenase is also seen at the level of translation. The TIMP gene is regulated independently: while phorbol diesters, interleukin 1, and retinoids up-regulate this gene, transformation and cytoskeletal regulators down-regulate it. The net expression of the proteolytic activity of the metalloproteinases is balanced by activation of the zymogen forms by enzyme cascades and inhibition of the enzyme activities by inhibitors. There are two distinct cellular enzyme cascade systems involved in activation of the metalloproteinases - one that is plasminogen-dependent and a second that is plasminogen-independent. Thus, there are multiple pathways for regulating metalloproteinase gene expression and coordination at the levels of transcriptional, translational activation and inhibition. Supported by a contract from U.S. Department of Energy, OHER #DE-AC03-76-SF01012, and by a National Research Service Award from NIEHS #T32 ES07106.

Cellular Proteases and Control Mechanisms

Keynote Address

X 023 BASIC BIOLOGICAL REGULATION MECHANISMS BY PROTEOLYSIS AND ANTIPROTEOLYSIS,
Hans Fritz, University of Munich, Nussbaumstr. 20, D-8000 Munich 2, FRG

Proteinases are involved in basic physiological or pathophysiological processes such as formation and inactivation of biologically active peptides or hormones, blood clotting and fibrinolysis, immunological defense mechanisms (via the complement system), and elimination of metabolic products or invading organisms by phagocytes of the reticulo endothelial system (RES).

Kinins represent an example of a very potent tissue hormone which regulates among others the tonus and permeability of capillary blood vessels. Excess kinin formation may cause, however, interstitial edema formation and thus organ dysfunction or shock. Kinins are generated during activation of the clotting/fibrinolytic systems and overactivation of these systems (e.g. after multiple injuries or during severe infections) will additionally impair organ function by microembolism or bleeding due to disseminated intravascular coagulation.

Besides the humoral systems comprising primarily highly selective proteolytic cascade mechanisms, the digestive enzymes or oxidizing agents present in the lysosomes or phagolysosomes of various blood and tissue cells (phagocytes) have to be especially envisaged as potentially dangerous for the organism. If released into the extracellular space due to severe inflammatory stimuli, these cellular factors and especially the lysosomal proteinases may extensively degrade tissue structures and humoral glyco-proteins and thus cause again organ dysfunction.

Under physiological conditions the proteinase activities from both the humoral cascade systems and the cell lysosomes are governed by potent natural antagonists, the proteinase inhibitors, which are ubiquitously present throughout the organism. These inhibitors are consumed during an inflammatory event via several routes which may lead to an acquired inhibitor deficiency or a socalled proteinase-proteinase inhibitor imbalance. Under such circumstances proteinases are able to cause serious toxic effects by generation of high amounts of pharmacologically active peptides and by digestion of structural elements and humoral factors outside the cells.

This up-to-date knowledge offers the rationale for the administration of proteinase inhibitors in medical therapy. They can be used, either to assist regulation of the humoral cascade systems by the natural inhibitors or as protectors of the extracellular compartments of the organism against the digestive proteinases of the lysosomes.

Late Addition

X 024 ROLES OF CHYMASE AND TRYPTASE IN MAST CELL GRANULES IN ALLERGIC INFLAMMATION, N.Katunuma and H.Kido, Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima,Tokushima 770,Japan

Chymase and tryptase are localized in the granules of rat mast cells. The chymase and atypical chymase were sequenced by our laboratory. The tryptase was purified from rat peritoneal mast cells with an endogenous inhibitor of it,named trypstatin by our group. These proteases are postulated to be important in IgE-mediated hypersensitivity reactions. We found that the release of histamine induced by anti-rat IgE was markedly inhibited by $F(ab')_2$ of anti-chymase IgG, boronic acid peptide derivatives, substrate analogs of chymase and low molecular weight inhibitor of chymase, such as chymostatin, but not by inhibitors for tryptase. Whereas, the release of histamine induced by Ca-inophore A23187 and compound 48/80 was not inhibited by chymase inhibitors. These results suggest that the antibodies and chymase inhibitors are incorporated into mast cell granules and inhibit chymase activity, resulting in inhibition of histamine release.

After degranulation, released chymase remains associated with the cell surface while released tryptase and trypstatin are found in the extracellular milieu. Tryptase released converted prothrombin to thrombin, as determined by increase in thrombin activity with a synthetic substrate and by the analysis of SDS-gel electrophoresis of products in the reaction of prothrombin with tryptase. The apparent Kcat/Km value for the conversion by tryptase was about the same level as that by Factor Xa, suggesting that the tryptase might contribute to fibrin accumulation in extra-vascular tissues. Trypstatin completely inhibited this conversion. Trypstatin was sequenced by our laboratory and was belongsto aprotinin family. These findings indicate the possible applications of inhibitors of chymase and tryptase in IgE-mediated allergic inflammation as therapoitic purpose.

Characterization and Structural Anlaysis

X 100 PURIFICATION AND CHARACTERIZATION OF DOG MASTOCYTOMA CHYMASE. IDENTIFICATION OF AN OCTAPEPTIDE CONSERVED IN CHYMOTRYPTIC LEUKOCYTE PROTEASES, George H Caughey, Nona F Viro, Stephen C Lazarus and Jay A Nadel, Cardiovascular Res. Institute, Univ.of CA, San Francisco, CA 94143.

Chymases are chymotryptic mast cell serine proteases which are released outside of the cell during exocytosis. Their biologic roles and relation to similar enzymes in other leukocytes are uncertain. We isolated a chymase from dog mastocytomas by gel filtration, high performance hydrophobic interaction and cation exchange chromatography. The purified enzyme had an M_r of 27000-30000 by gel filtration and gel electrophoresis, and a single N-terminal sequence. Like chymases from rat and human mast cells, the active site-titrated mastocytoma enzyme exhibited a high k_{cat}/K_m ($1.1 \cdot 10^5$ $M^{-1}s^{-1}$) at 25°C, employing succinyl-L-Val-Pro-Phe-4-nitroanilide, the best of several peptide 4-nitroanilide substrates screened. It was inhibited by diisopropyl fluorophosphate and soybean trypsin inhibitor, but not by aprotinin. The N-terminal 25 residues of mastocytoma chymase were 72% and 68% identical to the corresponding sequences of chymases from rat peritoneal and mucosal mast cells, respectively, and 64% identical to human cathepsin G and mouse cytotoxic T-lymphocyte chymotryptic protease. Mastocytoma chymase contained an octapeptide sequence which is common to all chymotryptic leukocyte proteases sequenced to date from four mammalian species, but was not present in either chymotrypsin or in dog mastocytoma tryptase. This octapeptide distinguishes chymases and other chymotryptic leukocyte proteases from serine proteases of coagulation and digestion. The molecular and functional significance of this distinctive structural feature remain to be determined.

X 101 PROTEIN DESIGN ON HUMAN PANCREATIC SECRETORY TRYPSIN INHIBITOR, John Collins, Hans Fritz*, Helmut Blöcker[+], Ronald Frank[+], Andreas Meyerhans[+], Konrad Schwellnus[+], Wolfgang Bruns[o], Jürgen Ebbers[o], Gerd Reinhard[o], Eugen Schnabel[o] Werner Schröder[o], Thomas W. Böldicke, Michael Szardenings, Michael Böcher, Gerhard Gross, Friedhelm Maywald, Joachim Reichelt[++] and Dietmar Schomburg[++]. GBF, Braunschweig, Mascheroder Weg 1 ([+]DNA Synthesis Group, [++]Instrumental Analysis/Molecular Modelling), *Institut für klinische Chemie und Biochemie, Universität München, [o]Pharmaforschung, Bayer AG, Wuppertal.

Using synthetic genes and site-directed mutagenesis hu PSTI and a series of variants wer constructed which should differentially inhibit trypsin, chymotrypsin (cathepsin G) and human or porcine elastases without inhibiting other serum proteases. The data base for the initial planning was the series ovomucoid, particularly third domain sequences of Laskowski. Expression problems were solved with secretion vectors and fully active, correctly processed PSTIs produced. Further variants are being planned with computer aided design based on crystallography data. The high absolute binding affinities and specificities could initially be rationalized by computer simulation based on modified porcine PSTI-protease complexes. Antisera and monoclonal antibodies to PSTI and to the active sites of PSTI and an elastase specific variant were produced which should be useful in pharmocokinetic studies and tumor diagnosis.

X 102 PERIPLASMIC PROTEASES OF E. COLI K12: A REASSESMENT; Robert A. Cook, Verna J. Lang and Indrasan Vaithilingam, University of Western Ontario, London, Ontario N6A 5C1. E.coli K12 grown to mid-log phase on Luria Broth contains as many as six proteolytic activities in the "periplasmic" fraction released by osmotic shock or spheroplast formation. These periplasmic proteases have been tentatively designated P1-P6 according to their elution order on DEAE-cellulose chromatography. Proteases P1, P2 and P4 have been purified to homogeneity and are metalloendoproteases capable of degrading α-casein, β-casein AND insulin B chain. Proteases P2 and P4 are monomers of molecular weight 94K and 100K, respectively, and are stabilized by Ca^{2+} (or Mn^{2+}). Protease P1, a dimer of 70K, exhibits a total dependence on Ca^{2+} for activity and is absent in cells grown to stationary phase. Protease P3, as well as Proteases P5 and P6 (eluted by high salt from DEAE-cellulose) have not yet been purified to homogeneity. Preliminary characterization studies indicate that P5 degrades only α-casein, exhibits a MW >150K and is probably a serine protease. Protease P6 degrades only insulin B chain and exhibits a MW of <70K. Analysis of E.coli K12 mutants defective in Protease III (ptr^-) indicate that Proteases P1-P6 are distinct from Protease III and that Protease III is, in fact, located in the cytoplasmic fraction. Immunological studies are presently underway to determine if Proteases P1-P6 represent unique gene products, as well as to quantitate protease levels at different growth stages in enriched and minimal media.

X 103 PRODUCTION OF A TISSUE INHIBITOR OF METALLOPROTEINASES BY BOVINE VASCULAR CELLS. Y. A. DeClerck, R. Bock, and W. E. Laug. Division of Hematology-Oncology, Childrens Hospital of Los Angeles and University of Southern California, Los Angeles, CA, U.S.A.

Inhibitors of collagenase play an important role in collagen turnover in tissues. We have investigated the production of these inhibitors by various cells derived from bovine vessels including endothelial cells of arterial, venous and capillary origin and arterial smooth muscle cells. While large amounts of collagenase inhibitor (800 mU/10^6 cells/24 hr) were produced by vascular smooth muscle cells, smaller amounts were detected in the medium conditioned by either arterial, capillary or venous endothelial cells (90, 1.7 and 1.1 mU/10^6 cells/24 hr respectively).

An inhibitor with a Mr of 28,500 was purified from serum free medium conditioned by bovine smooth muscle cells. This inhibitor had many characteristics similar to those of a collagenase inhibitor produced by human fibroblasts and a tissue inhibitor of metalloproteinases extracted from human amniotic fluid and rabbit bone.

The production of this inhibitor in bovine vascular smooth muscle cells markedly increased during cell proliferation, and in the presence of endothelial cells suggesting an enhancing effect of endothelial cells on vascular smooth muscle cells.

These data suggest that the large amount of this inhibitor produced by vascular muscle cells may be responsible for several physiopathological conditions such as the accumulation of collagen characteristically observed in conjunction with smooth muscle cells hyperplasia in atherosclerotic plaques, and the resistance of large arterial vessels to tumor invasion.

X 104 CHARACTERIZATION OF ENDOSOMAL CATHEPSIN D, Stephanie Diment, Melinda S. Leech, and Philip D. Stahl. Washington University Medical School, St. Louis. MO 63112

Rabbit macrophage endosomes contain a pepstatin-sensitive protease (Diment,S. and Stahl,P. (1985) J. Biol. Chem. 260, 15311). We have identified this protease as cathepsin D by immunoprecipitation with goat anti-cathepsin D IgG. Cathepsin D in endosomes and lysosomes was iodinated using endocytosed lactoperoxidase. The organelles were disrupted by homogenization and the membranes were washed in sequence with the following buffers: 5mM Tris pH 7.4 containing mannose 6-phosphate, 100mM sodium acetate pH 5.0 containing mannose 6-phosphate and EDTA, 100mM sodium bicarbonate pH 10.6. 50% of endosomal cathepsin D was dissociated from the membranes in these buffers, the remaining 50% required detergent extraction. In contrast, more than 90% of the lysosomal cathepsin D was washed from the membranes in the absence of detergent. To determine whether the membrane-associated form of cathepsin D (found in endosomes) is a precursor of the soluble lysosomal form, we biosynthetically labeled rabbit alveolar macrophages with ^{35}S-methionine, and subsequently washed the disrupted cells with the above buffers. Labeled cathepsin D was immunoprecipitated from buffer and detergent-extracted fractions after increasing chase times. During a one hour pulse, the enzyme was synthesized as a membrane-bound catalytically inactive 53kDa precursor. After 2 hours chase, cathepsin D was an active, 47kDa species. More than 50% of this form was membrane-bound. Mature 46kDa cathepsin D was formed after 8 hours chase and 50% of this was still membrane-bound. Our data provide evidence that cathepsin D is synthesized as a membrane-bound form, which is not anchored via mannose 6-phosphate receptors. This form is found in endosomes. Cathepsin D is subsequently processed to the soluble form found in lysosomes.

X 105 IDENTIFICATION, PARTIAL CHARACTERIZATION AND ROLE OF NEW BRUSH BORDER MEMBRANE NEUTRAL ENDOPEPTIDASES IN HUMAN AND RAT SMALL INTESTINE. RH Erickson, D Guan, M Yoshioka, YS Kim. GI Res. Lab, Dept of Med, Univ of CA & VA Hosp. San Francisco, Ca 94121

Two new phosphoramidon-insensitive neutral endopeptidases were identified and partially characterized in the brush border membrane of rat intestine using CBZ-Ala-Arg-Arg-MNA and azocasein or α-casein as substrates. Activities in the brush border membrane of both rat and human intestine were maximum at neutral to alkaline pH and inhibited by metal chelating and thiol reagents. Phosphoramidon, a known specific inhibitor of thermolysin-like endopeptidases, did not inhibit either enzyme. It was concluded that these endopeptidases are distinct from pancreatic proteases based on their sensitivity to various classes of protease inhibitors and by the fact that no pancreatic protease activities could be detected in crude mucosal cell homogenates or purified brush border membrane fractions. The enzyme hydrolyzing CBZ-Ala-Arg-Arg-MNA was shown to be different from that hydrolyzing azocasein or α-casein based on pH optimum, thermostability, sensitivity to inhibitors, molecular weight and differences in enzyme levels in rat and human brush border membranes. SDS-PAGE and gel filtration revealed that several native intact protein substrates such as α-casein, histone and fibrinogen were rapidly degraded to small molecular weight peptides and amino acids when incubated with rat or human brush border membrane preparations. During in vivo intestinal perfusion in rats, 11% of the total administered α-casein was hydrolyzed and absorbed by the intestine. The results of this study indicate that phosphoramidon-insensitive neutral endopeptidases in the intestinal brush border membrane may be of nutritional and physiological importance in protein digestion.

X 106 BETA-NGF-ENDOPEPTIDASE IS IDENTICAL TO EGF-BP TYPE A, Margaret Fahnestock*, James Woo*, June Snow+, Daniel Walz+ and William Mobley*, *SRI International, Menlo Park, CA 94542, +Wayne State University, Detroit, MI 48201, and *University of California, San Francisco, CA 94143.

The beta subunit of nerve growth factor (beta-NGF) from the mouse submaxillary gland is cleaved at a rare histidine/methionine bond to release an NH2-terminal octapeptide. The enzyme that performs this cleavage, beta-NGF-endopeptidase, shares immunological cross-reactivity with gamma-NGF and EGF-binding protein. To explore the relationship of beta-NGF-endopeptidase with the mouse kallikrein family we undertook protein sequence studies.

Beta-NGF-endopeptidase was purified by the method of Wilson and Shooter (J.Biol.Chem. 254:6002, 1979). The product migrated as a single major band on isoelectric focusing gels and SDS polyacrylamide gels. It was active in cleaving the NH2-terminal octapeptide from purified beta-NGF.

