PROCESSING ENZYMES: BIOCHEMISTRY, GENETICS AND CLINICAL RELEVANCE

Organizers: Nabil G. Seidah, Michel Chretien and Gary S. Jacob

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Keynote Address

B7-001 PROTEOLYTIC PROCESSING: A SAGA OF FIFTY YEARS, Hans Neurath, University of Washington, Department of Biochemistry, Seattle, WA 98195.

The roots of the regulation of enzymes, protein hormones and related biological messengers by proteolytic processing were planted at the end of the first half of the twentieth century when the basic principles of protein structure, enzyme kinetics and mechanisms of enzyme action were established. The activation of zymogen precursors of pancreatic enzymes, exemplified by the activation of trypsinogen, the phenomenon of "limited proteolysis", exemplified by the ovalbumin-plakalbumin conversion catalyzed by subtilisin, and the recognition of families of proteases possessing similar active site configurations are important landmarks of these early pioneering periods. Significant subsequent advances in experimental procedures and approaches, primarily in protein isolation, sequence analysis and x-ray crystallography, followed by the application of concepts and procedures of molecular and cell biology, established a basis of structural, functional and evolutionary comparison of the more complex, multi-domain regulatory proteases which by selective, site-specific, post-translational cleavage convert inactive precursors to their biologically active forms. Having witnessed and contributed to some of these developments, I shall attempt to high-light in a somewhat anecdotal fashion the exciting advances underlying our current knowledge of pro-hormone processing and related phenomena.

Molecular Biology of Pro-Hormone Convertases I

B7-002 THE SUBTILISIN/KEXIN FAMILY OF CONVERTASES: NEW DEVELOPMENTS AND FUTURE PERSPECTIVES. Nabil G. Seidah. Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montréal, 110 Pine Ave.W., Montréal, QC, Canada, H2W 1R7.

Recently, a family of subtilisin/kexin-like serine proteinases were molecularly characterized and shown to be responsible for the processing of a number of hormonal and protein precursors at either single or pairs of basic residues. So far the six known members have been named furin/PACE, PC1/PC3, PC2, PC4, PACE4 and PC5/PC6. Multiple mRNA forms have been isolated for all these convertases, but only in the case of PC4, PACE4 and PC5/6 does this presumably alternate splicing of their respective genes result in a diversity of C-terminal sequences. A similar phenomenon has also been observed in non-mammalian species such as *Drosophila melanogaster.* We demonstrated that the catalytic properties of the *Drosophila* isoforms dfurin1, dfurin1-CRR and dfurin1-X have similar cleavage selectivity towards a number of substrates, suggesting that the C-terminal variations affect either the sorting or the intracellular localization of these isoforms. In order to better define the structure-function activity of the mammalian convertases we began to use both site directed mutagenesis and domain deletion and/or swapping. With respect to the PCs in general, we first probed the functionality of the conserved R**RGD**L sequence in each of these convertases with respect to cellular trafficking and bioactivity. For PC2, we analyzed in details the effect of mutating the catalytically important Asp residue on the activation, transport and pro7B2 binding of proPC2 and cleavage preference of POMC by PC2. The residues in pro7B2 which are important for this Ca^{2+}-dependent binding were also analyzed. We have recently studied biosynthetically the difference between PC5 and PC6B and that betwen PACE4 and PACE4-C. Our results demonstrated that the intracellular fate of each isoform is different. Overexpression of furin, PC5 and PACE4 in the baculovirus system allowed the isolation of these enzymes in sufficient quantities and the study of their activity *in vitro*. We have accordingly addressed the question of the functional role of the Cys-rich domains in each of these convertases both *in vitro* and *ex vivo.* The anatomical distribution and cellular localization of these enzymes was studied in more details and what emerges is that each enzyme exhibits a unique distribution and mRNA regulation pattern. The cleavage specificity of each enzyme was probed with a number of precursor substrates that were shown to be co-localized and sometimes co-regulated in tissues and cells. In general we find that furin and PACE4 often have similar processing preferences and are localized not only neuronally but also in glial cells. In contrast, in the Central Nervous System, PC1, PC2 and PC5 are only found in neurons. The cloning of the promoter sequences of PC1, PC2 and PC5 showed that they are very different and that they contain unique regulatory sequences. In conclusion, although the members of convertase family exhibit some shared functions, they surely have evolved unique and divergent functions of their own.

B7-003 EVOLUTION OF STRUCTURE AND SPECIFICITY IN THE SPC FAMILY OF PROTEASES, Donald F. Steiner[1,2], Shu Jin Chan[1,2], Yves Rouillé[1,2], Grigory Lipkind[2], Qiuming Gong[1,2], Stephen Duguay[1,2], Tadashi Hanabusa[2], Machi Furuta[2], Kaare Lund[2] and Steven P. Smeekens[3], [1]Howard Hughes Medical Institute, [2]Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637 and [3]Khepri Pharmaceuticals, South San Francisco, CA 94080

Recent studies on the gene and protein structures of members of the kexin-like SPC family suggest that these enzymes have all diverged from a common ancestral protease having a subtilisin-related catalytic domain. Examples isolated from diverse metazoans going back to hydra suggest that divergence was an early event associated with development of greater complexity, primarily in intercellular signalling mechanisms. Divergence through gene duplication has resulted in the expression of at least 6 family members in mammals. We propose that this multiplicity of gene forms was necessary to satisfy several needs including: a) intracellular versus extracellular localization, b) localization to specific compartments in the secretory system to facilitate substrate selection, c) adaptations to differing compartmental conditions (pH, ionic composition, other proteins), d) greater site selectivity in processing precursors, i.e. differential processing, e) appropriate stability (or lack thereof) to limit proteolytic action. The evolutionary time and order of divergence for each SPC from its ancestral form remains to be determined. However, it is now clear that SPC2 is evolutionarily very ancient, having been found in all vertebrates and recently in mollusca and insects. In the protochordate, amphioxus, we have cloned at least 4 SPC family members. In terms of structure and activity, comparative and protein modeling studies indicate that the transition from the substrate selectivity of the subtilisins (generally hydrophobic preferences in P1, P2 and P4 positions) to that characteristic of the SPC's has necessitated the introduction of significant structural changes within an overall conserved catalytic domain. This transition must have occurred very early in organisms ancestral to both yeast and metazoans and may have been accompanied by the acquisition of the P domain. The nature and significance of these structural changes and evidence relating to the mechanisms regulating cleavage site selectivity will be discussed.

B7-004 INTRACELLULAR TRAFFICKING AND ACTIVATION OF THE FURIN PROPROTEIN CONVERTASE, Gary Thomas[1], Sean S. Molloy[1], Laurel Thomas[1], Bethan G. Jones[1], Eric. D. Anderson[1], Gseping Liu[1], Lei Wan[1], Judy, K. VanSlyke[1], Matt D. Fry[1], Craig Thulin[2] and Ken Walsh[2], [1]Vollum Institute, Oregon Health Sciences University, Portland, OR 97201 and [2]Department of Biochemistry, University of Washington, Seattle, WA.

Posttranslational proteolysis is a common mechanism required for the synthesis of biologically active peptides and proteins including peptide hormones, growth factors, viral envelope proteins, and bacterial toxins. One of the early events in precursor protein maturation is endoproteolysis at the carboxyl side of pairs of basic amino acids (especially -LysArg- and -ArgArg-). Genetic and biochemical studies unequivocally identified the gene required for the excision of the peptide mating hormone in Saccharomyces cerevisiae. This locus, KEX2, encodes a subtilisin related endoprotease (Kex2p) that cleaves on the carboxyl side of pairs of basic residues. Recently, a small but growing number of cDNA sequences have been identified that encode endoproteases which share significant homology with the Kex2p sequence [reviewed by Steiner et al., JBC 267:23435 (1992)].

One of these enzymes, furin, is a predominantly trans-Golgi network (TGN)-localized, calcium dependent, serine endoprotease which cleaves precursor proteins at the consensus sequence, -Arg-X-Lys/Arg-Arg- [Molloy et al., JBC 267:16396 (1992); Molloy et al., EMBO J. 13:18 (1994)]. Furin catalyzes the maturation of a wide variety of precursor proteins in both the exocytic (eg. pro-β-NGF and HIV-1 gp160) and endocytic (eg. bacterial toxins) pathways. Recent studies on the activation and intracellular routing of furin, particularly the role of phosphorylation/ dephosphoryation of the cytosolic tail of the endoprotease, will be presented.

