PROTEASES IN BIOLOGICAL CONTROL AND BIOTECHNOLOGY

Dennis Cunningham and George Long

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Keynote Address

E0 THE VERSATILITY OF PROTEOLYTIC ENZYMES, Hans Neurath, Department of Biochemistry, University of Washington, Seattle, WA 98195.

The enzymatic hydrolysis of peptide bonds is one of the most important post-translational modifications of proteins. It accompanies the biosynthesis of proteins from its earliest stage, i.e. the release of signal peptides from the nascent polypeptide chain, to the ultimate protein degradation. Intermediate, obligatory stages of limited proteolysis include a wide spectrum of physiological processes, catalyzed by specific proteases. Versatility is generated by variations in the specificity and mechanism of action of the protease as well as by the interaction and communication of domains of multifunctional proteases with tissue-specific cofactors and inhibitors. An element of rational analysis can be introduced into the seemingly endless versatility of proteases by considerations of their domain structure and their biological evolution. An intrinsic aspect of such an analysis is the relation of the gene organization of the proteases to their molecular structure. This presentation will focus on these issues.

Hemostasis — I

E1 **THE MOLECULAR GENETICS OF FIBRINOGEN** Gerald R. Crabtree, Stanford University, Stanford, California 94301.

Although not a protease itself, fibrin polymerization and dissolution are initiated by thrombin and plasmin respectively, and protolytic cleavage is an essential element of fibrin function. The structure of the three fibrinogen genes demonstrates a remarkable degree of modeling of each fibrinogen gene since their initial duplication nearly 1 billion years ago. The only common structural features of these three genes are the demarkation of the coiled-coil domain of fibrinogen by intervening sequences. Thus the coiled-coil was part of the primitive fibrinogen precursor which existed nearly 1 billion years ago and is probably the most ancient and immutable structure of the fibrinogen genes. Interestingly, the COOH terminus of the coiled-coil is a site of protolytic cleavage by plasmin suggesting that these introns occur at exposed regions of the fibrinogen molecule.

The genes for the α, β and γ chains of fibrinogen are represented by a single copy in the human and rat genome. These three genes are clustered in a 60 kb segment on the long arm of human chromosome 4. During the acute phase response to injury, transcriptional rates for each of the three fibrinogen genes increases 6 to 12 fold. Despite the close linkage, each gene has its own transcriptional unit as well as an independent promoter. An analysis of the function of deletion mutants transfected into hepatic and non-hepatic cell lines indicates that a sequence between 50 and 130 base pairs upstream of the transcription initiation site is essential for transcription of the fibrinogen genes. This sequence does not appear to be involved with tissue-specific expression since a mutant containing the sequences downstream of -150 is expressed well in Chinese Hamster ovarian cells and 3T3 cells.

E2 REGULATION OF NATURAL ANTICOAGULANT PATHWAYS OF BLOOD COAGULATION. C.T. Esmon, K. Harris, P.C. Comp, N.L. Esmon, P.P. Nawroth, and D.M. Stern. Oklahoma Medical Research Foundation, Oklahoma City, OK 73104.

Protein C is the vitamin K-dependent precursor of the serine protease activated protein C (APC). Available clinical and basic studies suggest that APC is the terminal enzyme in a pathway involved in the regulation of blood coagulation. This anticoagulant activity is mediated through the proteolytic inactivation of Factors Va and VIIIa. The pathway consists of two major steps: 1) protein C activation, and 2) expression of anticoagulant activity. Both the activation and expression steps in the pathway are under control by several distinct mechanisms; activation involves complex formation between thrombin and an endothelial cell surface protein, thrombomodulin. Upon complex formation with thrombomodulin, the macromolecular specificity of thrombin is altered, inhibiting procoagulant reactions such as fibrinogen clotting and platelet activation, while enhancing protein C activation several thousand-fold. Thus, when thrombin is bound to thrombomodulin, the enzyme functions as an anticoagulant. Thrombomodulin expression is subject to regulation by a variety of agents. Interleukin 1 is especially effective at reducing thrombomodulin activity, suggesting that initiation of this anticoagulant system may be down-regulated during inflammatory responses. Expression of activated protein C anticoagulant activity involves a second vitamin K-dependent factor, protein S. Protein S is required for binding of activated protein C to either platelet or endothelial cell membranes with subsequent expression of the potential for rapid factor Va inactivation. Three separate regulatory mechanisms may contribute to the control of expression of APC anticoagulant activity. First, protein S is found both in plasma and in platelets where functional protein S is expressed after platelet activation. This suggests that platelet activation may facilitate expression of anticoagulant activity under some circumstances. Second, protein S exists in at least two forms in the plasma, free and in complex with C4b-binding protein, a regulatory protein of the complement system. This complex lacks anticoagulant activity and may play a role in regulating expression, since the distribution between free and bound protein S is often shifted in response to inflammatory stimuli. Third, protein S function requires the presence of cell surface binding sites. On aortic endothelium, these surface binding sites disappear in concert with thrombomodulin when cells are exposed to interleukin 1. Thus, recent advances suggest that this pathway plays a major role in controlling the hemostatic balance, and this balance can be perturbed by modulation of both humoral and cellular factors.

Hemostasis — II

E3 REGULATION AND CONTROL OF FIBRINOLYSIS, Désiré Collen, Center for Thrombosis and Vascular Research, University of Leuven, Belgium, and Departments of Biochemistry and Medicine, College of Medicine, University of Vermont, Burlington, VT 05405

Mammalian blood contains an enzymatic system capable of dissolving blood clots, which is called the fibrinolytic enzyme system. The fibrinolytic system comprises a proenzyme, plasminogen which can be converted to the active enzyme plasmin, which will degrade fibrin. Plasminogen activation is mediated by plasminogen activators which are classified as either tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Inhibition of the fibrinolytic system may occur at the level of the activators or at the level of generated plasmin.

Plasmin has a low substrate specificity and when circulating freely in the blood will degrade several proteins including fibrinogen, Factor V and Factor VIII. Plasma does however contain a fast-acting plasmin inhibitor, α_2-antiplasmin, which will inhibit free plasmin extremely rapidly (t½ : 0.1 s) but which reacts much slower (10^3-fold) with plasmin bound to fibrin. A "systemic fibrinolytic state" may however occur by extensive activation of plasminogen (conc. in plasma 2 µM) and depletion of α_2-antiplasmin (conc. in plasma 1 µM). Clot-specific thrombolysis therefore requires plasminogen activation restricted to the vicinity of the fibrin.

Two physiological plasminogen activators, t-PA and single chain u-PA (scu-PA) induce clot-specific thrombolysis, however via entirely different mechanisms. t-PA is relatively inactive in the absence of fibrin, but fibrin strikingly enhances the activation rate of plasminogen by t-PA. This is explained by an increased affinity of fibrin-bound t-PA for plasminogen and not by alteration of the catalytic efficiency of the enzyme. The high affinity of t-PA for plasminogen in the presence of fibrin thus allows efficient activation on the fibrin clot while no significant plasminogen activation by t-PA occurs in plasma. scu-PA has a high affinity for plasminogen (K_m = 0.3 µM) but a low catalytic rate constant (k_{cat} = 0.02 s^{-1}). scu-PA does however not activate plasminogen in plasma in the absence of a fibrin clot, due to the presence of a competitive inhibitor, identified as Gc-globulin. Fibrin-specific thrombolysis appears to be due to the fact that fibrin reverses the competitive inhibition, but this does not seem to occur via specific binding of scu-PA or Gc-globulin to fibrin. We have not found any synergism between scu-PA and t-PA for thrombolysis.

E4 PROTEOLYTIC REGULATION OF FACTOR VIII, David N. Fass, Section of Hematology Research, Mayo Clinic/Foundation, Rochester, MN 55905

Factor VIII participates in a number of interactions involved in its activation, inactivation, expression of its cofactor activity, and maintenance of its normal procofactor circulatory form. The proteins with which it interacts include: factor IXa, factor X, activated protein C and Willebrand factor. To function as a coenzyme, factor Xa or thrombin must activate factor VIII through proteolytic cleavage. Non-protein ligands comprise phospholipids and as yet undefined cell surface receptors on platelets and reportedly endothelial cells. The porcine factor VIII molecule as it is isolated contains an N-terminal heavy chain, 82 kD, and a C-terminal light chain, 76 kD. In circulation the factor VIII is bound to Willebrand factor through light chain. Activation by thrombin or Xa cleaves light chain and heavy chain which remain associated. It is thought that the factor VIII/Willebrand factor interaction is ablated by the thrombin or Xa proteolytic activation. The activated factor VIII appears to rapidly lose its activity unless the factor VIIIa is participating in a complete factor X activating complex with factor IXa, lipid, Ca^{++}, and factor X. The factor VIII light chain appears to mediate the lipid binding. The factor IXa and factor X binding sites in factor VIII are presently unknown. Activated protein C inactivates factor VIII by cleavage of the heavy chain. This inactivation cleavage does not occur at a physiological rate either in the absence of lipid or in the presence of lipid but in the absence of factor VIII light chain.

E5 THE FIBRINOLYTIC SYSTEM OF CULTURED ENDOTHELIAL CELLS, David J. Loskutoff, Scripps Clinic and Research Foundation, La Jolla, CA 92037

Abnormal thrombus formation and dissolution are associated with several cardiovascular diseases including atherosclerosis and both thromboembolic and hemorrhagic conditions. The walls of blood vessels undoubtedly contribute to the pathogenesis of these disorders. More specifically, vascular endothelial cells have been shown to influence both the coagulation and fibrinolytic systems. We have been studying cultured bovine aortic endothelial cells (BAEs) as a model of endothelium. The fibrinolytic activity of these cells results from the production of multiple, functionally distinct forms of plasminogen activator (PA), including both urokinase-like (u-PA) and tissue-type (t-PA) molecules. The overall fibrinolytic activity of these cells changes with their growth state, and in response to the presence of a variety of agents including activated protein C. Although it has been suggested that such changes reflect changes in PA, our recent and unexpected finding that these cells also synthesize an unusually stable fibrinolytic inhibitor, makes accurate interpretation of such results difficult. For example, do the altered fibrinolytic states in these cells following various treatments, or for that matter, in blood in certain human diseases, reflect changes in PA, inhibitor or both? The inhibitor is a major product of BAEs, accounting for 2-12% of the total protein secreted by the cells in a 24 hour period. It is a single-chain glycoprotein of M_r 50,000, isoelectric point 4.5-5.0, and migrates in the β1 region when analyzed by agarose zone electrophoresis. It is an antiactivator and can neutralize the activity of both u-PAs and t-PAs. Inhibition is associated with the formation of an enzyme-inhibitor complex that survives SDS-PAGE. Preliminary kinetic analysis of the interaction between the purified PAI and t-PA established an apparent K_{ass} of $5x10^7 M^{-1} sec^{-1}$ and a k_d of 10^{-14}M. The PAI is active after incubation in the presence of 0.1% SDS, or at pH 2.7, but is rapidly inactivated by H_2O_2 and other oxidants. Activity can be restored by treating the inactive PAI with methionine sulfoxide reductase suggesting that the loss of activity is caused by oxidation of a critical methionine residue. Interestingly, both a latent form of PAI (representing 95% of the total) and an active form (5%) can be detected in cell samples. The latent form can be converted into the active one by treatment with SDS and other denaturants. Antiserum to the BAE PAI removes the rapidly acting PAI activity from human platelets, serum, and plasma. These results indicate that the BAE PAI and the PAIs present in these blood samples are immunologically related, and suggest that the β1-PAI may be the physiologically relevant inhibitor of PA in the vascular system. The PAI gene has been cloned and sequenced. Its relation to other protease inhibitors will be discussed.

E6 EXPRESSION OF BLOOD CLOTTING ENZYMES ON NATURAL AND SYNTHETIC MEMBRANES, Kenneth G. Mann, Dept. of Biochemistry, University of Vermont, Burlington, VT 05405
The hemostatic process involves complex interactions of the blood vessel wall, the platelet and the blood coagulation factors. In concert, these components lead to localization, amplification, and modulation of the blood coagulation response. The interaction of vessel wall subendothelium with platelets and coagulation factors, leading to triggering and localization of the clotting reaction, is a subject of active investigation, and involves metabolic, as well as binding events. In association with these surface and cell-mediated processes, the activation of coagulation zymogens and the formation of protein-enzyme complexes lead to the rapid generation of procoagulant activity. Most of the enzymes involved in the process, and their precursors, have been isolated, and the nature of the specificity of the proteolytic cleavages involved in the blood coagulation reactions are well understood. More recently, progress has been made in the isolation and elucidation of the nonenzyme cofactor proteins which contribute to the regulation of the blood coagulation process. Four principal enzymatic complexes involving the vitamin K dependent proteins can be identified in the blood coagulation system. These include the "extrinsic" activator, Factor VIIa, and tissue factor; the Factor X activator, composed of Factor IXa, and the cofactor, Factor VIII; the "prothrombinase" complex, composed of Factor Xa and activated Factor V, and the protein C activator, composed of thrombin and thrombomodulin. In each of these complexes, calcium-mediated phospholipid interactions are central to complex formation. The significance of complex formation can be represented by analysis of the prothrombinase complex, since it has been the most extensively studied. Factor Xa is the enzymatic component of the prothrombinase complex, and functions by cleaving the prothrombin molecule at two locations. Factor V (as Factor Va), "phospholipid" and calcium ions serve as cofactors to the reaction, and at physiologic concentrations lead to an amplification in reaction rate by 300,000 fold. In plasma, Factor V circulates as a "pro" cofactor which can be activated by proteolytic cleavage to produce the active cofactor, Factor Va. Factor Va serves to bind Factor Xa to the "phospholipid" surface. The amplification in prothrombin conversion, which occurs as a result of cofactor interaction, results from both concentration of reagents and alteration in the efficiency of Factor Xa as an enzyme. Factor Va also serves to bind Factor Xa to the platelet membrane, and it is likely that the platelet membrane, as well as phospholipid surfaces exposed by damage of cells, provide for localization of this protein-enzyme complex. Once complex formation has occurred, in addition to amplificaton of the reaction rate, modulation of the reaction is obtained because of protection of complexed Factor Xa to inhibition by antithrombin III and protection of complexed Factor Va, to proteolytic inactivation by activated protein C.

Evolution and Structure-Function Relationships of Proteases

E7 THE THREE DIMENSIONAL STRUCTURES OF ASPARTIC PROTEINASES AND THEIR INHIBITORS: APPLICATIONS TO THE RENIN-ANGIOTENSINOGEN SYSTEM, Blundell, T.L., Foundling, S.I., Hemmings, A.M., Pearl, L.H., Sibanda, B.L., Watson, F.E., Cleasby, A. and Wood, S.P., Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, University of London, Malet Street, London, WC1E 7HX, UK.

High resolution X-ray analysis of the aspartic proteinase, endothia pepsin, has defined the active site in terms of two symmetrically arranged sequences each containing an aspartic (32 and 215) essential for activity and a well organized water structure. Complexes with angiotensinogen sequences (P6 to P3') containing reduced and hydroxylated analogues of the scissile peptide bond (P1 -P1') (synthesized by Dr. M. Szelke and co-workers) have been studied by X-ray difference Fouriers. They demonstrate hydrogen bonds between enzyme and inhibitors, and close complementarity between sidechains and specificity pockets.

These X-ray studies have been used as a basis for modelling human and mouse renins and their interactions with substrate angiotensinogens. Their implications for designing effective inhibitors for human renin which may have applications as an antihypertensive will be discussed.

E8 GLANDULAR KALLIKREINS AS GROWTH FACTOR PROCESSING ENZYMES: STRUCTURAL AND EVOLUTIONARY CONSIDERATIONS, Ralph A. Bradshaw, Joan C. Dunbar and Paul J. Isackson, Department of Biological Chemistry, California College of Medicine, University of California, Irvine, CA 92717

Serine proteases are known to play a regulatory role in a broad range of biological activities including digestion, blood clotting and prohormone processing. Some, such as trypsin, have a rather general activity in terms of substrates utilized while others have a much more limited spectrum of substrates, reflecting their regulatory functions even though their bond cleavage specificities are similar. A family of serine proteases exemplifying this category are the glandular kallikreins. In the mouse submandibular gland, at least 30 members are present with approximately 80% homology to each other. Two of these kallikreins are associated with β-nerve growth factor (β-NGF) in a high molecular weight complex (7S) that is secreted in large amounts in the saliva. The γ-subunit is an active arginine esteropeptidase which is bound to the C-terminal arginine residue of mature β-NGF. It apparently removes a dipeptide, Arg-Gly, before forming this complex and has been touted as the agent responsible for processing some 20 kDa of amino terminal protein characterizing the β-NGF prohormone. cDNA cloning and sequence analysis of the α-subunit demonstrated that it is also a member of this family with greater whan 80% homology to the γ-subunit. However, several novel changes characterize the α-subunit sequence that, by comparison with the sequences and structures of other serine proteases, should render it catalytically inactive. These include an Arg→Gly change that prevents cleavage of the activation peptide, the deletion of the highly conserved N-terminal I/V-I/V-G-G sequence, and the change of two critical amino acids near the active site. Indeed, there is a complete lack of detectable catalytic activity and the protein is not modified by diisopropyl fluorophosphate. The mechanism by which the γ-subunit recognizes NGF is unknown. In contrast to the mouse submandibular gland, the guinea pig prostate, which also produces large amounts of β-NGF, contains only one form of glandular kallikrein which does not appear to be associated with NGF although it is highly homologous to γ-NGF and other glandular kallikreins. There is apparently no α-subunit either. These findings suggest that β-NGF is not released from pro β-NGF by these soluble kallikreins as has been suggested previously. Supported by USPHS research grant NS19964.

E9 STRUCTURE-FUNCTION RELATIONSHIPS IN THE REFOLDING OF SERINE PROTEINASES, Albert Light, Jeffrey N. Higaki, Chester Duda, and Thomas W. Odorzynski, Department of Chemistry, Purdue University, West Lafayette IN 47907

We study the refolding of pancreatic serine proteinases because these are the smallest members of the class, and the amino acid sequences, disulfide pairing, enzymatic properties, and three-dimensional structure are now known in great detail. We refold fully reduced bovine trypsinogen and chymotrypsinogen as the mixed disulfide derivative of glutathione with yields of 50% and half-times of 60-75 min at 4°C. Trypsinogen with one and two disulfides missing, because of a prior limited reduction and alkylation, still refolded with the same yield but took two and four times longer, respectively. We concluded that these two disulfides were nonessential in stabilizing intermediates and that more than one folding pathway could be followed. The values of the hydrodynamic volumes of intermediate species in the refolding of trypsinogen suggested that non-native disulfides dominate until the final compact structure of the zymogen is reached. Both neochymotrypsinogen and the corresponding neotrypsinogen (two-chain structures with Tyr 146-Thr 147 cleaved), as the mixed disulfide derivative of the purified fragments, refolded with 25% and 10% yields, respectively. The rates of formation of the regenerated zymogens (activatable molecules) and the regain of the original molecular weight of 25000 (SDS-gel electrophoresis) were the same, giving strong support to the hypothesis for independent folding of the serine proteinase domains (polypeptide fragments).

Proteases in Development, Cancer and Emphysema — I

E10 INTERACTIONS OF SERINE PROTEASES WITH CULTURED FIBROBLASTS,
Dennis D. Cunningham, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA 92717

The mechanisms by which serine proteases interact with cells is of great interest in view of their ability to regulate key cellular activities. For example, certain serine proteases can promote division of cells or regulate cell migration either directly or by altering their attachments to each other and to their substratum. Moreover, proteolysis has been implicated in cellular and extracellular matrix damage associated with several pathological conditions. Our studies on the interactions of several serine proteases with cultured fibroblasts have revealed binding mechanisms that differ from the usual pattern of reversible binding to cell surface sites followed by internalization and degradation.

Thrombin and elastase specifically bind to sites on the surface of cultured fibroblasts; neither protease is detectably internalized after this binding. However, the mechanism of binding is different for each protease. Thrombin, as well as DIP-thrombin, binds reversibly with a Kd of 10^{-9} M to cell surface sites with an Mr = 150,000. In contrast, elastase, but not DIP-elastase, binds irreversibly to an integral membrane component and forms a covalent complex of Mr = 54,000. The linkage to elastase appears to involve an ester bond with its catalytic site serine.

The interactions of a number of regulatory serine proteases with cultured fibroblasts are mediated by three protease nexins (PN-1, PN-2 and PN-3). The PNs are protein protease inhibitors that are synthesized and secreted by a variety of cultured cells. They form complexes with certain proteases in the extracellular environment; the complexes bind back to the cells and are rapidly internalized and degraded. The PNs seem ideally suited for regulating serine proteases at and near the cell surface. For example, the reaction between thrombin and PN-1 is accelerated by the surface of fixed fibroblasts. This acceleration appears to involve the thrombin binding sites, mentioned above, as well as cell surface or extracellular matrix glycosaminoglycans.

E11 THE ROLE OF PLASMINOGEN ACTIVATOR IN TUMOR INVASION AND METASTASIS.
Liliana Ossowski, Lab. of Cellular Physiology and Immunology, Rockefeller University, New York, N.Y. 10021. Elaine L. Wilson, Dept. of Clinical Science and Immunology, Cape Town Medical School, Observatory, Cape Town, South Africa.

We have used human carcinoma - HEp3 to test whether plasminogen activator produced by this tumor is required for invasion and metastasis. HEp3 grows and metastasizes efficiently in 2 different hosts: the chick embryo and the nude mouse. The kinetics of growth and metastasis is quantitatively predictable, enabling the detection of even small effects by exogenous factors. We tested the effect of antibodies to plasminogen activator (PA), which specifically block the catalytic activity of human urokinase type PA, by administering the antibodies to each of the two different, tumor bearing hosts - the chick embryo and the nude mouse. In the chick embryo the antibodies did not affect tumor growth at the primary site - the chorioallantoic membrane - but strongly inhibited metastasis to the lungs (1). In the nude mouse the antibodies reduced local invasiveness (as determined by histology and the rate of local tumor recurrence) and reduced metastasis to lymph nodes.

To identify the stages of metastasis in which PA produced by the tumor might play a role, we have further explored the HEp3 - chick embryo system. We arbitrarily separated the process into 3 steps: the invasion of CAM mesenchyme, the entry into blood vessels and the exit from the blood vessels into distant organs. In this series of experiments, the catalytic activity of the tumor PA was blocked by anti-PA antibodies while the PA synthesis was down-regulated by DMSO (2). To examine the role of PA in the invasion of CAM (early stage), we modified the chorioallantoic membrane in such a way as to permit invasion to occur either easily or with difficulty. The results of these experiments will be discussed in the context of our findings in nude mice.

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E12 PROTEINS REGULATING CELLULAR INVASION, Daniel B. Rifkin, New York University Medical School, New York, NY 10016.

In the initial stages of angiogenesis, endothelial cells display several properties in common with invasive tumor cells as they penetrate through the surrounding basement membrane and move into new tissue spaces. Therefore, we postulated that certain initiators of angiogenesis should induce the production of specific proteinases in endothelial cells. We have purified two molecules; one from human placenta and one from a human hepatoma cell line each of which induces the synthesis of plasminogen activator and collagenase from capillary endothelial cells at 1 ng/ml. Addition of these stimulators of protease production induces increased cell division and motility at concentrations of 0.1 - 1 ng/ml. We have also found that several proteinase inhibitors block the movement of invasive cells through a basement membrane. These include inhibitors of metallo and serine proteinases.