The first thirty amino acids of beta-NGF endopeptidase were determined. We report that the beta-NGF-endopeptidase sequence is identical to the sequence of EGF binding protein type A reported by Anundi et al (Eur. J. Biochem. 129:365, 1982) and coded for by the gene designated mGK-22 (Drinkwater et al, Biochemistry, in press). We conclude that a single protein may exhibit both EGF-binding and beta-NGF-endopeptidase activities. This raises the issue of whether the EGF binding protein type A is actually the physiologically significant binding protein in the EGF complex.

X 107 SYNTHETIC PEPTIDES EXPRESSING THE PLASMIN-BINDING ACTIVITY OF α_2-ANTIPLASMIN. Glen L. Hortin, Douglas M. Tollefsen, and Kam F. Fok, Washington University School of Medicine, St. Louis, MO 63110 and Monsanto Corporation, Chesterfield MO 63017

α_2-Antiplasmin (AP) serves as an important regulator of fibrinolysis by acting as the major physiological inhibitor of plasmin. Inhibition of plasmin by AP occurs as a two-step reaction. First, there is reversible, second-order association of plasmin and α_2-antiplasmin in which lysine-binding sites of plasmin interact with a complementary site of AP. Then, the active site of plasmin complexes covalently with the reactive site of AP. A segment of 26 amino acid residues in AP which mediates the initial interaction between plasmin and AP has been identified by Sasaki and coworkers (J. Biochem. 99, 1699-1705). This segment of AP bears a distinctive post-translational modification, sulfation of its single tyrosine residue. We have prepared synthetic peptides corresponding to all or part of the 26-residue segment of AP to examine the role of sulfate and specific amino acid residues in the binding of AP to plasmin. Affinity of peptides for plasmin was determined by their ability to inhibit the interaction of AP with plasmin. The synthetic 26-residue peptide with the following sequence (G-D-K-L-F-G-P-D-L-K-L-V-P-P-M-E-E-D-Y-P-Q-F-G-S-P-K) bound to plasmin with a K_d of 10 μM, slightly less than the reported value (5.5 μM) for the sulfate-containing peptide derived from AP. Thus, the sulfate group is not essential for binding to plasmin but may influence affinity. Peptides lacking 1 or 6 residues at the C-terminus were prepared and found to have detectable but much weaker affinity for plasmin. These peptides had to be added at more than 12-fold higher concentrations to produce the same effect as the 26-residue peptide. These results indicate that the C-terminal lysine residue is a major site of interaction with plasmin, but additional sites distant from this lysine residue also contribute to the interaction.

X 108 MULTINUCLEAR NMR STUDIES ON SERINE PROTEASE TRANSITION STATE ANALOGUES. F. Jordan and F. Adebodun, Dept. of Chemistry, Rutgers University, Newark, NJ 07102.

^{31}P NMR studies were performed on mono- and diisopropylphosphorylchymotrypsin, trypsin and subtilisin. Novel results on both specied included: the pK_a of the active center triad (reflecting the ionization of His), and the mobility of the P atom and its distance from the metal site (occupied ordinarily by Ca^{2+}, replaced for these purposes by lanthanide ions) from relaxation data. Distances obtained by molecular modelling of the crystal structure using X-ray coordinates will be compared to those deduced from solution NMR data. ^{11}B NMR studies were performed on phenylboric acid, a transition state analogue, in the absence and presence of chymotrypsin and subtilisin. Addition of enzyme affected both chemical shift and linewidth, characterizing a system under fast-exchange conditions. The physical properties, including mobility, of enzyme-bound B atom and its hybridization state could be deduced. ^{11}B NMR is a novel method for monitoring macromolecular active sites.

Supported by the Rutgers Busch Fund and Research Council.

X 109 INSULIN-DEGRADING ENZYME IS THE METALLOENDOPROTEASE INVOLVED IN THE DIFFERENTIATION OF L_6 MYOBLASTS, Celik Kayalar and William T. Wong, University of California, Los Angeles, CA 90024.

The cultured myoblasts of the rat skeletal muscle cell line, L_6, proliferate until confluency, fuse to form myotubes and express a number of muscle-specific proteins. We have shown that this differentiation process is blocked by specific metalloendoprotease inhibitors (1). We now demonstrate that metabolizing L_6 myoblasts and their cell extracts degrade insulin to acid-soluble fragments mainly by a non-lysosomal pathway. About 90% of the insulin-degrading activity resides in the cytoplasm and is due to a 110 kd enzyme known as the insulin-degrading enzyme (IDE)(2).

The same metalloendoprotease inhibitors that block the differentiation of L_6 myoblasts also inhibit their insulin-degrading activity in a) metabolizing cells, b) cell extracts, c) immunoprecipitates of cytoplasmic extracts obtained by an anti-IDE monoclonal antibody.

These results suggest that the insulin-degrading enzyme in L_6 myoblasts is the metalloendoprotease whose activity is required for the initiation of the morphological and biochemical differentiation in this system.

We also show that the fluorogenic tetrapeptide, 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide (3) can be used as a synthetic substrate for the insulin-degrading enzyme of L_6 myoblasts.

1. Baldwin, E., and Kayalar, C. (1986) Proc. Natl. Acad. Sci. USA. 83, 8029-8033.
2. Duckworth, W.C., Heinemann, M.A., and Kitabchi, A.E. (1972) Proc. Natl. Acad. Sci. USA. 69, 3698-3702.
3. Kam, C-M., Nishino, N., and Powers, J.C. (1979) Biochemistry 18, 3032-3038.

X 110 MOLECULAR ANALYSIS OF THE CYLINDER TYPE 19S RNP WHICH IS HOMOLOGOUS TO THE MULTICATALYTIC PROTEINASE. P.Kloetzel,P.Falkenburg,Ch.Haass, B.Dahlmann*; University of Heidelberg, *University of Duesseldorf.

All eukaryotic cells so far analysed contain 19s particles which share a cylinder like shape and are composed of a set of proteins whose mw ranges from 19kD to 36kD. By electronmicroscopy, immunoblotting, RNA identification and enzyme assays we show that the rat MCP and the Drosophila 19s RNP are immunologically related RNPs with similar proteolytic activity. The RNP particles of both species contain small sized RNA and show common, evolutionary conserved epitopes. Enzyme assays demonstrate that the 19s RNP of Drosophila cleaves synthetic peptide substrates as well as 14C-methylcasein with similar specific activity as the rat MCP. In addition, the caseinolytic activity of the 19s RNP is inhibited by antisera raised against the Drosophila and the rat liver 19s particles. Using various fractionation procedures we have been able to identify several subpopulations of the 19s RNP and our data show that this HMW proteinase undergoes tissue and development specific alterations in its protein composition and its subpopulations. By screening an λgt11 cDNA expression library of Drosophila we have been able to isolate a cDNA clone encoding one of the subunits of the MCP. The molecular characterization and sequence will be presented. Since the 19s RNP is in part tightly associated with polyribosomes and undergoes development dependent alterations the MCP may be involved in development specific posttranslational processes.

X 111 IDENTIFICATION OF THE BONDS CLEAVED DURING THE ENZYMATIC INACTIVATION OF HUMAN ALPHA-1-ANTICHYMOTRYPSIN, Lawrence F. Kress, Marian Kruzel and Joseph J. Catanese, Roswell Park Memorial Institute, Buffalo, NY 14263.

Incubation of human α_1-antichymotrypsin (α_1-AC) with catalytic amounts of α-protease from Crotalus atrox (Western diamondback rattlesnake) venom resulted in inactivation of the inhibitor. Electrophoretic analysis showed that intact α_1-AC (64 kDa) had been converted by limited proteolysis to a 60 kDa inactive inhibitor. In samples treated with SDS/mercaptoethanol and heated to 100°C, both the native and the inactive forms of α_1-AC migrated as broad bands and were poorly resolved. The peptide resulting from the cleavage remained tightly bound to the inactivated inhibitor core, and was observed only in samples which had been treated with SDS/mercaptoethanol at 100°C. In samples treated with SDS/mercaptoethanol at room temperature, native α_1-AC migrated as expected (64 kDa), but the inactivated inhibitor migrated to a position corresponding to 50 kDa, and no peptide band was apparent. The 50 kDa band was also detected under these treatment conditions in stored samples of α_1-AC which had partially lost inhibitory activity.

The products from the inhibitor inactivation reaction were reduced and carboxymethylated and separated on Sephacryl S-200 in the presence of SDS. Amino acid sequence analyses indicated that the inactivating cleavage had occurred at Ala_{360}-Leu_{361} (the P_2-P_3 residues) in the reactive site loop of α_1-AC . A non-inactivating cleavage occurred at Asn_8-Leu_9.

X 112 STRUCTURE-FUNCTION RELATIONSHIP OF PIG CALPASTATIN, M. Maki, E. Takano, T. Murachi and M. Hatanaka, Institute for Virus Research and Department of Clinical Science, Faculty of Medicine, Kyoto University, Kyoto 606, Japan.
Structure-function relationship of pig calpastatin was investigated. Calpastatin is an endogenous inhibitor protein specifically acting on calpains (Ca^{2+}-dependent cysteine endopeptidases). We recently cloned and sequenced the cDNA for pig heart calpastatin, and determined the amino acid sequence of the molecule as deduced from its nucleotide sequence. Various deletion mutants of one of four internally repetitive domains (Domain 3, approximately 140 amino acid residues) were created by in vitro site-directed mutagenesis of a cloned cDNA fragment, and expressin in E. coli. Deletion of a conserved region in either amino-terminal or carboxy-terminal side caused a loss of inhibitory activity drastically against calpain I (low-Ca^{2+}-requiring form) and to a lesser degree against calpain II (high-Ca^{2+}-requiring form). No significant inhibitory activities were observed in mutants deleted further up to a central conserved region. Substitution of two amino acids in this region of wild type Domain 3 protein caused a drastic loss of activities against both calpains. The creation of lowered-affinity inhibitors enabled us to perform a conventinal kinetic analysis and showed a mode of competitive inhibition mechanism. These results suggested that the central region of each domain of calpastatin was an area for direct interaction either with the catalytic site of calpain or with its closest vicinity. Supported by grants from the Ministry of Education, Japan and from the Muscular Dystrophy Association, U.S.A. (to T.M.).

X 113 CHARACTERIZATION OF THE MUTUAL BINDING SITES OF HMW KININOGEN AND PLASMA KALLIKREIN, Werner MÜLLER-ESTERL, Hans HOCK, Dominic CHUNG, Dept. Clin. Biochem., University of Munich, Munich, F.R.G. and Dept. of Biochemistry, University of Washington, Seattle, WA 98195 U.S.A.

High-molecular-weight kininogen (H-K) circulates in plasma in form of binary complexes with (pre)kallikrein or factor XI($_a$). A single binding site is exposed by the H-K light chain which binds to the heavy chain portion of the coagulation factors. We have initiated the characterization of the mutual binding sites of H-K and prekallikrein. Using recombinant DNA techniques, a stretch of 39 amino acids was identified in H-K which represents the primary binding site(s) for prekallikrein. Synthetic peptides mimicking the binding region effectively compete with H-K for prekallikrein binding.

From a panel of mouse monoclonal antibodies against plasma kallikrein, a single antibody (PK-6) was found to protect plasma kallikrein from binding to H-K. PK-6 of subtype $IgG_1\kappa$ significantly prolongued the partial thromboplastin time (PTT) in a dose-dependent fashion when Fletcher plasma was supplemented with purified prekallikrein. By contrast, PK-6 did not interfere with the amidolytic activity of the enzyme in a chromogenic substrate assay. Hence, PK-6 is likely to recognize an epitope located at or next to the binding site for H-K on the kallikrein heavy chain. Preliminary evidence suggests that this epitope harbors disulfide loops and lysine residue(s) intimately involved in the complexing of the H-K light chain.

X 114 IS THE SLOW OBSERVED BINDING OF THE TRANSITION-STATE ANALOG INHIBITOR LEUPEPTIN TO CATHEPSIN B EVIDENCE FOR A CONFORMATIONAL CHANGE? R.M. Schultz, P. Varma-Nelson, K.A. Kozlowski, A.T. Orawski, P. Pagast, R. Ortiz and A. Frankfater, Dept. Biochem., Loyola Univ. of Chicago, Stritch Sch. of Medicine, Maywood, IL 60153.

The inhibition of cathepsin B by leupeptin shows a lag phase for approach to steady-state inhibition, which has been used as evidence for the existence of a slow inhibitor induced conformation change leading to tighter inhibitor affinity (hysteretic mechanism) [Baici and Gyger-Marazzi (1982) Eur. J. Biochem. 129, 33-41]. Peptide aldehydes such as leupeptin bind to cysteine proteases as transition-state analogs, and the conclusion of a hysteretic mechanism has been used to further argue that the leupeptin-cathepsin B interaction may be an example of a substrate induced-fit conformation change leading to transition-state stabilization. However, ^1H NMR shows that leupeptin exists in 3 different covalent forms in equilibrium in aqueous solutions, the active form of the inhibitor, I_A, represents only 2% of the total leupeptin in solution. In addition, conversion of the hydrate form to I_A is fast relative to the rate of inhibition. Based on the solution concentration of I_A, the rate constant for I_A association to cathepsin B is 1.2×10^7 $M^{-1}s^{-1}$, approaching diffusion rate control. Other examples of the slow binding of transition-state analog inhibitors may also be over-interpreted, the observed slow on rates simply due to the low concentrations of the active form of the inhibitor. (Supported by NIH grant CA 44659.)

X 115 BIOCHEMICAL CHARACTERIZATION AND POSSIBLE FUNCTIONS OF HUMAN AND MOUSE T CELL SPECIFIC SERINE PROTEINASES, Markus M. Simon, Hans-Georg Simon, Michael D. Kramer and Uli Fruth, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-7800 Freiburg, FRG

A T cell specific serine proteinase has been highly purified from both a mouse and a human $CD8^+$ T lymphocyte clone (TLC). The mouse enzyme, termed MTSP-1, cleaves the model peptide substrate Pro-Phe-Arg nitroanilide (NA) with high efficiency at pH 8 to 8.5. For the human enzyme, termed human TSP, the optimal peptide substrate is Gly-Pro-Arg-NA with an pH optimum of 10.5-11. MTSP-1 is a disulfide linked homodimer with a molecular weight of $\sim$ 60 KD. HuTSP is a disulfide linked dimer with a molecular weight of $\sim$ 50KD. Both enzymes are not expressed in resting T cells but can be induced in both $CD4^+$ and $CD8^+$ lymphocytes by antigen/lectin in vitro. Moreover, MTSP-1 and HuTSP were detected in a number of independent murine or human $CD4^+$ and $CD8^+$ TLC, respectively, and were found to be released from effector cells upon ligand binding to the CD3/T cell receptor complex. Subcellular fractionation of mouse $CD8^+$ TLC revealed that MTSP-1 is exclusively associated with cytoplasmic granules. In contrast, HuTSP activity seems to be localized in the nuclear and perinuclear areas of $CD8^+$ human TLC which have rarely detectable cytoplasmic granules. Functional studies performed in the mouse system, using purified MTSP-1, a tailor made inhibitor for MTSP-1 and a MTSP-1 specific rabbit antiserum suggest that the enzyme participate in cytolysis possibly by processing structures involved in the lytic event. Furthermore, MTSP-1 was shown to induce B lymphocytes for DNA synthesis and to be able to degrade proteoglycan constituents of extracellular matrices and fibronectin known to be involved in adhesive and ligand-binding functions. The data suggest that MTSP-1 (HuTSP) is an important element involved in functions and regulatory processes mediated by T lymphocytes.