B7-005 THE MOLECULAR BIOLOGY AND STRUCTURAL FEATURES OF FURIN IN MAMMALS, Wim J.M. Van de Ven, John W.M. Creemers, Torik A.Y. Ayoubi and Anton J.M. Roebroek, Center for Human Genetics, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium.

Production of a broad spectrum of regulatory proteins in eukaryotes occurs via an intricate cascade of biosynthetic and secretory processes. Often, these proteins are initially synthesized as parts of higher molecular weight, but inactive, precursor proteins. Specific endoproteolytic processing of these proproteins is required to generate the regulatory proteins in a mature and biologically active form. Such endoproteolysis generally occurs at cleavage sites consisting of particular sequence motifs of basic amino acids, often paired basic residues. One of the responsible enzymes is furin which is most likely involved in the processing of precursors of proteins that are secreted via the constitutive secretory pathway. Furin is encoded by the *FUR* gene, which is expressed in a wide variety of tissues, if not in all. Expression of the *FUR* gene is directed by alternative promoters, house-keeping (GC-rich) as well as regulated (TATA containing) promoters, suggesting that their differential use may be a mechanism to modulate levels of the furin enzyme. Furin is predicted to contain a "prepro" domain, a subtilisin-like catalytic domain, a middle domain, a cysteine-rich region, a transmembrane anchor and a cytoplasmic domain. Via homology modelling, a 3-D structure of the catalytic domain of furin was proposed. Using site-directed mutagenesis, mutants of the subtilisin-like enzyme furin were studied. Such studies indicated that the three residues of the catalytic triad of furin, Asp46, His87 and Ser261, are critical not only for substrate processing but also for maturation of furin. Endoproteolytic removal of the propeptide appeared to be a prerequisite for efficient transport of furin out of the endoplasmic reticulum. Furthermore, evidence has been obtained that maturation of furin occurs through an intramolecular autocatalytic process. Analysis of carboxy-terminal deletion mutants revealed that the segment encompassing residues E449 to E469 of the "middle" domain, which is more than 100 residues downstream of the predicted catalytic domain, contains residues which seem to be critical for processing activity but that the more carboxy-terminal cysteine-rich region, the transmembrane region and the cytosolic tail are dispensable. Mutations in the substrate-binding region of human furin revealed that particular negatively charged residues in or near the substrate-binding region of furin are critical for cleavage activity and specificity of the enzyme for multiple basic residues in the substrate.

Molecular Biology of Pro-Hormone Convertases II

B7-006 GENE EXPRESSION AND REGULATION OF THE SUBTILISIN/KEXIN-LIKE FAMILY OF PROHORMONE CONVERTASES. Robert Day. Clinical Research Institute of Montreal, 110 Pine Ave West, Montreal, Quebec, Canada, H2W 1R7.

The family of subtilisin/kexin-like enzymes, generally refered to as the prohormone convertases, are responsible for the processing of precursor proteins at single or pairs of basic residues. The members of this family include furin (PACE), PC1 (PC3), PC2, PC4, PACE4 and PC5 (PC6). Using Northern blot analysis and *in situ* hybridization histochemistry, we have examined the mRNA distribution of each prohormone convertase in the central nervous system, the pituitary and in the periphery. While distinct patterns of expression are observed, we frequently observe the co-expression of at least two prohormone convertases at the cellular level. Using double labeling *in situ* hybridization we studied the co-localization of various peptides and hormones with the convertases in the brain and pituitary. In the paraventricular nucleus, we observed the co-localization of vasopressin mRNA with PC1 and PC2, while oxytocin mRNA was colocalized with PC1 and PC5. In the pituitary, we examined the expression of each convertase in various cell types. As an example, we observed that anterior lobe corticotrophs express PC1, PACE4 and furin, but not PC2 or PC5. Other tissues, including the heart, thyroid, adrenal, gut, and testis were also examined. These cellular localization studies provide important clues as to the potential precursor substrate for each enzyme. Our results demonstrate that PC1 and PC2 are primarily expressed in neuronal and endocrine cells, in contrast to furin which is ubiquitously expressed. It has been suggested that furin is important for the processing of precursors negotiating the constitutive pathway of secretion while PC1 and PC2 are important for the processing of precursors entering the regulated secretory pathway. In light of these correlations, we asked the question as to whether a regulated pathway of secretion and the expression of PC1 and PC2 can ever be dissociated. To address this issue, we studied the expression of the prohormone convertases in the recently established HYA.15.6.T.3. (6T3) cell line (Matsuuchi and Kelly, J Cell Biol 112:843-852). This cell line was obtained by the fusion of the mouse corticotroph AtT-20 cells with a mouse myeloma 4T001. The 6T3 cell lost the capacity to form dense core secretory granules as well as the regulated secretion. Interestingly, PC1 and PC2 are still expressed in 6T3 cells, suggesting that these convertases can be expressed in cells independently from the regulated pathway of secretion. This maintained expression of the convertases allowed us to probe the factors governing the efficiency of processing within the regulated pathway. Furthermore we observed that treatment of 6T3 cells with cyclic AMP results in the restoration of both the secretory granules and the regulated secretory pathway. We are presently examining potential mechanisms for such an induction of a regulated secretory pathway in 6T3 cells. In the pituitary, we have previously shown that PC1 and PC2 mRNA levels are affected by dopaminergic stimulation, glucocorticoids and thyroid status. At the molecular level, no transcription factor has yet been identified which regulates the PC1 and PC2 gene expression. Recently, we cloned the cDNA of a novel mouse LIM-homeodomain protein (mLIM-3) that is specifically expressed in the adult pituitary anterior and intermediate lobes. We also demonstrated the nuclear localization of mLIM-3 in AtT-20 cells, suggesting that it may mediate transcriptional processes. Since the prohormone convertases are also abundantly expressed in the pituitary, we tested the effects of mLIM-3 on their mRNA and protein levels. The results suggest the involvement of mLIM-3 in the differential regulation of the levels of the proprotein convertases. The mechanisms of this differential regulation are being studied.

B7-007 STRUCTURE AND FUNCTION OF FURIN-LIKE PROCESSING ENDOPROTEASES, Kazuhisa Nakayama, Institute of Biological Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

The maturation of biologically active peptides and proteins often involves the limited endoproteolysis of larger, inactive precursors. In neuronal and endocrine cells, precursors of bioactive peptides are cleaved at pairs of basic amino acids within the regulated pathway of secretion. By contrast, in non-neuroendocrine cells various secretory and membrane proteins are produced from precursors through cleavage at sites often marked by the consensus sequence, Arg-X-Lys/Arg-Arg (RXK/RR), within the constitutive secretory pathway. Recently, six different mammalian pro-protein processing endoproteases structurally and functionally similar to the prototypical processing protease, yeast Kex2, have been identified. These include ubiquitously expressed furin, PACE4 and PC6 (also known as PC5), neuroendocrine-specific PC2 and PC3 (also known as PC1), and testis-specific PC4. Furin, PACE4 and PC5/6 are thought to function as processing endoproteases within the constitutive secretory pathway, whereas PC2 and PC1/3 function within the regulated pathway.

By co-transfection experiments and *in vitro* experiments using purified recombinant furin, we have shown that furin is able to cleave a variety of pro-proteins at RXK/RR sites. These include growth factors, serum proteases, receptors, viral envelope glycoproteins, and bacterial exotoxins. Further analysis using various prorenin mutants with amino acid substitution(s) around the cleavage site as substrates has delineated the following sequence rules of pro-protein processing by furin:

1) At position -1, an Arg residue is essential.
2) At least two out of the three basic residues at positions -2, -4, and -6 are required for efficient cleavage (the presence of the basic residues at all the three positions results in most efficient cleavage).
3) At position +1, a hydrophobic aliphatic residue is not favorable.

Although PACE4 and PC5/6 can also cleave pro-proteins at RXK/RR sites, their substrate specificity appears to be more restricted than that of furin. Taken together with the fact that human colon carcinoma LoVo cells, which have a mutation of furin, are incapable of cleaving pro-proteins at RXK/RR sites, these data suggest that furin is the endogenous cellular protease responsible for pro-protein processing within the constitutive secretory pathway in most, if not all, cells.

B7-008 CHARACTERIZATION OF A NATURALLY SECRETED FORM OF PACE/*furin* AND ITS IMPLICATION IN EXTRACELLULAR PROCESSING. Alnawaz Rehemtulla[1], Chris J. Rhodes[2], Valerie M Gordon[3], Steve H. Leppla[3] and Randal J. Kaufman[1]. [1]Howard Hughes Medical Institute, University of Michigan, Ann Arbor, [2] Joslin Diabetes Center, Harvard Medical School, Boston, [3] National Institute of Dental Research, Baltimore.