Proteases in Development, Cancer and Emphysema — II

E13 PROPERTIES AND ACTIONS OF PROTEASE NEXIN I, Joffre B. Baker, Barbara L. Bergman, Anil Bajpai and Robert Gronke, Department of Biochemistry, University Kansas, Lawrence, KS 66045

Human foreskin fibroblasts synthesize and secrete several novel serine proteinase inhibitors, called protease nexins (PNs), which both form SDS-resistant complexes with their proteinase targets and mediate their cellular uptake and degradation. PN secretion could provide a mechanism for specific cellular regulation of extracellular proteinases at or near cell surfaces. PNI is the first of the PNs to be purified. Although the sequence of 30 amino acid residues at its N-terminus is not homologous to sequences in other known proteins, several of its properties indicate that it belongs to the anti-thrombin III/α-1-proteinase inhibitor family (1). In cultures of foreskin fibroblasts secreted PNI desensitizes the cells to mitogenic stimulation by thrombin and regulates the action of secreted fibroblast urokinase. Fibroblasts secrete urokinase in the single chain (fibrin-dependent) form. This does not interact with PNI. However, on exposure to exogenous plasmin it is converted to 2-chain (fibrin-independent) urokinase. The latter is rapidly inhibited by PNI (kassoc ~2×10^5 M^{-1} S^{-1}). Recently we have detected on fibroblasts cell surface binding sites for 2 chain urokinase. Most of these sites are cryptic, apparently because they are occupied by endogenous urokinase. ^{125}I-urokinase is neither internalized nor rapidly released, and is inaccessible to PNI at concentrations of the inhibitor at or above the concentrations of PNI in fibroblast culture medium. The combined properties of PNI and the urokinase binding sites may serve to localize urokinase activity to the cell surface.

The anti-urokinase activity of PNI suggests that it could protect tissue matrices from degradation mediated by this plasminogen activator. Human fibrosarcoma cells rapidly destroy vascular smooth muscle extracellular matrices *in vitro*. This process, which is largely mediated by secreted urokinase, is significantly inhibited by 2 nM PNI and virtually completely inhibited by 0.2 μM PNI (2).

PNI inhibits thrombin about 80 fold more rapidly than do the major plasma thrombin inhibitors, but it is at such a low concentration in plasma ($\leq 1 \times 10^{-11}$ M) that it is not a significant plasma thrombin inhibitor unless it is localized at a site of thrombin activation or action. Intriguingly, platelets carry a PNI-like factor on their surfaces. At or below nM concentrations of thrombin this factor accounts for a large fraction of the specific binding of thrombin to platelets.

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E14 THE RECEPTOR FOR THE UROKINASE PLASMINOGEN ACTIVATOR. Francesco Blasi and Patrizia Stoppelli, International Institute of Genetics and Biophysics, CNR, via Marconi 10, 80123, Naples, Italy.

Normal human monocytes and several human cell lines express a receptor for the urokinase-type of plasminogen activator (uPA). The properties of this receptor are: it binds uPA with an affinity of about 10^{-10}M, does not internalize the ligand, does not interefere with the enzymatic activity of uPA,which is concentrated at the cell surface, and is regulated by differentiation signals. Cross-linking experiments show that the receptor is a molecule of about 40,000 dalton under both reducing and non-reducing conditions.

uPA is synthesized as an inactive single-chain pro-uPA which can be activated by proteolytic digestion into a two-chains uPA. The receptor binds both uPA and pro-uPA with about the same affinity. Active uPA can be fragmented into a 33,000 dalton active C-terminal portion and a 17,000 dalton amino terminal fragment (ATF), deprived of activity. The receptor binds ATF but not the 33,000 dalton uPA form. Thus the amino acids sequence responsible for receptor binding is located on the ATF, and is thus separated from the catalytic portion.

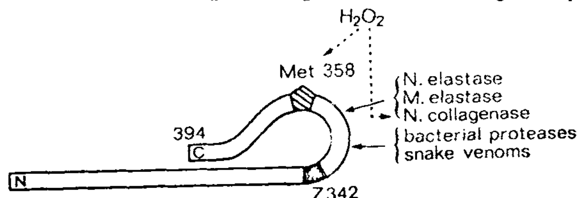
Human monocytes and cell lines that express the uPA receptor do not synthesize uPA, or do so at a rather low rate. Human A431 cells, which synthesize high levels of uPA in the pro-uPA form, do not bind uPA. Immunofluorescence and surface iodination studies, however, show that these and other cells possess a surface-bound pro-uPA. A short acid treatment of A431 cells uncovers uPA binding sites with the above-described properties, and dissociates surface pro-uPA. Kinetic studies show that secretion of pro-uPA by A431 cells precedes receptor binding.

uPA activity has been proposed by several investigators to be required for migration of both normal and malignant cells. The discovery of uPA receptors in both monocytes and tumor cells is in agreement with this proposal, and provides a biochemical mechanism. Receptors may be needed either to concentrate the uPA enzymatic activity on the cell surface to ensure that only receptors-expressing cells benefit of the uPA action; alternatively, they may be needed to provide the site of activation of the inactive pro-uPA; finally, the uPA receptors may confer to uPA a resistance to circulating uPA inhibitors. The saturation of all available sites in A431 cells is rather puzzling. This mayrepresent an important component of the malignant phenotype. The concomitant occurrence of unregulated synthesis of uPA and expression of the uPA receptor may provide malignant cells with a localized enzymatic function required to migrate and invade neighbor tissues, accounting for at least part of the malignant phenotype.

E15 α1-ANTITRYPSIN, THE SERPINS AND TISSUE DAMAGE, Robin W. Carrell, Molecular Pathology Laboratory, Clinical School of Medicine (University of Otago), Christchurch Hospital, Christchurch, New Zealand

α1-Antitrypsin is the archetype of a family of serine proteinase inhibitors (serpins)[1] that share a common, highly ordered, tertiary structure[2]. A dominant feature is a six stranded β-sheet, one strand of which is exposed to form an external loop containing the reactive centre. Variations in the single amino acid at the reactive centre explain the primary differences in inhibitory activity of the serpins.

The molecular pathology of the common S and Z variants of α1-antitrypsin is due to the loss of salt bridges. Z-mRNA in cell-free and surrogate oocyte studies, undergoes normal translation and processing to just prior to entry to the Golgi[3a,b]. Here aggregation occurs and only 15% is secreted. The physiological target of α1-antitrypsin is neutrophil elastase and homozygous deficiency therefore predisposes to cumulative damage to lung elastin, resulting in emphysema.



The exposed reactive centre loop of the serpins is vulnerable to proteolytic cleavage. This provides an incidental molecular switch which allows irreversible interaction of the inhibitors. This has been utilized by reptile and bacterial proteases and also explains the observed inactivation of the plasma serpins that occurs in the massive elastase release of the shock syndromes[4].

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Proteases in Biological Control and Biotechnology

E16 PROTEINASE-PROTEINASE INHIBITOR IMBALANCE IN THE DEVELOPMENT OF PULMONARY EMPHYSEMA, James Travis, Department of Biochemistry, University of Georgia, Athens, Georgia 30602

Proteolytic events resulting in the activation of several biological systems, including coagulation, fibrinolysis, complement activation, and protein catabolism, are normally under the control of both locally produced and circulating proteinase inhibitors. This balance can be perturbed, however, resulting in uncontrolled proteolysis, tissue damage, and possibly death. At least four possible means of disturbing the balance between proteinase and proteinase inhibitors are known. These include a), genetic defects in inhibitor synthesis, b), excessive production of tissue proteinases, c), enzymatic inactivation of inhibitors by specific proteinases, and d), chemical inactivation by modification of critical amino acid residues of the inhibitor. The best example of inhibitor imbalance can be found in the system controlling the activity of neutrophil elastase, a powerful enzyme which can degrade elastin, collagen, and proteoglycan. This enzyme is the target for the circulating plasma protein referred to as α-1-proteinase inhibitor (α-1-PI), and it is well known that its decreased production can be correlated with the development of familial emphysema. However, this genetic defect can only explain the onset of the disease in 3% of individuals affected, and other mechanisms must, therefore, also be responsible for its development. Data from this and other laboratories indicate that cigarette smoking causes massive recruitment of phagocytic cells to the lung, resulting in the excessive production of a), proteolytic enzymes which may titrate all of the active α-1-PI in the vascular bed of this organ, b) non-serine proteinases which use α-1-PI as a virtual substrate and inactivate the protein by cleavage either at or near the reactive site, and c), powerful oxidizing agents which convert the reactive site methionyl residue of -1-PI into the methionyl sulfoxide derivative, resulting in a severe reduction in the affinity of the modified inhibitor for elastase. Site specific mutation of methionine to valine at the reactive site of α-1-PI, using recombinant DNA technology, has resulted in the production of an oxidation resistant, elastase specific inhibitor. This could prove therapeutically useful if, as suspected, oxidation plays a major role in the reduction of normal inhibitor activity during phagocytosis.

Protein Secretion and Signal Peptidases

E17 SIGNAL PEPTIDE CLEAVAGE MUTANTS OF YEAST INVERTASE
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Eukaryotic cells consist of many functionally distinct subcellular compartments, each containing its unique subset of proteins. During cell growth many newly synthesized polypeptides have to be transported from their site of synthesis to diverse locations inside or outside the cell. Most of the proteins destined for secretion are initially made with an NH_2-terminal extension, the signal peptide. Translocation of these preproteins into the lumen of the endoplasmic reticulum is accompanied by removal of the signal peptides. Not much is known about the enzyme(s) that mediate(s) this proteolytic cleavage in eukaryotic cells.

The importance of signal peptide cleavage was addressed by constructing yeast invertase (SUC2) mutations that interfere with signal peptide processing. Oligonucleotide-directed mutagenesis was applied to alter the signal peptide cleavage site of wildtype invertase from ala-ser (SUC2) to ile-ser (suc2-s11), val-pro (suc2-s12) or ala-pro (suc2-s13). All three mutations display a similar phenotype:

(1) mutant invertase accumulates inside the yeast cell

(2) the rate of mutant invertase secretion is more than 50-fold slower than wildtype invertase

(3) the secreted periplasmic mutant invertases are underglycosylated and

(4) their signal peptides are still attached upon arrival in the periplasm.

We have chosen the mutant invertase gene suc2-s12 to identify extragenic suppressor mutations that allow improved secretion of s12-invertase. Since s12-invertase is secreted, albeit slowly, direct selection for trans-acting suppressor mutations has not been successful. Consequently, we have developed a screening procedure that involves an invertase activity overlay plate stain. So far, a screen of ten million colonies identified two plasmid-unlinked suppressor mutations. One specifically improves the secretion rate of s12-invertase. Cells carrying the second suppressor mutation exhibit faster transport rates and increased levels of secreted mutant and wildtype invertase.

E18 EFFORTS TOWARD PURIFICATION OF HEN OVIDUCT SIGNAL PEPTIDASE, Mark O. Lively and R. Keith Baker, Department of Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103

Tubular gland cells from the magnum region of oviducts from laying hens synthesize and secrete as much as one gram of egg white protein per day. These cells are an excellent source of rough endoplasmic reticulum (RER) and microsomes prepared from them contain an active signal peptidase that correctly processes nascent secretory proteins in vitro. Hen oviduct signal peptidase (HOSP) is an integral membrane protein in the RER (1) that can be solubilized in an active form using a variety of detergents including Nonidet P-40 (NP-40), sodium deoxycholate, or octyl-β,D-glucopyranoside. Enzymatic activity is detected by post-translational proteolysis of the signal peptide from the precursor of human placental lactogen prepared by cell-free protein synthesis. While two prokaryotic signal peptidases have been isolated from E. coli (2,3), complete purification of a corresponding eukaryotic enzyme has not yet been described.

We have developed a protocol for partial purification of HOSP. Seventy to eighty milliliters of sedimented, crude RER microsomes are obtained from 20 hens. Treatment of these membranes with 0.1 M sodium carbonate, pH 11.5, removes 90% of the associated protein without apparent loss of HOSP activity. The resulting vesicles contain only integral membrane proteins, including HOSP, and the majority of these proteins are solubilized in 2.5% (w/v) NP-40 at pH 8.2. This represents approximately 2 grams NP-40 per gram membrane protein. Detergent-solubilized HOSP is active from pH 5 to 10.

Chromatography of solubilized HOSP on DEAE-cellulose at pH 8.2 results in the removal of more than 70% of contaminating proteins. HOSP is recovered in the unretained fraction, dialyzed to pH 9.4 and applied to a chromatofocusing column equilibrated at that pH. The peptidase binds to this column and is eluted between pH 8.3 and 8.0 in a decreasing pH gradient. When fractions from the focusing column are examined by polyacrylamide gel electrophoresis in the presence of SDS, signal peptidase activity seems to correlate with a protein of M_r 33,000 among fewer than 20 other proteins present. We estimate that this protocol yields at least a 650-fold purification of HOSP from crude microsomes. (This work is supported by National Institutes of Health grant GM 32861.)

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E19 BACTERIAL LEADER PEPTIDASE: STRUCTURE, FUNCTION AND BIOGENESIS, Paul B. Wolfe, Department of Biological Chemistry, University of Maryland at Baltimore, School of Medicine, Baltimore, MD 21201

Bacterial leader (signal) peptidase, the enzyme responsible for removal of amino-terminal leader (signal) peptides from precursors of secreted and membrane proteins, has been purified to homogeneity from overproducing strains (1). Structural studies have shown that this enzyme spans the inner membrane with a small amino-terminal domain exposed on the cytoplasmic surface and a large carboxyl-terminal domain exposed to the periplasm (2). Models of the orientation of leader peptidase in the membrane suggest a possible mechanism for its unique substrate specificity.

Sequence analysis and peptide mapping have revealed that the peptidase assembles into the membrane without removal of a leader (signal) sequence (2). In vivo studies of the biogenesis of leader peptidase indicate that its insertion into the membrane requires the electrochemical potential, can occur post-translationally, and is accompanied by a conformational change (3). Furthermore, proper assembly of the peptidase requires the products of the secA and secY (prlA) genes. These loci have been shown to be essential for the correct localization of secreted and outer membrane proteins. Thus, leader peptidase, a membrane protein without a cleaved leader peptide, uses the same export pathway as pre-secretory proteins (4).

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Intracellular Protein Processing

E20 THE UBIQUITIN- AND ATP- DEPENDENT PATHWAY FOR INTRACELLULAR PROTEIN DEGRADATION.
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The dynamic turnover of cellular proteins has been recognized for a long time, however the underlying biochemical mechanisms involved in the selective degradation of intracellular proteins have begun to be elucidated only recently. A major fraction of the selective nonlysosomal protein breakdown appears to be carried out by the ubiquitin- and ATP-dependent proteolytic pathway. Ubiquitin, an abundent 76 amino acids residues polypeptide, is covalently linked via an isopeptide bond to a variety pf proteolytic substrates in an ATP requiring reaction. One or more ubiquitin polypeptides are conjugated at their C-terminal glycine residues to free ε-amino groups of lysines in the substrate polypeptide. Formation of a ubiquitin-protein conjugate may be the initial event in the degradation of the substrate. Immunochemical analysis of the turnover of ubiquitin-protein conjugates further corroborated the notion that ubiquitin conjugation to the substrate is an obligatory intermediate step in the degradation of the substrate. We have chracterized the enzymatic steps involved in the formation and degradation of ubiquitin-protein conjugates(for reviews see references 1,2). Recently we have characterized a cell-cycle arrest mutant with a thermolabile ubiquitin activating enzyme (3). The cells are consequently thermosensitive to selective degradation of short lived and abnormal amino acids analogs containing proteins (4). Studies on the regulation of the ubiquitin mediated pathway revealed that tRNA is an essential comopnent of the system and is required for conjugation of ubiquitin to specific proteolytic substrates(5). In nuclei of eukaryotic cells, ubiquitin is found conjugated to an internal lysine in the H2A histone molecule, forming a ubiquitin-H2A semihistone conjugate (uH2A). uH2A occurs in a subset of nucleosomes where it substitutes one or both of the nucleosomal H2A histone molecules. It occurs preferentially in nucleosomes at the 5' end of transcribed genes and is probably involved in regulation of gene expression at the chromatin structure level (6).

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E21 SITE-SPECIFIC MUTAGENESIS OF cDNA CLONES EXPRESSING A POLIOVIRUS PROTEINASE, Bert L. Semler, Victoria H. Johnson, Patricia G. Dewalt and Mary Frances Ypma, Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, CA 92717

Poliovirus, like all other members of the picornavirus family of animal viruses, contains a single strand RNA genome of messenger polarity (i.e., plus-stranded) that encodes a giant precursor polyprotein. The precursor polyprotein is proteolytically cleaved to produce all of the known viral-specific proteins. The cleavage of polio precursor polypeptides occurs at specific amino acid pairs that are recognized by viral and possibly host cell proteinases. Most of the polio-specific cleavages occur at glutamine-glycine (Q-G) pairs that are recognized by the viral-encoded proteinase, 3C (formerly called P3-7c). In order to carry out a defined molecular genetic study of the enzymatic activity of protein 3C, we have made cDNA clones of the poliovirus genome. The cDNA region corresponding to protein 3C was inserted into an inducible bacterial expression vector. This recombinant plasmid (called pIN-III-C3-7c) utilizes the bacterial lipoprotein promoter to direct the synthesis of a precursor polypeptide that contains the amino acid sequence of protein 3C as well as the amino- and carboxy-terminal Q-G cleavage signals. These signals have been previously shown to allow autocatalytic production of protein 3C in bacteria transformed with plasmid pIN-III-C3-7c. We have taken advantage of the autocatalytic cleavage of 3C in a bacterial expression system to study the effects of site-specific mutagenesis on its proteolytic activity. One mutation that we have introduced into the cDNA region encoding 3C is a single amino acid insertion near the carboxy-terminal Q-G cleavage site. The mutant recombinant plasmid (designated pIN-III-C3-μ10) directs the synthesis of a bacterial-polio precursor polypeptide like the wild type construct (pIN-III-C3-7c). However, unlike the wild type precursor, the mutant cannot undergo autocatalytic cleavage to generate the mature proteinase 3C. Rather, the precursor is able to carry out cleavage at the amino-terminal Q-G site but not at the carboxy-terminal site. Thus we have generated an altered poliovirus proteinase that is still able to carry out at least part of its cleavage activities but is unable to be a suitable substrate for self-cleavage at its carboxy-terminal Q-G pair. We have recently substituted the cDNA from pIN-III-C3-μ10 into a plasmid containing a full-length cDNA copy of the poliovirus genome (plasmid pEV104). We have previously shown that pEV104 will produce infectious poliovirus after transfection into cultured primate cells. The effect of the above mutant proteinase on the production of infectious poliovirus is presently being tested in several eukaryotic vectors following transfection into cultured human and monkey cell lines.

E22 MOLECULAR ARCHITECTURE OF A HORMONE PRECURSOR-CONVERTING ENZYME,

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Endoproteolytic cleavage of precursors at pairs of basic amino acids, especially -LysArg-, is a ubiquitous event in the production of peptide hormones and neurosecretory peptides. We have shown (1) that the product of the KEX2 gene of the yeast Saccharomyces cerevisiae is a novel endopeptidase specific for cleaving substrates on the carboxyl side of pairs of basic residues and is required for the production of secreted peptides whose maturation involves scission at -LysArg- sites (e.g. the mating pheromone precursor, prepro-α-factor). We cloned the KEX2 gene, determined its entire nucleotide sequence, and used expression vectors containing the galactose-inducible GAL1 promoter to overproduce the KEX2 protein in yeast 200-500-fold (2,3). The enzyme has been partially purified from such a source. Striking features of the KEX2 enzyme are its relatively large apparent MW, its tight membrane association, and the absolute dependence of its catalytic activity on Ca^{2+} (2,3). Although the KEX2 endoprotease is apparently a thiol enzyme with certain similarities to the Ca^{2+}-dependent neutral thiol proteases of animal cells (so-called calpains), it can be distinguished from calpains and other thiol proteases by virtue of its resistance to inactivation by derivatives of the trans-epoxysuccinic acid inhibitor, E64, and by cystatin C. In contrast, H_2N-Ala-Lys-Arg-chloromethylketone (ALACK) is a potent irreversible inhibitor. [^{125}I]Tyr-ALACK covalently tags a ~100,000 MW polypeptide that copurifies with KEX2 enzyme activity. Labeling displays the characteristics of the catalytic activity (e.g. requires Ca^{2+}) and a large C-terminal deletion of the KEX2 gene constructed in vitro produces a shorter labeled species in vivo, establishing that the KEX2 gene encodes the protease. The primary structure of the 814-residue KEX2 polypeptide deduced from the DNA sequence includes two markedly hydrophobic regions (a potential signal sequence at the N-terminus and a putative transmembrane domain near the C-terminus), a cysteine-rich region, several consensus sites for addition of Asn-linked oligosaccharide, a Ser/Thr-rich domain, and potential Ca^{2+}-binding sites. In tunicamycin-treated cells or after digestion with endo H, the [^{125}I]Tyr-ALACK-labeled band is smaller (~91,000 MW), indicating that the native enzyme does carry N-linked oligosaccharide. The properties of a series of C-terminal deletions suggest that the putative transmembrane domain is not solely responsible for the membrane localization of the enzyme and that the Ser/Thr-rich region may be modified extensively with O-linked sugars. Cells in which the KEX2 gene is deleted are still viable.
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Complement Activation and Chemotaxis

E23 COMPLEMENT FACTORS AND LEUKOCYTE ACTIVATION, Tony E. Hugli, Member, Department of Immunology, Scripps Clinic and Research Institute, San Diego, CA. 92037

Bioactive fragments C3a, C4a and C5a are derived from complement components C3, C4 and C5 and have been chemically characterized as a family of molecules. These hormone-like factors activate leukocytes via surface receptor-mediated mechanisms. It is generally recognized that C3a and C4a interact most strongly with basophils and eosinophils while the factor C5a promotes neutrophil and monocyte activation. Leukocyte activation by the anaphylatoxins includes such behavioral responses as chemotaxis, aggregation and adherence phenomena. Biochemical events occurring inside the activated cells include enhanced oxygen metabolism (e.g. oxygen burst), granular secretion, calcium mobilization, enhanced lipid metabolism and membrane remodelling events such as receptor up-regulation. Together the cellular reactions to these humoral effector molecules define an important host defense response. Although the anaphylatoxins (e.g. C3a, C4a and C5a) share chemical features and have a common genetic origin, the cellular receptors to these factors are distinct membrane components that lead to differential biologic activities. For example, C3a and C4a share common mast cell and basophil receptors while C5a interacts with a unique population of leukocyte receptors. Under physiologic conditions, C5a binds avidly to a neutrophil receptor and the ligand is subsequently internalized and degraded inside the cell. Internalization of the receptor-ligand complex removes receptors from the cell surface rendering the cell insensitive to the ligand, thereby modulating or "down regulating" the functional response. Preliminary indications are that C3a (C4a) interacts with mast cell or basophil receptors and stimulates a cellular response without eliciting internalization of the receptor-ligand complex.

The C5a receptor on human neutrophils has been identified as a 40-44,000 M.W. single chain component of the neutrophil membrane. Labelled C5a (^{125}I) can be cross-linked to the leukocyte receptor using bifunctional reagents and the complex has been isolated and characterized. A corresponding study of the putative C3a receptor on rat mast cells has led to some rather remarkable results. Human or rat C3a can be cross-linked to a 20-25,000 M.W. component on the mast cell surface; however the ligand is rapidly proteolysed during binding even at 0°C. C3a degradation is not a post internalization event but rather represents enzymatic conversion operative on the cell surface. When the protease inhibitor SBTI is added to the mast cells prior to offering ligand (C3a), ability to cross-link the C3a to a membrane component is lost suggesting the putative "receptor" on mast cells may in fact be a protease. As comparisons of cellular receptors to anaphylatoxins on various cell types continue it will eventually be possible to identify whether major biochemical differences exist between anaphylatoxin receptors on mast cells, leukocytes and monocytes.