X 116 FLUORESCENT PROBES OF HYDROPHOBIC BINDING DOMAINS IN HUMAN NEUTROPHIL ELASTASE S. Tyagi, Q.L. Ying, S. Simon, S. Sinha, A. Janoff, and J. Cheronis, SUNY Stony Brook, New York 11794 and Cortech, Inc., Boulder, Colorado 80301

Human neutrophil elastase (HNE) is believed to bind to elastin via a combination of electrostatic and hydrophobic interactions. We have probed the hydrophobic binding domains of HNE by employing a number of polarity-sensitive covalent modifications and noncovalent probes. Pyrene glyoxal (PYG) is an arginine-specific reagent which inhibits HNE. We have isolated a PYG-HNE complex and have determined its emission spectrum with 340 nm excitation. The emission maxima of PYG were shifted about 5 nm to shorter wavelengths and the ratio of the intensities around 378 nm and 395 nm was decreased in PYG-HNE compared to free reagent. These changes are both consistent with a more nonpolar environment of the pyrene ring in HNE-PYG. Oleic acid has been reported to be a noncovalent inhibitor of HNE. Upon addition of oleate to PYG-HNE, the emission maxima are further shifted to the blue, and the ratio of the intensities around 378 nm and 395 nm is further decreased, indicating that the polarity of the environment of the pyrene ring system has been further decreased by the fatty acid. We have also probed the hydrophobic binding domains of HNE with the noncovalent fluorescent probe, 8-oleyloxypyrene-1,3,6-trisulfonate (HPTS-oleate). The relative intensities of monomer and eximer emission bands of HPTS-oleate as well as Scatchard binding analysis based on emission intensity changes are consistent with binding of two molecules of HPTS-oleate per molecule of HNE. The endogenous fluorescence of HNE arising from tryptophan side chains is quenched in the presence of HPTS-oleate, suggesting that tryptophan may contribute to the hydrophobic binding domains.

X 117 PROTEASES ASSOCIATED WITH THE RAT LIVER NUCLEAR SCAFFOLD. Zoltan A. Tokes, Anthony K. Kong and Gary A. Clawson*, Dept. Biochemistry, Cancer Center, University of Southern Calif., Los Angeles, Dept. Pathology, The George Washington University Med. Center Washington, D.C.*

The inner portion of nuclear envelop is supported by a peripheral structural meshwork consisting predominantly of lamins A, B, and C. These polypeptides are present in a highly polymerized and essentially insoluble state during interphase of cell cycle. Lamins are major constituents of nuclear scaffold, NS. Another potentially significant functional component of NS is the 46 kD nucleoside triphosphatase, NTPase, which is thought to participate in nucleo-cytoplasmic transport of mRNA. Recently, (Clawson et al., Exp. Cell Res., 1988, in press) labeled the 46 kD component of NS with azido-ATP and purified it by SDS-polyacrylamide gel electrophoresis. After trypsin digestion, peptides were purified and microsequenced. Their sequences appeared to derive from the N-terminal region of lamins A/C, suggesting that the 46 kD azido-ATP binding molecules are derived from lamins A/C by proteolytic processing. Consequently, a search for NS associated proteases was initiated. Using sulfanilamide-azocasein assay, 100 ug of highly purified NS preparation had 85-135 ng trypsin-equivalent activity, and this activity was not changed with additional washings of NS. SDS-substrate gel electrophoresis, using gelatin in the polyacrylamide gels, revealed two types of proteases which were either Ca^{++} dependent or independent. Short term auto-digestion of NS transiently increased the NTPase activity, coupled with a concurrent and preferential decrease in lamins. These observations are consistent with an internal proteolytic processing of NS constituents to enhance NTPase activity.

X 118 ISOLATION OF RECOMBINANT HUMAN HEPARIN COFACTOR II AND REACTIVE SITE VARIANTS, Morey A. Blinder, Jayne C. Marasa, and Douglas M. Tollefsen, Washington University, St. Louis, MO 63110.

Heparin cofactor II (HCII) is a protease inhibitor of the "serpin" superfamily which inhibits thrombin by forming a stable, equimolar complex. Formation of the complex is accelerated ~1000-fold by dermatan sulfate or heparin. The reactive site sequence of HCII has been identified as Pro(P2)-Leu(P1)-Ser(P1'). A full-length cDNA for HCII has been isolated in lambda gt11, and a partial cDNA has been subcloned into the plasmid pTG-920 and expressed in *E. coli*. A protein of Mr 66,000 with thrombin inhibitory activity has been identified and purified to homogeneity from cell lysates. Immunoblot analysis identified the protein as HCII. Purified recombinant HCII inhibited hydrolysis of a synthetic peptide substrate by thrombin in a heparin and dermatan sulfate dependent manner and formed a stable complex of Mr 92,000 with ^{125}I-thrombin on SDS-PAGE. Single nucleotide substitutions of the HCII cDNA were constructed to yield reactive site variants with the sequences Pro-Arg-Ser and Pro-Met-Ser in which the P1 and P1' residues were identical to those in antithrombin III and α-1-antitrypsin, respectively. Recombinant HCII (Leu→Arg), but not HCII (Leu→Met), inhibited thrombin in a manner similar to that of native recombinant HCII. However, the reactive site variants did not rapidly inhibit coagulation factor Xa or pancreatic elastase, which are target proteases for antithrombin III and α-1-antitrypsin. These results imply that the protease specificity of HCII is not defined exclusively by the P1 and P1' residues of the reactive site.

Molecular Biology; Processing Mechanisms

X 200 IDENTIFICATION OF TWO PROTEASES POTENTIALLY INVOLVED IN IL-1β PROCESSING. Roy A. Black, Shirley R. Kronheim, Carl J. March, and Thomas P. Hopp. Immunex Corporation, 51 University Street, Seattle, Washington 98101 USA.

Interleukin 1-β (IL-1β) is a 17.4 kD polypeptide hormone that is involved in a wide range of immunoregulatory and inflammatory responses. It is synthesized as part of a 33 kD inactive precursor, from which the active "mature" form is derived by proteolysis. A number of known proteases were tested but do not generate this fragment. We have purified potential IL-1β processing enzymes from extracts of the human myeloid cell line, KG-1, by following their ability to process recombinant precursor IL-1β. A cathepsin L-like protease of KG-1 cells produces the 17.4 kD mature product while cathepsin G or a related protease produces a slightly larger fragment. Both cathepsin L and cathepsin G are externalized in stimulated leukocyte cultures, and precursor IL-1β appears to be processed extracellularly. Addition of inhibitors of cathepsin L and cathepsin G to leukocyte cultures failed to reduce IL-1β production, however, suggesting that these cultures may produce additional proteases capable of IL-1β processing.

X 201 CLONING AND CHARACTERIZATION OF TWO CYTOTOXIC T LYMPHOCYTE SERINE PROTEASE GENES, R. Chris Bleackley, Corrinne G. Lobe, Chris Upton, Chantal Fregeau, Brenda Duggan, Nancy Ehrman, Michael Meier, Jennifer Shaw, Grant McFadden and Verner H. Paetkau, University of Alberta, Edmonton, Alberta, Canada. T6G 2H7

A number of cDNA's specifically expressed in cytotoxic T cells were isolated by differential hybridization (1). Sequence analysis of two of these revealed that they encoded serine proteases, thus suggesting a cascade mechanism operating in T cell mediated lysis (2). Further analysis has revealed that the expression of both of these proteases correlates with the increase in cytotoxicity upon T cell stimulation, and that the proteins are sequestered within cytoplasmic granules (3). Therefore we believe that they play a key role in the cytolitic mechanism.

Genomic clones corresponding to both genes have now been isolated and sequenced. The intron/exon organization revealed that the catalytic triad residues were encoded by separate exons, however the positions of two of the introns was most unusual. Analysis of nuclease sensitivity indicates the presence of two hypersensitive sites in the 5' flanking regions of each gene. The sequences surrounding these potential regulatory sites have now been determined and compared.

1) Lobe, C.G., Havele, C. and Bleackley, R.C. Proc. Natl. Acad. Sci. U.S.A. 83:1448-1452 (1986). 2) Lobe, C.G., Finlay, B., Paranchych, W., Paetkau, V.H. and Bleackley, R.C. Science 232:858-861 (1986). 3) Redmond, M.J., Letellier, M., Parker, J.M.R., Lobe, C.G., Havele, C., Paetkau, V. and Bleackley, R.C. J. Immunol. (in press).

X 202 **SIGNAL PEPTIDE PROCESSING AND YEAST CELL GROWTH REQUIRE THE *SEC11* GENE PRODUCT,** Peter C. Böhni and Randy W. Schekman, Department of Biochemistry, University of California, Berkeley, Ca. 94720.

Most secretory proteins initially are synthesized with an amino terminal extension, the signal peptide. Translocation of these precursor polypeptides into the lumen of the endoplasmic reticulum (ER) is accompanied by proteolytic removal of the signal peptide and possible addition of core oligosaccharides. Inefficient signal peptide processing of several glycoprotein precursors results in accumulation of these preproteins in the ER lumen. Hence, a lesion in signal peptidase function is expected to cause a delay in the secretion of proteins that contain cleaved signal sequences. Among the pleiotropic secretion defective mutants of *Saccharomyces cerevisiae*, *sec11* fulfills this prediction. At 37°C, the restrictive growth temperature, *sec11* cells accumulate core glycosylated (thus, completely translocated) forms of invertase and acid phosphatase, each retaining an intact signal peptide. In contrast, other *sec* mutants in which protein transport from the ER to the Golgi apparatus is blocked show no defect in signal peptide cleavage.

The *SEC11* gene has been cloned from a YEp13 based genomic DNA library by complementation of the growth defect of *sec11* cells at 37°C. Northern blot analysis and DNA sequencing identified an 850 base transcript that potentially encodes a 167 amino acid polypeptide carrying an amino terminal, hydrophobic stretch reminiscent of a signal peptide. The 18.8 kDa protein contains one consensus glycosylation site and has an estimated pI~9.5. Disruption of the *SEC11* gene by one step gene displacement in a diploid yielded inviable haploid null mutant spores.

SEC11 may encode a subunit of the signal peptidase that is required for precursor processing but not for penetration of precursors into the ER lumen.

X 203 YEAST KEX2-LIKE PROTEASE FOUND IN HEPATIC SECRETORY VESICLES CONVERTS PROALBUMIN TO ALBUMIN, Stephen O. Brennan and Robert J. Peach, Pathology Department, Christchurch School of Medicine, Christchurch Hospital, Christchurch, New Zealand

The yeast KEX2 protease is the only enzyme that has a proven role in the activation of polypeptide hormones through cleavage at pairs of basic residues. The enzyme that fulfils this role in higher eukaryotes has yet to be unequivocally identified. In this investigation, a KEX2-like calcium-dependent protease has been identified in rat hepatic Golgi and secretory vesicles. The enzyme is membrane bound, has a pH optimum of 5-6 and converts proalbumin to albumin. More importantly, like the KEX2 protease, it meets two other exacting criteria defined by specific mutations in humans. Namely, it does not process proalbumin Christchurch (-1 Arg→Gln) which lacks one of the requisite basic residues and, whilst not itself a serine protease, it is inhibited by the reactive center variant, α_1-antitrypsin Pittsburgh (358 Met→Arg).

X 204 MEMBRANE PROTEASES INVOLVED IN ANTIGEN PROCESSING. Benjamin M. Chain and Marie-Anne Shaw. Dept. of Biology, University College, London WC1 6BT.

An essential feature in the activation of T helper cells is the involvement of the antigen presenting cell (APC). In order to be recognised by a T cell, antigen must be taken up by an APC, the expressed on the cell surface in conjunction with Class II MHC molecules. In the case of protein antigens, the APC must also process the antigen, and this is believed to involve some limited form of proteolysis. On the basis of earlier studies, we have suggested that processing may involve proteases at the cell surface. We have now identified a number of classes of membrane associated proteases in enriched plasma membrane preparations from a murine antigen presenting B cell lymphoma. Preliminary characterisation of the enzymes will be presented, together with functional data indicating that at least one of these enzymes is capable of processing ovalbumin in a cell free system to an immunologically active fragment.

X 205 GLYCOSYLATION AND PROCESSING OF HUMAN INTER-α-TRYPSIN INHIBITOR RELATED POLYPEPTIDES IN SACCHAROMYCES CEREVISIAE, Rathin C. Das and Donna J. Lehman, Biotechnology Products Division, Miles Inc., Elkhart, IN. 46515

Inter-α-trypsin inhibitor (ITI) is a serine protease inhibitor of molecular weight about 180 kd, and is found in human plasma and serum. ITI is proteolytically processed to generate a lower molecular weight species of 30 kd. This lower molecular weight form is also known as HI-30. Both ITI and HI-30 reportedly inhibit the proteolytic enzymes trypsin, chymotrypsin and neutrophil elastase.

Recently, the isolation of an mRNA-derived cDNA clone of HI-30 from human liver has been reported. Interestingly, this cDNA encodes for not only HI-30 but also for another serum protein, α-1-microglobulin (protein HC), which is found fused at the amino terminus of HI-30. The two protein sequences are separated by two arginine residues.

In order to understand the mechanism of HI-30 processing in eucaryotes, the isolated human liver cDNA was cloned in a yeast 2μ-based replicative vector. Either the cDNA which codes for the fused (protein HC:HI-30) protein, or a fragment of it which essentially codes for the HI-30 fragment, was fused to prepro-α-factor sequence under the control of the α-factor promoter. Northern blot analysis of total yeast RNA revealed HI-30 gene-specific transcripts in the transformants. Furthermore, both gene fusions expressed functional trypsin activity of which a significant amount is secreted into the growth medium. Analysis of the yeast secreted material using ITI antibody revealed proteolytic processing of HI-30 from α-1-microglobulin. Evidence was also obtained that such processed form of HI-30 is indeed glycosylated.

X 206 CHARACTERIZATION OF THE NEUROPEPTIDE PROCESSING ENZYME. Lakshmi Devi, New York University Medical Center, New York, NY 10016.

Many bioactive peptides and hormones are synthesised from large precursors in which pairs of basic amino acids are interposed between these peptide sequences. The production of the biologically active peptides occur by sequential trypsin-like endopeptidase and carboxypeptidase B-like exopeptidase activities. The study of substrate specificity of the endopeptidase has been complicated by the difficulty involoved in the purification of the enzyme and the substrates. To circumvent this problem, we have introduced a foreign cDNA, encoding prodynorphin, into a mouse anterior pituitary cell line, AtT-20_{D16V}. These cells post-translationally process endogenous proopiomelanocortin to mature ACTH by cleavages at 'Lys-Arg' residues. I transformed the AtT-20 cells with a recombinant plasmid containing mouse metallothionein promoter and rat prodynorphin cDNA. Stable transformants were analysed for the presence of prodynorphin precursor. Chromatography of the cell extract from the stably transformed cell line, AtT-20/ZRD 19 on Sephadex G-75 revealed the presence of low molecular weight [Leu]enkephalin containing peptides as well as larger peptides. Analysis of the low molecular weight peptides on reverse phase HPLC showed the presence of dynorphin A, dynorphin B and B-neo-endorphin, all products of cleavages at 'Lys-Arg' residues.

These results suggest that the processing enzyme that post-translationaal processed the endogenous proopiomelanocortin to mature ACTH is capable of processing prodynorphin to mature dynorphins. Thus, the enzymes involoved in the post-translational processing of peptide hormones and neuropeptides are not specific for a particular peptide precursor.

X 207 MOLECULAR MECHANISMS OF RESISTANCE IN BARLEY AGAINST POWDERY MILDEWS, Jan B. Andersen, Per L. Gregersen, Hans Thordal-Christensen and Viggo Smedegaard-Petersen, Dept. Plant Pathology, Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark.

Investigations of induced resistance in barley against powdery mildews have provided a good insight into the dynamics of the resistance mechanisms during the first hours after inoculation with fungal pathogens (1,2,3). These investigations have provided a model system for further studies of the molecular mechanisms underlying the resistance. These studies are performed by constructing cDNA libraries of host lines expressing resistance and host lines not expressing resistance. These libraries are differentially screened in order to isolate clones that differ between libraries. Different combinations of host lines and pathogenic and non-pathogenic fungal races are also taken into consideration in order to distinguish between resistance induced by pre-treatment with another fungus and resistance governed by powdery mildew resistance genes. Differences in gene expression will be related to their significance in the expression of resistance providing additional information on the dynamics of resistance reactions in barley plants.

1. Cho, BH & V Smedegaard-Petersen (1986): Phytopathol. 76: 301-305.
2. Thordal-Christensen, H & V Smedegaard-Petersen. Plant Pathol. in press.
3. Thordal-Christensen, H & V Smedegaard-Petersen. J. Phytopathol. in press.

X 208 IDENTIFICATION OF HTLV-I GAG PROTEASE AND ITS SEQUENTIAL PROCESSING OF THE GAG GENE PRODUCT.