PACE (furin) is a membrane associated endoprotease that efficiently cleaves precursor proteins on the C-terminal side of the consensus sequence Arg-X-Lys/Arg-Arg. PACE plays a role in the post-translational maturation of a number of growth factors, serum proteins, viral glycoproteins and cellular proteins. Recent evidence suggests that PACE mediates activation of various bacterial toxin components. To elucidate whether PACE can process bacterial toxins, we demonstrated that mutant Chinese hamster ovary cells, selected for toxin resistance, cannot process the substrate pro-von Willebrand factor. Expression of PACE in these cells restored sensitivity to bacterial toxins as well as the ability to process pro-von Willebrand factor.

Cleavage of bacterial toxins at a canonical PACE cleavage site is thought to occur extracellularly (e.g. anthrax toxin) as well as in an endosomal compartment (e.g. *Pseudomonas* exotoxin), although it is not known whether a secreted form of PACE retains functional activity. We have identified a site at which PACE is autocatalyticaly cleaved to release the catalytic domain from the carboxy terminus containing the transmembrane domain and the Golgi retention signal.. The secreted protein is functional and retains specificity for the Arg-X-Lys/Arg-Arg sequence. Mutation of the cleavage site prevented secretion of PACE, although the mutant molecule was able to process pro-von Willebrand factor. We propose that a secreted form of PACE is involved in the processing of extracellular substrates such as anthrax toxin.

Biochemical and Enzymatic Properties of the Mammalian Convertases

B7-009 MONOBASIC PROCESSING ENZYMES, Yemiliya Berman[1], Luiz Juliano[2], and Lakshmi A. Devi[1], [1]New York University Medical Center, New York, NY10016, [2]Escola Paulista de Medicina, Sao Paulo, Brazil.

Dynorphins are biosynthesized by posttranslational processing of prodynorphin at dibasic and monobasic sites. An enzymatic activity capable of processing at a monobasic processing site has been previously reported in rat brain and bovine pituitary. This enzyme is termed "the dynorphin converting enzyme" since it converts dynorphin B-29 (leumorphin) to dynorphin B-13 (rimorphin). The dynorphin converting enzyme has been purified approximately 30,000-fold from the neurointermediate lobe of the pituitary using hydrophobic chromatography on Phenyl-Sepharose, preparative isoelectrofocussing chromatography and preparative gel electrophoresis under non-denaturing conditions. The purified enzyme exhibits a pI of about 5.1 and a molecular weight of about 180,000 daltons. The protease inhibitor profile of the purified enzyme suggests that the enzyme requires thiol groups for activity; the enzyme activity is inhibited by parachloromercuribenzene sulfonic acid and not inhibited by serine, aspartyl, or metalloprotease inhibitors. The enzyme activity has been characterized as to its ability to cleave intramolecularly quenched fluorogenic peptide substrates containing monobasic, dibasic and multibasic residues. The relative efficiency of the cleavage of these substrates by the dynorphin converting enzyme is distinct from the efficiency of cleavage by furin or propeptide convertase 1 (PC1, also known as PC3). The substrate specificity studies with the dynorphin converting enzyme suggest that the enzyme follows the consensus predicted for the processing of peptides at monobasic sites. These data support the notion that the dynorphin converting enzyme activity is a monobasic processing enzyme and is distinct from the subtilisin-like processing enzymes.

The tissue distribution of the dynorphin converting enzyme suggests that this enzyme is involved in the biosynthesis of a number of peptides. In the adult rat, the activity is present at high levels in the brain, ileum, pituitary , and adrenal. Lower levels of the activity are present in other tissues. In the rat brain, the enzyme activity is present in high levels in the striatum, hippocampus, and hypothalamus. The areas of the brain that have high levels of Dyn B-13 are found to have high levels of the dynorphin converting enzyme activity. Also, the dynorphin converting enzyme activity is in a secretory compartment that is structurally and functionally similar to the secretory compartment containing the neuropeptides and other peptide processing enzymes. Taken together the cellular and subcellular distribution of the enzyme activity suggests a broad role for the dynorphin converting enzyme in the biosynthesis of dynorphins, neuropeptides, and other regulatory peptides by cleavage at monobasic sites.

B7-010 THE APPLICATION OF THE BACULOVIRUS EXPRESSION SYSTEM TO THE PRODUCTION OF LARGE QUANTITIES OF PC1 AND PC2 AND THEIR MOLECULAR CHARACTERIZATION, Alain Boudreault[1], Normand Rondeau[2], Nabil G. Seidah[3], Michel Chrétien[2] and Claude Lazure[1], [1]Neuropeptides Structure and Metabolism Laboratory and J.A. de Sève Laboratories of [2]Molecular and [3]Biochemical Neuroendocrinology, Clinical Research Institute of Montréal (affiliated to the University of Montréal) 110 Pine Ave. West, Montréal, Québec, Canada H2W 1R7.

Prohormones and proproteins are often cleaved at pairs of basic residues in order to release the biologically active moieties by specialized endoproteolytic enzymes collectively known as prohormone convertase. In order to study *in vitro* their biochemical and enzymic properties, two full length cDNAs corresponding to murine prohormone convertase-1 (mPC1) and -2 (mPC2) were inserted individually into the baculovirus nuclear polyhedrosis virus expressed in infected *Spodoptera frugiperda* (Sf9) cells. The recombinant enzymes were recovered from the cell medium at a level of 0.5-1 mg/L. By immunoprecipitation and metabolic labeling, secreted mPC1 was shown to be present in two major molecular forms. The highest molecular weigth form represents the enzymatically active enzyme whereas the second one represented a COOH-terminal truncated form. Radiosequencing of either forms yielded identical NH_2-terminal sequences resulting from processing at the predicted zymogen activation site. For mPC2, two molecular forms were also obtained, exhibited minimal enzymatic activity and resulted from improper cleavage within the pro-segment. All secreted proteins are N-glycosylated whereas proteins within the cells accumulate in an unglycosylated form. Treatment with endoglycosidases suggests that mPC1 contains complex-type oligosaccharides while mPC2 contains high-mannose-type oligosaccharides. Cell incubations in the presence of tunicamycin result in a drastically reduced yield of enzymes in the medium suggesting that glycosylation is important for secretion and/or activation of these enzymes. Furthermore, in insect cells, mPC1 is shown to be sulfated contrary to mPC2 which appears in this expression system not to be. We also demonstrate using this recombinant mPC1 that its enzymatic activity can be finely modulated by the presence of copper ions and its intracellular form is changing in the presence of copper. This modulation of enzymatic activity could be due to the interactions of excess copper with the lone Cys residue and an His residue present in the active site. Finally, together with recombinant vaccinia virus PC1, the enzymic properties of recombinant mPC1 were examined using various synthetic fluorogenic substrates and inhibitors.
This study was supported by the Medical research council of Canada.

B7-011 THE REGULATION OF PROHORMONE CONVERTASES 1 AND 2, Iris Lindberg, Xiaorong Zhu, and Yi Zhou, Dept. of Biochemistry and Molecular Biology, Louisiana State University School of Medicine, New Orleans LA 70112.

During transit through the secretory pathway, prohormones are cleaved by a sequential series of processing steps which begin in the trans-Golgi network and continue within maturing secretory granules. Processing occurs though the action of the prohormone convertases, members of a family of enzymes related to the bacterial enzyme subtilisin. We have been interested in regulatory mechanisms which control the activity of two of the prohormone convertases, namely PC1 (also known as sPC3) and PC2. One known controlling mechanism for serine proteinases is regulation of zymogen activation. ProPC1 appears to exhibit autocatalytic activation early in the secretory pathway, while proPC2 activation, which occurs at a much later stage of the pathway, may involve the neuroendocrine protein 7B2 since both proteins are associated immediately after synthesis. A second potential regulatory mechanism involves conversion of the various forms of PC1 and PC2 to smaller forms which exhibit varying biochemical properties and specific activities. For example, PC1 is cleaved to a 66 kDa form at a brefeldin-blockable stage of the secretory pathway. This step represents an activation reaction since the 66 kDa form exhibits a higher specific activity than the parent 87 kDa molecule. The 66 kDa form of PC1 also exhibits a higher calcium requirement and increased sensitivity to certain proteinase inhibitors than 87 kDa PC1. Thirdly, PC activity may also be controlled by the presence of inhibitors within the secretory pathway. The neuroendocrine protein 7B2 represents such an inhibitor for PC2, and peptide mapping studies indicate that inhibitory potency resides in a small domain close to the carboxy-terminus. In *in vitro* experiments, recombinant rat 27 kDa 7B2 can completely block cleavage of recombinant proenkephalin by immunopurified PC2. Fourthly, enzyme activity may also be regulated by inactivation; this phenomenon may pertain to PC1 within secretory granules since chromaffin granule 66 kDa PC1 is essentially inactive. The interaction of activated, correctly processed enzyme forms with prohormones and other secretory pathway proteins in the correct subcellular compartment appears to be required for the effective production of bioactive peptides.