E24 TRANSDUCTION MECHANISMS OF CHEMOATTRACTANT RECEPTORS ON LEUKOCYTES: RECEPTOR-MEDIATED ACTIVATION OF A POLYPHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C BY A GUANINE NUCLEOTIDE REGULATORY (N) PROTEIN, Ralph Snyderman, Charles D. Smith and Margrith W. Verghese
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Chemoattractants stimulate several important responses in polymorphonuclear leukocytes (PMNs) including directed motility, activation of the respiratory burst, and initiation of lysosomal enzyme secretion. Specific receptors for the chemoattractants C5a, leukotriene B_4 and N-formylated oligopeptides, e.g. fMet-Leu-Phe, are present on the surface of PMNs. The binding characteristics of fMet-Leu-[^{3}H]Phe to PMN membranes indicated that its receptor exists in high and low affinity forms which are interconvertible by guanosine di- and triphosphates, suggesting that this receptor interacts with an N protein. Although isolated PMN membranes have functional N proteins and adenylate cyclase activity, fMet-Leu-Phe neither stimulated nor inhibited cyclase activity. Incubation of PMNs with fMet-Leu-Phe led to the rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and production of inositol trisphosphate (IP_3), indicating that occupancy of the oligopeptide chemoattractant receptor activates a phospholipase C producing IP_3 and 1,2-diacylglycerol. These products elevate intracellular [Ca^{2+}] and activate protein kinase C, respectively. We postulated that chemoattractant receptors might use an N protein to activate polyphosphoinositide degradation. To test this hypothesis, we utilized <u>Bordetella pertussis</u> toxin (IAP), which ADP-ribosylates and inactivates certain N proteins. Treatment of PMNs with IAP selectively inhibited fMet-Leu-Phe-elicited responses, including PIP_2 hydrolysis, IP_3 production, Ca^{2+} mobilization, lysosomal enzyme secretion, superoxide production and chemotaxis. Similar responses induced by lectins, Ca^{2+} ionophores, or active phorbol esters were unaffected by IAP. Thus, an IAP-sensitive N protein appears to couple occupancy of the fMet-Leu-Phe receptor to activation of a phospholipase C. To further define the mechanism of this coupling, we developed a system to study receptor-mediated PIP_2 metabolism in plasma membranes isolated from human PMNs. PIP_2 hydrolysis was stimulated by fMet-Leu-Phe only if GTP was also present. In addition, nonhydrolyzable analogs of GTP, which can directly activate N proteins stimulated PIP_2 hydrolysis. In contrast to those from control PMNs, membranes isolated from cells treated with IAP did not demonstrate fMet-Leu-Phe plus GTP-induced PIP_2 hydrolysis. The activated N protein appears to activate the phospholipase C by reducing its Ca^{2+} requirement from superphysiological to intracellular concentrations of Ca^{2+}. Elevation of intracellular cAMP levels by increased [Ca^{2+}] appears to provide feedback signals which terminate cellular activation by inhibiting PIP_2 hydrolysis. This model for cellular regulation appears to be applicable to other chemoattractant receptors (e.g. C5a), and perhaps other receptors which elevate intracellular [Ca^{2+}] through stimulated PIP_2 hydrolysis.

E25 ELUCIDATION OF A SEQUENCE IN THROMBIN WITH GROWTH FACTOR PROPERTIES, George D. Wilner, Rachel Bar-Shavit, and Arnold J. Kahn, Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110, and Pediatric Research Institute, St. Louis University School of Medicine, St. Louis, MO 63104
In contrast to fibroblasts, the exposure of G_0/G_1-arrested J774 cells, a murine macrophage-like tumor cell line, with either active or esterolytically inactivated (iPR_2P) α-thrombin results in a mitogenic response as measured by increased ^{3}[H]-TdR incorporation. This response to thrombin is optimal at 10 nM, and is specifically blocked by hirudin, a high affinity thrombin inhibitor. When prethrombin 1 (a single chain precursor of thrombin) is cleaved with cyanogen bromide, a fragment containing thrombin B chain residues 338-400 is produced which, like the parent thrombin molecule, is mitogenic for J774 cells, but not for fibroblasts. Limited tryptic digests of this fragment retain the ability to stimulate macrophages, a function that can be mimicked by a synthetic tetradecapeptide homologue of this sequence (residues 367-380), but not by any of a series of well known growth promoters, including PDGF, EGF, NGF, FGF and murine CSF-1. The mitogenic effects of this peptide are not limited to J774 cells, but can be expressed in other macrophage-like tumor cell lines, including $P388D_1$, RAW, and PU5, and also in certain non-transformed cloned bone marrow macrophages. In addition to increased ^{3}[H]-TdR incorporation, the synthetic peptide stimulates a dose-dependent increase in total protein/culture well, cell number, and a 3-fold increase in quiescent cells entering S phase, as determined by cell sorter analysis. We conclude that thrombin molecule contains a macrophage growth factor domain which is separate and distinct from its active center. Thus, thrombin, in addition to its major role in hemostasis and thrombosis, may also have important functions in such basic processes as the inflammatory response and monocytopoiesis.

E26 MECHANISMS OF COMPLEMENT ACTIVATION AND CONTROL, Robert J. Ziccardi, Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037.

Complement is a group of serum proteins that together play a vital role in the host defense against infection. Stimulation of the complement system triggers sequential biochemical reactions, which are accompanied by the generation of numerous biologically active mediators of inflammation, ultimately leading to the destruction and clearance of invading organisms. Activation of the classical complement pathway is initiated by the first complement component (C1). After an activating substance, such as an immune complex, binds and activates C1, C1 then activates the second (C2) and fourth (C4) complement components thereby triggering the complement cascade. C1 in itself is an intriguing biochemical model involving specific protein-protein interactions, induced conformational changes, and activation by limited proteolysis. The molecular principles involved in C1 activation and its physiological control will be described. C1 is a 16S metalloprotein composed of 22 polypeptide chains and having a molecular weight of 750,000. Under physiological conditions, C1 consists of two reversibly interacting proteins, C1q and $C1r_2s_2$. During C1 activation, the C1r and C1s subunits are converted from proenzymes to active serine proteases as the result of limited proteolysis. Each 85,000 dalton C1r and C1s polypeptide chain is cleaved into two disulfide-held chains of 57,000 and 28,000 daltons. C1q is an allosteric effector for the activation of $C1r_2s_2$, a process that occurs slowly in the fluid phase and rapidly on a specific activator, such as an immune complex, where the association of C1q with $C1r_2s_2$ is strengthened. The natural substrate of activated C1r is C1s, while activated C1s cleaves C4 and C2. The larger products of this reaction (i.e., C2a and C4b) then combine to form the enzyme that activates C3 (i.e., C3 convertase).

Activated C1 is regulated by the serum glycoprotein C1-inhibitor (M_r 104,000), which firmly binds to the C1r and C1s subunits, thereby blocking their enzymatic activities, while at the same time dissociating $C1r_2s_2$ from C1. The resulting activator-bound C1q now expresses biologically reactive sites that were not exposed in macromolecular C1. C1-inhibitor is very efficient in controlling activated C1, which has a half-life of only thirteen seconds in its presence under physiological conditions. C1-inhibitor also controls the fluid phase activation of native C1 which is reversibly bound to C1-inhibitor in normal human serum. Finally a mechanism of feedback inhibition has been recently described whereby activated C3 and C4 inhibit the turnover of C1 by immune complexes. This control is physiologically important in that it prevents excessive complement activation by low levels of immune complexes, thereby limiting host tissue destruction.

Modeling of New Proteases

E27 Catalytic and Substrate Specificity Studies of Trypsin Via Site-Specific Mutagenesis

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Atomic resolution structure of a protein can provide valuable information about its function. However, the view is only a single static picture of a dynamic sequence of events. An approach to better understanding structure/function relationships in proteins is to modify amino acids that appear to be important for the function of the protein and then biophysically characterize the modified protein. Using site-specific mutagenesis, amino acids believed to be important in the function of trypsin have been replaced with alternate residues to critically evaluate the role of charge, steric constraints and enzyme bound water in enzyme catalysis, substrate specificity, protein conformation and stability. Codon replacements have been made in the cloned rat trypsinogen gene and the resultant genes expressed in bacterial and mammalian cells. The heterologously expressed enzymes have been purified to homogeneity and their properties studied. Substrate binding pocket mutants in trypsin show altered substrate specificities (kcat/Km): (216Gly→Ala)(Arg>>Lys); (226Gly→Ala)(Lys>>Arg) and (216,226Gly→Ala)(Arg>Lys). Mutant trypsins with Ala at position 226 exhibit substrate induced conformational changes. Other modifications that affect substrate specificity, i.e., trypsin (189Asp→Lys) and (189Asp→Glu) are being characterized. Asp102, a putative catalytic residue of serine proteases, has been replaced in trypsin with Asn. Trypsin (102Asp→Asn) shows a 10^3-10^4 fold decrease in activity relative to trypsin on peptide and ester substrates at neutral pH. This decrease can be accounted for predominantly by changes in kcat values. At alkaline pH's a hydroxide ion catalyzed substrate hydrolysis becomes significant to yield kcat values 1-10% that of the wild type enzyme at pH 9. X-ray diffraction quality crystals of the Asn102 mutant have been obtained by vapor diffusion against PEG using the hanging drop technique. The crystals (space group $P2_12_12_1$ with a=40.3, b=46.0 and c=172.2 Å) diffract to 1.7 Å. The structure of the Asn102 mutant is determined at 2.3 Å resolution. Both monomers in the crystallographic asymmetric unit have the same conformation. The Asn residue mimics the wild type Asp102 in its H-bonds with the backbone nitrogen atoms of Ala56, His57 and the Oγ of Ser214. His57 exhibits two discrete conformational states. In one, the placement of His in the active site resembles that seen in trypsin. However, Asn102 cannot act as a H-bond acceptor causing His57 to serve as a proton donor to Ser195. This would account for the decrease in enzymatic activity. The second state has the imidizole rotated out of the active site with no direct H-bond to either Asn102 or Ser195. In this conformation a water molecule can be accommodated between the Ser195 and Asn102 and may account for the observed specific base hydrolysis of substrate at alkaline pH. Ser195 shows two conformational states which may be coupled to the His57 rotation and further accounts for the reduced activity of the mutant enzyme.

E29 SUBTILISIN: A MODEL FOR PROTEASE ENGINEERING AND SECRETION, James A. Wells*, Richard R. Bott*, David B. Powers*, Mark H. Ultsch*, Scott D. Power**, Robin M. Adams**, Brian Cunningham**, Thomas P. Graycar**, David A. Estell**, *Biocatalysis Department, Genentech Inc., **Research Department, Genencor, Inc., South San Francisco, CA 94080

Subtilisin is a serine endoprotease that is secreted in large amounts by bacillus species. A large body of enzymatic, comparative, and structural studies make subtilisin an excellent model for protease engineering by site-directed mutagenesis.

The gene for this enzyme from B. amyloliquefaciens has been cloned and the protease has been expressed in a secreted form in B. subtilis(1). Despite a known x-ray crystal structure (2) the link between structure and function is often weak. To enlarge the structure-function data base, a cassette mutagenesis method has been developed to facilitate extensive amino acid replacements at particular functional sites in the molecule (3).

Subtilisin has been engineered by cassette mutagenesis to be more resistant to oxidative inactivation (4). Oxidative inactivation is believed caused by oxidation of a highly conserved methionine (Met-222) adjacent to the catalytic Ser-221 (5). Unexpectedly, all 19 substitutions at Met-222 produce active enzymes albeit with widely ranging specific activities. Non-oxidizable side chain replacements confer resistance to oxidative inactivation by H_2O_2.

The enzyme has a very broad substrate specificity. Exhaustive mutagenesis at a number of subsites in the molecule has produced (i) enzymes with dramatically altered substrate specificities (ii) enzymes that are more catalytically efficient (kcat/km) than wild type enzyme against particular substrates (iii) an enzyme which has a greater activity against a general protein substrate.

Finally, as a serine protease, hydrogen bonding is important in stabilizing the transition state complex in subtilisin. Mutations have been introduced into subtilisin to quantitate the importance of hydrogen bonding in transition state stabilization (6). Some of these mutations have proved useful in determining that the catalytic activity of subtilisin is important in the processing of its percurser, preprosubtilisin (7

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Proteolytic Mechanisms in Injury and Disease

E30 COOPERATIVE BINDING OF FACTOR VII TO TISSUE FACTOR IS MODULATED BY PHOSPHATIDYLSERINE, Ronald R. Bach, Mt. Sinai Medical School, New York, NY 10029

Factor VII, a plasma serine protease, binds to tissue factor, an integral membrane glycoprotein, forming a membrane bound proteolytic complex. This 1:1 stoichiometric complex is potent initiator of coagulation and may play an essential role in normal hemostasis as well as disease-related thrombosis. Positive cooperativity is observed when the complex forms on the surface of vesicles containing phosphatidylserine (PS). This induction of cooperativity by PS is coincident with an increase in the proteolytic efficiency of the complex, suggesting that the appearance of PS on the cell surface may modulate tissue factor-initiated coagulation. The cooperative binding data suggest that tissue factor may be a dimer in acidic membranes. The formation of tissue factor dimers, when the protein is solubilized in Triton X-100, has been directly demonstrated by sedimentation equilibrium. This cooperative association of an enzyme and its essential cofactor may represent a novel mechanism for regulating cell surface proteolytic activity. Cells may initiate coagulation in response to an injury which perturbs the asymmetric distribution of PS in the plasma membrane.

E31 INHIBITION OF TUMOR CELL-MEDIATED EXTRACELLULAR MATRIX DESTRUCTION BY PROTEASE NEXIN I, Barbara L. Bergman, Randy W. Scott, AnilBajpai, Sherry Watts and Joffre B. Baker, Department of Biochemistry, University of Kansas, Lawrence, KS 66045
Human fibrosarcoma (HT1080) cells, in contrast to normal fibroblasts, rapidly hydrolyze the glycoprotein, collagen and elastin extracellular matrix (ECM) synthesized by cultured rat aortic smooth muscle cells [Jones and DeClerk, Cancer Res. 40, 3222-3227 (1980)]. Here it is shown that protease nexin I (PNI), a fibroblast-secreted inhibitor of urokinase, plasmin and certain other serine proteinases, effectively inhibited the HT1080 cell-mediated degradation of this ECM. PNI at 2.0 nM significantly inhibited matrix destruction for 1-2 days, and at 0.2 μM caused a virtually complete inhibition that persisted for the entire 10 day period of observation. Inhibition of ECM destruction was accompanied by a transient arrest of HT1080 cell proliferation that took place during the first 3 days following PNI addition. PNI did not inhibit the growth of normal fibroblasts and also did not inhibit the growth of HT1080 cells that were seeded onto plastic dishes rather than onto ECM. Like many types of malignant cells, HT1080 cells release large amounts of urokinase. Antibody against this plasminogen activator partially protected ECM from HT1080 cell-mediated hydrolysis, indicating that it may have been a target of PNI. One potential physiological function of PNI could be to help maintain the integrity of connective tissue matrices, protection that malignant cells could overcome by secreting proteinases in excessive amounts. Further studies using fluorescent antibodies indicate that PNI is present at the cell surface of normal human fibroblasts. A proteinase would have to overcome a threshold of inhibition in order to degrade ECM.

E32 CATALYSIS OF PROTEASE NEXIN-THROMBIN COMPLEX FORMATION BY FIBROBLASTS, David H. Farrell and Dennis D. Cunningham, University of California, Irvine, Irvine, CA 92717
Protease Nexin (PN) is a protein protease inhibitor which is secreted by human fibroblasts in culture. PN inhibits certain serine proteases such as thrombin by forming a covalent complex with the protease; the complex then binds back to the cell surface and is internalized and degraded. Interestingly, the rate of complex formation between PN and thrombin is greatly increased by heparin. This finding suggested that a cell surface glycosaminoglycan may catalyze the complex formation. Here we demonstrate that fixed human fibroblasts do indeed catalyze the formation of PN-thrombin complexes. We have further characterized the identity of the component(s) responsible for this catalytic activity. This activity is not inhibited by protamine, implying that the component is not heparan sulfate. Experiments with diisopropylfluorophophate-inactivated thrombin (DIP-Th) show that the cell surface DIP-Th binding site may be involved in the catalysis. The activity is inhibited by about 60% at concentrations of DIP-Th which saturate the DIP-Th binding site. Further studies are in progress using specific glycosidases to remove the activity from the cell surface. These are paralleled by measurements using purified glycosaminoglycans to catalyze PN-thrombin complex formation without cells. In addition, we have found that antithrombin III-thrombin complex formation, which is catalyzed by endothelial cells, is not accelerated by the fibroblasts. From these results, we have concluded that the catalytic effect of the fibroblasts on PN-thrombin complex formation may reflect the physiological role of PN in regulating proteases in interstitial fluid, just as antithrombin III regulates thrombin activity in the vasculature.

E33 THE ROLE OF POLYMORPHONUCLEAR LEUKOCYTE PROTEINASES IN CONNECTIVE TISSUE BREAKDOWN DURING THE REVERSE PASSIVE ARTHUS REACTION.
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The classical reverse passive Arthus reaction (RPA) performed in the skin of rats was modified to allow for the determination of polymorphonuclear leukocyte (PMN) infiltration and measurement of the resulting damage to blood vessel walls (hemorrhage). Following initiation of the reaction by intradermal injection of antiovalbumin serum and intravenous injection of ovalbumin, vascular permeability (^{125}I-albumin) was observed to peak at about 2 hours. PMN infiltration, monitored by measurement of the tissue myeloperoxidase content, increased steadily between 2 and 8 hours. The time course of PMN infiltration was nearly identical to that of hemorrhage development (^{59}Fe-erythrocyte accumulation). Idomethacin administered by intraperitoneal injection 30 minutes prior to initiating the RPA had no effect on vascular permeability increase but suppressed PMN accumulation and hemorrhage development. If indomethacin was given 2 hours after the RPA was begun, no effect on any of the RPA parameters was noted. Dexamethasone suppressed all three parameters when given at minus 30 minutes or plus 2 hours. Catalase administered intravenously at plus 2 hours inhibited hemorrhage and PMN accumulation, as did trasylol, alpha1-antiproteinase and soybean trypsin inhibitor. These results indicate that the damage to blood vessels during a severe RPA reaction is a direct consequence of PMN activity, presumably caused by extracellular release of PMN enzymes in response to immune complexes formed in vessel walls.

E34 ACTIVATION OF COAGULATION RELEASES ENDOTHELIAL CELL MITOGENS. C. Gajdusek, S. Carbon, P. Nawroth, and D. Stern. Univ. of Washington, Seattle, WA; and Oklahoma Medical Research Foundation, Okla. City, OK 73104.
Recent studies have indicated that endothelial cell function includes elaboration of growth factors and regulation of coagulation. We demonstrate that Factor Xa, a product of the coagulation mechanism generated prior to thrombin, induces enhanced release of endothelial cell mitogens, linking these two functions. Mitogenic activity generated by cultured bovine aortic endothelial cells in response to Factor Xa included PDGF-like molecules based on a radioreceptor assay. Effective induction of mitogens by Factor Xa required the integrity of the enzyme's active center and the presence of the γ-carboxyglutamic acid-containing domain of the molecule. Factor Xa-induced release of mitogens from endothelium occurred in serum-free medium and was not altered by hirudin or antibody to Factor V. Generation of enhanced mitogenic activity in response to Factor Xa was unaffected by the presence of actinomycin D (0.24 μg/ml) and was not associated with increased hybridization of RNA from treated cells to a v-sis probe. Release of mitogenic activity was dependent on the dose of Factor Xa, being half-maximal at 0.5nM and reaching a maximum by 5nM. Radioligand binding studies demonstrated a class of endothelial cell sites half-maximally occupied at a Factor Xa concentration of 0.8nM. When Factor X was activated on the endothelial cell surface by Factors IXa and VIII, the Factor Xa formed resulted in the induction of enhanced release of mitogenic activity. These data suggest a new mechanism by which the coagulation system can locally regulate endothelial cell function and vessel wall biology prior to thrombin-induced release of growth factors from platelets.

E35 **INHIBITION OF MAMMALIAN COLLAGENASES BY THIOL-CONTAINING PEPTIDES.** Robert D. Gray, Robert B. Miller and Arno F. Spatola, University of Louisville, Louisville, KY 40292
Mammalian collagenases are zinc proteases which cleave native type I collagen at $-Pro-Gln-Gly_{775}-Leu(Ile)_{776}-Ala-Gly-Gln-Arg-$. Cysteine and other simple thiols are weak inhibitors of collagenases. In an effort to improve potency and specificity, we have synthesized several thiol-containing peptides which are analogues of the carboxyl side of the scissile peptide bond. The analogues shown below inhibit cleavage of acid soluble calf skin collagen by partially purified collagenases both from rabbit V2 tumor homogenates and from pig synovial membrane culture medium. Approximate inhibitory potencies of these compounds were determined by subjecting the reaction mixtures to SDS-PAGE and estimating the degree of collagenolysis by scanning densitometry.

	Approximate IC_{50} (uM)
$HSCH_2CH(CH_3)CO-Ala-OEt$	100
$HSCH_2CH(CH_2Ph)CO-Ala-OEt$	50
$HSCH_2CH(CH_2CH(CH_3)_2)CO-Ala-OEt$	4-10
$HSCH_2CH(CH_2CH(CH_3)_2)CO-Ala-Gly-OEt$	1-10
$HSCH_2CH(CH_2CH(CH_3)_2)CO-Ala-Gly-Gln$	4-10

Additional derivatives have been prepared and are now being evaluated. Supported by NIH AM 31364 and ACS Institutional Grant IN-111.

E36 ONE- AND TWO-CHAIN TISSUE PLASMINOGEN ACTIVATOR, ENZYMATIC ACTIVITY AND INHIBITION BY PEPTIDE DERIVATIVES OF ARGININE CHLOROMETHYL KETONE, George D.J. Green, Friedrich Miescher Institute, Postfach 2543, CH-4002 Basel, Switzerland.
Tissue plasminogen activator (tPA) is secreted in a one-chain molecular form. In the presence of very small amounts of plasmin this form is rapidly converted to the two-chain form of the enzyme. This conversion is accompanied by changes in the enzymatic properties of the enzyme. Activity towards small amidolytic substrates and plasminogen is increased and also changes in the reactivity towards naturally occuring inhibitors are observed. These changes could be due either to changes in specificity or reactivity of the active site. In this study the susceptibility of both forms of the enzyme towards peptide derivatives of arginine chloromethyl ketones is reported. The specificity of both forms of the enzymes appears to be very similar, suggesting little conformational change of the binding site between the two forms. In all cases studied the one-chain form was approx. 20 times less reactive than the two-chain form. A similar difference in activity was found towards small synthetic amide substrates. A decrease in reactivity with 1-chain tPA towards the natural substrate, plasminogen, was also found. The magnitude of this activity change may be similar to that found for the synthetic reagents. The presence of stimulators of tPA activity such as fibrin fragments and polylysines do not affect the activity differences between the tPA forms.

E37 PROTEASES MODULATE VIRAL INFECTION: POLY(A^+) ENRICHED RNA MEDIATES SYNTHESIS OF A PROTEASE ACTIVE PROTEIN IN CYTOTOXIC LYMPHOCYTES DIFFERENTIATING IN RESPONSE TO HEPATITIS B VIRUS SURFACE ANTIGEN. Anwar A. Hakim. Loyola University Medical Center. Maywood. Illinois 60153.

The cause of hepatic injury during viral hepatitis infection remains unknown. Earlier studies (Hakim, Cancer Biochem. Biophys. 4:175-185,1980) demonstrated an estradiol-mediated protease release among the induced biochemical changes in human neoplastic cells.The present investigations show the following: 1. Peripheral blood lymphocytes(PBL) from healthy donors with(HBs Ab-sero positive) circulating antibodies to the hepatitis B virus surface antigen(HBsAg) are more cytotoxic to a hepatocellular carcinoma PLC/PRF/5 cells, and to a myeloid-induced K-562 cells than PBL from adults without (HBsAb-sero-negative) the antibodies. 2. Vaccination,and/ or incubation with HBsAg increased the cytotoxic activities, and Poly(A^+)enriched RNA and Protease synthesis of PBL from HBsAb-sero positive adults. 3. Isolation and characterization of a Poly(A^+) enriched RNA mediating an in vitro synthesis of a Proteas active protein(s) and of two proteins with molecular weights of 23,500 and 27,000 daltons similar to the viral surface antigens. RNA gel blot hybridization showed that the lymphocyte Poly(A^+) enriched RNA hybridizes with ^{32}P-labelled DNA of the viral particles (Danes particles) sedimenting from sera of patients with Chronic active hepatitis B infection. It is suggested that in blood, the viral particles alter the PBL mRNA biosynthesis and its biofunctions. Several types of virus including Measles virus are able to infect PBL in vitro. In the present study, the gel blot hybridization analysis indicated that hepatitis B virus surface antigens are capable of altering the PBL synthetic activities to encode viral specified proteins.

E38 DEREGULATION OF PLASMA PROTEINASE ACTIVITY IN TUMOR-BEARING ANIMALS IS THE RESULT OF THE HOST RESPONSE TO THE TUMOR, David A. Hart, University of Calgary, HSC, Calgary, Alberta, T2N 4N1.