Masakazu Hatanaka and Seok Hyun Nam,
Institute for Virus Research, Kyoto University, Kyoto 606 JAPAN

We constructed a full length provirus of HTLV-I and studied its infectivity and replication competence (K. Mori et al., J. Gen. Virol. 68, 499-506, 1987). The cells transfected with the full provirus DNA, produced the gag gene product of HTLV-I that are processed to the ordinary forms of p19,p24 and p15. We determined the nucleotide sequence of a region between gag and pol genes of this provirus and found an open reading frame encoding a sequence of 234 amino acids and identified as a HTLV-I protease gene (S.H.Nam and M. Hatanaka, BBRC, 136, 129-135, 1986). By constructing variousely modified gag genes, we examined the order of sequential processing of the gag gene product by the protease. We also studied a catalytic site of the protease by site-directed mutagenesis.

X 209 IDENTIFICATION OF PREPROTACHYKININ SPECIFIC PROCESSING ENZYMES.

V.Y.H. Hook, T. Krieger and H.-U. Affolter; Dept. of Biochemistry, USUHS, Bethesda, MD and Brain Res. Inst., Univ. of Zurich, Switzerland. The neuropeptides substance P, neurokinin A and neuropeptide K are derived from a large precursor, that must be specifically cleaved to form the bioactive peptides. In contrast to bovine, human and rat tissues show no tissue specific splicing of the tachykinin gene product. Therefore the mechanism of tissue specific production of tachykinin peptides must occur at the level of precursor processing. To identify the endopeptidase(s) that cleave this precursor, authentic 35S-(Met) beta-preprotachykinin was synthesized by in vitro translation, using a human cDNA derived mRNA (SP6 in vitro transcription system). Processing enzyme activity was found in adrenal medulla granules, which have been shown to contain substance P. Sequential cleavage of substrate followed by processing of resultant intermediate products seems to require distinct activities. Substrate cleavage was abolished by pepstatin, whereas further processing of intermediate peptide was inhibited by chymostatin. Additionally, biochemical characterization of these processing activities revealed, that multiple preprotachykinin specific processing enzymes are present in these granules.

X 210 THE DEGRADATION OF THREE CHIMERIC POLYPEPTIDES IN *ESCHERICHIA COLI* IS ASSOCIATED WITH THEIR SECRETION,

Ibrahim Ibrahimi, Yvonne Kuys and Reiner Gentz, Eur. Mol. Biol. Lab., Heidelberg, F.R. Germany and F. Hoffmann, La Roche and Co., CH-4002 Basel, Switzerland.

We investigated the stability of fusion proteins composed of the signal peptide (SP) of the heat labile enterotoxin of *E. coli* (EltB) and each of chloramphenicol acetyltransferase (CAT), dihydrofolate reductase (DHFR) and gamma interferon (IFNγ). The targeting of these chimeric proteins to the secretion apparatus in *E. coli* lead to their degradation by membrane associated proteases. Four lines of experimental evidence are presented in support of this suggestion. First, the chimeric polypeptides having a functional signal peptide were not detected in the precursor or processed forms *in vivo*. when a mutation was introduced in the signal peptide resulting in loss of recognition by the secretion apparatus, the chimeric proteins accumulated at high levels in the cytoplasm of the cell. Second, both the wild type and mutant polypeptides accumulated in a purified and reconstituted *in vitro* translation system from *E. coli* and were equally susceptable to digestion by an exogenous protease. Third, the chimeric polypeptides lacking the signal peptide accumulated in a stable form *in vivo*. Fourth chimeras whose synthesis and secretion were initiated at the permissive temperature and completed at the restrictive one in a SecA mutant of *E. coli*, accumulated in the unprocessed form suggesting that degradation is tightly linked to the secretion apparatus.

X 211 Molecular cloning and expression in heterologous Cos cells of rat and human enkephalinase, B. Malfroy, C. Gorman, P. Schofield, P. Seeburg, W.J. Kuang, H. Kado-Fong, D. Gies, and T. Mason, Genentech, Inc., So. San Francisco, CA 94080.
cDNA clones encoding rat and human enkephalinase (neutral endopeptidase, EC 3.4.24.11) have been isolated in λgt10 libraries from rat brain and kidney, and from human placenta, respectively. The rat and human enzymes are 95% homologous. Enkephalinase is a 742 amino acid protein, which possesses a single transmembrane spanning domain near the N. terminal of the molecule, but lacks a signal sequence. Because enkephalinase has its active site located extracellularly and is thus an ectopeptidase, we suggest that the N-terminal transmembrane region of the enzyme anchors the protein in membranes and that the majority of the protein, including the carboxy terminus, is extracellular. When transfected with an expression plasmid containing the rat or human enkephalinase cDNA, Cos 7 cells expressed recombinant enkephalinase which was enzymatically active and displayed properties similar to those of the native enzyme.

X 212 SECRETION OF MUTANT ALPHA-1-PROTEINASE INHIBITORS, *A.A. McCracken, *K.B. Kruse, and J.L. Brown, *University of Nevada-Reno, 89557
University of Colorado Health Sciences Center, Denver, 80262
The decreased A1PI serum level of individuals homozygous for the A1PI/Z variant, is due to a defect in secretion. The A1PI/Z gene exhibits two point mutations resulting in a Val213 to Ala (Crystal et.al., 1986) and a Glu342 to Lys (Jepsson, 1976). We have studied the secretion of genetically altered and normal A1PI synthesized in COS cells transfected with cloned human A1PI genes. This model system correctly duplicates the secretion defect seen in individuals homozygous for the A1PI/Z allele. The absence of the Val213 to Ala substitution does not alter the secretion of A1PI/M or A1PI/Z, indicating that this is a silent mutation. Crystallographic studies (Loebermann et.al., 1984), show a salt bridge between Glu342 and Lys290. The disruption of this salt bridge by the "Z" mutation may be the reason for the secretion defect. We constructed three mutants to test this hypothesis. Removal of the salt bridge by altering the Lys290 to Glu in A1PI/M reduced secretion to 73% of normal. Replacing the salt bridge by altering the Lys290 to Glu in A1PI/Z reduced secretion to 43% of normal. Loss of the salt bridge in A1PI/Z due to the Glu342 to Lys substitution resulted in 15% of normal secretion. These data indicate that the salt bridge is required for normal secretion, but that the charged residue at position 342 is the main factor in the A1PI/Z secretion defect.

X 213 STRUCTURE AND DEVELOPMENTAL EXPRESSION OF THE ADIPSIN GENE: EVIDENCE FOR TWO DISTINCT UPSTREAM REGULATORY ELEMENTS, Hye Yeong Min and Bruce M. Spiegelman, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115.
Adipsin is a novel serine protease whose expression is linked to adipocyte differentiation. In addition, adipsin levels have been shown to be extremely low in genetically obese mice. We have isolated, mapped and sequenced the mouse adipsin gene and have demonstrated developmental regulation of the gene by 5' flanking sequences. The gene spans 1.7 kb and contains five exons. While the basic exon structure characteristic of serine protease genes is conserved in adipsin, there is a fusion of two exons that are separate in pancreatic serine protease genes. Utilizing a viral enhancer, we have demonstrated that adipocyte-dependent expression is conferred to a transfected bacterial chloramphenical transferase (CAT) gene by the presence of as little as 400 bp of adipsin upstream sequence. Deletions of the 5' end of the adipsin promoter sequence reveal that there is a negative regulatory element, between -300 and -400 relative to the transcription start site, that functions to reduce expression of the adipsin gene in preadipocytes. Further deletion shows the presence of a tissue-specific promoter element between -100 and -38; particularly notable within those sequences is a dyad symmetry spanning 44 nucleotides. Deletion of this sequence results in diminished CAT expression in transfected adipocytes. These results suggest that at least 2 distinct regulatory elements function in the 5' flanking region of the adipsin gene to regulate its expression. Experiments are in progress to elucidate the nature of the nuclear proteins from preadipocytes and adipocytes that interact with these 2 regulatory elements to control developmental regulation of the adipsin gene.

X 214 **A β-AMYLOID cDNA CLONE CONTAINS A DOMAIN HOMOLOGOUS TO SERINE PROTEINASE INHIBITORS**

P. Ponte, J. Schilling, S. Hilliker, P. Gonzalez-DeWhitt, D. Hsu, F. Fuller, and B. Cordell, California Biotechnology, Inc., 2450 Bayshore Parkway, Mt. View, CA 94043

β-amyloid is the major protein component of the neuritic plaques and cerebrovascular amyloid characteristic of the brains of people with Alzheimer's disease. This protein has been isolated as an approximately 4500 Dalton species which exhibits a ragged amino terminus upon amino acid sequencing [1-3]. Previously, a human brain cDNA which encodes β-amyloid within the structure of a larger protein (called here β-amyloid-D) has been described[4]. Here we present another β-amyloid cDNA, β-amyloid-I, which is distinguished from β-amyloid-D by the presence of a 168 nucleotide insert. The 57 amino acid peptide predicted from the insert has extensive homology with several well-characterized serine proteinase inhibitors. Using synthetic oligonucleotides, the expression of these two transcriptional forms of β-amyloid has been examined in RNA preparations from a variety of samples. In an initial survey, β-amyloid-I mRNA and β-amyloid-D mRNA appear to be differentially regulated.

In order to address questions regarding the capacity of this insert to function as a proteinase inhibitor, we are attempting to produce the insert peptide in both bacterial and yeast expression systems.

1. Glenner, Biochem. Biophys. Res. Commun. 120: 885 (1984), 2. Glenner, Biochem. Biophys. Res. Commun. 122: 1131 (1984), 3. Masters, Proc. Natl. Acad. Sci. USA 82: 4245 (1985), 4. Kang, Nature 325: 733 (1987).

X 215 **CLONING AND SEQUENCING OF A SEGMENT OF THE HUMAN GENE FOR PLASMINOGEN ACTIVATOR INHIBITOR TYPE-1 (PAI-1),**

Alnawaz Rehemtulla, David I. Hoar and David A. Hart, University of Calgary HSC, Calgary, Alberta, Canada T2N 4N1.

Regulation of fibrinolysis is essential in maintaining hemostasis and in processes such as inflammation, tissue remodelling and neoplasia. Plasminogen activator inhibitors (PAI) play a prominent role in achieving control of fibrinolytic enzymes. PAI type 1 (PAI-1) has been shown to be produced by endothelial cells and platelets and may be most important in maintaining hemostasis of the fibrinolytic system in the vasculature. In order to study regulation of gene expression of PAI-1 we have initiated a study of genomic DNA for this inhibitor using a library constructed by inserting 35-40 Kb fragments of human DNA into a Cosmid-shuttle vector. The library was divided into 12 pools, each of which were screened using an oligonucleotide (21 bp) synthesized from the published cDNA sequence. Pool 5 was found to contain a 7 Kb EcoRI fragment that hybridized with the probe by Southern blotting. The DNA from pool 5 was transfected into an E. coli host and 10,000 colonies were screened using the probe. Four strongly hybridizing colonies were isolated and all were shown to contain cosmids with a 7 Kb ECoRI fragment that was recognized by the probe. Sequence analysis revealed an exon which matched perfectly with the PAI-1 cDNA sequence and the exon was flanked by introns with appropriate donor and receptor splice signals. Additional analysis of the gene is in progress to determine the domain structure of this molecule and its relationship to other related proteinase inhibitors. (Supported by the AHFMR and the Canadian Arthritis Society).

X 216 A UNIQUE STRUCTURAL DOMAIN OF A YEAST ASPARTYL PROTEASE DIRECTS PROTEIN SECRETION,

Susan K. Welch and Vivian L. MacKay, Zymogenetics, Inc. Seattle WA 98103.

The BAR1 gene of Saccharomyces cerevisiae codes for a secreted (exported) aspartyl protease, called Barrier activity, which functions during mating. The deduced primary translation product is a 587 aa protein with a putative signal peptide and 9 potential N-linked glycosylation sites. The protein is organized into 3 structural domains. The N-terminal domains are homologous to the 2 conserved domains typical of pepsin-like proteases, and mutation of an ASP predicted to be at the active site destroys Barrier activity (1). The Barrier protease differs from other pepsins in having an additional C-terminal domain of about 190 aa. This 3rd domain is rich in SER and THR and has 4 potential N-linked glycosylation sites. Examination of activity and cellular localization of a series of Barrier proteases from which segments of the 3rd domain have been deleted suggests that this domain may have a role in directing export of the protein. Deletion of 61 aa has little effect on export or activity of the protein. Deletion of 162 aa results in a protein which retains biological activity, but is detectable only in cell pellets. ELISA and Western blot data show an apparent acumulation of this truncated protein inside the yeast cell. Deletion of 198 aa results in an inactive Barrier protein detectable only in cell pellets. Although we have not strictly ruled out differences in protein stability of the truncated proteins, our data strongly suggest that the C-terminal domain of the Barrier protein is important in directing it through the export pathway. We have also demonstrated that a Barrier protein composed almost entirely of the 3rd domain (lacking 367 of 372 aa of the catalytic domains) is readily exported. The 3rd domain of the Barrier protein has also been used to direct the export of heterologous proteins to the culture medium.

Many enzymes exported from other fungi include a SER-THR-rich domain similar to the 3rd domain of the barrier protein, suggesting that such regions have a similar function in these proteins.

1. MacKay, V.L., Welch, S.K., Insley, M.Y., Manney, T.R., Holly, J., Saari, G.C., Parker, M.L., PNAS, in press.

Control Mechanisms

X 300 TUMOR CELLS METALLOPROTEINASES: LOCALIZATION WITHIN CELL STRUCTURE AND THEIR INHIBITION BY A RECOMBINANT TISSUE INHIBITOR OF METALLOPROTEINASE, Ofelia Alvarez and Yves DeClerck, Childrens Hospital of Los Angeles and University of Southern California, Los Angeles, CA 90027.

There has been many experimental evidences suggesting that collagenases produced by tumor cells play an important role in tumor invasion and metastasis.
In order to locate these enzymes, we analyzed their activity in tumor cell extracts and serum-free conditioned medium from several tumor cell lines including a human fibrosarcoma (HT1080), a human melanoma (Bowe), Lewis lung carcinoma, and c-Ha-ras transfected rat embryo cells. Collagenolytic activity for interstitial collagen was detected in the cytosol of all four cell lines while type IV collagenase was present in both cytosol and membrane compartments as well as in the conditioned medium. In addition, a significant degree of gelatinase activity was found secreted by all cell lines.
We investigated the inhibitory effect of a recombinant Tissue Inhibitor of Metalloproteinases (rTIMP) upon the collagenolysis and gelatinolysis of these cell extracts and conditioned medium. Whereas this inhibitor had minimal inhibitory activity (10-20%) on the proteolytic activity of HT 1080 and Bowe melanoma cell lines, it completely inhibited the proteolytic activity of the c-Ha-ras transfected cells. Furthermore, metalloproteinases resistant to rTIMP were identified in the HT 1080 and Bowe melanoma cells by gelatin substrate polyacrylamide gel electrophoresis.
Our data suggest that although rTIMP may inhibit the collagen degradation of some tumor cells, the collagenolytic activity of other cell lines may be in part resistant to such inhibitor.

X 301 FETAL ASTROCYTE AND GLIOMA CELL METALLOPROTEINASE ACTIVITY IS REGULATED BY ENDOGENOUS INHIBITORS, Gerard Apodaca, James T. Rutka, Karyn Dwyer, Jane R. Giblin, Mark L. Rosenblum, Michael J. Banda, and James H. McKerrow, University of California, San Francisco, CA 94143.

Fetal astrocytes and five glioma cell lines were found to secrete a number of metalloproteinases whose activities were regulated by inhibitors produced by the same cells. The conditioned medium (CM) of these cells did not degrade azocoll in suspension, but several proteolytic activities, inhibitable by 1,10 phenanthroline, were detected on SDS-polyacrylamide gels containing gelatin. Both cell types secreted three gelatinases (M_r 65,000, 60,000, and 58,000). Two of the glioma lines secreted an additional gelatinase (M_r 92,000). Treatment with 12-0-tetradecanoylphorbol-13-acetate (TPA) stimulated the secretion of the gelatinases and induced new activities in all of the cells. Anti-collagenase IgG precipitated M_r 52,000 and 47,000 proteinases in the CM of TPA-treated glioma lines. When the CM of one of the glioma cell lines was concentrated and fractionated on a preparative gel, inhibitory activity was separated from a proteinase of M_r 65,000 with type IV collagenolytic activity. Tissue inhibitor of metalloproteinases (TIMP) was detected in the CM of all of the cells by substrate gel analysis and immunoprecipitation of [^{35}S]-methionine labeled proteins with anti-TIMP IgG. The glioma lines also secreted various amounts of smaller inhibitors of metalloproteinases (IMPs) (IMP-1 at M_r 22,000, IMP-2 at M_r 19,000, and IMP-3 at M_r 16,500). TPA stimulated the secretion of TIMP in all of the cells and induced IMPs in some of the glioma lines. When gel filtration chromatography of concentrated CM was used to separate inhibitors from proteinases, the isolated proteinases had activity against the glycoprotein and collagen components of an *in vitro* model of the extracellular matrix. The secretion of metalloproteinases by astrocytes may be important in facilitating astrocytic migration during development and in pathologic conditions such as inflammation or invasion of astrocytic neoplasms. These enzymes are probably regulated by various factors, including the cosecretion of endogenous inhibitors.