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B7-012 **SUBTILISIN: AN ENZYME MADE FOR ENGINEERING,** James. A. Wells[1], Marcus Ballinger[1], David Matthews[3], and Paul Carter[2], [1]Department of Protein Engineering and [2]Cell Genetics, Genentech, 460 Pt. San Bruno Blvd., South San Francisco, CA 94080. [3]Present address: Arris Pharmaceuticals, 385 Oyster Pt. Blvd., Suite 3, So. San Francisco, CA 94080.

Subtilisin, a simple serine protease secreted from species of bacillus, has been engineered to cleave fusion proteins at designed sites. The wild-type enzyme has broad specificity making it impractical for site-specific proteolysis. By removing the catalytic His64 the enzyme becomes exquisitely specific for substrates containing a P2 or P1' histidine by substrate-assisted catalysis. This coupled with additional mutations in the P1 binding site make the enzyme useful for site-specific proteolysis. Additional specificity requirements of the enzyme were explored using "substrate phage"--a phage display technique which allows many substrate sequences to be tested simultaneously so that the optimal ones can be identified. This method has been extended to analyze the substrate specificity of furin, a mammalian hormone processing enzyme. More recently we have introduced mutations into subtilisin, some of which were derived from the sequence of KEX2, that were designed to impart specific cleavage of dibasic residues. Dibasic sequences are often used as processing sites for many mammalian hormones. Indeed, it was possible to produce a subtilisin variant having high specificity for dibasic residues. Further analysis by the substrate phage method should allow us to identify the best dibasic sequences to install into fusion proteins for most efficient processing. These designed proteases should be practically useful tools for biotechnologists. Furthermore, these studies bear witness to the functional plasticity of subtilisin whose specificity can be engineered almost at will.

Evolutionary Models of Mammalian Convertases

B7-013 PROPROTEIN PROCESSING IN YEAST, Robert S. Fuller[1,2], Alison Bevan[1,2], Charles Brenner[2,3], Jason Brickner[1,2], Hiroto Komano[1,2], Laura Marschall[2], Kevin Redding[2,4] and Nathan Rockwell[1,2], [1]University of Michigan, Ann Arbor, MI 48109, [2]Stanford University, Stanford, CA 94305, [3]Brandeis University, Waltham, MA 02254, [4]University of Geneva, Geneva, Switzerland.

Baker's yeast (*Saccharomyces cerevisiae*) has served as a productive model system for elucidating pathways of proteolytic maturation and secretion of proteins in eukaryotic cells. Yeast Kex2 protease, which is localized to a late Golgi compartment, cleaves pro-α-factor and other proproteins carboxyl to pairs of basic residues, principally -Lys-Arg- and -Arg-Arg-. Kex2 is the prototype of a family subtilisin-related eukaryotic proprotein processing enzymes that includes furin, PC2 and PC1/3. We examined the primary sequence specificity of Kex2 protease using peptidyl methylcoumarinamides and internally quenched fluorogenic peptides. These results support a model wherein Arg at P1 is required for a high k_{cat} (~25 s^{-1}), whereas substitutions at P2 primarily affect K_M. The wild-type (-Lys-Arg-) cleavage site and all 19 substitutions for the P2 Lys were constructed in a form of pro-α-factor having a single α-factor repeat and a single Kex2 cleavage site. Efficiencies of cleavage of these molecules measured *in vivo* using a quantitative mating assay closely paralleled the effects of similar substitutions on k_{cat}/K_M values for cleavage of small peptide substrates by purified enzyme. The correlation was particularly strong for peptide substrates having a P4 residue. These results provide direct evidence that Kex2 protease functions *in vivo* under k_{cat}/K_M conditions.

Localization of Kex2 protease in a late compartment of the yeast Golgi complex requires a retention/retrieval signal in the cytosolic tail that includes important aromatic residues Tyr_{713} and Phe_{715}. A screen for mutations that suppressed the effects of point mutations at Tyr_{713} on retention of Kex2 protease in the pro-α-factor processing compartment led to the identification of 7 allele-specific suppressors. Linkage analysis showed that these suppressors define three genes involved in Kex2 retention that may encode or affect the function of a receptor for the retention signal.

A null mutation in the *kex2* gene leads to cold-sensitive growth (failure to grow below 20°C). Isolation of multicopy suppressors of *kex2* cold-sensitivity led to the identification of *MKC7*, which encodes a novel aspartyl protease precursor most closely related to the *YAP3* gene product, previously identified as a multicopy suppressor of the pro-α-factor processing defect of *kex2* null mutants. Synergistic effects of mutations in *kex2*, *mkc7* and *yap3* on growth at low and high temperatures implies the existence of redundant functions, which are important for yeast growth, that are carried out by the three proteases.

B7-014 THE *DROSOPHILA MELANOGASTER* FURIN-LIKE PROTEINASES, Anton J.M. Roebroek, John W.M. Creemers, Torik A.Y. Ayoubi, and Wim J.M. Van de Ven, Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven, Herestraat 49, B-3000 Leuven, Belgium.

Studies on *FUR*-like genes in *Drosophila melanogaster* led to the identification of two genes, *Dfur1* and *Dfur2*. The *Dfur1* gene encodes 13 exons dispersed over more than 100 kbp. It is transcribed into four different mRNAs (7.6, 6.5, 4.5 and 4.0 kb), generated by alternative splicing and poly-adenylation, encoding three structurally different *Dfur1* isoforms, Dfurin1 (892 residues), Dfurin1-X (1269 residues) and Dfurin1-CRR (1101 residues). The structural difference is found carboxy-terminal of the 'PRO', 'CATALYTIC' and 'MIDDLE' domains being so characteristic for furin and all other members of the family of subtilisin-like proprotein processing enzymes. The compact *Dfur2* gene encodes 16 exons within a region of about 9 kbp. The single 6.0 kb *Dfur2* mRNA encodes Dfurin2 (1680 residues). All four *Drosophila* furin-like proteinases contain a carboxy-terminal transmembrane domain like mammalian furin, but only Dfurin1-CRR and Dfurin2 also a cysteine-rich region preceding this transmembrane domain. The common ancestral origin of the members of the family of subtilisin-like proprotein processing enzymes is not only reflected by amino acid sequence similarity in the characteristic domains, but also by conservation of intron positions in the parts of the genes encoding these domains. Several intron positions present in the *Dfur1* gene and/or the *Dfur2* gene are found to be conserved in the related mammalian genes *FUR*, *PC1/PC3*, *PC2* and *PC4*, of which the genomic organization is known. Both *Dfur1* and *Dfur2* seem to be expressed during the whole life-span of *Drosophila*. RNA *in situ* hybridization experiments revealed expression of the different *Dfur1* isoforms and *Dfur2* in non-overlapping sets of tissues during *Drosophila* embryogenesis, indicative for distinct physiological functions. In gene transfer experiments in which the *Dfur1* isoforms and Dfurin2 were co-expressed with substrates having a multiple basic or dibasic cleavage site, the *Drosophila* furin-like proteinases displayed cleavage specificity similar to mammalian furin.

B7-015 THE *BLI-4* LOCUS OF THE NEMATODE *CAENORHABDITIS ELEGANS* ENCODES STRUCTURALLY DISTINCT KEX2/SUBTILISIN-LIKE ENDO-PROTEASES, ESSENTIAL FOR EARLY DEVELOPMENT AND ADULT MORPHOLOGY, Colin Thacker, Martin Srayko, and Ann M. Rose, University of British Columbia, Vancouver, BC V6T 1Z3, Canada.