Plasma proteinase activity (PPA) is regulated by a balance between enzyme activation and inhibition. Homeostasis is critical for maintenance of several plasma systems including coagulation, fibrinolysis and complement. Since many tumors, and activated host cells, produce high levels of proteinases, such enzymes could lead to systemic alterations in the regulation of PPA. Altered PPA has been reported to occur in human cancers as well as some murine leukemias (Hart et. al., Haemostasis, 1985). In order to understand this process in more detail, the impact of the transplantable murine B16 melanoma on the regulation of PPA was studied. Utilizing an ^{125}I-caseinolytic assay, plasma from tumor-bearing animals was found to exhibit levels of PPA that were 200-300% of control values. The onset of increased levels of activity was found to occur at a specific time following tumor transfer. The level of elevation was independent of tumor burden and the phenotype of the B16 subline used. Invasive and metastic lines induced changes comparable to the parent cell line. The induction of elevated PPA was however dependent on the site of the tumor. Since animals with elevated PPA lived as long as animals without elevated PPA, one can conclude that the induction of elevated PPA does not contribute to the mortality of the mice and that the elevated PPA is due to an altered host system rather than the tumor. Analysis of plasma from patients bearing melanomas may provide insights into whether similar phenomenon occur in humans. (Supported by the ACB [H-277] and the AHFMR).

E39 INTERACTIONS BETWEEN THROMBIN AND THROMBOMODULIN
J. Hofsteenge and S.R. Stone, Friedrich Miescher Institute, P.O.Box 2543, CH-4002 Basel, Switzerland.

The purpose of this study is to examine the mechanism by which thrombomodulin (TM) changes the enzymic specificty of thrombin. The effect of TM on the interaction of different inhibitors and substrates with thrombin has been examined; in addition, inhibition experiments with fragments of the polypeptide chain of thrombin have been performed. TM has a minimal effect on the irreversible inhibition of thrombin by tripeptide chloromethyl ketones. The rate of inhibition of thrombin by antithrombin III is stimulated 3.8-fold in the presence of TM. The binding of TM is competitive with respect to that of hirudin. Hirudin becomes a slow-binding inhibitor in the presence of TM. The effect of TM on the kinetics of fibrinogen cleavage indicates that it acts as a competitive inhibitor with respect to fibrinogen. The activation of Protein C is inhibited at high concentrations of TM which suggests the existence of at least two binding sites for TM on thrombin. CNBr fragments of thrombin were examined as inhibitors of the activation of Protein C by the thrombin-thrombomodulin complex. One large fragment as well as a proteolytic subfragment were found to be inhibitory. The exact mechanism of inhibition is not yet clear. The results will be discussed in relation to possible interaction sites between thrombin and TM.

E40 THROMBIN INDUCES THE RELEASE OF PROSTACYCLIN (PGI_2) BY HUMAN ENDOTHELIAL CELLS BY ELEVATING INTRACELLULAR CALCIUM, Eric A. Jaffe, Jürgen Grulich, and Babette B. Weksler, Cornell University Medical College, New York, NY 10021

Incubation of Quin 2-loaded HUVEC with thrombin caused a rapid, transient, and dose-dependent rise in cytoplasmic free calcium ($[Ca^{2+}]_i$) and a parallel elevation in 6-keto-$PGF_{1\alpha}$ production with ED_{50} = 0.71 ± 0.12 and 0.68 ± 0.18 units/ml respectively. DFP- and PPACK-thrombin were inactive. In contrast, incubation of Quin 2-loaded cultured BAEC with thrombin failed to elevate either $[Ca^{2+}]_i$ or 6-keto-$PGF_{1\alpha}$. Thrombin-induced rises in $[Ca^{2+}]_i$ and 6-keto-$PGF_{1\alpha}$ were not blocked by incubation with EGTA or the mitochondrial uncoupler 1799, suggesting that calcium is released from a non-mitochondrial, intracellular pool. Thrombin-induced rises of $[Ca^{2+}]_i$ or 6-keto-$PGF_{1\alpha}$ were not induced or suppressed by 8-Br-cAMP or calmodulin inhibitors. Restimulation of HUVEC with ionomycin ± EGTA 5 min after an initial stimulation with thrombin (2 u/ml) induced a second rise in $[Ca^{2+}]_i$ and further production of 6-keto-$PGF_{1\alpha}$ whereas restimulation with a second dose of thrombin was without effect. If the initial thrombin stimulation was terminated by adding PPACK within 10-60 sec of stimulation, the initial rises in $[Ca^{2+}]_i$ and 6-keto-$PGF_{1\alpha}$ production were somewhat blunted. Restimulation with thrombin under these circumstances induced a second rise in $[Ca^{2+}]_i$ and additional 6-keto-$PGF_{1\alpha}$ production that were greatest when the initial thrombin stimulus was shortest. These observations suggest that thrombin stimulates HUVEC to release PGI_2 by elevating $[Ca^{2+}]_i$ and that in vivo EC may be stimulated to produce PGI_2 by thrombin multiple times though each stimulation probably lasts for only a short time.

E41 ELECTROPHORETIC AND IMMUNOCHEMICAL CHARACTERIZATION OF TISSUE PLASMINOGEN ACTIVATOR. Bruce A. Keyt, Reed J. Harris, Thomas Doherty, Rodney G. Keck and Andrew J.S. Jones, Dept. of Medicinal and Analytical Chemistry, Genentech, Inc. South San Francisco, CA. 94080

Recombinant tissue plasminogen activator (rTPA) and Bowes melanoma derived TPA were analyzed by dodecyl sulfate gel electrophoresis (SDS-PAGE),immunoblotting and amino acid sequencing. Samples were prepared by reduction with dithiothreitol and alkylation with iodoacetate. Both rTPA and melanoma TPA displayed qualitatively similar patterns of discrete protein bands which were resolved by SDS-PAGE, electro-eluted and identified by amino acid sequencing. However, additional uncharacterized protein bands were seen in melanoma TPA. Electrophoretically distinct forms of TPA were due to glycosylation variants (type I and type II), cleavage after arginine at 275 (1 chain and 2 chain) and partial cleavage at arginine 27. Proteolytic cleavage at Arg 275 was catalyzed in vitro by plasmin, whereas the cleavage at Arg 27 required trypsin for completion. rTPA was quantitatively converted to the two chain form with immobilized plasmin on Sepharose as monitored by SDS-PAGE and chromogenic activity with S2288 substrate. Two chain rTPA was reduced and carboxymethylated (RCM), then chromatographed on G75 superfine Sephadex. RCM (1-275) and RCM (276-527)were separated and used to prepare polyclonal anti-RCM (1-275) and anti-RCM (276-527), respectively. Polyclonal antiserum was also raised to synthetic peptide (1-27) conjugated to soybean trypsin inhibitor or bovine serum albumin. These antisera were purified by immunoabsorption to immobilized TPA and eluted at low pH. The purified antibodies are currently being used to further characterize recombinant TPA and melanoma TPA with respect to structural determinants and fibrinolytic activity.

E42 A NOVEL METHOD FOR SPECIFIC RADIOLABELLING OF MEMBRANE PROTEINS, Alice L. Lau, James A. Thompson and Dennis D. Cunningham, University of California, Irvine, Irvine, CA 92717.

A procedure was developed to radio-label cell surface components to a very high specific activity. This procedure utilizes a water-soluble form of the Bolton-Hunter reagent, sulfo-succinimidyl-3-(4-hydroxyphenyl)propionate (sulfo-SHPP). When labelled with 125-I, this reagent can be used to specifically radio-label membrane proteins exposed at cell surfaces. Comparison of this procedure with the lactoperoxidase technique on erythrocytes demonstrates that sulfo-SHPP labels membrane proteins to a higher specific activity with much less cytoplasmic labelling. This allows for better resolution and identification of membrane proteins after PAGE. Sulfo-SHPP labels exposed amino-groups of membrane proteins; these sites can be saturated with unlabelled reagent thus preventing subsequent labelling with 125-I sulfo-SHPP. In addition, with blockage of exposed amino-groups on intact cells with unlabelled sulfo-SHPP, the inner surface of the membrane can be specifically labelled after lysis of the cells. This procedure can be used to label membrane proteins to a high specific activity, determine the spatial orientation of proteins in the membrane by labelling either side of the membrane, or allow for the identification of cell surface proteolytic cleavages.

E43 PROTEINASE INHIBITOR ACTIVITY IN SCLERODERMA SERA. E.C. LeRoy and T. Tidwell. Medical University of South Carolina, Charleston, SC.

A deficiency of proteinase inhibition has been demonstrated in the sera of some patients with scleroderma (systemic sclerosis). Control and scleroderma serum inhibition of trypsin esterase activity using an L-BAPNA substrate has been measured at both high and low substrate concentrations; differences were found only at low concentrations measuring initial reaction velocity. α_1PI was found to account for 90% of total serum inhibition. The molar ratio of α_1PI to trypsin inhibited at high substrate concentration (10^{-3} M) was 1.1 for both normal and scleroderma sera. A kinetic analysis at low substrate concentration (10^{-4} to 10^{-5} M) of five sera gave the molar ratios in three which were the same as those found at high substrate concentrations. In two of the five sera, both from scleroderma patients, the ratio was 2.5 and 2.2, (i.e., twice as much α_1PI was necessary to reduce trypsin activity). These preliminary observations provide a sensitive, kinetic approach for determining the mechanism of deficient α_1PI in some patients with scleroderma.

E44 PROTHROMBIN FRAGMENT I, which contains 10 GLA RESIDUES , IS CHEMOTACTIC FOR MONONUCLEAR CELLS. J. DAVID MALONE AND MICHAEL RICHARDS. ST. LOUIS UNIVERSITY AND V.A. MED CENTER, ST. LOUIS, MO 63106.

Studies with γ carboxyglutamic acid (gla) indicate that it evokes a chemotactic response in human peripheral mononuclear cells. This response is not shared by polymorphonuclear leucocytes (PMN), which are derived from the same colony forming units (CFU's) and respond to the same colony stimulating factors (GM-CSF's). The difference in response is quite striking since PMN's respond to a broad range of chemoattractants. Gla, in which the post-translational modification is Vitamin K dependent, is found in osteocalcin, the venom of certain sea snails, and proteins of the clotting cascade. In particular it is present in prothrombin and Factors II, VII, IX and X.

Prothrombin is without chemotactic activity, however Fragment I, which contains 10 gla residues, is chemotactic for mononuclear cells. Optimum concentration for chemotaxis in a Boyden Chamber assay is 10^{-7} M. The response is concentration dependent over the range 10^{-11} M to 10^{-6} M. The data suggest that cleavage products of prothrombin may be important in the recruitment of mononuclear cells to areas of tissue inflammation and repair. In addition the data suggest that gla may be an important part of the recognition signal for cell recruitment.

E45 AN ACTIVE FIBRINOLYTIC ENZYME FROM SOUTHERN COPPERHEAD VENOM. F.S. Markland, K.N.N. Reddy, and A.L. Guan, Dept. of Biochemistry and Comprehensive Cancer Center, University of Southern California, School of Medicine, Los Angeles, CA 90033

A fibrinolytic enzyme has been purified from *Agkistrodon c. contortrix* (southern copperhead) venom. Purification of the enzyme presently employs four steps: ion exchange chromatography on CM-cellulose, molecular sieve chromatography on Sephadex G-100, affinity chromatography on benzamidine-Sepharose to remove serine proteinase contaminants, and preparative immobiline isoelectric focusing. The enzyme is directly fibrinolytic on human blood clots or purified human fibrin, and does not require plasmin for activity. Physicochemical studies reveal that the enzyme has a molecular weight of 25,000 ± 2,000, is a glycoprotein, and has an isoelectric pH of 6.6 - 6.8. The enzyme apparently has two isoelectric forms with identical amino-terminal sequences. These forms may derive from minor sequence differences or carbohydrate alterations. The enzyme is devoid of fibrinogen clotting activity and does not hydrolyze a series of p-nitroanilide containing serine proteinase substrates. The enzyme is inhibited rapidly by 10 mM EDTA and tetraethylenepentamine suggesting that it is a metalloproteinase. Although the enzyme is inhibited by α_2-macroglobulin, it is not inactivated by soybean or lima bean trypsin inhibitors, trasylol, or diisopropylfluorophosphate.

In vivo studies in a rabbit model of subacute venous thrombosis, in collaboration with J.J. Bookstein, University of California, San Diego, reveal that the purified enzyme has excellent thrombolytic activity with minimal toxicity. These studies were supported in part by Cortech, Inc., of Denver, Colorado and USPHS Grant No. HL31389, awarded by the National Heart, Lung, and Blood Institute, DHHS.

E46 BINDING OF PLASMINOGEN ACTIVATOR (UROKINASE) TO HUMAN BREAST CANCER CELL MEMBRANE
GK Needham[1]*, AL Harris[2], GV Sherbet[2] and JR Farndon[1], Dept of Surgery[1] and cancer Research Unit[2], University of Newcastle upon Tyne, United Kingdom.

Plasminogen activators (PA) are implicated in the processes of tumour invasion and metastasis by mechanisms as yet unknown. Membrane bound rather than free PA may be the form of the enzyme required for anchorage independent growth of cells[1] and it has been observed that membrane associated PA is higher in secondary tumours than their primaries although cytosolic levels are similar[2].

Here, a study has been made into the binding of high molecular weight (54,000) urokinase to membranes prepared from human breast carcinomas. Not all tumours exhibited specific binding but high affinity (Kd approx 5×10^{-11} to 10^{-9}) specific binding was achieved in other cases with equilibrium attained in 30 minutes at 30°C. Endogenous PA has been assayed in both cytosol and membrane of the tumours. We intend to correlate the data with the estrogen receptor and epidermal growth factor receptor status.

The binding of urokinase may present a local inactivation mechanism, a means of modifying the membrane or an incorporation of active enzyme to facilitate plasminogen activation near the cell's surface.

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E47 HORMONAL REGULATION OF COLLAGENASE AND COLLAGENASE INHIBITOR IN OSTEOBLASTIC CELLS
N. Partridge, J. Jeffrey, H. Welgus, S. Teitelbaum & A. Kahn, St. Louis & Washington Univ., St. Louis, MO

Collagenases that specifically cleave native collagen at neutral pH have been implicated in the maintenance and turnover of connective tissue. The cellular origin of this enzyme in bone has remained equivocal. However, recent studies of our own and others have indicated that the osteoblast appears to be the cell in bone responsible for production of collagenase (C'ase). In the present study, the production and regulation of C'ase and collagenase inhibitor (CI) were investigated in two osteogenic sarcoma cell lines; the rat clonal line, UMR-106-01, and the human line, SaOS-2. C'ase and CI were measured by ELISA or functional assays. UMR-106-01 cells produced undetectable levels of C'ase under basal conditions. However, several bone-resorbing agents caused the release of C'ase into the medium by these cells. After 24 h, parathyroid hormone (PTH) produced C'ase levels of 529±27 ng/mg cell protein. By comparison, 3 other agents, $1,25(OH)_2D_3$, PGE_2 and retinoic acid caused a lesser increase in C'ase concentrations. As well as being the most potent stimulator of C'ase production by these cells PTH caused a 2-fold increase in CI secretion, suggesting coordinate expression of these two proteins by an osteoblastic cell. In contrast to the rat line, the human SaOS-2 cells, while capable of producing CI, did not secrete C'ase. Also, the only agents shown to promote CI production by these cells were a phorbol ester and epidermal growth factor. Thus osteoblasts, in addition to producing abundant collagen, also may synthesize C'ase and CI, at least when exposed to some bone-seeking agents. If so, osteoblasts like osteoclasts must be considered important contributors in the resorptive phase of bone remodelling.

E48 THROMBIN STIMULATED METABOLISM OF INOSITOL-CONTAINING PHOSPHOLIPIDS,
Daniel M. Raben, Kathleen Yasuda, and Dennis D. Cunningham, Department of Microbiology, College of Medicine, University of California, Irvine, CA 92717.

The release of arachidonic acid (AA) and its subsequent metabolism has been implicated in the mitogenic stimulation of cultured cells. Recent studies have indicated that the presence of AA in certain diacylglycerols (DAG) and its subsequent release in an important aspect of transmembrane signalling events involving inositol-containing phospholipids. Furthermore, the DAG serves as a substrate for the synthesis of phosphatidylinositol (PI).

We have examined the relationship among a) the release and metabolism of AA from phospholipids; b) the metabolism of inositol-containing phospholipids; and c) the mitogenic stimulation by thrombin. These studies were conducted on a subclone of Chinese hamster embryo fibroblasts (IIC9) which show a large mitogenic response to low concentrations of thrombin in the absence of other growth factors. Our results demonstrate that thrombin stimulates a rapid, dose-dependent release of AA and its metabolites from IIC9 cells. Thrombin also stimulates the synthesis of PI. This stimulation of PI is due to a cyclooxygenase metabolite of AA. Furthermore, the release and metabolism of AA and the early stimulation of PI synthesis are not necessary for thrombin-stimulated cell division. In addition, studies on the effect of chymotrypsin on thrombin-stimulated events indicate that thrombin stimulates at least two pathways for the metabolism of inositol-containing phospholipids. One of these pathways is mediated by a GTP binding protein designated as G_i while the other pathway does not involve this G protein.

E49 FACTORS IN LEECH SALIVA AND SALIVARY GLAND EXTRACTS WHICH COUNTERACT HEMOSTASIS AND LEUCOCYTE ACTIVITY

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Dilute saliva (DS) of *Hirudo medicinalis* and aqueous extracts of its salivary glands contain a battery of factors which inhibit blood clotting, platelet aggregation and leucocyte activity. DS is obtained by inducing the animal to bite and suck through a membrane, the other side of which contains a stimulant solution of dilute arginine in saline. After the "meal," the saliva is "milked" by forcing through the mouth. The process may be repeated several times. The high molecular fraction of DS was obtained by gel filtration on Sephadex G-10. Isoelectric focusing of this fraction showed the presence of six components. DS and salivary gland aqueous extracts contain hirudin (thrombin inhibitor), eglin (chymotrypsin, leucocyte elastase and cathepsin G inhibitor), apyrase, collagenase and hyaluronidase. Partial separation of these components was obtained by gel filtration on Fractogel TSK HW-55. DS and the gland extracts inhibit platelet aggregation induced by collagen, ADP and epinephrine. DS inhibits the generation of oxygen free radicals by activated human polymorphonuclear leucocytes. This activity is due to eglin.

E50 SELECTIVE INHIBITION OF PROTEOLYTIC ENZYMES CATHEPSIN B, UROKINASE AND THROMBIN IN A B16 MELANOMA MOUSE LUNG COLONIZATION MODEL FOR METASTASIS, Richard M. Schultz, Lawrence E. Ostrowski, A. Ahsan, B.P. Suthar and P. Pagast, Dept. of Biochemistry, Loyola Univ. Schl. of Medicine, Maywood, IL. 60153.

Peptide aldehyde transition-state analog inhibitors of are used to selectively inhibit targeted proteases implicated as having a role in tumor cell metastasis. The K_i values of these inhibitors determined <u>in vitro</u> against cathepsin B, plasmin, urokinase, thrombin and Factor Xa show they are highly selective by orders of magnitude. The inhibitors are introduced into C57BL/6 mice concurrently with B16 F10 melanoma cells, and the number of metastatic foci in the lung determined. Mini-pump infusion of leupeptin at a steady-state plasma concentration of $3x10^{-6}$ M ([I] = $1000xK_i$) over 5 days gave no decrease in lung colonization by the B16 tumor cells. Ep475, a second inhibitor of cathepsin B, at a steady-state plasma concentration of $3x10^{-7}$ M also did not inhibit foci formation. Thus a secreted cathepsin-B-like enzyme does not have a significant role in B16 tumor cell lung colonization. Selective inhibitors of plasminogen activator with general structure R-Glu-Gly-Argininal did not inhibit lung colonization. The selective thrombin inhibitor D-Phe-Pro-Argininal dramatically and significantly <u>increased</u> B16 melanoma colonization of mice lungs (>300 foci vs. 12 in control). This result implicates thrombin as having a role in preventing metastasis. Supported by NIH grant CA 34530.

E51 PARTICIPATION OF ENDOTHELIAL CELLS IN THE PROTEIN C-PROTEIN S ANTICOAGULANT PATHWAY: THE SYNTHESIS AND RELEASE OF PROTEIN S. D. Stern, J. Brett, K. Harris and P. Nawroth. Oklahoma Medical Research Foundation, Oklahoma City, OK 73104, and Dept. Pathology, Columbia University, New York City, NY 10032.

The protein C-protein S anticoagulant pathway is closely linked to the endothelium. In this report the synthesis and release of the vitamin K-dependent coagulation factor protein S is demonstrated. Western blotting, following SDS-PAGE of Triton X-100 extracts of bovine aortic endothelial cells grown in serum-free medium, demonstrated the presence of protein S. A single major band was observed at $M_r \sim 75,000$, closely migrating with protein S purified from plasma. Extracts from cells treated with cycloheximide did not demonstrate this band, indicating that de novo synthesis was required. Using a radioimmunoassay, endothelium was found to release 180 fmole/10^5 cells/24 hrs and contain 44 fmoles/10^5 cells of protein S antigen. Protein S released from endothelium was functionally active based on its ability to promote activated protein C-mediated Factor Va inactivation on the endothelial cell surface. Warfarin decreased secretion of protein S antigen by > 90% and increased intracellular accumulation by almost two-fold. Morphologic studies demonstrated that protein S was present in the golgi of endothelium. A pool of intracellular protein S could be released rapidly by the calcium ionophore A23187 (5 μM). This effect was dependent on the presence of calcium in the culture medium and could be blocked by $LaCl_3$, suggesting that cytosolic calcium flux was responsible for protein S release. These results demonstrate that endothelial cells, but not subendothelial layers of the vessel wall, can synthesize and release protein S, indicating a new mechanism by which the inner lining of the vessel wall can contribute to the prevention of thombotic events.

E52 Protease Inhibitors Prevent Glomerular Necrosis in Experimental Glomerulonephritis.
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University of North Carolina at Chapel Hill, Chapel Hill, NC 27514

It has been well documented that proteolytic enzymes play a major role in the initiation of inflammation resulting from immune complex deposition. With this observation as a background we investigated a series of protease inhibitors of the amidine type on the murine model of immune complex mediated necrotizing glomerulonephritis. Control mice given horse apoferritin (HAF) daily for 12 days developed proliferative glomerulonephritis with massive glomerular HAF, IgG and C3 deposits and extensive glomerular necrosis. While none of the inhibitors reduced glomerular hypercellularity, immune complex localization or neutrophil attraction, one of the compounds, bis(5-amidino-2-benzimidazolyl)methane (BABIM) did produce a striking reduction in glomerular necrosis. Animals given daily i.p. injections of BABIM of either 20 or 5 mg/kg on days 7-12 showed a 90% reduction in glomerular necrosis as compared to untreated controls. A proposed site of action of BABIM is the inhibition of neutral lytic proteases released by the neutrophils.

E53 STRUCTURE AND FUNCTION OF LYSOSOMAL CYSTEINE PROTEINASES AND THEIR INHIBITORS,
V. Turk, J. Stefan Institute, Ljubljana, Yugoslavia.
We have isolated and sequenced human cathepsin B, L, and H as well as a series of low molecular weight inhibitors of these enzymes from human tissues. The human enzymes show a large degree of homology to cysteine proteinases from bovine, rat, and plant sources. The proteinase inhibitors fall into three families. The stefin family, which consists of inhibitors with no disulfide bonds, the cystatin family, which consists of molecules with disulfide bonds, and kininogen which consists of moelcules with inhibitor squences in themiddle of a larger protein. The structures, homologies and evolution of these enzymes inhibitors will be illustrated.

E54 STRUCTURAL AND FUNCTIONAL HOMOLOGIES BETWEEN PROTEASE NEXIN I AND C1-INHIBITOR
William E. Van Nostrand and Dennis D. Cunningham, University of California, Irvine CA 92717

We show that protease nexin I and C1-inhibitor share structural and functional homologies. Previous studies have shown that protease nexin I is synthesized by many cultured cells and inactivates certain proteases at the cell surface, whereas C1-inhibitor is a plasma inhibitor named for its inhibition of certain complement proteases. Cyanogen bromide peptide mapping studies revealed numerous homologies between these two protease inhibitors. Specificity studies have shown that both inhibitors can covalently complex the same proteases. Enzymatic inhibition studies on Factor XIIa and plasma kallikrein showed that protease nexin I was a potent inactivator of each of these important regulatory proteases. We have been unable to detect appreciable quantities of protease nexin I in plasma. Furthermore, cultured human fibroblasts did not secrete C1-inhibitor. Thus, these two inhibitors may control similar regulatory proteases in different compartments of the body.