X 302 REGULATION OF PLASMINOGEN ACTIVATOR INHIBITOR EXPRESSION BY HUMAN K562 CELLS, Allan Arndt, Alnawaz Rehemtulla and David A. Hart, University of Calgary HSC, Calgary, Alberta, Canada T2N 4N1.

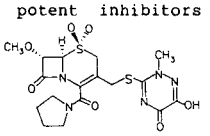
Plasminogen activators (PA's) are involved in the activation of plasminogen to yield the general protease plasmin. They have been shown to be involved in such diverse processes as inflammation, tissue remodelling and fibrinolysis. PA specific inhibitors (PAI) are essential in regulating PA activity in such processes. A human cell line (K562) was used as a model to study PA/PAI regulation. Zymographic analysis of conditioned media (CM) revealed the presence of a 52Kd fibrinolytic band that corresponded to urokinase (UK). Treatment of cells with Phorbol Myristate Acetate (PMA) induced a PAI detected by reverse zymography, with a M_r = 43,000. CM from PMA treated cells contained PAI which reacted preferentially with UK. The PAI was not adherent to a heparin-sepharose affinity column. Interleukin-1 (IL-1) alone had no effect on PA/PAI production but treatment with PMA followed by IL-1 markedly enhanced PAI production. PMA is known to induce the appearance of a macrophage phenotype in K562 cells. Therefore PMA may also induce IL-1 receptors, thereby sensitizing the cell population to the mediator. Interestingly, IL-1 alone has been shown to influence PAI regulation in endothelial cells in a similar fashion. This may indicate similar regulation of different subtypes of PAI depending on cell lineage. The PAI was purified to near homogeneity by adherence to ConA-sepharose followed by anion exchange and reverse phase chromatography with a Pharmacia FPLC system. Presently immunological and nucleic acid probes are being used to characterise the PAI at the molecular level. (Supported by the AHFMR and the Canad. Arth. Soc.).

X 303 Cleavage and inactivation of α_1-antitrypsin by metalloproteinases released from neutrophils Ian C. BATHURST*, Margret C.M. VISSERS, Peter M. GEORGE Stephen O. BRENNAN and Christine C. WINTERBOURN *Chiron Corporation, 4560 Horton Street, Emeryville, CA. 94608 Christchurch School of Medicine, Christchurch, New Zealand

Human neutrophils, when stimulated with phorbol myristate acetate or with fMet-Leu-Phe in the presence or absence of cytochalasin B. released metalloproteinases which catalytically inactivated the plasma serine proteinase inhibitor, α_1-antitrypsin. Inactivation, measured as loss of elastase inhibitory capacity, was accompanied by cleavage of a Mr 4,000 peptide from the C-terminus. Cleavage of a α_1-antrypsin by cell supernatants was inhibited by EDTA, o-phenanthroline and dithiothreitol, but not by inhibitors of serine or thiol proteinases. Gelatinase and collagenase were separated from the medium of stimulated neutrophils. Both preparations cleaved and inactivated α_1-antitrypsin, cleavage occurring close to the reactive centre, at the Phe-Leu bond between positions P_7 and P_6. Cleavage by purified gelatinase was very slow and could account for only a minor fraction of the activity of neutrophil supernatants. The collagenase preparation was more active. However, the unusual cleavage site, and the ability of fMet-Leu-Phe-stimulated neutrophils to cleave α_1-antitrypsin without releasing collagenase, suggests that collagenase is not responsible for cleavage by the cells. Our results demonstrate that by releasing metalloproteinases, neutrophils could proteolytically inactivate α_1-antitrypsin at sites of inflammation. This provides an alternative to the previously documented mechanism of inactivation of neutrophil-derived oxidants.

X 304 INHIBITION OF LUNG CONNECTIVE TISSUE DAMAGE IN HAMSTERS BY A B-LACTAM INHIBITOR OF NEUTROPHIL ELASTASE by R. J. Bonney, P. Dellea, K. Hand, D. Osinga, D. Fletcher, A. Maycock, P. Finke, W. Hagmann and J. Doherty, Merck Sharp & Dohme Research Laboratories, Rahway, NJ 07065

Cephalosporins with substituents in the 7 position and an ester linkage in the 4 position have been shown to be potent inhibitors of human leukocyte elastase (HLE) (Nature 322:194, 1986). L-659,286



a prototype of this class is a selective inhibitor of HLE with a Ki of 0.4 μM. This inhibition is time-dependent, rapid and reversible very slowly (t1/2 > 3 days). In hamsters, the intratracheal (i.t.) administration of HLE causes damage to the hamster lung vasculature as measured by leakage of red blood cells into the air spaces of the lung. This damage can be quantitated by the amount of hemoglobin in the bronchial alveolar lavage fluid. L-659,286 and ∝1PI administered i.t. 30 min prior to HLE inhibits lung damage with ED_{50}s of 20 μg and 1000 μg/animal respectively. In addition, L-659,286 will block lung damage caused by HLE even when administered 20 min after HLE whereas, ∝-1PI is inactive in this regimen.

X 305 INTERACTION BETWEEN NEUTROPHIL PROTEINASES AND HEPARIN COFACTOR II. Frank C. Church and Charlotte W. Pratt, Univ. of North Carolina, Chapel Hill, NC 27599.

Because neutrophil proteinases inactivate some proteinase inhibitors, the interaction of neutrophil elastase and cathepsin G with heparin cofactor II (HC), which is homologous to α_1-proteinase inhibitor and antithrombin III, was examined. The ability of HC, a heparin-dependent inhibitor of thrombin, to inhibit neutrophil proteinases was also investigated. HC inhibits cathepsin G with a rate constant of 10^7 M^{-1} min^{-1}, but it does not inhibit elastase. Catalytic amounts of either elastase or cathepsin G inactivate HC by proteolysis. The first proteolytic event converts native HC (M_r 94,000 on SDS PAGE) to a species (M_r 76,000) that retains full ability to inhibit the amidolytic activity of thrombin. The second proteolytic step produces a species (M_r 68,000) that has lost all antithrombin activity. Under conditions where inhibition of cathepsin G occurs within seconds (2 μM HC, 100 nM cathepsin G) the loss of HC antithrombin activity occurs over 30 min, suggesting that small amounts of uninhibited proteinase, or cathepsin G released from the HC-proteinase complex, are responsible for the proteolytic inactivation of HC. Heparin and dermatan sulfate alter the pattern of proteolysis of HC and accelerate the loss of antithrombin activity. The effect of glycosaminoglycans on the susceptibility of HC to proteolysis is not correlated with the effect of heparin or dermatan sulfate on proteinase activity, as measured by synthetic substrate hydrolysis. The results indicate that neutrophil proteinases might modulate antithrombotic activity in the presence of glycosaminoglycans during inflammation.

X 306 REGULATION OF CALCIUM-DEPENDENT PROTEASES: AN ASSAY FOR ENZYME ACTIVATION. D.E. CROALL, Department of Physiology, University of Texas Southwestern Medical Center at Dallas. Dallas, Texas 75235-9040. Several distinct mechanisms regulate the calcium-dependent proteases, CDP-1 and CDP-2 (E.C.3.4.22.17). Both enzyme activities are modulated by a protein inhibitor and an endogenous stimulatory protein. Each protease is isolated as a proenzyme that requires calcium-dependent autoproteolytic processing for enzyme activation and also to decrease the calcium requirement for proteolytic activity 20-50 fold. The activated, calcium sensitive forms of each enzyme can be distinguished from the proenzyme forms by electrophoresis under denaturing conditions because the small regulatory subunit of each protease is converted from 30 kDa to 17 kDa. We have developed an immunoblot assay to directly assess activation of CDPs by measuring conversion of the regulatory subunit from 30 kDa to 17 kDa. Rat erythrocytes were used to demonstrate calcium- and ionomycin-dependent activation of CDP-1. The extent of enzyme activation was dependent on time, ionophore concentration and calcium concentration. Thus, in this model system, CDP-1 activation occurs in response to increased intracellular calcium. This is the first direct demonstration of proteolytic activation of CDP in a cell. Proteolysis of filamin and talin (P235) occurs when platelets are treated with calcium ionophores or dibucaine (Fox et al., JBC 260:1060, 1985). This proteolysis has been attributed to the endogenous CDP. We have now demonstrated directly the activation of endogenous platelet CDPs in response to ionophore or dibucaine. This assay will allow us to identify factors involved in stimulating enzyme activation in vivo in numerous cell types and thereby contribute to our understanding of the physiological roles of this proteolytic system.

X 307 MODULATION OF THE COLLAGENASE INHIBITOR TIMP mRNA LEVELS BY ANTI-SENSE RNA INFLUENCES THE INVASIVENESS OF MOUSE 3T3 CELLS, R. Khokha[1], P. Waterhouse[1], S. Yagel[2], P.K. Lala[2], G. Norton[1], D.T. Denhardt[1]. [1]Cancer Research Laboratory and the [2]Department of Anatomy, University of Western Ontario, London, Ontario, Canada.

A murine cDNA clone, 16C8, was isolated in our laboratory by differential screening of two cDNA libraries with probes reflecting the mRNA content of quiescent vs. serum-stimulated Swiss 3T3 cells. The sequence of the encoded protein has substantial homology to the sequence encoding human tissue inhibitor of metalloproteinases (TIMP) and human erythroid potentiating activity (EPA) and is likely the murine equivalent of human TIMP/EPA (NAR, 14, 8863, 1986). Synthesis of the 0.9-kb mRNA is stimulated by PDGF, FGF, EGF, double-stranded RNA and TPA; the product of the mRNA is an Mr 22,500 secreted glycoprotein. TIMP is a specific inhibitor of neutral metalloproteinases such as collagenase, stromelysin, gelatinase and proteoglycanase; it is a major regulatory factor in cell-mediated collagen type I degradation. Elevated collagenase and TIMP levels have been found at the site of tissue remodelling and during scar formation. Studies of Reynolds and his collaborators have shown an inverse correlation between TIMP levels and the invasive potential of the murine and human tumour tissues. We show here that expression of anti-sense TIMP RNA results in a down-regulation of TIMP RNA levels in Swiss 3T3 cells and causes previously non-invasive 3T3 cells to become invasive as assessed by the amnion invasion assay; the TIMP down-modulated cells are also tumorigenic and metastatic in nude mice.

X 308 IDENTIFICATION OF A GENE FROM *E. COLI* INDUCED SPECIFICALLY IN RESPONSE TO DIAMIDINE PROTEASE INHIBITORS, Christine C. Dykstra[1], Phillip Ponder[1], and Richard R. Tidwell[2], [1]Laboratory of Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, N.C. 27709 and [2]Department of Pathology, University of North Carolina School of Medicine, University of North Carolina-Chapel Hill, Chapel Hill, N.C. 27514.

Diamidine compounds have been shown to act as potent and often specific inhibitors of trypsin-like proteases. Their small size makes them useful as molecular probes. We took advantage of the observation that protease inhibitors can stimulate the general production of the proteases they inhibit to search for an inducible gene in *E. coli*. Gene fusions are a way to identify genes related by a common regulatory network. Our goal is to identify inducible genes that respond to inhibitors specific for specific classes of protease inhibitors and then look for genes that might regulate them in a common manner. We have isolated a Mud(*Ap,lac*) operon fusion in *E. coli* that is induced specifically in response to bacteriostatic doses of 8 out of 26 amidine derivatives tested. A two-fold induction is seen with BABIM, the most potent inhibitor of trypsin, while a greater than 10-fold induction is seen with SB-59, RL-101 and RL-164, compounds with different enzyme specificities. 40-061, a direct analog of SB-59 with blocked amidine groups is devoid of anti-protease activity and does not induce the fusion Fusions to either *dnaK* (a heat-inducible gene) or *recA*(a DNA-damage inducible gene) are not induced in response to the eight inducing compounds. Mutations in *lon*, a gene affecting ATP-dependent proteolysis, suppress the induction by BABIM. The Mud(*Ap, lac*) fusion does not map to any known *E. coli* protease genes. We are in the process of identifying fusions inducible by other protease inhibitors and mutations that abolish the inducibility of this fusion as well as characterizing the current gene fusion.

X 309 EXPRESSION OF PLASMINOGEN ACTIVATOR INHIBITOR-1 IN CULTURED HUMAN VASCULAR ENDOTHELIAL CELLS R.D. Gerard, F.X. Riedo, K.R. Chien and R. Munford Departments of Internal Medicine and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235

The specific, fast-acting inhibitor of plasminogen activators, PAI-1, is synthesized and secreted by vascular endothelial cells in response to a variety of stimuli. Using a cDNA clone as a probe for Northern blots, we have examined the synthesis of mRNA encoding PAI-1 in response to two agents previously demonstrated to increase secretion, bacterial endotoxin (LPS) and tumor necrosis factor (TNF). LPS was found to increase the level of PAI-1 mRNA in primary human endothelial cell cultures within hours at doses as low as 1 ng/ml. The maximal increase in PAI-1 mRNA was five-fold. Deacylation of the endotoxin by a human neutrophil enzyme was found to reduce the stimulatory activity of LPS to background levels. TNF was also found to increase the level of PAI-1 mRNA in a dose-dependent fashion. The results of nuclear "run on" transcription assays indicate that the increase in PAI-1 mRNA is primarily due to a decrease in the rate of mRNA degradation rather than an increase in the frequency of transcription initiation.

X 310 THROMBIN MODULATES AND REVERSES NEUROBLASTOMA NEURITE OUTGROWTH, David Gurwitz and Dennis D. Cunningham, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717.

Previous studies have shown that neuroblastoma cells and several types of primary neuronal cells in culture rapidly extend neurites when switched from serum-containing to serum-free medium. The present studies on cloned neuroblastoma cells show that thrombin blocked this spontaneous differentiation at 2 nM with a half-maximal potency of 50 pM. This required the catalytic activity of thrombin and was reversed upon thrombin removal. Thrombin also caused cells in serum-free medium to retract their neurites at equally low concentrations. Two other serine proteases, urokinase and plasmin, did not block or reverse neurite extension even at 100-fold higher concentrations. A specific assay for thrombin indicated that thrombin detected in serum-containing medium from neuroblastoma cultures was derived from serum and that it was likely responsible for much of the known capacity of serum to maintain neuroblastoma cells in a nondifferentiated state. This conclusion was supported by the finding that heparin addition reduced the thrombin concentration in serum-containing medium and stimulated neurite outgrowth from neuroblastoma cells in serum-containing medium. Studies on the ability of thrombin to modulate neurite outgrowth by other agents showed that it blocked and reversed the neurite outgrowth activity of two thrombin inhibitors: protease nexin-1 (which is identical to the glial-derived neurite promoting factor) and hirudin. Thrombin, however, did not block the neurite promoting activity of dibutyryl cAMP or prostaglandin E1. These results suggest a specific role for thrombin in control of neurite outgrowth.

X 311 ROLE OF PROCESSING IN REGULATION OF RENIN RELEASE, Willa A. Hsueh, Tatsuo Shinagawa and Yung-Shun Do, University of Southern California Medical Center, Los Angeles, Ca. 90033

The enzyme renin regulates a cascade of events which ultimately controls blood pressure and sodium-potassium balance. The postraslational processing of renin in man may be altered by certain physiologic states or pathological conditions so that circulating prorenin and active renin concentrations are modified. In order to define biochemical mechanisms involved in conversion of prorenin to renin, we purified human renal renin and recombinant prorenin. Pure active renin has a molecular weight (MW) of 44,000 with 22,000 and 18,000 MW subunits. Aminoterminal sequencing reveals that a 43 amino acid prosegment is clipped after a dibasic residue Lys^{-2}-Arg^{-1}-Leu^{1}. Recombinant prorenin has a MW of 47,000 and, in general, has no enzyme activity. However, prorenin can be activated either without a change in molecular weight (which can occur during prolonged cold storage), or exposure to low pH. This activation appears to be reversible and related to a conformational change of the enzyme. Prorenin is activated by cleavage of the first 32 amino acids of the prosegment or by cleavage of the complete prosegment. This activation is irreversible. Whether the latter or all of these mechanisms play a role in renin activation in vivo remains to be determined. Nevertheless, this information will be useful in designing inhibitors of renin and in developing methods to regulate renin processing and release.