The *bli-4* gene from *Caenorhabditis elegans* encodes gene products related to the kex2/subtilisin-like family of proprotein convertases. *bli-4* encodes four protein products that share a common amino terminus, but that differ at the carboxy termini. Characterization of genomic and cDNA clones indicate that the individual isoforms arise by alternative splicing. The four protein isoforms have been designated blisterase A, B, C and D. The structure of the predicted proteins suggest that *bli-4* may represent a unique member of the proprotein convertase family. Blisterase A and B exhibit an overall structural similarity to PC1/3, PC2 and PC4, examples of proprotein convertase enzymes whose expression is tissue regulated. Blisterase C is similar in structure to PACE4, whereas blisterase D has an overall similarity to furin. Both PACE4 and furin show a constitutive expression tissue pattern. Currently, fourteen independent mutations within the *bli-4* gene have been isolated. Thirteen of the mutant alleles result in lethality, causing arrest of development in late embryogenesis. The mutation that originally identified the locus, *e937* is completely viable yet exhibits blistering of the adult cuticle. We have determined the molecular lesions within three *bli-4* lethal mutants. The molecular lesions occur within exons common to all of the *bli-4* isoforms, suggesting that this class of lethal mutation represent defects that result in the complete lack of blisterase activity. Two of the lethal mutants, alleles *h1010* and *q508* contain mutations that the result of the insertion of a transposon or the deletion of coding nucleotides within the 'middle' or 'P-domain', respectively. The third mutant, *h199*, is the result of an amino acid substitution (His-127 →Leu) N-terminal to the protease domain. The only viable *bli-4* mutation, *e937*, shows incomplete penetrance and causes blistering of the adult cuticle. Molecular analysis of this allele has revealed a deletion of 3.5 kb within the *bli-4* gene. The deletion removes an exon unique to blisterase A and results in the abrogation of expression of this isoform, accompanied by an increase in expression of blisterase B, C and D transcripts. The blistered phenotype observed for *e937* suggests that blisterase A activity is required only for the production and/or maintenance of the adult cuticle. However, we have been able to rescue the lethal mutant phenotype of at least three lethal alleles using a 'mini-gene' composed of sequences that can only encode blisterase A. Furthermore, a mini-gene encoding only blisterase B is able to dramatically reduce the blistered phenotype of *e937*. We conclude from these observations that the *bli-4* isoforms may have an overlapping functional redundancy, and this may explain the incomplete penetrance of blistering in *e937* mutant animals. We are currently focusing on determining the identity of the blisterase substrates. Towards this end, we have begun to clone other genes in which mutations result in blistering of the adult cuticle, as well as perform a search for extragenic suppressors of the lethal mutations.

Carboxypeptidase-E and Metalloendopeptidases

B7-016 THE MINIGLUCAGON-GENERATING ENDOPEPTIDASE, Dominique Bataille, Philippe Blache, Alain Kervran, Dung Le-Nguyen, INSERM U 376, CHU Arnaud-de-Villeneuve, 34295 Montpellier Cedex 05, France

Glucagon, the 29-aminoacid (AA) hyperglycemic hormone from α cells of the endocrine pancreas, displays its biological activities *via* G-protein-linked receptors present in liver and several other tissues. We have shown that glucagon is processed at the level of several of its target tissues into a C-terminal fragment, glucagon-(19-29) or miniglucagon, which displays original biological properties. The main role of miniglucagon seems to be a local modulation of the activity of the mother-hormone *via* a specific receptor-second messenger system involving G-proteins and the plasma membrane calcium pump. In insulin-secreting β cells, miniglucagon inhibits the glucagon-induced insulin secretion; in heart cells, miniglucagon displays a negative inotropic effect, opposite to the positive inotropic effect of glucagon. We observed that the post-secretory processing of glucagon into miniglucagon is due to the presence, at the level of the cell surface of glucagon-target tissues, of a protease, refered to as "miniglucagon-generating endopeptidase" (MGE). On the basis of its capacity of processing glucagon into miniglucagon using a specific miniglucagon-immunoassay, we have isolated MGE under the form of a 100-KDa protein, the molecular structure of which was partially characterized. The enzymatic properties of purified MGE indicated that it belongs to a newly described family of thiol-dependent metalloendopeptidases. A partial AA-sequence was determined as follows : REARPTTEKS. From this sequence, an immunogen was synthetized, leading to the generation of a MGE-specific antibody. Western blots analysis allowed us to show that MGE is present in significant amounts in glucagon-target tissues such as liver, pancreas, gastric mucosa, heart or kidney; MGE is also present in thyroid, adrenals, hypothalamus and pituitary, other possible sites of glucagon action. Thanks to the determined AA sequence and the specific MGE-antibody, we are currently performing experiments using classical approaches (use of synthetic oligonucleotide sequences and polymerase chain reaction) and more specific approaches (presuming the existence of consensus sequences in this family of proteases), for molecular cloning of the cDNA coding for MGE and determining its complete molecular structure. MGE belongs to a new family of processing enzymes with original characteristics. MGE has, in addition, the features of a membrane-bound extracellular protease the role of which is likely to process a circulating hormone, releasing locally a fragment which modulates the biological action of the mother-hormone on its target tissue. This novel biological regulatory process evidenced from our data appears to be of general interest justifying the interest for further studies concerning the molecular and biological features of MGE and possible other similar proteases involved in extracellular processing of biologically active peptides, inside the wider field of prohormone / hormone processing enzymes.

B7-017 PURIFICATION, CHARACTERIZATION AND CLONING OF THE HORMONALLY REGULATED TRH-DEGRADING ECTOENZYME, Karl Bauer, Lutz Schomburg, Birgit Schauder, Max-Planck-Institut für experimentelle Endokrinologie, Feodor-Lynen-Str. 7, 30625 Hannover, Germany

Thyrotropin-releasing hormone (TRH) is an important extracellular signal substance which acts as a hypothalamic releasing factor stimulating the release of the adenohypophyseal hormones and functions as a neurotransmitter/neuromodulator in the central and peripheral nervous system. The inactivation of TRH after its release is catalyzed by an ectoenzyme localized preferentially on neuronal cells in the brain and on lactotrophic pituitary cells. This enzyme exhibits a very high degree of substrate specificity as well as other unusual properties. The activity of the adenohypophyseal enzyme is stringently controlled by estradiol and thyroid hormones, indicating that this ezyme itself may serve regulatory functions.

After solubilization by limited trypsin digestion we succeeded in purifying this enzyme (200 000 fold) from rat and pig brain. Peptide fragments were then generated by enzymatic digestion or cyanogen bromide cleavage, purified by reverse-phase HPLC and sequenced. PCR-amplification and screening of cDNA libraries from rat brain and pituitary led to the identification and isolation of a cDNA that encodes a protein of 1025 amino acids. The analysis of the deduced amino acid sequences was consistent with the identification of the enzyme as a glycosylated, membrane-ancohred Zn-metallopeptidase and transient transfection of COS-7 cells with this cDNA led to the expression of an active ectopeptidase that displayed the characteristics of the TRH-degrading ectoenzyme. Furthermore, Northern blot analysis demonstrated that the mRNA levels paralleled the tissue distribution of the enzyme and that in pituitary tissue the transcript levels rapidly increased when the animals were treated with triiodothyronine and decreased after injection of estradiol. Supported by the Deutsche Forschungsgemeinschaft.

B7-018 PROTEOLYTIC PROCESSING OF THE ALPHA SUBUNIT OF RAT ENDOPEPTIDASE-24.18 BY FURIN, Guy Boileau, Department of Biochemistry, University of Montreal, Montreal, Canada.

Endopeptidase-24.18 (EC 3.4.24.18, meprin, endopeptidase-2, PABA-peptide hydrolase, E-24.18) is a zinc-metallopeptidase expressed in the brush border membranes (BBM) of rodent kidney and human intestine. E-24.18 is a member of the astacin family of proteases which includes several developmentally regulated proteins. *In vitro*, E-24.18 can hydrolyse a variety of peptides including bradykinin, substance P and luteinizing hormone-releasing hormone. More recently, it has been shown to be involved in the degradation of the cellular matrix. E-24.18 is a multisubunit metallopeptidase. The functional unit of rat and mouse E-24.18 seems to be disulfide-linked α/ß dimers which can then associate through non-covalent interactions to form tetramers $(\alpha/\beta)_2$. However, soluble α_2 dimers have also been observed *in vivo* and in transfected cells. Molecular cloning has shown that both subunits have a similar structural domain organization except for a stretch of 55 amino acid residues that is found upstream the transmembrane domain in the α subunit but not in the β subunit. This segment of α subunit contains the sequence R-P-K-R_{655} which corresponds to the R-X-K/R-R consensus sequence for specific cleavage by furin. In order to investigate the involvement of this putative cleavage site in the secretion process of endopeptidase-24.18 α subunit, we expressed in COS-1 cells rat α subunits in which residues R_{655} or S_{656} were mutated to valine or leucine, respectively. In contrast to the wild-type protein, the αR655V and αS656L mutants were not secreted in the culture media. Moreover, when cells expressing the α subunit were infected with a furin-encoding vaccinia virus, immunoblotting showed a shift of the major cell-associated form of endopeptidase-24.18 α subunit from 98 kDa to 88 kDa and an increase in the amounts of secreted α subunit. This shift in molecular mass was not observed with the mutant α subunits. As observed for the 98 kDa species, the 88 kDa cell-associated protein was sensitive to endoglycosidase H treatment suggesting that the proteolytic cleavage occured in the endoplasmic reticulum or in an early Golgi compartment. Similar experiments using PACE4 and PC5 instead of furin showed that these enzymes were not able to generate the 88 kDa species. We conclude that furin is most probably the cellular enzyme involved in the proteolysis resulting in secretion of rat endopeptidase-24.18 α subunit.