E55 CELL LINEAGE-DEPENDENT POLYMORPHISM OF GELATINASES IN HUMAN PHAGOCYTES.
T. Vartio and T. Hovi, University of Helsinki, Helsinki, Finland.
Human peripheral blood granulocytes, monocyte-derived macrophages and U937 and RC2A promonocytic cell lines produce a proteolytic enzyme (gelatinase) that rather specifically degrades denatured but not native type I collagen. The enzyme can be isolated from the growth medium or extracts of these cells by affinity chromatography on gelatin-Sepharose. Isolated gelatinases were run in SDS-PAGE under reducing or nonreducing conditions. The polypeptides were analyzed either by protein staining or immunoblotting using antiserum against the M_r 95 000 gelatin-binding protein produced by cultured human macrophages (1). Under reducing conditions the gelatinases from granulocytes, macrophages and promonocytic cells had a molecular weight of M_r 95 000. Nonreduced gelatinase from the cells of the mononuclear phagocyte lineage migrated to the position of M_r 92 000. The corresponding granulocyte protein appeared as three polypeptide bands M_r 225 000, M_r 130 000 and M_r 92 000. All these polypeptides reacted with the antiserum made against the M_r 95 000 macrophage protein. The results suggest that the gelatin-degrading enzyme produced by human blood granulocytes and monocyte/macrophages exists in several immunologically cross-reactive polymorphic forms which are different in these two cell-lineages.
1. Vartio, T., et al. J. Biol. Chem. 257: 8862-8866, 1982.

E56 STIMULATION OF HUMAN LEUKOCYTE ELASTASE ACTIVITY BY BILE ACIDS AND DETERGENTS
M. Vered and S.R. Simon, SUNY at Stony Brook, New York 11794

We have measured the hydrolysis of elastin and of the chromogenic substrate succinyl-$(Ala)_3$nitrophenylanalide (SLAPN) by human leukocyte elastase (HLE) in the presence of the bile acid conjugates, taurocholate (TC) and glycocholate (GC), or the steroidal zwitterionic detergents, CHAPS and CHAPSO. All these compounds stimulate HLE activity with bell-shaped activation profiles which peak in the range 1-25 mM, around the critical micelle concentrations. At the optimal activating concentrations, the order of stimulation of SLAPN hydrolysis is CHAPSO (12X)>CHAPS (11X)>TC (7.5X)>GC (5X). Both the hydrophobic steroidal ring system and the very polar sulfate group are important for stimulation: the unconjugated bile acid, cholate stimulates activity less than 1.5X and the aliphatic amphiphiles, Zwittergent 3-14 and 3-16 are without effect. Only leukocyte elastase is stimulated by the steroidal detergents, while pancreatic elastase is unaffected. Lineweaver-Burk analysis of activation of SLAPN hydrolysis by CHAPS revealed no effect on K_M but an 11-fold increase in V_{max}. The inhibition of HLE-mediated elastin hydrolysis by α-1-protease inhibitor (α-1-PI) was diminished in the presence of CHAPS, as reflected in an increase in the apparent dissociation constant for α-1-PI, K_{Iapp}, from $0.29 \pm 0.11 \times 10^{-9}$ M to $0.20 \pm 0.09 \times 10^{-8}$ M in the presence of detergent. Conversely, inhibition of HLE-mediated SLAPN or elastin hydrolysis by pneumococcal extracts (M. Vered et al., Am. Rev. Resp. Dis. 130: 1118 (1984)) was enhanced by CHAPS. No marked conformational changes in HLE induced by optimally activating concentrations of CHAPS can be detected by UV circular dichroism. These studies are consistent with roles of both hydrophobic and ionic interactions in regulation of HLE catalytic activity.

E57 METALLOPROTEASES OF HUMAN CARTILAGE IN RELATION TO OSTEOARTHRITIS, J. Frederick Woessner, Jr., Walid Azzo and David D. Dean, Univ. Miami Sch. Med., Miami, 33101

Methods have been developed for the extraction of metalloproteases from human articular cartilage. A tissue inhibitor of metalloproteases (TIMP) is also present in these extracts. Two proteases have been purified on the basis of their ability to degrade proteoglycan monomers. One protease has a pH optium of 7.2 and a molecular weight in latent form of approximately 56,000. A second protease has an unusual pH optium of 5.3. This protease has been purified 4000-fold starting from 20 g of cartilage. It is found in a latent form of 55,000 and an active form of 35,000 daltons. Both proteases are activated by aminophenylmercuric acetate. The acid protease is inhibited strongly be chelating agents, TIMP and ovostatin but not by phosphoramidon. It cleaves the B chain of insulin at Ala_{14}-Leu_{15} and Tyr_{16}-Leu_{17}. Both proteases appear to have zinc at the active center and both are stimulated by calcium. TIMP has been only partially purified, it has a molecular weight of approximately 28,500. Methods have been developed to measure each protease and the inhibitor in the presence of one another in crude tissue extracts. The results to date indicate that both proteases are elevated at least 3-fold in osteoarthritis, but that TIMP shows little change in activity. We earlier showed that collagenase was also elevated in osteoarthritis. The combined results indicate that various metalloprotease activities are elevated in the disease process, without a concomitant increase in inhibitor. (NIH Grant AM-16940)

E58 Occurence of PMN-elastase as well as Degraded Alpha-1-Antiprotease Inhibitors in Bronchoalveolar Fluids of Adult Respiratory Distress Syndrome, Shiu Y. Yu, Steven K. Harmon, Sally Tricomi, Alpha A. Fowler and Thomas M. Hyers, St. Louis V A Medical Center and St. Louis Univ.Schol.of Med. St. Louis, Mo. 63125.
A possible role for PMN elastase in acute lung injury in ARDS was examined. Since elastase is the major protease secreted by PMN leukocytes, PMN elastase and alpha-1-antiprotease inhibitor (a-1-Pi) in brochoalveolar fluids (BAL) of subjects with ARDS (19 subjects), smoker-controls (16 subjects) and non-smoker-controls (16 subjects) were studied. Higher amounts of antigenic PMN elastase were found in BAL of ARDS as compared with those of controls; 1,336+228 vs. 45+30ng of PMN elastase/mg of BAL protein, p>0.01.
In studies of antigenic a-1-Pi in BAL by SDS-PAG electrophoresis followed by immunoelectrophoretic detection after electrophoretic transfer from gel to nitrocellulose-papers, we found significant amounts of degraded products of a-1-Pi (M.W.of 20K, 30K and 43K dalton) as well as degraded and intact products of enzyme-inhibitor complexes of a-1-Pi (M.W. of 94K,80K and 67K dalton) in BAL of ARDS in addition to free a-1-Pi. But the degraded products of a-1-Pi were not detected in those of controls.
Although elastase activity, assayed by using C-14-elastin as substrate, was not detected in BAL of either ARDS or controls, the presence of significant amounts of both PMN elastase and degraded products of a-1-Pi in BAL suggests that proteolytic injury by elastase may have taken place in lungs of ARDS. (Supported by V A Medical Research and NIH grant HL-30572)

Activation of Cell Activities, Degenerative Diseases and Protease Inhibitors

E59 PROTEASE ACTIVATION OF CELLULAR ACTIVITIES: POSSIBLE ROLES FOR BOTH RECEPTOR OCCUPANCY AND ENZYMIC ACTIVITY, D. H. Carney, Division of Biochemistry, University of Texas Medical Branch, Galveston, TX 77550
Several proteases including trypsin, thrombin and plasminogen activators have been reported to initiate a variety of cellular activities which include platelet activation, fibroblast chemotaxis and cell proliferation. Studies with DIP- and PMS-thrombin have shown that receptor occupancy by proteolytically inactivated (nonmitogenic) thrombin molecules do not inhibit stimulation of proliferation by enzymically active thrombin. This could suggest that receptor occupancy is not involved in mitogenesis. Other studies, however, have shown that initiation of cell proliferation by thrombin appears to require both receptor occupancy and enzymic activity. In this workshop we will examine positive and negative evidence for involvement of specific receptors in these activities. We will also examine the specific regions of thrombin which interact with cells and are responsible for stimulating chemotaxis and proliferation. Recent studies suggest that two separate regions of the thrombin molecule might be involved. Lastly, we will update progress in characterization of receptors for these proteases and present recent findings about the possible signals which might be generated by receptor occupancy and by enzymic activities. In particular, recent studies from at least 3 laboratories have shown that phosphoinositide turnover is stimulated by thrombin as a part of the mitogenic signal. Thus, it is important to determine how phosphoinositide turnover is stimulated by thrombin and to compare this stimulation with that observed in cells after addition of other growth factors.

E60 SELECTIVE DEGRADATION OF FOREIGN AND ABNORMAL PROTEINS
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Escherichia coli, like mammalian cells, rapidly degrade highly abnormal proteins by a soluble pathway that requires ATP. *E. coli* contain at least 8 soluble endoproteases, one of which, protease La (the product of the *lon* gene), requires ATP for function. This novel enzyme catalyzes the initial cleavages in proteins. It is a serine protease that cleaves proteins and ATP in a coupled process. Two ATPs are utilized per peptide bond cleaved. It is a tetramer composed of identical subunits (M_r=94,000). Much progress has been made in understanding its mechanism through the use of fluorogenic substrates. These studies indicate that ATP-dependent proteolysis involves a multistep process: 1) ATP binding occurs initially and allows peptide bond cleavage. 2) ATP hydrolysis then follows. 3) This reaction cycle is repeated until small peptides are generated. 4) In addition, protein substrates activate this protease. Thus, unfolded proteins bind initially to a regulatory site distinct from the active site. Activation of the enzyme by substrates may help prevent inappropriate proteolysis *in vivo*. The cellular content of protease La is also precisely regulated. It is a heat-shock protein whose transcription is controlled by the *htpR* locus. Synthesis of protease La and other heat-shock proteins increases under various conditions that cause the appearance of large amounts of abnormal proteins, including production of foreign polypeptides. Induction of this (and perhaps other) proteases by substrates can help prevent the intracellular accumulation of the abnormal polypeptides.

E61 Human Protease Nexin-I: Immunological Characterization and Biosynthetic Studies,
Daniel J. Knauer and Eric W. Howard, Department of Developmental and Cell Biology, University of California, Irvine, CA 92717.

Protease nexin-I (PN-I) secreted by human fibroblasts is a 47,000 Mr serine protease inhibitor of limited specificity. PN-I mediates the cellular binding, internalization and degradation of the protease to which it has become linked during the inactivation step. We have now developed a monospecific, polyclonal antibody to highly purified PN-I from human fibroblasts. Anti-PN-I IgG blocks the linkage formation between PN-I and its protease substrates, and also blocks the cellular binding of the complexes once formed.

This antibody will also immumoprecipitate PN-I:Protease complexes, but will not react with AT-III:Protease complexes, thus establishing immunological non-identity between PN-I and AT-III. In addition, the antibody will not recognize either the bovine or human endothelial cell-secreted protease inhibitors, but will recognize "PN-I like" molecules from fibroblasts of rodent origin.

Finally, we have used this antibody to immunoprecipitate metabolically ^{35}S-labeled PN-I from human fibroblasts. PN-I comprises about 5% of total cell-secreted protein, and is heavily glycosylated. Short-term pulse experiments using ^{35}S-Methionine, followed by treatment of immunoprecipitates with endogylcosidases suggest that PN-I contains 15% to 17% carbohydrate. Steady state labeling experiments indicate that PN-I levels in the medium might regulate PN-I synthesis and release.

E62 UBIQUITIN-LYSOZYME CONJUGATES: IDENTIFICATION AND CHARACTERIZATION OF AN ATP-DEPENDENT PROTEASE FROM RABBIT RETICULOCYTE LYSATES. R. Hough, G. Pratt and M. Rechsteiner, University of Utah, Salt Lake City, UT 84112

Ubiquitin-lysozyme conjugates have been used as substrates to identify an ATP-dependent protease from rabbit reticulocyte lysates. The protease, which has been partially purified by DEAE chromatography and glycerol gradient centrifugation, has an apparent molecular weight of 600 kilodaltons based on sedimentation and gel filtration. It degrades lysozyme molecules within conjugate complexes only in the presence of hydrolyzable nucleoside triphosphates, but it does not degrade free lysozyme molecules even in the presence of added ubiquitin, lysozyme-ubiquitin conjugates and nucleoside triphosphates. Degradation of lysozome conjugates is independant of added upiquitin and occurs in fractions incapable of ubiquitin conjugation. Proteolysis is maximal at pH 7.8, inhibited by hemin, N-ethylmaleimide or aurintricarboxylic acid and proceeds with an apparent Arrhenius activation energy within the range 27 ± 5 kcal per mole. These properties are similar to those observed for the degradation of ubiquitin-lysozome conjugates in lysates indicating that the partially purified protease catalyzes the 'second' ATP-utilizing reaction identified previously (Hough and Rechsteiner, Proc. Nat. Acad. Sci. USA 81:90 (1984); Hershko et al., Proc. Nat. Acad. Sci. USA 81:1619 (1984); Tanaka et al., J. Cell Biol. 96:1580 (1983).

E63 THE PROTEINASE INHIBITOR ALPHA-1-ANTICHYMOTRYPSIN IS DETECTABLE IN THE HUMAN CENTRAL NERVOUS SYSTEM BY IMMUNOHISTOCHEMICAL TECHNIQUES.
Deanna L. Justice, Roy H. Rhodes and Zoltàn A. Tökès. Depts. of Biochemistry and Pathology, Univ. of Southern California, School of Medicine, Comprehensive Cancer Center, Los Angeles, CA 90033.

Alpha-1-antichymotrypsin, Achy, is a 68kd glycoprotein and a potent inhibitor of enzymes, like cathepsin G, that preferentially cleave proteins at hydrophobic regions. It is synthesized primarily by the hepatocytes, but breast epithelial cells, certain melanomas and HeLa cells also produce it. Its synthesis is induced by estradiol in cancer cells. We report the presence of this proteinase inhibitor in the human central nervous system (CNS). Paraffin-embedded normal fetal, neonatal and adult CNS tissue from 9 autopsy specimens were stained with monospecific antibodies and avidin-biotin-peroxidase complex. Tissue sections included spinal cord, medulla oblongata, cerebellum, mesencephalon, basal ganglia, hippocampus, and neocortex. Positive non-granular, diffuse staining of Achy appeared throughout numerous neurons and glial cells in specific regions of the CNS, including the thalamus, putamen, hippocampal cortex, substantia nigra, grey matter of the cerebral cortex, various cranial nerve nuclei, inferior olivary nucleus, and cerebellar cortex. In addition, a strong granular cytoplasmic staining was apparent in the choroid plexus epithelium. Ventricular ependymal cells demonstrated moderate to strong non-granular staining. Midgestational fetal brains were negative. Specimens from newborn patients in general were negative. Exception was the choroid-plexus epithelium which had a strong apical and granular staining. These observations, thus, establish a widespread distribution of this proteinase inhibitor in the CNS and raise the question of Achy's potential significance in the maintenance of functional neural networks.

E63 ALTERED DISTRIBUTION OF PROTEINASE INHIBITORS IN HUMAN PROSTATE ADENOCARCINOMAS. Kiyoaki Kitajima, Donald G. Skinner, Gary Lieskovsky and Zoltàn A. Tökés. Departments of Biochemistry and Urology, Comprehensive Cancer Center, Univ. Southern California, School of Medicine, Los Angeles, CA 90033 and Nihon University, Tokyo, Japan.

The expression of proteinase inhibitors α-1-antichymotrypsin, (Achy), α-1-proteinase inhibitor, (API), α-2-macroglobulin, (AMG), and inter-alpha-trypsin inhibitor, (IATI), was investigated using immunohistological techniques on frozen human tissue specimens. For control unrelated serum proteins haptoglobin, (Hpg) and alpha-1-acid glycoprotein (AGP) were studied. The purpose was to examine deviations in the availability of these proteins due to malignancy. Antibodies to AGP stained predominantly at the apical surface of normal epithelial cells. In tumor specimens, the expression of AGP was significantly diminished. Hpg was detectable in some of the basal cells and in the basal region of the cytoplasm of all normal epithelial cells. In adenocarcinoma specimens, Hpg was found in intercellular spaces. AMG was undetectable in epithelial and basal cells. IATI was also not detectable in malignant cells. Only faint positive staining was seen for IATI in normal epithelial cells. The most remarkable change in distribution due to malignancy was observed with API and Achy. API shifted position from primarily the apical cytoplasmic regions in normal epithelial cells to what appeared as extracellular compartments in malignant specimens. Achy was located predominantly in the basal cytoplasmic regions of normal and hyperplastic epithelial cells and was below the level of detection in all of the prostate cancer specimens. The altered distribution of these normal proteins, in particular of proteinase inhibitors, may be related to the breakdown of three-dimensional secretory structures, destabilization of cell interactions and to the cellular disorganizations seen in prostatic carcinoma.

E64 THE USE OF ASSOCIATION RATE CONSTANTS IN DETERMINING THE SPECIFICITY OF PROTEINASE INHIBITORS, James Travis, Dept. of Biochemistry, University of Georgia, Athens, Georgia, 30602.

A major problem in determining the specificity of a proteinase inhibitor lies in the fact that many have overlapping activities against several enzymes. For this reason, many inhibitors have, in the past, been referred to as having slow, poor, progressive, or rapid inhibition of a given proteinase. Unfortunately, such terms give no meaning as to whether an inhibitor can react rapidly enough to be of physiological importance. In this laboratory we have attempted to determine the target enzyme controlled by a specific inhibitor by measuring second order association rate constants during enzyme-inhibitor interactions. The results indicate that although several enzymes can be inhibited by a single inhibitor the rates of interaction may differ by factors as large as 100,000. Thus, it is highly unlikely that the slowly reacting inhibitors with a given enzyme have any physiological role, except under extreme conditions where the normal inhibitor may be saturated. Using six of the well known plasma inhibitors we have determined that each has one specific target enzyme and that nearly all secondary reactions with other enzymes are too slow to be of physiological significance. When this data is compared with the in vivo distribution of a given enzyme with all of the plasma inhibitors the results compare favorably, indicating that association rate kinetics are invaluable in determining inhibitor specificity.

E65 MULTIPLE PATHWAYS FOR REGULATING THE EXPRESSION OF METALLOPROTEINASES. Zena Werb, Laboratory of Radiobiology and Environmental Health, and Department of Anatomy, University of California, San Francisco, CA 94143.

The degradation of the connective tissue scaffolding of tissues in degenerative diseases owes largely to the activities of metal-dependent proteinases that include interstitial collagenase, stromelysin, gelatinase and macrophage elastase. These proteinases share the common properties of induced synthesis in response to specific stimuli, activation from precursor forms, enzymatic function in the pericellular or extracellular environment, and specific inhibition by the tissue inhibitor of metalloproteinases (TIMP). The expression of these proteinases, therefore, is the net result of induction of the expression of mRNA for the enzymes, translation into proenzymes, secretion into extracellular spaces, sequestration of proenzymes by matrix proteins, activation by endogenous activators or by exogenous enzymes including kallekrein and plasmin generated by the activity of plasminogen activators, inhibition of active proteinases by TIMP and α_2-macroglobulin, clearance of enzyme-inhibitor complexes, and susceptibility of the extracellular matrix substrate. In the case of synovial cells, the majority of the regulation appears to be at the pretranslational level and at the level of activation of proenzymes. In the case of capillary endothelial cells, the massive synthesis of TIMP provides an additional level of control. Tumor cells such as HT1080 and SV40-transformed fibroblasts appear to express proteinases constitutively and are also inducible for additional proteinases. Supported by U.S. Department of Energy DE-AC03-76-SF01012.

E66 RELEVANCE OF PEPTIDASES AND PROTEASES TO CLINICAL ENZYMOLOGY, Paul L. Wolf, Department of Pathology, University of California, San Diego, La Jolla, CA 92037

This paper is an overview of relevant peptidases and proteases which are significant in clinical enzymology and medicine. Decubitus ulcers are caused by multiple factors including pressure leading to compression ischemia, infection with fever, moisture and immobility. Proteolysis occurs with necrosis of tissue. Necrotic tissue must be debrided since necrotic tissue leads to further proteolysis. Fibrinolysis and desoxyribonuclease are useful in the treatment by degrading denatured proteins. Proteolytic enzymes are important in the humoral regulation of blood pressure, by degrading or producing vasoactive peptides such as bradykinin or angiotensin. Two endopeptidases, renal kallikrein and renin are important in this field. Thrombin, plasmin and elastase are serine proteinases which may cause a proteolysis syndrome in septic patients, associated with coagulopathies. Human granulocytes contain endoproteinases which are released from leukocytes in inflammatory diseases due to bacteria, viruses and fungi and in collagen vascular diseases which are responsible for tissue breakdown. The invasive and metastatic behavior of malignant cells is associated with specific proteinases produced by the neoplastic cells. Proteinases are also important in follicular rupture resulting in ovulation. The plasminogen activating enzyme system is significant in this area. Myofibrillar proteolysis is associated with an increased excretion of 3-methylhistidine in the urine. During fever myofibrillar proteolysis may result from interleukin-1, calcium and prostaglandin E_2. In muscular dystrophy proteinases have been implicated in the catabolism of myofibrillar proteins. These include cysteine proteinases, chymotrypsin and trypsin-like serine proteinases.

Proteases in Control of Normal Biological Processes

E67 THE ADHESION PLAQUE PROTEIN, TALIN, IS RELATED TO P235, A MAJOR SUBSTRATE OF THE CALCIUM-DEPENDENT PROTEASE IN PLATELETS, M. Beckerle, T. O'Halloran, D. Croall*, and K. Burridge, Univ. of North Carolina, Chapel Hill, NC and Univ. of Texas, Dallas, TX.

Talin is a 215kD vinculin-binding protein which is localized in fibroblasts at adhesion plaques where actin filament bundles associate with the plasma membrane. Talin is extremely susceptible to proteolysis at a single site, a characteristic that led us to consider the events of platelet activation in which selective proteolysis is thought to be physiologically significant. One of the proteins that is cleaved during platelet activation is a 235kD protein, P235. Because P235 is remarkably similar to talin in its purification and biophysical properties, we investigated the possible relationship between the two proteins. By Western blot analysis of platelet extracts probed with anti-talin antibody, P235 was demonstrated to be antigenically related to talin. The two proteins also exhibit similar one-dimensional partial proteolytic maps when digested with Staphylococcal V8 protease, elastase, or chymotrypsin. Moreover, both talin and P235 bind radioiodinated vinculin in a blot-overlay assay suggesting a functional relationship between the two proteins as well. One of the most striking characteristics of P235 is its susceptibility to cleavage by the Ca^{++}-dependent protease(CDP). If talin were closely related to P235 one might expect it likewise to be a substrate for the protease. When talin and P235 are incubated with CDP plus Ca^{++}, both proteins are cleaved to yield similar-sized domains of 190-200kD and about 46kD. Proteolysis of a structural component of the adhesion plaque could provide a mechanism for either strengthening or disrupting the stability of substrate-membrane-actin interactions *in vivo*.

E68 PRODUCTION OF INHIBITORS OF COLLAGENASES BY BOVINE AORTIC ENDOTHELIAL CELLS.
Yves A. DeClerck. Childrens Hospital of Los Angeles, Los Angeles, University of Southern California, Los Angeles, CA 90027, USA.

Tissue inhibitors of collagenase play an important role in the control of collagen turn-over in tissues. We have investigated the ability of bovine aortic endothelial cells to secrete inhibitors of collagenases. Serum free medium conditioned by confluent cultures of endothelial cells was found to inhibit the degradation of type I collagen by collagenases from tadpole, rabbit fibroblasts and human fibroblasts but not by bacterial collagenase. This inhibitory activity was suppressed by treatment of the cells with cycloheximide suggesting a direct production of inhibitors by endothelial cells. Gel filtration chromatographic analysis of endothelial cells conditioned medium revealed the presence of at least 2 types of inhibitors. Whereas one inhibitor was identified as Tissue Inhibitor of Metalloproteinases (TIMP), the second with a molecular weight in the range of 70,000-75,000 has to be characterized.