X 312 PEPTIDES OF BOROARGININE AS INHIBITORS OF TRYPSIN-LIKE ENZYMES, Charles Kettner, Lawrence Mersinger and Gerald Siefring, E. I. du Pont de Nemours & Co., Central Research & Development and Medical Products Depts., Experimental Station, Wilmington, DE 19898.
Peptide boronic acids are effective inhibitors of serine proteases which hydrolyze bonds at a neutral residue. This group of inhibitors has been extended to proteases specific for basic residues by the synthesis of boroArginine peptides. Initial studies with inhibitors corresponding to the sequence of inhibitors of plasma kallikrein and thrombin have shown that they far exceed other synthetic inhibitors in binding. The most effective inhibitor of thrombin, H-(D)Phe-Pro-boroArg-OH, inhibits thrombin 96-99% at 5nM indicating a Ki <50-fold lower. The corresponding N-Ac & Boc analogs and the pinanediol boronic acid esters gave comparable levels of inhibition. Similar results have been obtained for H-(D)Phe-Phe-boroArg-OH in the inhibition of plasma kallikrein. A comparison of the effectiveness of kallikrein inhibitors in the inhibition of the intrinsic and extrinsic blood coagulation pathways in vitro indicates much greater effectiveness in the inhibition of the intrinsic pathway. This is expected for agents selectively inhibiting kallikrein since it is involved in the initiation of the intrinsic pathway.

X 313 THE REGULATION OF PROTEOLYTIC ACTIVITY DURING BLISTER FORMATION IN HUMAN SKIN - EVIDENCE FOR THE INVOLVEMENT OF ALPHA-2-MACROGLOBULIN (MG) AND INTER-ALPHA-TRYPSIN-INHIBITOR (ITI), M.D. Kramer, C. Justus & W. Tilgen, Uni-Hautklinik, Voss-Str.2, D-6900 Heidelberg, FRG.

Proteinases are thought to act as final mediators of tissue destruction during blister formation in human skin. Proteolytic tissue degradation is likely to be controlled by proteinase inhibitors. MG and ITI are proteinase inhibitors each inactivating a variety of human proteinases.

We raised a panel of monoclonal antibodies recognizing either MG or ITI which were used for immunohistological localisation of ITI or MG in normal human skin and for quantification of the inhibitors in fluid of artificially induced blisters by means of moAb-based enzyme immuno-assays.

We found that MG and ITI are localised to the infrabasal part of normal human skin. By immunoassays and western-blotting we obtained evidence for the presence of both inhibitors in fluid of suction blisters induced artificially on normal human skin.

The presence of MG and ITI in normal epidermis and their appearence in pathological situations where proteolysis is experienced suggests their regulatory function in human skin. These findings are of particular interest considering the biological effectsof proteolytically modified forms of MG and ITI which may contribute to biological phenomena following blister formation.

X 314 ACTIVATION OF A CELLULAR PROTEOLYTIC FACTOR BY POLIOVIRUS PROTEINASE 2A, Hans G. Kräusslich, Martin J. H. Nicklin and Eckard Wimmer, State University of New York, Stony Brook, N.Y. 11794.

Poliovirus infection rapidly and effectively turns off host cell protein synthesis, and this event is accompanied by proteolytic cleavage of a large cellular polypeptide (termed p220 corresponding to its molecular weight of 220 x 10^3 Mr). Polypeptide p220 is a component of the cellular cap binding complex (eIF-4F). Its destruction by proteolysis is thought to be a major factor in the killing of the host cell by poliovirus. We have shown that cleavage of p220 can be induced in vitro by poliovirus proteinase 2A that was obtained by in vitro translation or by expression in E.coli. The viral enzyme, however, does not cleave p220 directly but activates a second, cellular, factor that degrades p220. This factor can be induced in HeLa- and L-cells and in rabbit reticulocytes. Studies with proteinase inhibitors indicate that the cellular proteolytic factor is different from the known ribosomal proteinase cathepsin R.

X 315 PERIODIC STABILIZATION AND DESTRUCTION in vitro OF MITOTIC CYCLINS FROM EARLY EMBRYOS. Frank C. Luca and Joan V. Ruderman, Duke University, Durham, NC 27706.

Within minutes of fertilization, embryos of the marine clam Spisula begin to synthesize two new proteins, cyclin A (55 kd) and cyclin B (50 kd). Both cyclins accumulate across the cell cycle until the end of mitosis, when they abruptly disappear. Cyclin A disappears a few minutes before the metaphase-anaphase transition; cyclin B drops right at the onset of anaphase. This periodic rise and fall in cyclin levels is important: cyclin A is a mitotic inducer (Cell 47, 861) and high levels of B keep cells in M phase. All available evidence indicates that the rapid, scheduled loss of the the cyclins at the end of each cell cycle is due to proteolytic destruction.

We have been able to reproduce in vitro some aspects of the rise and fall in cyclins that are seen in vivo. Cells were labeled with 35-S-methionine and homogenized midway through the cell cycle, just as cells were entering M-phase. 12,000g supernatatants were prepared, incubated at 18C, and aliquots were taken at various intervals and analysized by gel electrophoresis followed by autoradiography. In all cases, a discrete period of cyclin stability in vitro was followed first by the disappearance of cyclin A and then by the loss of B. In some lysates, cyclin disappearance was followed by resynthesis and restabilization. Lysates could be frozen and thawed repeatedly without any detectable loss of any of these activities. This in vitro system should enable us to pick apart and manipulate the various elements controlling the periodic destruction of the cyclins.

X 316 INDUCTION OF GENE TRANSCRIPTION OF PLASMINOGEN ACTIVATOR INHIBITOR (PAI)-1 and PAI-2 BY TUMOR NECROSIS FACTOR IN HUMAN FIBROSARCOMA CELLS, Robert L. Medcalf, Eva Van den Berg,* Egbert K.O. Kruithof and Wolf-Dieter Schleuning, Hematology Division, Department of Medicine, University Hospital Centre, CHUV, CH-1011, Lausanne and * Gaubius Institute TNO, 2313AD Leiden, The Netherlands.

Tumor necrosis factor/cachectin (TNF), is a monokine which mediates characteristic phenotypic features of the inflammatory response. Experimental septicaemia is associated with high circulating levels of TNF and its fatal consequences can be blocked by anti-TNF IgG. Disseminated intravascular coagulation (DIC), characteristic of septicaemia, is associated with an increased biosynthesis of the tissue factor of blood coagulation induced by TNF. To explore a possible effect of TNF on the fibrinolytic system, we have studied the human fibrosarcoma cell line HT-1080 which constitutively expresses tisssue-type plasminogen activator (t-PA), urokinase-type plasminogen activator, plasminogen activator inhibitor (PAI)-1 and PAI-2. Over a 48 hour time course, TNF causes a sustained increase of PAI-1 and PAI-2 biosynthesis, as demonstrated by pulse-chase labelling and radioimmunoassays. By run-on transcription assays it is shown that this increase is a result of a 10-fold and 20-fold increase of transcription rates of PAI-1 and PAI-2 respectively. Similar increase in both mRNA steady state levels is also observed. TNF had no effect on u-PA biosynthesis, however t-PA biosynthesis was suppressed. Our results indicate that the accumulation of intra- and extra vascular fibrin deposits often observed in chronic and acute inflammation, may be the direct result of TNF mediated stimulation of PAI biosynthesis. On the other hand, TNF may render tissues resistant to PA mediated extracellular matrix degradation during invasive or metastatic growth.

X 317 MOLECULAR STUDIES ON PLASMINOGEN ACTIVATOR INHIBITOR BY SITE DIRECTED MUTAGENESIS Huda E. Shubeita and Robert D. Gerard, Departments of Internal Medicine and Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX 75235

Plasminogen activator inhibitor (PAI-1) is a 50,000 Mr glycoprotein which is highly homologous to other members of the serine protease inhibitor (serpin) family and is synthesized and secreted by vascular endothelium. It inactivates plasminogen activator (PA) by the formation of a stable, covalent 1:1 complex of the two proteins. The best current model for the inactivation of PA by PAI-1 postulates that the inhibitor serves as a competitive substrate for the enzyme in a manner similar to that of alpha-1 antitrypsin inhibition of elastase. A loop of amino acid residues in the serpin molecule (termed the reactive center) that closely resembles sequences present in the normal substrate fits into the active site of the enzyme. Upon proteolytic cleavage of the inhibitor, a covalent bond forms between the protease and its inhibitor resulting in the inactivation of PA. Two regions on each of the molecules have been implicated in their interaction. On the PA molecule, the active site is known to bind the reactive center of the PAI-1 protein, and the kringle domains are proposed to bind at a second site on PAI-1. The functional contributions of the various structural regions of the two molecules to their interaction is being assessed by the use of mutant PAI-1 and PA proteins constructed by site directed mutagenesis. Since the specificity of the serpins for their respective proteases lies primarily within the reactive center, substitution mutants of the P1 and P1'residues were constructed. The inhibitor muteins are being tested for their ability to bind and inhibit different serine proteases. "Second site" mutants in the PAI-1 protein are also being constructed to disrupt potential charged-pair interactions between PA and PAI-1.

X 318 LOCALIZATION OF PROTEASE NEXIN II ON THE EXTRACELLULAR MATRIX OF HUMAN FIBROBLASTS, William E. Van Nostrand and Dennis D. Cunningham, University of California, Irvine, CA 92717.

Protease Nexin II (PN II) is a protein proteinase inhibitor that is synthesized and secreted by a variety of cultured nonvascular cells. PN II forms denaturation-resistant 1:1 complexes with three serine proteinases: the epidermal growth factor binding protein (EGF BP), the gamma subunit of nerve growth factor and trypsin. Proteinase:PN II complexes bind back to the cells which secrete PN II and are rapidly internalized and degraded. PN II also inhibits chymotrypsin; this inhibition appears to be resersible since denaturation-resistant chymotrypsin:PN II complexes are not observed. We recently found that PN II is localized on the extracellular matrix (ECM) of human fibroblasts. Using a polyclonal antibody to PN II, we developed an ELISA which showed that PN II is present on the surface of these cells. Furthermore, the ECM contains approximately 70% of the cell surface-associated immunoreactive PN II. When ECM was incubated with ^{125}I-labeled EGF BP and analyzed by SDS-PAGE and autoradiography, complexes were observed which were identical to ^{125}I-EGF BP:PN II complexes. In addition, when solubilized ECM components were separated by SDS-PAGE, transferred to nitrocellulose and incubated with ^{125}I-chymotrypsin, the labeled proteinase bound to a protein with an Mr identical to PN II. ECM-bound PN II may play a role in protecting the ECM from proteolytic degradation.

X 319 ASSOCIATION OF PROTEASE NEXIN-1 WITH THE FIBROBLAST SURFACE BLOCKS ITS ABILITY TO FORM COMPLEXES WITH UROKINASE, Steven L. Wagner, Alice L. Lau and Dennis D. Cunningham, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717.

Protease nexin-1 (PN-1) is a protein protease inhibitor that is secreted by a variety of cultured cells and rapidly forms complexes with thrombin, urokinase, plasmin and trypsin. The complexes bind back to the cells, providing a localized mechanism for controlling these proteases at the cell surface. Recent studies showed that PN-1 is localized to the extracellular matrix (ECM) of human fibroblasts and that the ECM accelerates the inactivation of thrombin by PN-1.

The reactivity of cell surface-bound PN-1 with thrombin and urokinase was compared in the present studies by incubating each ^{125}I-protease with human fibroblasts under conditions that they did not secrete PN-1. ^{125}I-thrombin-PN-1 complexes were detected bound to the cell surface, but ^{125}I-urokinase-PN-1 complexes were not detected either bound to the cells or released from them. To directly determine if association of PN-1 with the cell surface changed its specificity, "endogenous" cell surface-bound PN-1 was inactivated using a PN-1 monoclonal antibody, and purified PN-1 was added to the cells. The newly-bound PN-1 formed complexes with ^{125}I-thrombin but not ^{125}I-urokinase, demonstrating that this association blocked its ability to form complexes with urokinase.

Proteases in Disease; Therapeutic Applications

X 400 THE *SCHISTOSOMIATIUM DOUTHITTI* CERCARIAL PROTEASE IS BIOCHEMICALLY DISTINCT FROM THAT OF *SCHISTOSOMA MANSONI*, Payman Amiri, Judy Sakanari, Paul Basch, and James H. McKerrow, University of California, San Francisco and Stanford University, Palo Alto, CA.

The cercarial acetabular gland protease of *Schistosomatium douthitti*, an organism which causes "swimmer's itch" cercarial dermatitis, was identified and characterized. Gelatin substrate gel electrophoresis shows that there is a single major proteolytic species in *S. douthitti* cercarial extracts. Like the cercarial protease of *Schistosoma mansoni* (a causative agent of human schistosomiasis), it has a significant elastase activity, and can degrade a model of dermal extracellular matrix. It has a pH optimum of 9 and calcium dependence of 1 mM. However, in contrast to the corresponding *S. mansoni* enzyme which is a 30,000 kD serine proteinase, the *S. douthitti* elastase is a 50,000 kD metalloproteinase. A cDNA probe for the *S. mansoni* proteinase does not hybridize to *S. douthitti* sporocyst RNA. Failure of "swimmer's itch" cercariae to reach the bloodstream in humans is therefore not due to lack of a potent "penetration protease." However, differences in the structure of the *S. douthitti* enzyme may contribute to the differences noted in the human immune response to animal or bird host schistosomes.

X 401 PROTEINASE-INHIBITOR ACTIVITY IN SERA OF PATIENTS WITH CARCINOMA OF THE CERVIX UTERI. Luis Benítez, Rafael Freyre, Mario Villalobos, Leticia Pastrana, Investigaciones Oncológicas, IMSS, Apartado 12-921, 03020 México, D.F. México.
It has been clearly established that proteinases play an important role in tumor progression; on the other hand, serum alpha-1-proteinase inhibitor (API) is greatly increased in patients with different malignancies. We and others have found that despite the increment of API, its inhibitory capacity is diminished. Our study was aimed to investigate the API relative activity in different stages of carcinoma of the cervix where increased serum proteinase levels have been reported. Patients with cervical dysplasia, carcinoma *in situ* and invasive carcinoma were studied. API was determined by laser nephelometry, serum trypsin inhibitory capacity by the method of Liebermann and TIC/API ratio was calculated. Dysplasias had no statistical differences with controls; however 82.0% of the *in situ* patients and 87.3% of the invasive group, had API values above 2 SD of the mean. No significant increase of trypsin inhibitory capacity (TIC) was observed in any of the groups but the TIC/API ratio fell to 0.54 in carcinoma *in situ* and 0.56 in invasive carcinoma, thus indicating an almost 50% reduction of their proteinase inhibitory function. These findings suggest that diminished proteinase-inhibitor activity might play a role favouring tumor progression.