B7-019 STRUCTURE, FUNCTIONAL PROPERTIES, TISSULAR AND SUB-CELLULAR LOCALIZATION OF TWO NOVEL METALLOPEPTIDASES EXHIBITING A SPECIFICITY FOR BASIC RESIDUES, Paul Cohen[1], Annik Prat[1], Thierry Foulon[1], Valérie Chesneau[1], Adrian Pierotti[1,2], Sandrine Cadel[1] and Dominique Segretain[3], [1] Biochimie des signaux régulateurs cellulaires et moléculaires, Université Pierre et Marie Curie, URA 1682 du CNRS, 96 boulevard Raspail, 75006 Paris, France,[2] Caledonian University, City Campus, Cowcaddens Road, Glasgow G4OBA, Scotland, United Kingdom, [3] Histochimie, Faculté de Médecine, Université René Descartes, 45 rue des Saints-Pères, 75600 Paris, France.

An endoprotease and an aminopeptidase-B were isolated, characterized and their cDNAs were cloned and sequenced. The first one appears to be a Zn^{2+}-endopeptidase of 1161 amino acid residues with the HFLEH cannonical Zn^{2+}-binding site and an acidic stretch of 71 amino acids containing 80% Glu and Asp. Since it exhibits *in vitro* a marked selectivity for peptide bonds on the N-terminus of R moieties in dibasic stretches, it was called NRD convertase (Nardilysin : E.C.3.4.24.61) and was shown to belong to the pitrilysin family with 35-50% homology with *E.coli* protease III (E.C.3.4.99.44) and insulysin (E.C.3.4.99.45). The aminopeptidase-B component is a 72kDa metallo-exopeptidase which is able to remove Lys and Arg from naphtylamide derivatives and from the N-terminus of a series of peptide substrates. A combination of immunochemistry and *in situ* hybridization studies on rat brain and testis allowed to define the regions of expression of the corresponding genes. They indicated that both NRD convertase and ApB are expressed transiently and exclusively in the germ line at late stages of spermatogenesis. Since electron microscopy examination of immunogold labeled producing tissues allowed a more precise definition of the subcellular localization of both enzymes, new hypothesis on their possible biological role(s) will be discussed.

C-Amidation Enzyme and Glutaminyl-Cyclase

B7-020 GLYCINE-EXTENDED PROGASTRIN PROCESSING INTERMEDIATES EXERT GROWTH-PROMOTING EFFECTS THROUGH A NOVEL RECEPTOR SUBTYPE, Chris J. Dickinson, Catherine Seva, Mitsuru Kaise, Jung Park, John DelValle, and Tadataka Yamada, Departments of Pediatrics, Physiology, and Internal Medicine, University of Michigan Medical Center, Ann Arbor, MI 48109-0658.

Gastrin, a hormonal stimulant of gastric acid secretion, is initially synthesized as a large precursor that is processed to form mature carboxyl-terminally amidated gastrin (G-NH_2). Diminished production of G-NH_2 from its immediate precursor, glycine-extended gastrin (G-Gly) in developing tissues and in some neoplasms results in high tissue concentrations of G-Gly. Since G-Gly has very weak effects on gastric acid secretion but its concentrations are elevated in conditions associated with cellular proliferation, we undertook studies to examine whether G-Gly has a growth-promoting effect independent of the G-protein linked gastrin/CCK_B receptor that mediates the effects of G-NH_2. For these studies we used AR4-2J cells, an exocrine pancreatic cell line known to exhibit a proliferative response to G-NH_2. G17-Gly induced dose-dependent increases in [^{3}H]-thymidine uptake ($EC_{50} \approx 10^{-11}$M) as well as ornithine decarboxylase activity ($EC_{50} \approx 10^{-10}$M) in a fashion comparable to the effects of G17-NH_2. The gastrin/CCK_B selective receptor antagonists L365,260 (10^{-6}M) and PD-134308 (10^{-6}M) completely inhibited G17-NH_2-stimulated proliferative responses while neither antagonist diminished the effects of G-Gly. In contrast, the somatostatin analog, octreotide (10^{-7}M) suppressed the actions of both G17-NH_2 and G17-Gly. G17-Gly also stimulated a dose-dependent (Kd$\approx 10^{-9}$M) increase in H^+-K^+-ATPase gene expression in isolated canine parietal cells that could not be inhibited by L365,260 or PD-134308. Competitive binding assays with ^{125}I-Leu$^{15}G_{2-17}$-Gly demonstrated high (Kd$\approx 10^{-9}$M) and low affinity (Kd$\approx 10^{-6}$M) binding sites on AR4-2J and isolated canine gastric parietal cells. ^{125}I-Leu$^{15}G_{2-17}$-Gly binding to AR4-2J and canine parietal cells was dose-dependently inhibited by G_{2-17}-Gly, but not by G17-NH_2, CCK8, a glycine-extended form of hexapancreatic polypeptide, nor by L365,260 or PD-134308. However, inhibition of ^{125}I-Leu$^{15}G_{2-17}$-Gly binding was seen with G_{5-17}Gly, G_{12-17}-Gly and G_{1-14} albeit at much higher doses than with G_{2-17}-Gly, suggesting a role for the amino- and carboxyl- termini of G17-Gly in binding to its receptor. Gastrins extended beyond the carboxyl-terminal glycine residue (G_{12-17}-GlyArgArg and G_{12-17}-GlyArgArgSerAla) did not inhibit ^{125}I-Leu$^{15}G_{2-17}$-Gly binding. ^{125}I-Leu^{15}-G17-NH_2 also bound AR4-2J and parietal cells but, this binding was completely displaced by G17-NH_2, L365,260, PD-134308, and CCK8, but not by G_{2-17}-Gly (10^{-6}M) nor G_{5-17}-Gly (10^{-6}M). We noted that G17-NH_2 induced a dose-dependent increase in [Ca^{2+}] .in parietal cells but G17-Gly did not. G17-Gly also had no effect on intracellular cAMP accumulation, but stimulated an increase in cellular tyrosine phosphorylation that was inhibited with genistein (10^{-6}M). These results lead us to conclude that amidated gastrin and its precursor, G-Gly, have biological actions that are mediated by separate and distinct receptors, emphasizing the important role that peptide processing plays in determining the physiological function of hormones.

Cellular Aspects of Processing Enzymes I

B7-021 THE BIOGENESIS OF SECRETORY GRANULES AND THE TRAFFICKING OF INTEGRAL MEMBRANE PAM. Sharon L. Milgram* and Betty A. Eipper, Dept. of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and *Dept. of Physiology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599