We also demonstrated the inhibition of "classic" collagenase and type IV specific collagenase from tumor cells by these inhibitors.

Our data therefore indicate the production of potent inhibitors of collagenase by bovine aortic endothelial cells, and suggest that these inhibitors may be important in the control of collagen degradation by tumor cells.

E69 NEUROPEPTIDE PROCESSING AT A SINGLE ARGININE RESIDUE: CONVERSION OF LEUMORPHIN (DYN B-29) TO DYNORPHIN B AND DYNORPHIN B-14 BY A THIOL PROTEASE, Lakshmi Devi and Avram Goldstein, Addiction Research Foundation, Palo Alto, CA 94304

Dynorphin B (rimorphin) is formed from dynorphin B-29 (leumorphin) by the action of a thiol protease from rat brain membranes, in a single step. This represents a "single-arginine cleavage" between threonine-13 and arginine-14 of the substrate. We have observed that in addition to dynorphin B, dynorphin B-14 is formed from dynorphin B-29. Among the various general protease inhibitors tested, none except p-chloromercuri benzene sulfonic acid inhibited the formation of the two products. In addition, both temperature and pH had identical effects on the formation of dynorphin B-14 and dynorphin B. The inhibitory potencies of ACTH, peptide E and dynorphin A were identical for the formation of the two products. These results suggest that the same enzyme is responsible for the formation of Dyn B-14 and Dyn B. This represents a novel mechanism of processing at a "single-arginine residue", in that a single enzyme appears to be responsible for the formation of two products by alternatively cleaving to the NH_2-terminal and COOH-terminal side of arginine.

E70 ROLE OF ATP HYDROLYSIS IN THE DEGRADATION OF PROTEINS BY PROTEASE LA
Tim Edmunds and Alfred L. Goldberg, Harvard Medical School, Boston, MA 02115

The ATP requirement for protein degradation in Escherichia coli appears to be due to protease La, the lon gene product. This protease hydrolyzes proteins and ATP in a linked process. Recent studies indicate that degradation of small peptides requires only ATP binding to the enzyme, but degradation of proteins to acid-soluble products requires ATP hydrolysis.

To clarify the function of ATP hydrolysis, we used SDS gels to study the degradation of casein in the presence of ATP or non-hydrolyzable ATP analogs. With ATP, casein is degraded rapidly to acid-soluble products, and no intermediates are found even when the reaction is slowed by lowering the temperature. With the analogs (AMPCPP, AMPPCP and AMPPNP), two large (19 and 20,000d) fragments are produced and released from the enzyme. With AMPCPP, casein is rapidly degraded to these fragments, which can be further degraded to acid-soluble peptides. These data suggest that the degradation of proteins by protease La is initiated at only a limited number of sites and that ATP hydrolysis then allows rapid procession along the substrate without release of polypeptide intermediates.

E71 PURIFICATION AND PROPERTIES OF MICROSOMAL SIGNAL PEPTIDASE, Emily A. Evans, Reid Gilmore, and Günter Blobel, Rockefeller University, New York, NY 10021

We have purified to homogeneity microsomal signal peptidase from canine pancreas. Stripped microsomal membranes were selectively solubilized and subjected to anion and cation exchange chromatography, hydroxylapatite chromatography, sucrose gradient velocity sedimentation and gel filtration. An improved post-translational assay was used to monitor signal peptidase activity. Properties of the purified microsomal signal peptidase were studied and will be discussed.

E72 GENETIC ANALYSIS OF UBIQUITIN FUNCTION, Daniel Finley, Engin Özkaynak, Andreas Bachmair, Stefan Jentsch, John McGrath and Alexander Varshavsky, Department of Biology, M.I.T., Cambridge, MA 02139

Ubiquitin is a protein found in eukaryotic cells either free or covalently joined to a variety of protein species [1]. Conjugation of ubiquitin to short-lived proteins is essential for their selective degradation in vivo, as shown in particular by our previous studies of the mouse cell line ts85, a cell-cycle mutant, which has a thermolabile ubiquitin activating enzyme and is consequently defective for the conjugation of ubiquitin at nonpermissive temperature [2,3].

Current studies focus on the multigene family that encodes ubiquitin in yeast (S. cerevisiae) [4]. We will discuss the phenotypes of various yeast ubiquitin mutants, the synthesis of ubiquitin in the form of a polyubiquitin precursor protein, transcriptional control of ubiquitin synthesis by the heat shock regulatory system, and the role of ubiquitin in the heat-shocked cell.

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E73 PURIFICATION AND CHARACTERIZATION OF TWO ACID PROTEINASES OF THE NEMATODE TURBATRIX ACETI. Rajendra D. Ghai*, Mathew von Wronski+, Don W. Bryant+ and Roger S. Lane+. *Res. Dept., Pharma. Div., CIBA–GEIGY Corp., Summit, NJ 07901 and +Dept. of Biochemistry, Univ. of South Alabama, Mobile, AL 36688.

Two acid proteolytic activities, one that hydrolyzes [methyl-^{14}C] hemoglobin to acid-soluble fragments (Proteinase A) and a second that hydrolyzes glutaryl-L-phenylalanine p-nitroanilide liberating p-nitroaniline (Proteinase B), have been detected in the free-living nematode, *Turbatrix aceti.* These two proteases have been isolated from the 100,000 x g supernatant of *T. aceti* extracts by acid precipitation, and gel exclusion chromatography, and separated from one another by affinity chromatography on diamino-dipropylamine-pepstatin-Sepharose CL-6B. Proteinase B is not adsorbed to immobilized pepstatin in 0.2 M NaCl at pH 3.5 whereas Proteinase A is specifically bound under these conditions; elution of Proteinase A is achieved with 1 M NaCl at pH 9. Proteinase A is a single-chain enzyme of molecular weight approximately 30,000 as estimated by gel filleration and SDS gel electrophoresis. It is optimally active towards hemoglobin as substrate at pH 3.0, is maximally stable at 4°C and pH 7.0, and is an aspartyl protease sensitive to inhibition by pepstatin (50% inhibition is obtained with about 1.0 ng pepstatin/ml). Non-denaturing polyacrylamide gel electrophoresis of proteinase A shows the presence of two active protease components, indicating the occurrence of isoenzymic forms. Proteinase B is of Mr ~ 80,000 and consists of two dissimilar polypeptide subunits with molecular weights of 30,000 and 19,000. It exhibits an optimum pH of 5.5 with glutaryl-L-phenylalanine p-nitroanilide as substrate, is maximally stable at 4°C and pH 6.0, and is a serine protease sensitive to inhibition by DFP and PMSF. The functional significance of these two proteases in nematode protein degradation is not yet known. (Supported by NIH Grant AG01002)

E74 PROPERTIES OF THE Ca^{2+}-DEPENDENT PROTEINASE AND ITS PROTEIN INHIBITOR, D.E. Goll, J.D. Shannon, W.C. Kleese, and S.K. Sathe, University of Arizona, Tucson, AZ 85721
A procedure has been developed for purifying the micromolar Ca^{2+}-requiring form (μM CDP), the millimolar Ca^{2+}-requiring form (mM CDP), and the protein inhibitor (CDPI) of the calcium-dependent proteinase from one sample of bovine skeletal muscle. Both μM and mM CDP autolyze when incubated with Ca^{2+}. The polypeptides and the Ca^{2+} required for one-half maximal activity of the four forms of CDP are as follows: 1) autolyzed μM CDP, 76-kDa and 28-kDa polypeptides, 0.6 μM Ca^{2+}; 2) μM CDP, 80-kDa and 28-kDa, 4 μM Ca^{2+}; 3) autolyzed mM CDP, 78-kDa and 18-kDa, 180 μM Ca^{2+}; and 4) mM CDP, 80-kDa and 28-kDa, 1000 μM Ca^{2+}. Peptide mapping and amino acid analyses show that both the 80-kDa and the 28-kDa polypeptides of μM and mM CDPs differ and probably originate either from different genes or from one gene whose transcription product is processed in alternative ways. CDPI inhibits all four forms of CDP. Binding studies using active CDPs indicate that approximately 30% of the CDP molecules are inactive and that these inactive molecules bind to CDPI with a lower affinity than active CDP molecules. These inactive CDP molecules account for the high inactivation ratios of 7 to 10 CDP molecules inactivated per 1 CDPI polypeptide. The true binding and inactivation ratio is 4 to 5 CDP molecules (either μM or mM) bound per 1 CDPI polypeptide. Immunohistochemical studies using monoclonal antibodies specific for μM or for mM CDP and polyclonal antibodies show that the CDPs and CDPI are not always located at the same places although all three proteins are found exclusively intracellularly. μM and mM CDP co-localize at least at the fluorescent microscope level of resolution. (Supported by NIH Grants AM-19864 and HL-20894; the MDA, and NSF Grant PCM-8118177.)

E75 INDUCTION OF COLLAGENASE IN U937 CELLS, Carol Lipsey Hersh, Raymond K. Yeh, James E. Callaway, Joseph Garcia, Jr., and Maureen Gilmore-Hebert, International Genetic Engineering, Inc., 1545 17th Street, Santa Monica, CA 90404
Collagenase production is induced in U937 tissue culture cells (a monocyte-like cell line) by the addition of phorbol myristate acetate (PMA) to the media at concentrations greater than or equal to 1.6 x 10^{-8} M. Control cultures in sera-free media alone do not produce the enzyme. The induced collagenase cleaves type I collagen into the classical 3/4 and 1/4 products. The induction of the enzyme appears to be the result of de novo synthesis rather than granule release. The production of collagenase is inhibited by actinomycin D and cycloheximide. Collagenase activity can be detected in induced cells 24 hours after the addition of phorbol ester ($8x10^{-8}$M). No enzyme is detected in media after incubation of cells for 1-2 hours with $8x10^{-8}$M phorbol ester. Collagenase production is also induced in U937 cells by endotoxins, interleukin 1, and latex beads. The media from induced U937 cells contains active collagenase. No increase in collagenase activity was detected after N-ethyl maleimide treatment of media collected after a 24 hour incubation with phorbol ester. A 150-fold purification of this enzyme was achieved using DEAE-Sephacel followed by wheat germ agglutinin-agarose chromatography.

E76 Expression of metalloproteinases which degrade gelatin and native type V collagen in resident and inflammatory cells. Margaret S. Hibbs and Andrew H. Kang, University of Tennessee Center for Health Sciences, Memphis, TN 38104.

While interstitial collagenase is responsible for the initiation of degradation of the interstitial collagens, it has no activity against types IV and V collagens. Recently we have purified and characterized a metalloproteinase secreted by human PMNL which degrades gelatin and native type V collagen. The proteinase shows a high degree of specificity for native and denatured collagens with little activity against other proteins. A similar proteinase was found to be secreted by macrophages. This proteinase has a molecular weight of 92,000 and is inhibited by tissue inhibitor of metalloproteinases. The macrophage proteinase is secreted by resident alveolar macrophages and monocytes differentiated in culture but not by peripheral blood mononuclear cells. Using substrate gel analysis, we have extended our observations to fibroblasts.The major metallogelatinase seen in fibroblast culture media has a M_r=67,000 and appears to be constitutively secreted. This proteinase is not recognized by antibodies to the 92,000 proteinase. AM_r=92,000 metallogelatinase which appears immunologically similar to the macrophage proteinase is expressed by fibroblasts in a highly regulated manner with significant secretion noted only after optimal activation of the cells. The differences in the expression and regulation of these proteinases may be important in extracellular matrix alterations in both physiologic and pathologic processes.

E77 FUNCTIONAL DIFFERENCES OF ONE-CHAIN AND TWO-CHAIN TISSUE-TYPE PLASMINOGEN ACTIVATOR WHICH MAY HAVE PHYSIOLOGICAL SIGNIFICANCE, Deborah L. Higgins, M. Christine Lamb, and Gordon A. Vehar, Dept. of Protein Biochemistry, Genentech, Inc., South San Francisco, CA 94080.

Tissue-type plasminogen activator (t-PA) is synthesized as a single polypeptide chain. It can be hydrolyzed to a two-chain protein at a site which is analogous to the "activation site" in most serine proteases. Dansyl-glutamyl-glycyl-arginyl chloromethyl ketone (DEGR-CK) was shown to inactivate both one-chain and two-chain human, recombinant t-PA which was obtained from the supernatants of mammalian cells in tissue culture. The interaction of DEGR-CK with t-PA was accompanied by an increase in the fluorescence intensity and a blue shift in the wavelength of maximum emission. The kinetics of the interaction of t-PA with DEGR-CK could be followed by both loss of activity and an increase in fluorescence. The second order rate constants (k_2/K_i) obtained with these two methods agreed quite well. The rate at which one-chain t-PA is inactivated by DEGR-CK is 15-fold lower than the rate with two-chain t-PA. The results demonstrated, however, that the hydrolysis of the one-chain protein to the two-chain form is not required for reactivity with DEGR-CK. Both one-chain t-PA and two-chain t-PA are active in fibrinolysis. However, their ability to interact differently with other plasma proteins may help regulate the lysis of clots.

E78 INHIBITION OF PROTEOLYTIC PROCESSING OF PRECURSORS TO C3 AND C4, Glen Hortin and Arnold W. Strauss, Washington University, St. Louis, MO 63110

A series of amines, recognized as lysosomotropic agents, inhibited the conversion of the single-chain precursors of the third and fourth components of complement (proC3 and proC4) to their mature forms in the human hepatoma-derived cell line HepG2. Processing of C3 and C4 was assessed by labeling cells with [^{3}H]leucine, isolating labeled C3 or C4 from culture medium by immunoprecipitation, and analyzing the immunoprecipitates by SDS-polyacrylamide gel electrophoresis. In the absence of the amines these cells secreted predominantly C3 and C4. When 0.2 mM chloroquine, 2 mM amantadine, 5 mM triethylamine, 5 mM cyclohexylamine, 5 mM benzylamine, 5 mM tyramine, 5 mM benzamidine, or 5 mM procainamide was included in culture medium, proC3 and proC4 became the predominant secretory forms. Methylamine, ammonium ion, and tris(hydroxymethyl)aminomethane at a concentration of 10 mM had a lesser effect. In pulse-chase experiments cleavage of proC3 was inhibited by addition of chloroquine to medium up to one hour after synthesis of the protein. Thus, the amines probably affected processing of these proteins in a distal compartment along the secretory pathway. Other agents, monensin and nigericin, known to raise the pH of acidic intracellular compartments also inhibited the cleavage of proC3 and proC4. Our results suggest that cleavage of proC3 and proC4 occurs within an acidic compartment along the secretory pathway, and that cleavage is inhibited when the pH of this compartment is raised.

E79 SELECTIVE PROTEASE ACTION ON A MAMMALIAN ENDOCELLULAR ENZYME: ASPARTATE TRANSAMINASE. Ana Iriarte, Holly Hartman, and Marino Martinez-Carrion, Virginia Commonwealth University. Richmond, VA 23298.

Proteases are selective for the NH_2-terminal region of heart aspartate transaminase. These regions overlap, through several hydrophobic contacts, the surface region of the accompanying subunit. Release of small molecular weight peptides from each subunit produces loss of catalytic ability. The action of trypsin, but not of other proteases, is inhibited by polyphosphates, including nucleotides. This is through electrostatic binding to an external region (probably Lys-19) of the transaminase distant from its active site. The action of proteases leads to inactivation due to a conformational change in the transaminase structure induced by the loss of the NH_2-terminal peptide. This is detected by spectroscopic and calorimetric studies on the protease-treated transaminase. After loss of the NH_2-terminal peptide(s) other proteases find it easier to attack the remaining core protein. Reversibility of the initial protease inactivation is being attempted using synthetic peptides covalently attached to the core transaminase through a controlled reversal of the initial proteolytic event. Efficiency of "resynthesis" is monitored following activity and by immunoelectrophoretic methods. It is felt that, whether the coenzyme pyridoxal phosphate is bound (holoenzyme) or not (apoenzyme), the initial proteolytic event induces a new conformational structure of the transaminase that is trigger-facilitator of subsequent proteolytic hits. (Supported by HL-22265).

E80 HUMAN LUNG MAST CELL TRYPTASE. David A. Johnson, Biochem. Dept. Quillen-Dishner College of Medicine, East Tenn. State Univ., Johnson City, TN 37614

Tryptase, a trypsin-like enzyme, is the major serine proteinase of human mast cells. It appears to exist as a tetramer of 125 kDa. The in vivo function of tryptase is unclear, but it must be involved in the limited proteolysis of a specific substrate because activity on denatured proteins such as casein is very limited. One hallmark of this enzyme is its resistance to inhibition by protein proteinase inhibitors, such as alpha-1-proteinase inhibitor, alpha-2-macroglobulin and protease nexin. Tryptase also fails to cleave the bait region of alpha-2-macroglobulin. The enzyme does not activate procollagenase or degrade proteoglycan, which are processes catalyzed by trypsin. Tryptase has a high affinity for heparin, another component of mast cells. In vitro studies show that heparin stabilizes the activity of tryptase.

Previously, tryptase has been isolated as a mixture of forms differing by approximately 500 in subunit molecular weight. These forms have been separated on a cellulose phosphate column by affinity elution with heparin. The high M. W. form was found to be 96% active while the low M. W. form was only 16% active. Incubation of the high M.W. form for 24 hrs at 37°C did not result in its autolysis to the low M.W. form. The high M.W. tryptase seems to be more active on dibasic synthetic peptide substrates. The activities and structures of these two forms will be compared.

E81 REGULATION OF RATTLESNAKE VENOM PROTEOLYTIC AND HEMORRHAGIC ACTIVITY BY INHIBITORS ISOLATED FROM OPOSSUM SERUM, Lawrence F. Kress, Joseph J. Catanese and Lowell G. Sheflin, Roswell Park Memorial Institute, Buffalo, N.Y. 14263

Rattlesnake bite in most mammalian victims results in extensive localized hemorrhage caused by metalloproteinases in the crude venom. However, opossums show little or no localized effects following venom injection, and the gelatinase and hemorrhagic activity of crude rattlesnake venom can be blocked in vitro by whole opossum serum. Fractionation of opossum serum led to the purification of an inhibitor designated oprin (opossum proteinase inhibitor) which formed inactive complexes with venom metalloproteinases, but did not react with HTa, the most active hemorrhagic toxin from C. atrox venom (Fed. Proc. 44, 1431 (1985)). A metalloproteinase inhibitor similar in size to oprin (Mr = 50,000) and differing in charge has now been isolated from opossum serum. This inhibitor blocks both the proteolytic and hemorrhagic activity of HTa. Opossum α_1-proteinase inhibitor (α_1PI) and α_1-antichymotrypsin have also been purified. These inhibit trypsin and chymotrypsin, respectively, but have no effect on venom metalloproteinases. Opossum α_1PI retains inhibitory activity in the presence of venom or venom metalloproteinases under conditions in which human α_1PI is totally inactivated. The presence in opossum serum of the metalloproteinase inhibitor, oprin, and of the specific hemorrhagic toxin inhibitor partly account for the resistance of the opossum to rattlesnake envenomation. The results with opossum α_1PI indicate that there are naturally occurring forms of this inhibitor which are resistant to inactivation by venom metalloproteinases.

E82 DAMAGE AND REMOVAL OF A QUINONE-BINDING PROTEIN OF THE CHLOROPLAST MEMBRANE: A MODEL SYSTEM TO STUDY MEMBRANE PROTEIN TURNOVER. David J. Kyle, Martek Corporation, 9115 Guilford Rd, Columbia MD 21046.

A 32 kDa chloroplast membrane protein (the QB-protein) is known to be involved in the transfer of electrons from Photosystem II to oxidized plastoquinone. Consequently, the enzymatic activity is analogous to the NADP reductase complex of mitochondria. The electron transfer catalyzed by the QB-protein occurs in two steps; the first results in the formation of a tightly-bound semiquinone anion radical, and the second results in the protonation and release of the doubly reduced plastoquinone. Under high intensity illumination the quinone pool is fully reduced, damage ensues at the level of the QB-protein and it is rapidly removed from the membrane. We suggest that when the quinone pool becomes overly reduced, oxygen can successfully compete with the oxidized quinone for the binding site on this enzyme and the resulting electron transfer produces superoxide at the active site. We further suggest that the resulting oxidation of an active site residue causes a conformational change to the protein rendering it more susceptible to protease attack. A rapid turnover of mitochondrial membrane proteins upon reduction of the ubiquinone pool suggests that this might be a universal phenomenon among quinone-binding proteins. Preliminary results indicate that a very stable, membrane-bound protease is involved which does not require ATP. Since the rate of QB-protein turnover is easily controlled (turnover is proportional to light intensity) we can use this system to identify molecular mechanisms involved in the identification and removal of aberant membrane proteins.

E83 HUMAN VASCULAR SMOOTH MUSCLE CELLS (HVSMC) PRODUCE INHIBITORS OF PLASMINOGEN ACTIVATORS (PA). W.E.Laug, A.T'Ang, C.Y.Cheng. Childrens Hospital of Los Angeles, Los Angeles, CA 90027, USA.

Medium conditioned by HVSMC inhibits urokinase (u-PA) and tissue type plasminogen activator (t-PA). Analysis of this medium by SDS-PAGE followed by reverse fibrin autography showed PA inhibitory activities in the molecular weight range of 50,000 to 55,000. These inhibitory activities were partially destroyed by acid treatment. Heparin-sepharose chromatography of the HVSMC conditioned medium allowed for the detection of a protease nexin like substance forming SDS resistant complexes with thrombin and PA. An additional, acid stable PA inhibitor with an approximate Mr of 50,000 was found in the unabsorbed fractions of the heparin-sepharose column. After purification by means of DEAE ion exchange and Con-A sepharose chromatography a preparation was obtained which was resistant to treatment with acid (pH 2.5, 1 hr.), trypsin, dithiothreitol, urea, guanidine-HCl and SDS. The inhibitor neutralized both u-PA and t-PA, plasmin and trypsin and in contrast to protease nexin, also pancreatic elastase. The production of two distinct PA inhibitors by HVSMC may be of importance in the pathobiology of thrombus formation in arteriosclerotic vessels as well as in the natural resistance of large vessels to tumor cell invasion.

E84 PROTEOLYTIC PROCESSING OF ENKEPHALIN PRECURSORS BY A CHROMAFFIN GRANULE TRYPSIN-LIKE PROTEASE. I.Lindberg, Dept. of Biochemistry, L.S.U. Med. School, New Orleans, LA 70119

Several years ago, we reported the identification and partial purification of a trypsin-like protease from bovine adrenal chromaffin granules which may be involved in the biosynthesis of enkephalins (BBRC 106, 186, 1982); this protease is inhibited by DFP, soybean and lima bean trypsin inhibitors, but not by TLCK, PCMB, or leupeptin. Current work has centered on defining the specificity of the reaction of this protease with purified enkephalin precursors. We have used the 5.3 kDal fragment of proenkephalin (which terminates in the sequence YGGFMRGL); the 8.6 kDal fragment of proenkephalin (terminating in YGGFM); and Peptide B (terminating in YGGFMRF). All of these precursors occur naturally within chromaffin granules. Digestion products were characterized by their retention times on HPLC as well as by radioimmunoassays directed against the carboxyl terminal enkephalins. The results indicate that the chromaffin granule protease is capable of correctly cleaving these precursors at pairs of basic residues to yield the intact enkephalins. Further cleavage of YGGFMRGL to YGGFMR was not observed. The enzyme does not appear to be a kallikrein; no generation of bradykinin from human low molecular weight kininogen was measurable by radioimmunoassay. These results provide further evidence for the involvement of this chromaffin granule protease in the biosynthesis of enkephalins.