X 402 VIRAL INFECTION INCREASES TRACHEAL PERMEABILITY RESPONSE TO SUBSTANCE P (SP) IN RATS BY DECREASING NEUTRAL ENDOPEPTIDASE (NEP). D.B. Borson, J. Brokaw, K. Sekizawa, D. McDonald, J.A. Nadel. Cardiovascular Research Institute, UCSF, San Francisco, CA, 94143.
To determine whether respiratory tract viral infections alter the effect of SP on vascular permeability, we studied the extravasation of Evans Blue dye in pathogen-free (PF) rats and rats that had recovered from spontaneously occurring infections by Sendai and Corona viruses and by Mycoplasma pulmonis. To determine whether NEP plays a role in modulating SP-induced effects and whether NEP plays a role in these diseases, we studied rats perfused with the NEP inhibitor, leu-thiorphan (Squibb). Rats were anesthetized with sodium pentobarbital and placed supine. Jugular veins were exposed, and 30 μg dye/kg body wt. was injected, followed 1 min. later by SP in saline (1 μg/kg; iv). Five min. later, the rats were perfused with citrate buffer (pH, 3), and the tracheas and esophagi removed and weighed. The dye was extracted in formamide and dye contents were determined spectrophotometrically. In the absence of SP, tracheal dye contents were similar in infected and PF rats (27±1 vs. 30±4 ng dye/mg wet wt.). SP increased permeability in the tracheas of infected rats more than in the PF rats (97±7 vs. 44±4 ng/mg; $p<0.05$, n=7). After leu-thiorphan (600 μg/kg; iv), the tracheal responses to SP were similar in the infected and PF rats (74±10 vs. 99±12 ng/mg; n=8). Responses in the esophagi were similar for both groups. Tracheal NEP activity was lower in the infected rats than in the PF rats (397±36 vs. 640±34 fmoles [^{3}H-Tyr, DAla^2], leu-enkephalin degraded.min^{-1}. mg protein^{-1}; $p<0.001$, n=6), whereas esophageal NEP was not different. These results suggest that viral infections decrease tracheal NEP activity chronically and specifically, thereby increasing permeability responses to SP. (Supported in part by PPG HL-24136).

X 403 CANCER URINE DERIVED ANTIPROTEINASE EDC1 INHIBITS THE GROWTH OF B LYMPHOBLASTS. R.K. Chawla and D.H. Lawson, Emory University School of med and VA Med. Center, Decatur, GA 30033.
A urinary glycoprotein, labeled EDC1, Mr 30 KDa, was originally isolated in our labs in a homogeneous form from the urine of a leukemic patient ED. Subsequently this molecule was detected in large quantities in urine of most patients with different types of advanced cancer. EDC1 is antigenically related to plasma inter-alpha trypsin inhibitor and inhibits trypsin, chymotrypsin, and granulocytic elastase. We previously reported that EDC1 inhibits the uptake of labeled thymidine by normal peripheral blood lymphocytes stimulated with phytohemagglutinin. We have now observed that EDC1 inhibits the growth of Raji cells (B lymphoblasts) in a dose dependent, reversible manner. Raji cells ($2x10^5$ cell / ml) were grown in 35 mm2 tissue culture dishes at 37° in RPMI 1640 supplemented with insulin, transferrin, selenium, and linoleic acid. Aliquots of EDC1 (0.3 pg to 3.0 μg /ml in 100 μl medium) were added daily for 7 days; a control group of cells received 100 μl of medium. In addition, all cells received 1 ml medium on day 4. On day 8 cells were counted on a hemocytometer. A significant decline in cell number was noted in cells treated with 0.03 μg, 0.3 μg, and 3.0 μg EDC1 per ml (8 dishes per concentration); growth (ave ± SD; % of control group) at these concentrations was respectively 66.2 ± 2.2, 41.7 ± 6.5; and 23.8 ± 4.8. A 50% decline in cell growth was observed at the final concentration of ~ 0.13 μg or ~4 pmol/ml of medium. EDC1 treated cells excluded trypan blue and resumed normal growth pattern after EDC1 was removed from the medium suggesting that EDC1 was not toxic to the cells. Other proteinase inhibitors, e.g., soybean trypsin inhibitor, lima bean trypsin inhibitor, and turkey ovo - mucoid did not affect the growth of Raji cells at comparable concentrations. Furthermore, no complex of EDC1 with transferrin or insulin was detected which suggested that EDC1 did not inhibit the growth of Raji cells by blocking their uptake of these nutrients/hormones. We propose that EDC1 inhibits the growth of Raji cells by complexing with a membrane associated proteinase which may be required for their proliferation or that EDC1 binds to a specific membrane receptor on these cells.

X 404 α_1-PROTEINASE INHIBITOR IN MONKEY LUNG INFLAMMATION. T. Emerson, T. Redens and M. Fournel. Cutter Biological, Miles Inc., Berkeley, CA 94710.

Acquired α_1-proteinase inhibitor (α_1-PI) deficiency and hence an imbalance in PMN neutrophil elastase and α_1-PI levels, has been implicated in lung damage during conditions of pulmonary inflammation. The present study was to test the hypothesis that i.v. prophylatic treatment with α_1-PI prevents or limits lung damage in a non-lethal monkey model of acute, locally induced pulmonary inflammation. Adult male cynomolgus monkeys (Macaca fascicularis) (6-8 kg) were anesthetized with ketamine. One group (n=5) received IV infusions of purified human α_1-PI (240 U/kg) 16 hrs prior to insult and the same dose at T-1 hr. Another group (n=5) received equal volume infusions of normal saline solution (NSS). Each monkey was instilled intrabronchially (right or left lung) with 200 ug/kg (1 mL/kg) FNLP dissolved in DMSO and NSS at time 0 (T-0). At T+6 hrs, a second instillation of FNLP was carried out and at T+7 hrs a brochio-alveolar lavage (BAL) of 15 mL NSS was completed. Results show improvements in several variables in the α_1-PI group compared to the NSS group as follows: Values are means ± SEM. 1) recovered BAL volume was less (2.7±0.4 vs 4.8±0.4 mL); 2) BAL total protein was less (4.4±0.7 vs 8.1±1.4 mg); 3) BAL active neutrophil elastase was less (97±21 vs 133±8 ng); and 4) BAL α_1-PI was greater (416±188 vs 54±5 ug). These data support our hypothesis that prophylactic treatment with α_1-PI improves indices of lung damage in a monkey model of local pulmonary inflammation.

X 405 CLEAVAGE AND INACTIVATION OF α_1-ANTITRYPSIN BY METALLOPROTEINASES RELEASED FROM NEUTROPHILS, Margret C.M. Vissers, Peter M. George, Ian C. Bathurst, Stephen O. Brennan and Christine C. Winterbourn, Department of Pathology, Christchurch School of Medicine, Christchurch Hospital, Christchurch, New Zealand.

Human neutrophils, when stimulated with phorbol myristate acetate or with fMet-Leu-Phe released metalloproteinases which catalytically inactivated the plasma serine proteinase inhibitor, α_1-antitrypsin. Inactivation, measured as loss of elastase inhibitory capacity, was accompanied by cleavage of a Mr 4,000 peptide from the C-terminus. Cleavage of α_1-antitrypsin by cell supernatants was inhibited by EDTA, o-phenanthroline and dithiothreitol, but not by inhibitors of serine or thiol proteinases. Gelatinase and collagenase were purified from the medium of stimulated neutrophils. Both preparations cleaved and inactivated α_1-antitrypsin, cleavage occurring close to the reactive centre, at the Phe-Leu bond between positions P_7 and P_6. Cleavage by purified gelatinase was very slow and could account for only a minor fraction of the activity of neutrophil supernatants. The collagenase preparation was more active. However, the unusual cleavage site, and the ability of fMet-Leu-Phe-stimulated neutrophils to cleave α_1-antitrypsin without releasing collagenase, leaves some uncertainty as to which metalloproteinase is responsible for cleavage by the cells. Our results demonstrate that by releasing metalloproteinases, neutrophils could proteolytically inactivate α_1-antitrypsin at sites of inflammation. This provides an alternative to the previously documented mechanism of inactivation by neutrophil-derived oxidants.

X 406 IMPAIRMENT OF SCIATIC NERVE REGENERATION BY PROTEASE INHIBITORS: INHIBITION OF SCHWANN CELL MIGRATION. N.Kalderon, J.P.Kirk, & A.Juhasz. Rockefeller Univ. NY, NY 10021

The Schwann cell is an essential supporting component of the repair process of an injured nerve. The temporal sequence of events in peripheral nerve regeneration in a model system, through a 10mm silicone chamber, has been established: (a) within one week postsurgery, an acellular fibrin bridge is formed between the nerve stumps; and (b) in the second week, extensive migration within this bridge of Schwann cells and other cells takes place, while the regenerating axons lag by 1-2 days behind the Schwann cells (Williams et al., J.Comp.Neurol., 218:460, 1983). Schwann cells, the proliferating populations, express high levels of plasminogen activator activity (Kalderon, PNAS, 81:7216, 1984). The experiments described in this report examine the idea that Schwann cells utilize proteolysis to invade, paving the way for the regenerating axons. For this purpose we are studying the effects of protease inhibitors on rat sciatic nerve regeneration through a 10mm silicone chamber. The nerve was cut and the stumps were sutured to a silicone tubing which was filled with a physiological salt solution. The inhibitors were injected into the chamber at 7 days postsurgery and their effect was examined a week later. Samples were cryostat sectioned through their horizontal axis and were analysed for: cell content, regenerating axons, etc. The plasmin inhibitors E-aminocaproic acid (7.6mM), D-Lys(Bz)-Lys(Bz)-LysCH_2Cl (1μM), and leupetin (0.1mM) impaired cell migration at 61%, 60%, & 43% correspondingly, as compared with control samples. These inhibitors did not show any adverse effect on Schwann cells in culture. It is concluded that plasmin activity is elaborated by the Schwann cell and is instrumental in nerve regeneration, since inhibition of this activity prevented cell migration; accordingly, axonal regrowth was delayed.

X 407 INCREASED TISSUE-TYPE PLASMINOGEN ACTIVATOR AND FIBRONECTIN IN HUMAN GRANULOSA CELLS CORRELATE TO THE FERTILIZING CAPACITY OF THE RESPECTIVE OOCYTES. J.C. Kirchheimer, J. Deutinger, G. Christ, A. Reinthaler, G. Tatra, B.R. Binder; Department of Clinical Experimental Physiology. University of Vienna, Austria

Plasminogen activators convert the abundant extracellular zymogen plasminogen into plasmin, an active protease that either directly or indirectly, can promote degredation of all components of the extracellular matrix. The plasminogen activators and their specific inhibitors regulated by gonadotropins have been shown to be involved in follicular rupture in rats, exhbiting increased plasminogen activator activity at the time of ovulation. In our study 49 follicles and the respective follicular fluid were aspirated from 20 women undergoing in vitro fertilization (IVF) after stimulation of the ovaries with hMG/hCG. The concentration of urokinase type plasminogen activator, uPA (RIA); tissue type plasminogen activator, tPA (ELISA); plasminogen activator inhibitor PAI (functional assay) and fibronectin, FN (ELISA) was determined in the lysates of the granulosa cells (Table 1) and in the respective follicular fluid and related to the fertilizing capacity of the respective oocytes.

		Fertilized	Non-Fertilized
uPA	(pg/mg protein)	186.4 ± 42.5	182.4 ± 35.9
tPA	(pg/mg protein)	180.3 ± 68.2	34.7 ± 15.5
PAi	(mU/mg protein)	89.8 ± 23.0	82.2 ± 19.3
FN	(ng/mg protein)	9.3 ± 1.4	5.9 ± 0.7

The data obtained show a significant increase in $p < 0.05$) of tPA and FN in lysates of granulosa cells from fertilized over non-fertilized oocytes. However, no changes of the parameters could be observedin case of the follicular fluids. Our results indicate that beside the hormonal profile, tPA and FN represent useful parameters for follicular maturation and oocyte quality.

X 408 THE INFLUENCE OF DEGLYCOSYLATION ON tPA CLEARANCE AND IN VIVO ACTIVITY IN RABBITS, D. Livingston, G. Kuzma, C. Oppenheimer, D. Lau, R. Purcell, N. Hsiung, W. Markland and R. Douglas, Integrated Genetics, Inc., Framingham, MA 01701.

The clearance of tPA administered at pharmacological dose may depend on recognition of one or several N-linked oligosaccharides in the molecule. We have examined this possibility by generating seven mutant forms of tPA which lack one or more of the three N-linked oligosaccharides. The cDNA for uterine tPA was altered by site-directed mutagenesis to change asparagine to glutamine at the sites of sugar attachment, thereby eliminating glycosylation at that site. The resulting mutant tPA's were expressed in and secreted by mouse C127 cells, the proteins purified from conditioned medium, and carbohydrate compositon determined. The purified proteins were assayed for direct, indirect amidolytic, and fibrinolytic activity. Lability to plasmin cleavage was assessed in addition to pharmacokinetic behavior in rabbits. Significant variation in systemic half-life, stability to proteolysis, and <u>in vivo</u> thrombolysis was observed among mutants, with less variation observed in assays for <u>in vitro</u> enzymatic activity.

X 409 SECRETION OF TRANSFORMATION-ASSOCIATED METALLOPROTEINASES OF RAT KIDNEY CELLS INFECTED WITH MOLONEY MURINE SARCOMA VIRUS, Motowo Nakajima and James C. Chan, The University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston, Texas 77030.

The 6M2 cell line is derived from the normal rat kidney cells infected with a temperature-sensitive mutant (ts110) of the Moloney murine sarcoma virus. The 6M2 cells express the transformation phenotypes at the permissive temperature; the phenotypes include a transformed morphology, an increased saturation density, anchorage-independent growth, production of the viral transformation proteins, and secretion of TGF-a. We analyzed the metalloproteinases secreted from the 6M2 cells using substrate-embedded polyacrylamide gel electrophoresis. In the serum-free medium conditioned by the 6M2 cells at the nonpermissive temperature (39°C), four major collagenolytic enzymes (Mr~86K, 82K, 69K, and 65K) and a fibronectinolytic enzyme (Mr~60K) were detected. At the permissive temperature (33°C) three more collagenolytic enzymes (Mr~78K, 34K, and 31K) appeared and the 60K fibronectinolytic enzyme activity significantly increased. A 34K proteinase was active on the fibronectin, however, these enzymes did not degrade casein, serum albumin, or hemoglobin and were completely inhibited by EDTA but not PMSF. The results indicate that the expression of certain connective-tissue-degrading metalloproteinases associates with the transformation by the v-mos oncogene. (Supported by NCI CA41524)

X 410 ROLE OF CANDIDA ALBICANS PROTEASE(S) IN INCREASING THE SUSCEPTIBILITY OF BURNED MICE TO LETHAL CANDIDOSIS. Alice N. Neely, Cindy M. Childress and Ian Alan Holder, Shriners Burns Institute, Cincinnati, OH 45219.

The role of C. albicans (Ca) protease(s) as virulence factors in burned hosts was studied by using a wild-type protease-producer (Ca^+) and its slow protease-producing mutant (Ca^-). Burned mice challenged IV with 10^5 CFU Ca^+ or Ca^- showed significant mortality differences, 80% vs. 0%, respectively. Treating Ca^+ infected mice with the protease inhibitor α2-macroglobulin improved survival 53% ($p<0.05$) and giving Ca^- infected mice an exogenous protease increased mortality 50% ($p<0.05$), suggesting that the mortality was related to the strain differences in protease production. Quantitation of Ca in the kidneys just prior to death of the Ca^+ group gave 5.00±0.32 vs 2.12±0.38 $\log_{10}$CFU renal Ca in the Ca^- group ($p<0.05$). Were the differences in renal fungal load and mortality due to the effect of Ca protease(s) on circulating host defense related proteins? Immunoglobulin (IgG) and transferrin (Tr) levels were measured in plasma of burned Ca^- and burned Ca^+ infected mice 6-72h post-burn and challenge. At 6-72h, IgG was significantly less in burned Ca^+ than in burned Ca^- infected mice. Tr was equally reduced in both groups at 6h; by 72h, Tr from burned Ca^- infected mice was slightly above normal, but in burned Ca^+ infected mice was still below normal ($p<0.05$). Thus, it appears that the Ca protease activity in the circulation of the burned host decreased the plasma level of these defense proteins and reduced burned Ca^+ infected hosts' ability to clear the Ca infection.

X 411 PROTEASE-MEDIATED DAMAGE TO HUMAN LUNG ALVEOLAR WALL, Muriel S. Palmgren, Richard D. deShazo, Robert M. Carter, and Sudhir V. Shah, Tulane Medical School, New Orleans, LA 70112.