Peptidylglycine α-amidating monooxygenase catalyzes the generation of COOH-terminal α-amides from peptidylglycine substrates via a two step reaction involving peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PAL). PHM and PAL are contained within a single precursor protein that can occur naturally in either integral membrane or soluble forms. In rat PAM-1, PHM is separated from PAL by the noncatalytic region Exon A; PAL is followed by a single transmembrane domain and the COOH-terminal, cytoplasmic domain. In AtT-20 cells, integral membrane and soluble forms of PAM undergo endoproteolytic processing in a subcellular compartment distal to the trans-Golgi network (TGN). In PAM-1, this processing results in the generation of soluble, monofunctional PHM while most of the PAL remains membrane-associated. When expressed indepedently, soluble monofunctional PHM and PAL were both targeted to secretory granules in AtT-20 cells, indicating that both catalytic domains contain all of the information necessary for entering the regulated secretory pathway. Integral membrane PAM proteins were also targeted to immature secretory granules and underwent endoproteolytic processing. However, the steady state distribution, the kinetics of processing, and kinetics of basal release of soluble PAM differed from that observed for integral membrane PAM and integral membrane PAM was less efficiently stored in secretory granules. When integral membrane PAM proteins truncated 9 amino acids after the transmembrane domain were expressed in AtT-20 cells, 30% of the expressed protein was targeted to secretory granules while the remainder was missorted to the cell surface. These data indicate that lumenal interactions and cytoplasmic signals act together to mediate the targeting of integral membrane PAM. Using site-directed mutagenesis of the cytoplasmic domain of integral membrane PAM, we identified an 18 amino acid region which contains the information required for the trafficking of PAM within the regulated secretory pathway. We also identified an internalization motif dependent on Tyr^{936} in the cytoplasmic domain. When this Tyr is changed to an Ala (PAM-1/Y936A), PAM proteins which reach the cell surface are not efficiently internalized. However, newly synthesized PAM-1/Y936A proteins exiting the TGN are efficiently cleaved and the soluble protein which is generated is secreted in response to secretagogue. Therefore, this internalization signal is not required for targeting of PAM to immature granules. The less efficient storage of integral membrane PAM proteins in secretory granules compared to soluble PAM proteins may be related to the inability of the integral membrane PAM to efficiently condense with the granule core during the maturation process. Integral membrane PAM proteins which are removed from the maturing secretory granule during the maturation process, may be retrieved and returned to the TGN via cytoplasmic signals. We are currently analyzing chimeric proteins to determine the relative contributions and roles of the lumenal and cytoplasmic signals mediating the targeting of integral membrane PAM.

C-Amidation Enzyme and Glutaminyl-Cyclase

B7-022 MOLECULAR AND CELLULAR BIOLOGY OF CARBOXYPEPTIDASE E, Lloyd D. Fricker[1], Lixin Song[1], Iris Lindberg[2], Oleg Varlamov[1] [1]Dept. of Mol. Pharm., Albert Einstein Coll. of Med., Bronx, NY 10461, [2]Dept. Biochem. and Mol. Biol., LSUMC, New Orleans, LA 70112

Carboxypeptidase E (CPE) is involved with the biosynthesis of numerous peptide hormones and neurotransmitters. A single gene for CPE gives rise to several forms of protein through differential post-translational processing of the precursor, proCPE. These forms include one which is soluble at pH 5.5 (S-CPE), a form which can be extracted from membranes with 1 M NaCl at pH 5.5 (M1-CPE), and a form which requires both 1% Triton X-100 and 1 M NaCl for extraction from membranes at pH 5.5 (M2-CPE). Like other peptide processing enzymes, CPE is known to be sorted into peptide-containing secretory vesicles of the regulated pathway. One mechanism that has been proposed to be important for sorting of regulated pathway proteins is Ca^{2+} and pH-induced aggregation. Aggregation of CPE is pH- and concentration-dependent, with very little aggregation occurring at pH 6 or above, or for protein concentrations below 0.3 μg/μl. At pH 5.0-5.5, the M2 form of CPE shows a greater tendency to aggregate than the other two forms. At pH 6, Ca^{2+} concentrations in the mM range increase the aggregation of M1- and M2-CPE, but not S-CPE. The aggregation of M2-CPE does not explain the apparent membrane binding of this protein since the aggregate is solubilized by 1% Triton X-100 at pH 5.5 or by pH 6.0, whereas M2-CPE is not extracted from membranes under these conditions. Taken together, these results are consistent with a model in which the decreasing pH and increasing Ca^{2+} levels in the *trans* Golgi network induce the aggregation of CPE, which contributes to the sorting of this protein into regulated pathway secretory vesicles.

Reserpine treatment of cultured bovine adrenal chromaffin cells has previously been found to increase CPE activity approximately 100% without altering the level of CPE protein (Hook, Eiden, Pruss, J.Biol.Chem., 260, 5991, 1985). We tested whether any of the mechanisms known to activate CPE could be responsible for this increase. Kinetic analysis showed the increase to be due to an elevated Vmax, with no change in the Km for substrate hydrolysis. Reserpine treatment had no effect on the level of immunoreactive CPE protein or on the level of CPE mRNA. Biosynthetic labeling of the cells with [^{35}S]methionine followed by either immunoprecipitation or affinity chromatography to isolate CPE showed no change in the rate of CPE biosynthesis upon reserpine treatment. Reserpine treatment also did not alter the ratio of soluble to membrane-bound forms of CPE in the cell. When purified CPE was incubated with reserpine-treated chromaffin cell extracts, a 50-80% increase in CPE activity was detected. This activation was not observed when CPE was incubated with control chromaffin extracts or incubated in the absence of cell extracts. Taken together, these results are consistent with a model in which CPE is activated by reserpine through a novel post-translational mechanism.

B7-023 STRUCTURE-FUNCTION ACTIVITY OF THE AMIDATION ENZYME PAM, Richard E. Mains, Department of Neuroscience, The Johns Hopkins University School of Medicine, Baltimore MD 21205.

Biologically active peptides are produced from large, inactive precursors by a series of endoproteolytic and exoproteolytic steps; the final steps in the production of a biologically active peptide often involve modifications of the NH_2- or COOH-terminus of the peptide. One modification is COOH-terminal α-amidation, which is essential to the biological activity of more than half of all bioactive peptides. The protein responsible for peptide α-amidation is a bifunctional enzyme called peptidylglycine α-amidating monooxygenase (PAM: EC 1.14.17.3). The NH_2-terminal domain of PAM is a Cu^{++}, O_2 and ascorbate dependent enzyme, peptidylglycine α-hydroxylating monooxygenase (PHM), while the middle of the protein is the second of the pair of enzymes, peptidyl-α-hydroxyglycine α-amidating lyase (PAL). Peptide α-amidation is performed by the sequential actions of PHM and then PAL. PAM is the product of a single gene whose RNA is subjected to alternate splicing, which determines the post-translational processing steps to which the PAM protein is subjected. The presence of alternate exon A (exon 16) is required for cleavage between PHM and PAL; the presence of alternate exon Ba (exon 25) is required to produce an integral membrane form of the enzyme. In the absence of the transmembrane domain, a tyrosine residue in the COOH-terminal is sulfated; in the presence of the transmembrane domain, specific serine and threonine residues in the COOH-terminal are phosphorylated in a tissue-specific manner. Phosphorylation of the COOH-terminal is important to the correct intracellular routing and recycling of integral membrane forms of PAM, while tyrosine sulfation has an inhibitory effect on the rate of secretion of the soluble enzyme. To determine whether active enzyme is formed in the ER or only after transit through the Golgi, a KDEL-anchored construct was examined, showing full enzyme activity while trapped in the ER. The two enzymes have been expressed separately in stably transfected cell lines. NH_2- and COOH-terminal truncations have been used to define the minimum region yielding enzyme activity. Exploration of key residues in PHM using site-directed mutations has been guided by biochemical studies on dopamine β-monooxygenase (DβM), a Cu^{++}, ascorbate and O_2 dependent monooxygenase with significant homology to PHM over a 291 amino acid domain. The catalytic core of PHM is only slightly longer than the PHM/DβM homology domain. Two histidine clusters are crucial to enzyme activity while a third histidine-methionine cluster is not. One of the 2 methionine residues conserved between DβM and PHM is necessary to PHM activity while the other methionine is not. The tyrosine corresponding to the *p*-cresol target in DβM is involved in peptide substrate binding. The enzymatically inactive histidine mutant forms of PHM still bind to the peptidylglycine substrate. A *Drosophila* PAM has been cloned which conserves several of the key features of the PHM active site.

B7-024 MOLECULAR BIOLOGY OF GLUTAMINYL CYCLASE, Ulrich Teichert, Thomas Pohl, Michael Kreutz, Tobias Böckers, and Joachim Spiess, Department of Molecular Neuroendocrinology, Max Planck Institute for Experimental Medicine, 37075 Goettingen, Germany.

Peptide hormones are synthesized via larger precursors which are co- and posttranslationally processed by proteases and modifying enzymes. It has been generally accepted that Kex 2-like enzymes are often involved in the processing of prohormones at dibasic cleavage sites. However, the molecular mechanism of cleavage at single basic residues, especially arginine residues, has not yet been established. It is possible that Kex 2-like enzymes, as well as aspartyl proteases participate in the processing at these sites.