E85 PHARMACOLOGICAL MODULATION OF PLASMINOGEN ACTIVATOR LEVEL IN P388D1- CONDITIONED MEDIUM G. Chow, A. Woronick, P. Kinkade, R. DeLeon and M. Matteo, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT 06877

P388D1, a continuous line derived from mouse macrophages, secretes proteases constitutively, at levels comparable to those secreted by elicited macrophages. These levels are reduced considerably by dexamethasone (DEX), while levels of constitutively secreted lysozyme are unaffected (Werb, Z., et al (1978) J. Immunol. 121). In this study, the effects of DEX, auranofin (AUR) and indomethacin (IND) on plasminogen activator (PA) activity of P388D1 conditioned media (CM) were compared. P388D1 cells were cultured in 5% CO_2 at 37°C in RPMI 1640 containing 10% acid-treated fetal calf serum +/- DEX, AUR or INDO. Conditioned media were harvested at 48 or 72 hrs, centrifuged, desalted and assayed for PA, inhibitor of PA (IPA) and lysozyme activities. PA activity was determined kinetically by the plasminogen-dependent hydrolysis of chromogenic peptide S-2251 or, qualitatively, by fibrinolysis. IPA was measured by the decrease in activity of exogenously added PA (from P388D1 CM) or urokinase. DEX (.01-1 μM) and AUR (1 μM) substantially decreased detectable PA levels, while INDO increased slightly or had no effect on PA. The drug effects were not caused by direct action on PA itself. Lysozyme levels were unaffected by the drug treatment. Conditioned serumless media were then prepared +/- DEX (.01 μM; 0.2% lactalbumin hydrolysate in RPMI 1640). Fractions from an HPLC anion exchange column of the +DEX CM were inhibitory to both PA (fraction from -DEX CM) and urokinase, suggesting the presence of a dexamethasone-induced inhibitor.

E86 MEMBRANE SECRETORY COMPONENT IS CLEAVED ON THE CELL SURFACE OF RAT HEPATOCYTES
Linda S. Musil and Jacques U. Baenziger, Washington U., St. Louis, MO 63110

Transcellular transport of polymeric IgA from serum to bile in rat hepatocytes is mediated by a 105Kd membranous form of secretory component (mSC). mSC (with or without bound IgA) is cleaved and released into the bile as a soluble 80Kd protein (fSC). We have used monolayer cultures of rat hepatocytes, which synthesize mSC and efficiently cleave it to fSC, to determine the cellular site of this conversion. Cleavage of [35S]Cys-mSC in hepatocytes is inhibited by leupeptin (80% inhibition at 50 ug/ml) but not by DFP, pepstatin, o-phenanthroline, or NEM. [35S]Cys-mSC accumulated in the presence of leupeptin is released as fSC following leupeptin removal and incubation at 37C. Small amounts of fSC are also produced by hepatocytes shifted to leupeptin-free media at 4C. Following leupeptin removal, production of fSC at 4C is abolished if cell surface mSC is first removed by trypsinization at 4C. Prior trypsin treatment does not prevent fSC production if the subsequent incubation is at 37C, suggesting that cleavage occurs at the plasma membrane. Since 80-90% of [35S]Cys-mSC in hepatocytes is intracellular based on insensitivity to trypsin digestion at 4C, lactoperoxidase catalyzed iodination of hepatocytes at 4C was used to selectively label surface mSC with 125I. Surface [125I]-mSC is converted to fSC by cells at 4C, indicating that cleavage occurs on the cell surface because: 1) [125I]-mSC remains trypsin sensitive at 4C and 2) there is a 70% decrease in fSC generation by addition of anti-SC antiserum. Warming of iodinated monolayers to 37C for 10 minutes results in complete internalization (trypsin insensitivity) of [125I]-mSC. If these cells are returned to 4C no cleavage of the intracellular [125I]-mSC is seen. In addition, plasma membrane [125I]-mSC is cleaved to fSC following dounce homogenization whereas [125I]-mSC internalized by cells prior to disruption is not cleaved. Thus, rat hepatocyte mSC is converted to fSC at the cell surface but not intracellularly. This most likely reflects localization of the protease on the cell surface. Supported by NCI grant R01-CA21923.

E87 PURIFICATION AND CHARACTERIZATION OF A HUMAN RHEUMATOID SYNOVIAL METALLOPROTEINASE THAT DIGESTS CONNECTIVE TISSUE MATRIX COMPONENTS.
Yasunori Okada, Edward D. Harris, Jr., and Hideaki Nagase: UMDNJ-Rutgers Medical School, Departments of Medicine and Biochemistry, Piscataway, New Jersey 08854

Two active forms of a metalloproteinase that digests proteoglycans and other connective tissue components were purified to homogeneity from the culture medium of human rheumatoid synovial cells. The relative molecular masses of the two forms were estimated to be 45,000 and 28,000 by SDS/polyacrylamide gel electrophoresis, whereas the latent precursor form was found to be Mr 51,000 by gel permeation chromatography. Both forms had optimal activity at pH 7.5-7.8, and their activity was inhibited by EDTA, 1,10-phenanthroline, tissue inhibitor for metalloproteinases and dithiothreitol, but not by other inhibitors for cysteine, serine and aspartic proteinases. Removal of Ca^{2+} from the enzyme solution resulted in a complete loss of activity which could be restored by the addition of 1 mM Ca^{2+}. The activity of the apoenzyme was restored by the addition of Zn^{2+}, Co^{2+}, or Mn^{2+} in the presence of 5 mM Ca^{2+} but not by each individual metal ion alone. The identical digestion patterns of reduced, carboxymethylated human transferrin and bovine serum albumin indicated both active forms of the enzyme have similar substrate specificity. The enzyme degraded cartilage proteoglycans, gelatin, type IV collagen, fibronectin, and removed the NH_2-terminal propeptides from chick type I procollagen. This enzyme may play a role in normal matrix turnover and in pathological destruction of the matrix.

E88 ACTIVE SITE MAPPING OF HUMAN COMPLEMENT PROTEINS D, C2 AND B WITH PEPTIDE THIOESTER SUBSTRATES AND SYNTHETIC INHIBITORS, James C. Powers, Chih-Min Kam and John E. Volanakis, Georgia Institute of Technology, Atlanta, GA 30332 (C.M.K. and J.C.P.) and U. of Alabama, Birmingham, AL 35294 (J.E.V.)

The specificity and reactivity of complement serine proteases, D, B, C2, Bb, and C2a, were determined using a number of peptide thioester substrates. The rates of thioester hydrolysis were measured using the thiol reagent 4,4'-dithiopydridine at pH 7.5. Each substrate contained a P_1 Arg residue, and the effects of various groups and amino acids in the P_2-P_5 positions were determined. Among peptide thioesters corresponding to the activation site sequence in B, dipeptide thioesters containing a P_2 Lys residue were the best substrates for D. C2 and fragment C2a preferred hydrolyzing peptides containing Leu-Ala-Arg and Leu-Gly-Arg, which have the same sequence as the cleavage site of C3 and C5. B was the least reactive among these complement enzymes and the best substrate was Z-Lys-Arg-SBzl with a k_{cat}/K_M value of 1370. The catalytic fragment of B, Bb, had higher activity toward these peptide thioester substrates. The best substrate for Bb was Z-Gly-Leu-Ala-Arg-SBzl with a k_{cat}/K_M similar to C2a and 10 times higher than the value for B. These synthetic substrates were also used to study the irreversible inactivation of D, B, C2 and fragments Bb and C2a by several classes of serine protease inhibitors. Two inhibitors with positively charged thiouryl groups on the isocoumarin ring, not only inhibited protein D but also inhibited C2, B, C2a, and Bb to a lesser extent.

E89 POSSIBLE ROLE OF CALPAIN I AND CALPAIN II IN DIFFERENTIATING MUSCLE, Judith E. Schollmeyer, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE
The variable distribution of the 80-kDa subunit of two calcium-activated proteases, calpain I and calpain II, has been examined in L_8 and L_6 myoblasts, and their nonfusing variants, fu-1 and M_3A using non-cross-reacting monoclonal antibodies to both subunits. Immunofluorescence results have shown that while the 80-kDa subunit of calpain I is localized in the cytoplasm of all the myoblasts, the 80-kDa subunit of calpain II appears to be predominantly associated with the plasma membranes of L_8 and L_6 myoblasts. The distribution of the 80-kDa subunit of calpain II in non-fusing myoblasts, fu-1 and M_3A, is generally cytoplasmic and diffuse. Immunoblot analysis of membrane and cytosol fractions of all the myoblasts using the monoclonal antibodies described above essentially confirms the immunofluorescence findings. Because calpain II exhibits a peripheral distribution in cells which are fusion-competent, L_6 and L_8 myoblasts but not in fu-1 and M_3A myoblasts, we suggest that calpain II may play a role in the Ca^{2+}-mediated fusion events of differentiating (prefusion) myoblasts.

E90 AN INSULIN-STIMULATED PROTEASE IN ADIPOCYTE PLASMA MEMBRANES POSSIBLY INVOLVED IN SIGNAL TRANSDUCTION, J. Seals and J. Quail, U. Mass. Med. Sch., Worcester, Ma 01605
A critical element to be identified in the mechanism of action of a hormone or growth factor is an activity closely linked to the ligand receptor that transduces the binding event into a cellular enzyme event, ideally one that triggers subsequent regulatory actions. In insulin action, several lines of evidence have led to the proposal that a proteolytic event might be a common regulatory signal linking receptor binding to cellular actions (J.B.C. 255, 6529, 1980). We have therefore attempted to identify a protease in rat adipocte plasma membranes (PM) whose activity is increased in the presence of insulin. First, protease activity of isolated PM was determined toward a general substrate, casein labeled with fluorescein. Total activity of control PM was about equally divided between serine and thiol protease activities at pH 7.4. EGTA partly inhibited the serine activity, indicating a Ca^{++}-dependent serine protease component representing 5-10% of the total activity. When these measurements were made in the presence of 5 nM insulin, the same components were identified. Total activity was only slightly increased ($9.0 \pm 5.7\%$), but the Ca^{++}-dependent serine protease component was significantly increased ($112.5 \pm 19.4\%$). Second, PM were labeled with ^3H-DFP followed by SDS-PAGE to resolve protease components. In control PM, a major band (58 kd) and nine minor bands were identified. Labeling of one band (62 kd) but no others was increased $35.7 \pm 12.2\%$ in the presence of insulin. These results are consistent with a PM-associated, Ca^{++}-dependent serine protease stimulated by insulin whose role, yet to be determined, might be in eliciting the cellular response to insulin.

E91 PLASMINOGEN ACTIVATOR ACTIVITIES IN THE DEVELOPING RAT PROSTATE, Akhouri A. Sinha[1,2] Richard D. Estensen[3], James E.H. Powell[1], and Michael J. Wilson[1,3], V.A. Medical Center[1], and Depts of Genetics and Cell Biology[2] and Laboratory Medicine and Pathology[3], University of Minnesota, Minneapolis, MN 55417
Plasminogen activator (PA) activities are increased during embryonic and fetal growth. We examined plasminogen activator activities in the rat prostatic complex of Sprague-Dawley rats of different ages from birth to sexual maturity to determine if this protease is associated with the establishment of tissue organization in this gland. PA activity/unit protein did not change significantly in prostatic complexes (which included the seminal vesicles) in animals 2-3, 15-16, and 21 days of age (this includes the period of acinar development, formation of lumina in ducts and acini, and cytodifferentiation of epithelial secretory cells). The individual lobes of the prostatic complex were dissected from 21 and 70 day old animals. PA activities/unit protein decreased 4 fold in the ventral prostate and 27 fold in the dorsolateral prostate (2 and 14 fold respectively when expressed per unit DNA) during growth from the sexually immature to the mature state. The substantially higher PA activities of the ventral prostate in the adult correlates with the finding of PA activities in its secretions. Thus, not only are PA activities elevated during prostatic development, but in the ventral prostate it is an expression of the differentiated secretory function of the gland. The control of PA expression for these two separate purposes remains to be established. (Supported in part by general research funds of the VA and NCI grant CA-22195)

E92 HUMAN BRONCHIAL LEUKOCYTE PROTEINASE INHIBITOR: EVIDENCE FOR A PRECURSOR.
C.E. Smith and D.A. Johnson, Biochem. Dept., East Tenn. State Univ., Johnson City, TN

The secretions of the upper respiratory tract contain a low M.W. (11 kDa) acid stable inhibitor known as bronchial leukocyte proteinase inhibitor (BLPI) that apparently functions to inhibit human neutrophil elastase (HNE) and cathepsin G (Cat G) released from extravascular leukocytes (Ki=10^{-10} M, 10^{-9} M resp.). BLPI is present in a variety of seromucous secretions including those of salivary glands. Since BLPI is a secretory protein, we have investigated the possibility that BLPI may be derived from limited proteolysis of a larger precursor. A simple quantitative immuno-blot assay for BLPI was developed and used to analyze human saliva for precursors of BLPI. Antigen (BLPI) is first blotted to nitrocellulose, then incubated with affinity purified rabbit antibody (anti-BLPI) followed by incubation with peroxidase tagged anti-rabbit IgG. A bright blue color results where the original antigen (BLPI) was present. Densitometry tracings of these blots allows quantitation of BLPI in the picogram range. This assay in conjunction with western blot analysis of cetyl-pyridinium Cl treated saliva show a possible BLPI precursor of ≈80 kDa. Gel filtration of the treated saliva on Sephacryl S-200 gives a small peak at 89 kDa that contains HNE inhibitory activity. Polyacrylamide gel electrophoresis (SDS and reduced) of this peak shows a single band at 70 kDa that is immunologically cross-reactive with BLPI and indicates that the HNE inhibitory activity observed, was not due to complexation of BLPI with other saliva components. These data suggest that BLPI is excreted as a precursor.
(Supported by The Health Effects Institute.)

E93 THE KINETICS OF THE INHIBITION OF THROMBIN BY HIRUDIN
Stuart R. Stone and Jan Hofsteenge, Friedrich Miescher Institute, P.O. Box 2543, CH-4002 Basel, Switzerland

The dissociation constant for hirudin was determined by varying the concentration of hirudin in the presence of a fixed concentration of enzyme and tripeptidyl p-nitroanilide substrate. The estimate of the dissociation constant determined in this manner diplayed a dependence on the concentration of substrate which suggested the existence of two competitive binding sites for the substrate. A high affinity site could be correlated with the binding of the substrate at the active site and the other site displayed an affinity for the substrate that was two orders of magnitude lower. Extrapolation to zero substrate concentration yielded a value of 20 femtomolar for the dissociation constant of hirudin. The dissociation constant for hirudin was markedly dependent on the ionic strength of the assay; it increased 20-fold when the ionic strength was increased from 0.1 to 0.4. This increase in dissociation constant was accompanied by a decrease in the rate with which hirudin associated with thrombin. This rate could be measured with a conventional recording spectrophotometer at higher ionic strength and was found to be independent of the binding of substrate at the active site.

E94 THE UBIQUITIN/ATP-DEPENDENT PATHWAY: EVIDENCE THAT IT EXISTS IN EYE LENS AND PLAUSIBLE ROLE IN CATARACT FORMATION,
Allen Taylor, Jessica Jahngen, Joanne Blondin. USDA Human Nutrition Research Center on Aging at Tufts Univ., 711 Washington St., Boston, MA

The sole function of the eye lens is to collect and focus light on the retina. Accordingly, the lens must remain clear throughout life. With increasing age the lens becomes clouded, and in over 25% of the elderly, opacified. Cataractous opacities are due in part to the accumulation, aggregation and eventual precipitation of damaged lens proteins. In many cell systems damaged proteins are rapidly catabolized by proteases. Thus, we endeavored to establish models in which to study the effects of aging on proteolysis and to determine if the precipitation of lens proteins is due to failure of proteolytic systems upon aging. Using UVA and UVB irradiation as a model of age related photooxidation, we show that 1) after 60 min aminopeptidase activity is diminished; 2) after 120 min protein aggregation is obvious and 3) 15mM ascorbate delays both of these damages.

Next, we monitored the initiation of lenticular proteolysis. Using cell free and in vivo lens systems, we show that the lens contains a fully functional ubiquitin-lens protein conjugating system which appears to be attenuated with age. This data suggests that impaired initiation of proteolysis via the ubiquitin pathway and impaired proteolytic capabilities may account for the accumulation of damaged proteins in cataractous opacities. Such photooxidative damage may be delayed with antioxidants.

Supported by grants to Allen Taylor from USDA/ARS, Fight for Sight, Mass Lions Eye Research Fund, Inc., N.I.H.

E95 SPECIFIC LABELING OF CELL SURFACE PROTEOLYTIC PRODUCTS, James A. Thompson, Alice L. Lau, and Dennis D. Cunningham, Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, CA 92717.

A procedure was developed to detect proteolytic cleavages at cell surfaces using a water-soluble form of the ^{125}I-labeled Bolton-Hunter reagent, sulfosuccinimidyl-3-(4-hydroxyphenyl) propionate (sulfo-SHPP). This method was developed in order to detect thrombin generated cell surface cleavage(s) as the initiating event(s) in the mitogenic stimulation of fibroblasts by thrombin. Membrane proteins were labeled before or after treatment with thrombin. Comparison of labeled proteins on an autoradiogram revealed thrombin substrates as bands that disappeared after treatment of cells with thrombin while products of proteolysis were bands that appeared. Since sulfo-SHPP labels free amino-groups, new amino-termini produced by proteolysis were preferentially labeled. In addition, the sensitivity of labeling of new amino-termini was increased by blockage of existing amino-termini and lysines by incubation of cells with unlabeled sulfo-SHPP before the generation of new amino-termini as a result of thrombin treatment. In this way, amino-terminal proteolytic fragments were specifically labeled and easily identified by autoradiography. Resolution was enhanced by fractionation of labeled membrane proteins by selective extraction with detergents before electrophoresis.

E96 PLASMINOGEN ACTIVATOR ACTIVITIES IN THE VENTRAL AND DORSOLATERAL PROSTATIC LOBES OF AGING FISCHER-344 RATS, Michael J. Wilson[1,2] Janet V. Ditmanson[2], and Richard D. Estensen[2], VA Medical Center[1] and Dept. Laboratory Medicine and Pathology[2] University of Minnesota, Minneapolis, MN 55417

Plasminogen activators (PA) are increased in oncogenesis. Since prostatic neoplasic changes occur with aging in rats and humans, we undertook a study of the ventral and dorsolateral prostatic lobes of Fischer-344 rats of different ages to determine whether altered PA activities result from aging alone or the pathology concurrent with aging. Protein and DNA content of the ventral was decreased only in 30 month old animals. The ventral prostates of 30 month old rats had widespread epithelial atrophy and small foci of intraglandular atypic hyperplasia and/or adenocarcinoma. There was no change in PA activity/unit protein or DNA in ventral prostates of 2-3, 5, and 16 month old animals. However, PA activities were about 20 fold higher in ventral prostates of 30 month old animals. On the other hand, there was no change in PA activities/unit protein or DNA in the dorsolateral prostate. There was also no prevalent epithelial atrophy nor neoplastic changes in this lobe. Thus, it appears that prostatic plasminogen activator activities do not change in association with aging alone, but with development of atypical tissue organization. (Supported in part by general research funds of the VA and NCI grant CA 22195).

Hemostatis, Posttranslational Modification, and Therapeutic Uses

E97 RAPID ISOLATION OF SERPIN LEAVING PEPTIDE: USE OF METHOD IN THE IDENTIFICATION OF A NEW REACTIVE CENTER VARIANT OF ANTITRYPSIN, Stephen O. Brennan and Robin W. Carrell, Molecular Pathology Laboratory, Pathology Department, Clinical School of Medicine, Christchurch Hospital, Christchurch, New Zealand

The tight association between the leaving peptide and post-complex antitrypsin was responsible for the erroneous N-terminal placement of the active site. The difficulty of isolating the leaving peptide also led to antitrypsin being described as a double-headed inhibitor. We report a one-step SDS/ethanol fractionation procedure for isolating the C-terminal leaving peptide.

This procedure was used in the identification of a new variant of antitrypsin, AT Christchurch 363 Glu->Lys. This substitution involves an alteration in charge at the P5' residue of the reactive center. Competition experiments showed that AT Christchurch reacted at the same rate as normal AT in the presence of limiting amounts of trypsin and leukocyte elastase. Both inhibitors were inactivated by catalytic amounts of papain: this inactivation was due to cleavage at the P7 position. There is no obvious clinical condition associated with this new antitrypsin, at least when present in the heterozygous state.

E98 STRUCTURE OF SERPINS AND DESIGN OF SPECIFIC INHIBITORS. Robin W.Carrell, Molecular Pathology Laboratory, Clinical School of Medicine (University of Otago), Christchurch Hospital, Christchurch, New Zealand

The ancestral serpin appeared 500 million years ago and subsequent divergence has given the family of inhibitors that control mammalian proteolytic cascades. Homology alignment of 14 members indicates a conserved tertiary structure with three areas of major variation: N-terminal extensions, the reactive centre and its associated loop peptide. Variation of the reactive centre (358 Met) of α1-antitrypsin alters its inhibitory specificity in a generally predictable way[1-4].

K assoc	HNE	PPE	Trypsin	Thrombin	Kallikrein	Xa
358 Met	$7x10^7$	$1x10^5$	$4x10^4$	$5x10^1$	$7x10^1$	$2x10^2$
358 Arg	$2x10^3$	Nil	$3x10^6$	$3x10^5$	$1x10^5$	$2x10^4$
	XIa	XIIf	Plasmin	CatG	C1S	
	$7x10^1$	Nil	$2x10^2$	$4x10^5$	Nil	
	$1x10^5$	$8x0^2$	$2x10^5$	$2x10^4$	Nil	

A range of such variants has now been produced principally in yeast[5,6]. The engineered inhibitors are not glycosylated; this does not affect function but does give a markedly decreased stability. There is a five-fold reduction in half-life due to increased catabolism and loss through the glomerular membrane. Antigenicity, though present, will probably not be a major problem for therapeutic use (N.Roosdorp, personal communication).

It is intended that discussion of the individual serpins will focus on the structural features responsible for their specificity with the aim of defining the type of changes that may be engineered - including alternatives to full glycosylation.

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E99 THROMBOLYSIS WITH TISSUE-TYPE PLASMINOGEN ACTIVATOR (t-PA) AND SINGLE CHAIN URO-KINASE-TYPE PLASMINOGEN ACTIVATOR (scu-PA), Désiré Collen, Center for Thrombosis and Vascular Research, University of Leuven, Belgium and Departments of Biochemistry and Medicine, College of Medicine, University of Vermont, Burlington, VT 05405

The thrombolytic efficacy and fibrin-specificity of natural and recombinant t-PA has been demonstrated in animal models of pulmonary embolism, venous thrombosis and coronary artery thrombosis. In all these studies intravenous infusion of t-PA at sufficiently high rates caused efficient thrombolysis in the absence of systemic fibrinolytic activation.
The thrombolytic efficacy and relative fibrinogen sparing effect of t-PA was recently confirmed in three multicenter clinical trials in patients with acute myocardial infarction. Intravenous infusion of 0.5 mg to 1 mg of t-PA per kg body weight over 1 to 3 hrs resulted in coronary reperfusion in approximately 70 percent of patients. It raised the plasma level about 1,000-fold but was associated with an average decrease of the plasma fibrinogen level by 30 percent.
Specific thrombolysis by scu-PA has also been demonstrated in animal models of pulmonary embolism, venous thrombosis and coronary artery thrombosis. Again intravenous infusion of scu-PA at sufficiently high rates caused thrombolysis in the absence of systemic fibrinolytic activation. We have treated six patients with acute myocardial infarction with scu-PA and obtained coronary reperfusion during intravenous infusion of 40 mg scu-PA over 60 min in four of the patients and during subsequent intracoronary infusion in one addditional patient. A decrease of fibrinogen to 25 percent of the preinfusion value was observed in one patient.

E100 Regulation of Prothrombinase Activity of Vascular Cells. Paula B. Tracy, Edwin G. Bovill and John C. Hoak, University of Vermont, Burlington, VT 05405.
We are investigating the interactions of factor Va (FVa) with human venous and arterial cells as related to its cofactor role in the prothrombinase (IIase) complex and its inactivation by the activated protein C (APC)/protein S (PS) regulatory complex. FVa performs its cofactor role in part by binding to the cell surface and forming the receptor to which factor Xa (FXa) binds. Venous and arterial endothelium derived from both fetal and adult vessels support II activation when FVa and FXa are added exogenously. Adult artery > adult vein > fetal vein > fetal artery with respect to eliciting IIase activity. Comparison of these data with platelet IIase activity indicate that ~50 platelets would express the same relative IIase activity as one adult arterial endothelial cell, whereas only six platelets would be required to elicit the IIase of one fetal venous endothelial cell. These comparisons were made based on studies using confluent endothelial cell monolayers. Endothelial cells examined at sub-confluence elicit 3-4 times more IIase activity than confluent cells. In addition, confluent arterial smooth muscle cells support II activation at rates comparable to the subconfluent arterial cells when compared on a per cell basis. We have shown also that fetal vein endothelium and arterial smooth muscle cells support the inactivation of FVa by APC. The rate of inactivation could be enhanced by the addition of PS. The rate and extent of FVa inactivation was greater on a per cell basis with smooth muscle cells than with cells from venous endothelium. These data would suggest that cells of vascular origin can amplify and modulate thrombin generation.