Neutrophil-derived proteases have been implicated in damage to the glomerular basement membrane in vitro and have been postulated to be responsible for kidney damage in vivo. We hypothesize that a similar mechanism operates when disease processes result in tissue infiltration with neutrophils and acute lung injury occurs. A common manifestation of such injury is non-cardiac pulmonary edema which appears to result from loss of integrity of the alveolar-capillary interface. In our experiments, supernatants from activated, human neutrophils were incubated for 24 hours with isolated fragments of human lung alveolar wall (HLAW) denuded of cells. The primary constituents of HLAW were interstitial collagen and basement membrane containing Type IV collagen. Collagen in these fragments was hydrolyzed as evidenced by release of hydroxyproline, an amino acid characteristically high in collagen. On the average, 9.8% ± 0.6% of the total hydroxyproline was released after this incubation. Inhibitors of proteases were added to the incubation mixtures in an attempt to determine the protease responsible for digestion of collagen (release of hydroxyproline). Neither alpha-1-trypsin inhibitor nor soybean trypsin inhibitor significantly reduced the release of hydroxyproline. However, metal chelators, EDTA and 1,10-phenanthroline, significantly inhibited the release of hydroxyproline by 34.7% and 32.1% respectively. We conclude that (1) human neutrophils contain proteases capable of digesting HLAW and (2) the most likely proteases with this activity are metalloenzymes. This activity is likely to be an important mechanism of acute lung injury.

X 412 SERIAL CHANGES IN TISSUE PLASMINOGEN ACTIVATOR (tPA), ITS INHIBITOR (PAI) AND OTHER FIBRINOLYTIC SYSTEM FACTORS UNDERGOING ABDOMINAL SURGERY, Ronald C. Roberts, Robert L. Kolts, Richard D. Sautter and Donna Dumar, Marshfield Medical Research Foundation, Marshfield, WI 54449.

Abdominal surgery patients treated prophylactically with low dose heparin (LDH) had significantly greater incidence of fibrinolytic "shutdown" within 24 hrs following surgery than those who did not receive LDH. Serial measurements of tPA activity, PAI activity, plasmin-α_2-plasmin inhibitor complexes, plasminogen and fibrinogen in plasma from before surgery to up to 4 days post-surgically in 16 patients with no LDH and 11 patients with LDH. PAI levels correlated most closely with prolonged whole blood euglobulin clot lysis times of the LDH group of all these assays. The PAI activity in the recovery room samples were borderline significantly higher ($P = 0.0523$) in patients receiving LDH than in those not receiving LDH. PAP levels and the other parameters measured did not differ between the two groups. tPA levels were lower and on post-op days 1 through 4 in the LDH group. These studies are focusing on the effect of low dose heparin therapy on the fibrinolytic system.

X 413 CHARACTERIZATION OF A PROTEASE EXCRETED BY THE INVASIVE STAGE OF THE "SUSHI WORM" ANISAKIS SIMPLEX, Judy A. Sakanari and James H. McKerrow, Department of Pathology, University of California, San Francisco, CA 94143.
Anisakiasis is a parasitic disease of humans caused by the accidental ingestion of a larval nematode found in raw fish dishes such as sashimi, sushi and ceviche. After ingestion, the worms can penetrate the mucosa and submucosa of the stomach and intestine, causing extreme pain, nausea and vomiting. The purpose of our study was to characterize the proteases that may be involved in the tissue invasion by these parasites. Live worms were used in an in vitro assay to determine if excretory products from the worms had proteolytic activity against tritium-labeled macromolecules (collagen, elastin and glycoprotein) derived from a rat endothelial cell line. Five living worms in PBS + gentamicin (100ug/ml) degraded 25% of the extracellular matrix in 12 hrs. Twenty-five per cent of the collagen and 35% of the trypsin-labile glycoproteins were degraded. A 21 Kd protease was identified from the media using substrate gel electrophoresis. It was inhibited by 1, 10 phenanthroline, an inhibitor of metalloproteinases. There was also endogenous inhibitor activity in cell-free extracts of homogenized worms that may be involved in the post-translational regulation of the protease.

X 414 RECOMBINANT HUMAN TISSUE INHIBITOR OF METALLOPROTEINASES (rTIMP) INHIBITS LUNG COLONIZATION AND HUMAN AMNION INVASION BY B16-F10 MURINE MELANOMA CELLS. R.M. Schultz, S. Silberman, B. Persky, A.S. Bajkowski, and D.F. Carmichael, Departments of Biochemistry, Pathology and Anatomy, Loyola Univ. of Chicago, Stritch Schl Medicine, Maywood, IL 60153, and Synergen, Inc., Boulder, Colorado 80301.
The human tissue inhibitor of metalloproteinases (TIMP) appears to be ubiquitous in human mesoderm tissues and has previously been shown to be identical to the collagenase inhibitor isolated from human skin fibroblasts. TIMP inhibits type I and IV specific collagenases and other neutral metalloproteinases that may be responsible for the degradation of extracellular matrix in tumor cell metastasis. In this work we have utilized recombinant human TIMP (rTIMP) obtained by expression of its cDNA gene in E. coli. The rTIMP, which is unglycosylated and has a molecular weight of 20,700, is shown to have similar inhibition properties as natural TIMP against human skin fibroblast collagenase. In an in vitro amnion invasion assay system, rTIMP inhibits the invasion of B16-F10 mouse melanoma cells through the human amnion membrane at an identical concentration to that reported previously for natural TIMP. The availability of large amounts of rTIMP allowed us to test its effect on metastasis in an in vivo animal model. Mice that are treated with intraperitoneal injections of rTIMP show a significant inhibition of experimental metastasis to the lung after receiving tail vein injections of B16-F10 cells. These results indicate an essential role for metalloproteinase(s) in the extravasation and invasion of tumor cells during metastasis. (Supported by NIH grant CA 44659.)

X 415 SUPPRESSION OF EXPERIMENTAL ARTHRITIS BY PROTEASE INHIBITORS, Richard R. Tidwell, Sonia K. Anderle, Katherine B. Pryzwansky, John H. Schwab, J. Dieter Geratz, University of North Carolina, Chapel Hill, NC 27514.
Intraperitoneal application of streptococcal cell wall-derived peptidoglycan-polysaccharide fragments (PG-APS) in Lewis rats leads to a recurrent destructive arthritis and to characteristic lesions in liver and spleen. The initial bone changes consist of marked tri-lineage hyperplasia of the hematopoietic elements. This is followed by neutrophilic and histiocytic infiltration of epiphysis and metaphysis and destruction of the bone trabeculae and of epishyseal plates. The synovium is acutely inflamed and proceeds to send an erosive pannus onto the joint cartilage. In liver and spleen deposits of PG-APS fragments stimulate formation of an abundance of granulomata many of which become confluent and develop central abscesses.

Intravenous injection of the potent trypsin inhibitor (K_i = 1.7 x 10^{-8}) bis(5-amidino-2-benzimidazolyl)methane (BABIM) in doses of 10 mg/kg body weight, initially given daily and then at 2-3 day intervals, leads to nearly complete suppression of the bone and joint lesions and markedly reduces the granulomatous process in liver and spleen. The results support the notion that trypsin-like enzymes are involved in the response to PG-APS fragments and that BABIM may be a representative of a new type of antiinflammatory agents.

X 416 ACTIVATION OF THE Ca PUMP ATPase OF INTACT RED BLOOD CELLS BY A23187, Frank F. Vincenzi, Ling Wu and Thomas R. Hinds, University of Washington, Seattle, WA 98195.

The well known Ca pump ATPase of plasma membranes has usually been studied in isolated membranes where it is expressed as a Ca^{2+} and Mg^{2+} dependent ATPase. In experiments presented here, ATPase activity was monitored by following the loss of ATP in intact human red blood cells (RBCs) (in the presence of 1mM iodoacetic acid [IAA] to prevent ATP resynthesis) exposed to the divalent ionophore, A23187 in the presence of Ca^{2+}. Under these conditions, within 2 minutes of the addition of ionophore, the loss of ATP from the cells obeyed pseudo first order decay with a rate constant of approximately 0.2 min^{-1} (half life approximately 3.5 min). In the absence of Ca^{2+} in the medium, A23187 induced no ATP loss. The rate constant for ATP loss differed in a characteristic manner, depending on the individual blood donor. The rate constants determined in intact cells from various donors correlated with the specific activities of the ATPase as determined in RBC lysates and/or isolated membranes from the same donors, respectively. The rate constant also differed in 'young' and 'old' RBCs (as selected by density). The rate constant for A23187-induced ATP loss was approximately 20% greater in young than in old RBCs. The results demonstrate that the activity of the plasma membrane Ca pump ATPase is activated by the influx of Ca in intact cellls, that within two minutes of such activation, the enzyme appears to not be saturated with the substrate, ATP and that the rate of ATP loss can be taken as a measure of the Ca pump ATPase activity of intact cells. Funded by the National Dairy Board administered in cooperation with the National Dairy Council.

X 417 **Differentiation Linked Secretion of Urokinase and Tissue Plasminogen Activator by Normal Human Haemopoietic Cells.** **E. L. Wilson and G. E. Francis*, Dept. of Cell Biology, N.Y.U. Medical Center, 550 First Avenue, New York, N.Y. 10016; *Dept. of Haematology, Royal Free Hospital, London.**

Previous studies have shown that leukemic cells from patients with acute myeloid leukemia secrete plasminogen activators either of the urokinase (u-PA) or the tissue plasminogen activator (t-PA) type. The type of enzyme secreted is of prognostic significance in that patients whose cells secrete t-PA only, fail to respond to combination chemotherapy. It was therefore decided to examine normal human bone marrow cells for the species of enzyme secreted. Bone marrow was fractionated by equilibrium density gradient centrifugation into cells at different stages of differentiation. It was found that the low density cells ($\pm$ 1.045 - 1.065 gcm^{-3}) produced exclusively t-PA whereas more mature higher density cells ($\pm$ 1.07 - 1.085 gcm^{-3}) produced a mixture of t-PA and u-PA. The cells that produced t-PA comprised two populations - the first with a density of $\pm$ 1.063 gcm^{-3} and the second with a density of $\pm$ 1.072 gcm^{-3}. The first population corresponds to the modal density for CFU gm while the second population corresponds to the density distribution profiles of myelocytes and promyelocytes. The cells that produced u-PA correspond to the density of neutrophil granulocytes and myelocytes and promyelocytes. Direct evidence for the cellular species secreting each enzyme type was obtained from cultures of clones in semi solid agar. Day 3 cultures secreted t-PA whereas day 11 cultures secreted u-PA. Early progenitors were therefore found to secrete t-PA exclusively and more differentiated cells produced u-PA.

X 418 PROTEASE INHIBITORS PROTECT AGAINST ANOXIC AND TOXIC CELL DEATH IN RENAL TUBULE CULTURES, Patricia D.Wilson and David Hreniuk, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854.

Monolayer primary cultures of individually microdissected renal tubules in defined, serum-free media retain differentiated functions and are sensitive to anoxic and toxic cell death. Susceptibility to anoxia (An), gentamicin (G) and cyclosporine (CsA) is time and dose dependent and varies according to nephron segment of origin. To determine whether cysteine protease inhibition could attenuate such injury freshly confluent cultures of rabbit and human proximal straight tubules (PST) and cortical collecting ducts (CCT) were subjected to 45 min An ($95\%N_2/5\%CO_2$) or were incubated in media containing G (1mg/ml) or CsA (100µg/ml) for 1 hr to 3 days. Parallel cultures were preincubated for 2 hrs in the presence of the active site directed, irreversible, non-cytotoxic, cysteine cathepsin inhibitor L-trans epoxy succinyl-leucyl amido(4-guanido)butane (E64, 100µg/ml). Cell death was measured by nigrosine dye uptake and (LDH) release. E64 attenuated induced by each agent. Percent of viable cells 6 hrs after An insult; after 4 hrs of CsA or 3 days of G was:

	An	An+E64	G	G+E64	CsA	CsA+E64
PST	14+5	52+15	46+12	78+14	20+8	75+19
CCT	49+9	78+12	65+10	93+8	64+15	97+9

Prior depletion of lysosomal enzymes by pretreatment with NH_4Cl offered no protection. We conclude that anoxic and nephrotoxic cell death induced by N_2, G and CsA is mediated, at least in part via cytoplasmic, calcium dependent cysteine proteases. Thus, protease inhibitors may have chemotherapeutic potential in prevention of acute renal failure.

X 419 DETECTION AND DISTRIBUTION OF SERINE PROTEASE AT THE SINGLE CELL LEVEL BY A CYTOCHEMICAL STAIN. COLIN P WORMAN, LUDWIG WAGNER. Dept. HAEMATOLOGY, UNIVERSITY COLLEGE HOSPITAL, GOWER ST.LONDON UK.

Previously, detection and characterisation of the cytolytic-associated serine esterase or putative protease enzyme has relied upon spectrophotometric or SDS gel assays on cell lysates of cytotoxic T cell lines and ex vivo cell preparations. These procedures require relatively large numbers of cells and a very high degree of purity in isolated cell populations. To obviate these constraints we have developed a rapid cytochemical staining method which can detect the presence/activity of this enzyme system at the single cell level that can be used on a wide variety of tissue/blood preparations and has shown to be invaluable where very limited cell numbers are available. This adapted staining method when used consecutively with a routine counterstain or monoclonal antibodies can readily identify the SE+ cell in a mixed population. Data will be given on the staining method and the normal distribution of the enzyme in haemic cells together with some aspects of in vitro and in vivo generation of this family of putative cytolytic esterases under varied stimuli. Preliminary results are indicating that this new cytochemical method for the detection of serine proteases has potential application in various fields from differential diagnosis of leukaemia to studying both natural and therapeutically generated lymphocyte-mediated cytotoxicity.

X 420 PURIFICATION OF A FAMILY OF METALLOPROTEASES FROM HUMAN CANCER CELLS, S. Zucker, J. Wileman, R.M. Lysik, B. Imhof, L.A. Liotta and H. Nagase, VA Med Center, Northport, NY, N.C.I., Bethesda, MD, and Univ of Kansas.
This study has characterized connective tissue degrading metalloproteases from highly metastatic human NCI-H82 small cell lung cancer cells and RWP-1 pancreatic cancer cells. The cytosol isolated from cancer cells propagated as solid tumors in nude mice each contained a gelatinolytic enzyme (Mr=160,000) that was subjected to purification by ammonium sulfate precipitation, followed by gelatin gel, zinc chelate Sepharose column, anion exchange and gel permeation chromatographies. Human cancer gelatinase was inhibited by metal chelators and serum, but not by serine or cysteine protease inhibitors. The gelatinase fractions also degraded type IV collagen, suggesting that a single enzyme is able to digest both substrates; but it did not degrade native type I collagen. A second cancer metalloproteinase of Mr=29,000 which primarily degraded proteoglycan substrates, was isolated from lung and pancreatic cancer cells. Lung cancer cells were more highly enriched in type IV collagenase-gelatinase activity, whereas pancreatic cancer cells demonstrated a dominant stromelysin-type enzyme. We used the above chromatographic procedures to identify a type I collagenase (Mr>400,000) and two distinct gelatinases (Mr=160,000 and Mr=29,000) from detergent solubilized cell membranes isolated from human pancreatic cancer cells. We propose that cancer cells contain a family of connective tissue degrading metalloproteases, including type I collagenase, type IV collagenase-gelatinase, and stromelysin that work to facilitate the process of cancer invasion. Cancer cells produce these metalloproteases constitutively and incorporate these enzymes into their plasma membranes where they have direct access to connective tissue stromal elements.

X 421 RECOMBINANT CATHEPSIN G EXERTS BROAD SPECTRUM ANTIBACTERIAL ACTION IN VITRO. W.M. Shafer* and V.C. Onunka, Emory University School of Medicine, Atlanta, GA.
Human PMN lysosomal cathepsin exerts bactericidal activity in vitro against E. coli, Neisseria gonorrhoeae, and S. aureus. Since it is a candidate for nonoxidative killing of these bacteria within phagolysosomes we have sought to define the region(s) of the molecule responsible for bactericidal action. Toward this end we subcloned a 870 bp EcoRI fragment known to direct the synthesis of a full-length cathepsin G molecule. When present in lambda gt11 the translational product is fused to betagalactosidase. We placed this RI fragment behind the T7 promoter in pT7.6 and the plasmid construct (pWS2w introduced into E. coli CSH 734 bearing pGP1.2 that directs the synthesis of T7 RNA polymerase. Synthesis of recombinant cathepsin G was achieved after heat induction and treatment with rifampin. Recombinant cathepsin G (Mr=20 KDa) was obtained after soluble extracts were subjected to affinity chromatography. As little as 5 µg of recombinant cathepsin G per ml were sufficient to achieve an ED_{50} against the test bacteria.