Alterations of Proteases by Evolution and Genetic Engineering

E101

Protein Engineering of Subtilisin: Proteases of Enhanced Stability

P. Bryan, Pantoliano, M., Rollence, M., Wood, J., Gilliland, G.
Finzel, B., Ladner, B., Hsiao, H. and Poulos, T.

We have devised a system for introducing mutations in vitro into random sites within the cloned subtilisin gene from Bacillus amyloliquefaciens and screening for proteases of increased stability. Libraries of variant protease genes were produced containing from 1 - ~10 base changes per molecule. Mutagenized genes were then introduced into Bacillus subtilis, and secreted proteases of enhanced stabilities were identified using a novel plate assay procedure which enabled us to screen >10^5 variants. A number of these with substantially increased resistance to thermal inactivation were further characterized kinetically and by differential scanning calorimetry to distinguish between classes of variants with increased intrinsic thermal stability vs. resistance to autodigestion. We have determined the DNA sequence of the most interesting variants and determined the amino and substitutions responsible for the altered phenotypes. X-ray crystallographic analysis is underway on a number of these. This approach has helped identify structural features of subtilisin critical to protein stability and aided in the design of other amino acid changes to further enhance stability.

E102 THE AUTOPROTEOLYTIC MATURATION OF SUBTILISIN, Scott D. Power, Robin M. Adams, Brian Cunningham and James A. Wells, GENENCOR Inc. and GENENTECH Inc., South San Francisco, CA 94080.

The secretion and maturation of the Bacillus amyloliquifaciens subtilisin has been studied in several Bacillus subtilis hosts. This secreted serine endoprotease appears first as a preproenzyme associated with the cell membrane. Mutations which alter a catalytically critical residue (i.e., Asp+32 -> Asn), or delete the COOH terminal portion of the protein (containing the active site Ser+221), block the maturaton of this precursor in Bacillus subtilis strains from which the chromosomal subtilisin gene has been deleted. Inactive subtilisin precursors can be processed by addition of active "helper" subtilisin in vivo or in vitro. Thus, the release of subtilisin from the membrane appears to be dominated by an autoproteolytic process which is novel among secreted proteins.

E103 PROTEOLYTIC PROCESSING OF HUMAN FACTOR VIII, Dan L. Eaton and Gordon A. Vehar. Genentech, Inc., South San Francisco, CA 94080.

Human factor VIII purified from concentrates consists of multiple polypeptides with M_r ranging from 80,000 - 210,000. Amino acid sequence data shows that the M_r 80,000 - 210,000 proteins represent the C- and N- terminal portions of the (cDNA predicted) single chain precursor of factor VIII, respectively. Thrombin treatment of factor VIII results in the cleavage of the M_r 80,000 - 210,000 proteins and the generation of subunits with M_r 73,000, 50,000 and 43,000. Generation of these subunits correlates with activation of factor VIII by thrombin. Factor Xa proteolysis of factor VIII initially results in the generation of subunits of M_r 73,000, 50,000 and 43,000, however, the M_r 50,000 and 73,000 subunits are subsequently cleaved to fragments of M_r 45,000 and 67,000. These latter cleavages correlate with inactivation of factor VIII by factor Xa. Inactivation of factor VIII by activated protein C (APC) correlates with the proteolysis of M_r 90,000 - 210,000 proteins and the generation of a M_r 45,000 fragment. Correlation of the above mentioned cleavages with activation or inactivation of factor VIII has allowed the tenative identification of regions of factor VIII that may be required for factor VIII coagulant activity.

E104 IMPORTANCE OF HYDROGEN BOND FORMATION IN STABILIZING THE TRANSITION STATE COMPLEX IN SUBTILISIN, David A. Estell*, Thomas P. Graycar*, Brian C. Cunningham*, James A. Wells**, *Research Department, Genencor Inc., **Department of Biocatalysis, Genentech Inc., South San Francisco, CA 94080.

Structural studies on serine protease have shown that hydrogen bonds are involved in stabilizing the charged tetrahedral intermediate in the transition state complex. However, little is known of the quantitative importance of these hydrogen bonds to transtion state stabilization. X-ray crystalographic studies of subtilisin (Robertus, J.D., Kraut, J., Alden, R.A. and Birktoft, J.J. (1972) Biochem. 11, 4293) have suggested that the amide side-chain from Asparagine-155 forms a hydrogen bond with the oxyanion produced on the substrate carbonyl oxygen in the tetrahedral intermediate. The importance of this interaction has been studied by kinetic analysis of purified mutant enzymes produced by site-directed mutagenesis of the B. amyloliquefaciens subtilisin gene. Replacement of Asn-155 with Thr, His, Gln or Asp caused a large decrease in substrate turnover, k_{cat} (200-4,000 fold). These translates to a large drop in transition state stabilization energy of 2.2 to 4.7 kcal/mol (Wells, J.A., Cunningham, B.C., Graycar, T.P. and Estell, D.A. (1986) Proc. Roy Soc. Ser B. in press). This data also suggest the hydrogen bond between the substrate and Asn-155 develops most strongly with the substrate bound in the transition state complex (E•S*) and only weakly, if at all, with the substrate bound in the Michaelis complex (E•S).

E105 THE 1.2A CRYSTAL STRUCTURE OF SUBTILISIN BPN', Barry C. Finzel, Andrew J. Howard and Michael W. Pantoliano, Genex Corporation, Gaithersburg, MD 20877

The three-dimensional atomic structure of the bacterial serine protease Subtilisin BPN' from B. Amyloliquefaciens has been refined crystallographically at 1.2A resolution. A precise crystal structure determination has revealed the existence of two previously unidentified cation binding sites spacially separated from each other and from the proteolytic active site. Integrated electron density and coordination geometry at these sites leads us to infer that these are calcium ion sites fully occupied in the monoclinic crystal form, even though no calcium or calcium salt was explicitly introduced during protein purification or crystallization. Studies indicate a marked dependence upon calcium ion concentration for protein stability and autolysis.

E106 CLONING AND SEQUENCE ANALYSIS OF cDNA FOR BOVINE CARBOXYPEPTIDASE E
Lloyd D. Fricker and Edward Herbert, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Rd., Portland, OR 97201

Carboxypeptidase E (enkephalin convertase) is a carboxypeptidase B-like processing enzyme which is involved in the biosynthesis of many neuropeptides (Fricker, 1985, Trends Neurosci. 8: 210-214). A cDNA clone which encodes this enzyme has been isolated from a bovine pituitary cDNA library. The sequence of this clone suggests an evolutionary relationship between carboxypeptidase E (CPE) and carboxypeptidases A and B. Although the overall homology is low, all of the amino acids thought to be essential for catalytic activity of carboxypeptidases A and B have been conserved in CPE. Of the 90 amino acids which have been conserved between bovine CPB, crayfish CPB, bovine CPA, and rat CPA, 35 have been conserved in bovine CPE. Major differences between CPE and the other carboxypeptidases include an additional 120 amino acids at the C-terminal of CPE, and numerous changes in amino acids near the substrate binding sites. These changes may reflect the different specificities of the enzymes: CPE is very specific for basic amino acids, and does not bind aromatic or aliphatic amino acids. Also, CPE is optimally active at pH 5.6, whereas the other carboxypeptidases are maximally active at a neutral pH. A comparison of these enzymes may lead to a better understanding of a structure-function relationship for carboxypeptidase activity.

E107 **CLONED EGLIN PREVENTS DEVELOPMENT OF SHOCK LUNG IN EXPERIMENTAL SEPTICEMIA, H. F. Welter, M. Siebeck, H. Wiesinger, U. Seemüller, M. Jochum and H. Fritz, University of Munich, D-8000 Munich 2, FRG**

The medical leech Hirudo medicinalis contains a potent inhibitor of neutrophil elastase and cathepsin G. This inhibitory mini-protein was produced recently by Rink et al. (1984) in transformed bacteria. The recombinant eglin has been used to elucidate pathomechanisms leading to the development of shock lung in experimental septicemia. Septicemia was induced in pigs by i.v. infusion of E.coli for 2 hours. Eglin was administered in the "therapeutic" group for 4 hours in a dose of 3.85 mg/kg x h. Compared to the untreated septicemia group with a mean survival time of 5.3 + 1.5 hours most of the eglin-treated animals survived the experimental period (30 hours). This therapeutic effect of eglin was corroborated by improvement of many of the parameters measured, especially by reduction of the consumption of plasma factors (antithrombin III, clotting factor XIII, α_2-macro-globulin) and only modest morphological alterations of the lungs. Obviously, unspecific proteolysis due to liberated neutrophil proteinases contributes significantly to the development of lung dysfunction in septicemia. Administration of suitable proteinase inhibitors offers an promising tool to protect the lungs against the inflammatory response of the organism induced by bacterial sepsis or septic shock.

E108 EXPRESSION AND MUTAGENESIS OF HUMAN TISSUE PLASMINOGEN ACTIVATOR, Mary-Jane Gething*, Esa Kuismanen+ and Joe Sambrook*. *University of Texas Health Science Center, Dallas, TX. +Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

We have used a variety of mammalian vectors to express to high levels a cloned copy of a cDNA coding for human plasminogen activator. Using oligonucleotide mismatch mutagenesis, we have constructed mutants at defined locations in the various domains of the protein. The properties of these mutants will be reported.

E109 Expression and Secretion of Bovine Calf Chymosin by Aspergillus nidulans

K. Hayenga, R. Berka, D. Cullen, G. Gray, S. Norton, M. Rey, and L. Wilson
Genencor, Inc., South San Francisco, CA 94080

We have constructed vectors containing the structural gene for calf chymosin which is functionally linked to the promoter, presumptive upstream activator sequence and terminator of the A. niger glucoamylase gene. This cassette was inserted into the unique Cla I site of the A. nidulans transformation vector pDJB3 (Ballance and Turner, Gene, in press).
A. nidulans transformants were selected by pyr 4 complementation. Active chymosin was identified in culture supernatants by western blot analysis and milk clotting activity. A. nidulans transformants secreted chymosin when grown on media containing either starch or xylose indicating that the A. niger glucoamylase promoter is not regulated in A. nidulans.
The A. nidulans derived chymosin is being further characterized by standard protein methodology.

E110 PRIMARY STRUCTURE OF HUMAN α_2-ANTIPLASMIN, W.E. Holmes, L. Nelles, H.R. Lijnen, D. Collen, Center for Thrombosis and Vascular Research, University of Leuven, Belgium

Fibrinolysis is a multicomponent enzymatic system. Limited proteolysis of the inactive zymogen, plasminogen, generates the serine protease plasmin which dissolves fibrin. In addition, plasmin degrades other proteins such as fibrinogen, factor V and factor VIII and, if uncontrolled, plasmic attack on these proteins can lead to hemostatic breakdown. Human plasma contains a very rapid and irreversibly acting inhibitor of plasmin, called α_2-antiplasmin. This primary physiological inhibitor is a single chain glycoprotein of MW 70,000 which reacts stoichiometrically with plasmin.
We have isolated cDNA clones encoding α_2AP with the use of adult human liver as a source of poly A^+ mRNA for the preparation of cDNA libraries in λgt11. Two degenerate and partially overlapping deoxyoligonucleotides encoding the α_2AP amino acid sequence EQPEIQVAHFPFKNNM were annealed and repaired using radioactive nucleotides to produce a probe for low-stringency hybridization to the cDNA library.
The amino acid sequence deduced from the cDNA of α_2AP demonstrates significant homology to other serine proteinase inhibitors (serpins) including α_1AT, AT III, ovalbumin and angiotensinogen. Its reactive site sequence is AR-MSLS as determined by NH_2-terminal and COOH-terminal amino acid sequence of the two α_2AP peptides obtained by nucleophilic dissociation of plasmin-α_2-antiplasmin complex. This sequence was confirmed by the overlapping cDNA sequence. The reactive site sequence thus is different from the R-SL or M-SI reactive site sequences of AT III and α_1AT respectively.

E111 MODELLING α_1-ANTITRYPSIN FUNCTION BY PROTEIN ENGINEERING, Sophie Jallat, Luc-Henri Tessier, Annie Benavente, Ronald G. Crystal* and Michael Courtney, Transgene S.A., 11 rue de Molsheim, 67000 Strasbourg, France and • NHLBI, Bethesda, MD 20205, U.S.A.

We have previously shown that a single point mutation at the reactive centre (P1 position) of recombinant human α_1-antitrypsin (α_1AT) can alter its specificity of inhibition from neutrophil elastase to thrombin (1) as well as the contact phase proteases Kallikrein and Factor XII (2). This involved a Met→Arg substitution at position 358, a mutation also found in a natural α_1AT variant which was associated with a severe bleeding disorder. It was also shown that a Met^{358}→Val substitution retains efficient antielastase function but is no longer susceptible to oxidative inactivation, a process thought to be important in vivo in the genesis of pulmonary emphysema. A series of further mutations have been performed in an attempt to target the inhibitor towards different serine proteases. In this approach alterations were made to generate potential cleavage sites for various proteases at the α_1AT reactive centre. For example, α_1AT (Met^{358}→Phe) does not inhibit either neutrophil or pancreatic elastase but does inhibit cathepsin G. α_1AT (Met^{358}→Leu), like the natural inhibitor, inactivates both elastase and cathepsin G but is fully resistant to oxidation. These properties suggest that α_1AT (Met^{358}→Leu) could be particularly effective in replacement therapy for cases of α_1AT deficiency disorders.

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E112 SITE-SPECIFIC MUTAGENESIS TO STUDY THE STRUCTURE AND FUNCTION OF FACTOR VIII, Randal J. Kaufman, Debra D. Pittman, Patricia Murtha, Louise Wasley, and John J. Toole, Genetics Institute, Boston, MA

The recent cloning of the human cDNA for factor VIII revealed an apparent domain structure for the protein which can be represented as A1-A2-B-A3-C1-C2. Recombinant DNA techniques have been used to remove large segments of DNA encoding the heavily glycosylated B domain from the full-length factor VIII cDNA. These constructs directed the synthesis of biologically active factor VIII when introduced into mammalian cells despite the deletion of up to 38% of the factor VIII molecule. The modified protein was activated by thrombin in a manner similar to the native molecule. These data demonstrate that the B domain is dispensible.

E113

CONTROL OF VIRAL PROTEASE ACTION. B. Korant, L. Ivanoff, T. Towatari, A. Cordova, C. Kettner, V. Turk and S. Petteway. Central Research Dept., DuPont Experimental Station, E328, Wilm, DE 19898, and Josef Stefan Inst., Dept. Biochem, 61111 Ljubljana, Yugoslavia.

Many viruses require intracellular protein cleavages in order to assemble progeny virus particles. With several virus groups, the viral genome encodes a protease function which participates in the site-specific endoproteolytic cleavages preceding assembly of viral precursor polypeptides. We are interested in designing specific inhibitors of viral proteases, and have adopted the strategy of synthesizing peptide mimics of the viral cleavage sites. As the peptide sequence is varied to closely match the viral concensus processing sequences, protease inhibitor and antiviral activity increase substantially. Protein cleavages of some viruses (influenza, poliovirus) are susceptible to inhibition by small proteins which are natural protease inhibitors, and we are also exploring whether cells engineered to contain unusual amounts of such inhibitors are altered in sensitivity to virus challenge. In support of these studies, we have cloned and expressed a picornavirus cysteine protease in bacteria, and identified three active site residues by a combination of protein chemistry and site-directed mutation. The picornaviral proteases are not closely related by amino acid sequence to each other or to other proteases, but have a high degree of structural similarity and appear to be analogs of papain formed by deletion events. The chemical basis for the high substrate selectivity of the viral enzymes is still unknown but may now be addressed by modifications to the protease and with synthetic substrates and inhibitors.

E114 CLEARANCE RATE DETERMINATIONS OF GLYCOSYLATED AND DEGLYCOSYLATED RECOMBINANT T-PA IN RABBIT, Glenn Larsen, Yitzhak Blue, Kimberlee Henson and Randal Kaufman, Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140

We have previously cloned and developed mammalian cells which express high levels of tissue plasminogen activator (t-PA). t-PA is a serine protease which is responsible for the dissolution of fibrin clots. In an attempt to understand the mechanism of rapid systemic clearance of t-PA when intravenously administered to rabbits, we conducted studies using t-PA derived from our recombinant cell lines. Two forms of t-PA were prepared in these studies, glycosylated and deglycosylated t-PA. Recombinant mammalian cells were metabolically labeled with ^{35}S-methionine and secreted t-PA was purified from conditioned media using a mouse monoclonal immuno-affinity column. To investigate the possible interaction of t-PA with carbohydrate-specific receptors of the liver, we prepared a deglycosylated form of t-PA. Comparison of the _in vivo_ clearance rates of glycosylated and deglycosylated t-PA were made and found to demonstrate no significant difference. The possibility of inhibitor mediated clearance was also investigated by treating the deglycosylated t-PA with DFP (diisopropyl fluorophosphate), a serine protease inhibitor which binds irreversibly to the active site. We observed a similar clearance rate of this preparation as compared to the forms of t-PA mentioned above. In summary, we have shown that t-PA produced in a recombinant mammalian cell line is cleared in rabbits at a rate similar to that observed for t-PA purified from melanoma cells. In addition the clearance rate of t-PA was not affected by the absence of carbohydrate or when the deglycosylated radiolabeled protein was treated with the active site inhibitor, DFP.

E115 BPTI FOLDING MUTANTS, C. Berman Marks, T. Kuntz*, H. Naderi*, P. Kosen*, R. Sheek*, H. Morehead*, S. Manogaran*, and S. Anderson. Genentech, Inc., South San Francisco, CA *University of California, San Francisco.

A heterologous E. coli expression/secretion system has been developed for bovine pancreatic trypsin inhibitor (BPTI) that yields native, correctly-folded BPTI. We have used this system to express BPTI with various amino acid substitutions that affect its folding. In E. coli, mutant proteins that lack either the 14-38 or the 30-51 disulfide bond are able to fold correctly. However, in vitro experiments indicate that 14-38 disulfide mutants refold more slowly than wild-type BPTI, while 30-51 disulfide mutatns refold more rapidly. The mutant proteins also exhibit different sensitivities to denaturation by heat or dithiothreitol. Presently, we are in the process of characterizing the solution structures of these mutant proteins using high-resolution 2-D NMR spectroscopy.

E116 OVOSTATIN: AN EGG WHITE PROTEINASE INHIBITOR HOMOLOGOUS TO α_2-MACROGLOBULIN. Hideaki Nagase, Departments of Medicine and Biochemistry, UMDNJ-Rutgers Medical School, Piscataway, NJ 08854

Ovostatin is a tetrameric egg white proteinase inhibitor with $\bar{M}w$=780,000. The quaternary structure of ovostatin and its inhibitory mechanism for proteinases are similar to those of plasma α_2-macroglobulin (α_2M). However, ovostatins from chicken and duck egg whites were shown to be distinct from human α_2M in a number of respects. Chicken ovostatin inhibited only metalloproteinases in 1:1 stoichiometry. A kinetic study of collagenase binding to chicken ovostatin gave the value of k_2/K_i=6.3x10^5 M^{-1} min^{-1}, indicating that ovostatin was equally as good a substrate for collagenase as type I collagen. Chicken ovostatin did not contain a thiolester. Therefore, there was no covalent linkage formed between ovostatin and an "entrapped" proteinase. On the other hand, duck ovostatin inhibited both metalloproteinases and serine proteinases in 1:1 stoichiometry, but not cysteine proteinases. However, duck ovostatin contained a thiolester in each subunit. Nevertheless, unlike many α_2Ms, CH_3NH_2-treated duck ovostatin could bind and inhibit proteinases. The NH_2-terminal sequences for the first 14 residues of duck and chicken ovostatins were identical and they were sufficiently simiar (about 40% of identical residues) to that of human α_2M. The results suggest that ovostatin and α_2M may have evolved from a common ancestor, but their evolutionary divergence has led to the development of distinct specificities in their action on different proteinases.

E117 GENE TRANSFER AND EXPRESSION OF HUMAN ALPHA-1-ANTITRYPSIN IN HETEROLOGOUS MAMMALIAN CELLS, Richard N. Sifers, Joyce A. Carlson, Vincent J. Kidd and Savio L.C. Woo, Howard Hughes Medical Institute, Dept. of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

The major function of the plasma protease inhibitor alpha-1-antitrypsin is to neutralize the hydrolytic activity of excessive neutrophil elastase in the lung. Most individuals are homozygous for the normal Pi^M allele, whereas individuals homozygous for the mutant Pi^Z allele have only 10-15% of the normal level of alpha-1-antitrypsin in their plasma. The substitution of lysine for glutamate at a specific site in the PiZ alpha-1-antitrypsin protein results in its accumulation within the endoplasmic reticulum of hepatocytes and subsequently its decreased secretion into the circulatory system. In order to define the molecular basis of this phenomenon, the normal (Pi^M) and the mutant (Pi^Z) alpha-1-antitrypsin genes were isolated from a cosmid genomic DNA library constructed with an SV2-dhfr expression vector. Both alpha-1-antitrypsin genes were transfected into CHO ($dhfr^-$) cells. Heterologous DNA in the stably transformed cells has been amplified by selection with methotrexate in the culture medium allowing the enhanced expression of the dhfr and alpha-1-antitrypsin genes. Using this strategy, we have developed a model for studying the intracellular transport defect of the PiZ alpha-1-antitrypsin protein.

Late Additions

E118 EARLY AND LATE PROCESSING STEPS IN THE BIOSYNTHESIS OF SECRETORY PEPTIDES. Christa Mollay and Gunther Kreil, Molecular Biology Institute, Austrian Academy of Sciences, Bilrothstrasse 11, A-5020 Salzburg, Austria

Signal peptidase has been partially purified from rat liver and dog pancreas microsomes. After several purification steps, including centrifugation in sucrose gradients containing detergents and reconstitution into phospholipid vesicles, only two polypeptides can be detected. In the presence of melittin or other membrane perturbing agents, these signal peptidase containing vesicles correctly convert prepromelittin to promelittin.
In our studies on the different processing enzymes catalyzing the conversion of pro-hormones and other pro-peptides to the mature products, we have used the skin of *Xenopus laevis* as a model system. It has previously been shown in several laboratories that amphibian skin synthesizes large amounts of peptides which are identical or homologous to mammalian hormones and/or neurotransmitters.
From skin secretion of *X. laevis*, a dipeptidyl aminopeptidase has been purified, which cleaves after proline, alanine and glycine residues. This enzyme is postulated to catalyze a late step in the liberation of caerulein (a homologue of mammalian cholecystokinin) and xenopsin (a peptide related to neurotensin) from their respective precursors. In addition, the enzyme catalyzing the formation of terminal amides has been partially purified from this secretion.

E119 ON THE MOLECULAR BIOLOGY OF HUMAN ENDOTHELIAL DERIVED SERINE PROTEASE INHIBITORS, Hans Pannekoek, Central Laboratory of the Netherlands, Blood Transfusion Service, Department of Molecular Biology, Plesmanlaan 125, 1066CX, Amsterdam, Netherlands

A heterologous antiserum, raised against bovine endothelial plasminogen activator inhibitor, was employed to screen a human endothelial cDNA expression library in *E. coli*, composed of 60,000 independent colonies. The 2.1 kb cDNA insert of an antigen-producing clone was found to hybridize with a human endothelial mRNA with a length of approximately 2,000 to 3,000 nucleotides. A synthetic oligonucleotide (24-mer), derived from the 5' region of the 2.1 kb cDNA insert, was used to detect homologous cDNA clones, containing approximately 2.3 kb of inserted cDNA. DNA sequencing revealed that the cloned cDNA's exhibit a striking homology with members of the serine protease inhibitor family, e.g., antithrombin III, α_2-antiplasmin and α_1-antitrypsin.