In contrast to the endoproteases identified as processing enzymes, modifying enzymes such as peptidylglycine α-amidating monooxygenase (PAM), glutaminyl cyclase (QC), lysyl hydroxylase or carboxypeptidase H require only short amino acid sequences as recognition sites. QC has been identified as a processing enzyme catalyzing the cyclization of N-terminal glutaminyl to pyroglutamyl residues. This cyclization probably is part of the biosynthesis of pyroglutamyl peptides such as thyrotropin-releasing hormone (TRH) and gonadotropin-releasing hormone (GnRH). The enzyme was found in secretory vesicles of (bovine) anterior pituitary from which it was purified. On the basis of the N-terminal protein sequence, DNA coding for QC was cloned and completely sequenced. In the meantime, QC has been characterized from other species in our and other laboratories and found highly conserved. Immunohistochemical experiments revealed that QC is co-localized with growth hormone in bovine anterior pituitary. Since many antibody chains have been shown to be blocked by pyroglutamyl residues, immune cells have been analyzed for the presence of QC. However, in contrast to observations in other laboratories, we did not find evidence for QC mRNA or enzymatic activity in purified activated B lymphocytes of the rat. Mouse hypothalamic GT 1-7 cells which have been demonstrated to produce GnRH and successfully used for the investigation of the GnRH biosynthesis have been shown to contain QC mRNA.

Cellular Aspects of Processing Enzymes I

B7-025 TRANSPORT, TARGETING, AND STORAGE OF INSULIN-CONTAINING PEPTIDES WITHIN THE REGULATED SECRETORY PATHWAY. Regina Kuliawat[1], Xue Fen Huang[1], and Peter Arvan[1,2] [1]Division of Endocrinology, Beth Israel Hospital Research North and [2]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA02215

Milieu-induced condensation is envisioned to play an important role in two alternative hypotheses which attempt to account for the targeting of proteins destined for storage in granules: *sorting-for-entry* into immature secretory granules (IGs) and *sorting-by-retention* within IGs. In β-cells of pancreatic islets, insulin expression is ≥ 2-orders of magnitude higher than other proteins stored in granules. Thus, most condensation within the secretory pathway can be viewed to represent changes within the biophysical state of insulin-containing peptides. We and others have obtained compelling evidence indicating that insulin formation from proinsulin occurs within IGs[A]. By coupling pulse-chase analysis to an in vitro assay that exploits sedimentation of peptides, we found that formation of insoluble aggregates of regulated secretory protein lagged behind the conversion of proinsulin to insulin, suggesting that condensation occurs within IGs[B]. To confirm this, we wished to assay proinsulin and insulin structural maturation *in vivo*. For this purpose we exploited disulfide accessibility to reduction with dithiothreitol (DTT). Pro(insulin) has three disulfides, two of which are interchain bonds in the insulin heterodimer. Using tricine-urea-SDS-PAGE, standards were resolved into 4 bands containing none, 1, 2, or all 3 native proinsulin disulfides. When pulse-labeled islets were exposed to low doses of DTT, newly-synthesized proinsulin developed mild resistance to disulfide reduction during the first 5 minutes of chase, consistent with oligomerization within the ER. However, even when accounting for a modest difference in DTT potency over the pH range 6.2 —> 5.4 (felt to represent progression from TGN to mature granules), no significant change in disulfide accessibility was observed until proinsulin was converted to insulin, when a dramatic increase in resistance to DTT was observed; this major biophysical change was indicative of formation of the nascent granule core. The data suggest that proinsulin conversion to insulin is necessary (although not sufficient) for optimal storage of this peptide hormone within secretory granules. This hypothesis is now being tested in a cellular transfection model, in which preliminary data supports the idea that conversion to insulin plays a major role in hormone storage in granules. Experimental manipulation of condensation conditions in vivo indicates that the intragranular ionic environment is another important factor in polymer assembly within IGs. Since condensation occurs after proinsulin has already entered IGs, the data emphasize the importance of the sorting-by-retention model.

[A] Huang, X.F., and P. Arvan. (1994) Formation of the insulin-containing secretory granule core... J. Biol. Chem 269: 20838-20844
[B] Kuliawat, R. and P. Arvan (1994) Distinct molecular mechanisms for protein sorting... J. Cell Biol. 126: 77-86

B7-026 RAB3A, ASSOCIATED PROTEINS AND INSULIN EXOCYTOSIS. Christopher J. Rhodes, Research Division, Joslin Diabetes Center and Harvard Medical School, Boston, MA 02215.

In pancreatic ß-cells, the proinsulin processing enzymes, PC1/3, PC2 and carboxypeptidase-H are co-secreted with mature insulin in a response to a secretagogue-stimulation, such as that from extracellular glucose. The signaling mechanism for glucose to induce insulin exocytosis is complex, but involves accelerated glucose metabolism that then instigates changes in ionic fluxes across the ß-cell plasma membrane, leading to a rapid rise in cytosolic Ca^{2+}. A rise in $[Ca^{2+}]_i$ has long thought to be the intracellular trigger to induce insulin exocytosis. However, the actual molecular mechanism whereby $[Ca^{2+}]_i$ promotes transport to, association with, and then fusion of insulin secretory granules to the plasma membrane for exocytosis is not clear. We have been examining the mechanism behind regulated insulin exocytosis by focussing on a key protein involved in the regulated exocytotic mechanism in neuroendocrine cells, the GTP-binding protein, Rab3A. Rab3A is thought to mediate exocytosis by an interaction of its effector domain with a putative effector protein. We have shown that Rab3A effector domain peptides specifically stimulated insulin exocytosis in electroporated insulin secreting cells ($K_{0.5}$ activation between 6-8μM) in a Ca^{2+}-independent manner (although the presence of Ca^{2+} slightly potentiated insulin exocytosis). By using an ^{125}I-radiolabeled photoactivated cross-linking Rab3A effector domain peptide, we identified two novel cytosolic proteins (REEP-1 (17kDa) and REEP-2 (14kDa)) which specifically interact with the Rab3A's effector domain. Competitive inhibition studies revealed this protein-protein interaction to be at a concentration equivalent to that required for Rab3A effector domain peptides to trigger insulin exocytosis (Ki between 6-8μM). Futhermore, the association of Rab3A effector domain with REEP-1 and -2 was pH- and Ca^{2+}-dependent (with a Ca^{2+}-requirement in the range required to trigger exocytosis ($K_{0.5}$ association 40-50μM Ca^{2+})). The intracellular localization of REEP-1 and -2 was cytosolic, as indicated by analysis of insulinoma subcellular fractions. However, in isolated rat islets under basal secretory conditions REEP-1 and -2 were membrane associated, but upon stimulation of exocytosis they were released into a cytosolic fraction. We are currently purifying REEP-1 and -2 in order to identify and subsequently clone them. Although as of yet identified, REEP-1 and -2 are most likely part of the regulated exocytotic machinery, and their dissociation upon stimulation of hormone release (likely from a pre-exocytotic protein complex) may be essential to the mechanism that triggers regulated exocytosis in pancreatic ß-cells. As well as general control of regulated exocytosis in neuroendocrine cells, the hypothetical steps that are required for transport of secretory granules to the plasma membrane, will be discussed.

B7-027 PROHORMONE PROCESSING IN PERMEABILIZED CELLS, Dennis Shields, Huaxi Xu, Yeguang Chen, Cary Austin, Wai Lam Wong, Joydeep Mitra and Arkady Elgort, Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461.

Many peptide hormones are synthesized as larger precursors which undergo selective endoproteolytic cleavage at paired basic residues to generate a bioactive molecule. Morphological evidence has implicated either the Trans Golgi Network (TGN) or immature secretory granules as the site of prohormone cleavage. To identify the cellular machinery that mediates prohormone sorting, proteolytic cleavage and formation of nascent secretory granules, we have established a permeabilized cell system from transfected rat anterior pituitary GH3 cells which express high levels of human prosomatostatin, proSRIF, (Xu and Shields, J.Cell Biol. 122: 1169-1184 1993). By incubating these cells at 20°C, both exit from the TGN and proSRIF processing are inhibited. Following the 20° block, the cells were permeabilized and proSRIF processing determined. Cleavage of proSRIF to the mature hormone was approximately 35-50% efficient, required incubation at 37°C and ATP hydrolysis, but was independent of GTP and cytosol. Cleavage of proSRIF to the mature hormone required ATP hydrolysis, and was inhibited by chloroquine, a weak base, or CCCP, a protonophore. This suggested that a proton gradient and/or an acidic pH facilitated by a vacuolar $[H^+]$-ATPase was required for prohormone processing. By utilizing bafilomycin A1, a specific inhibitor of vacuolar $[H^+]$-ATPases, proSRIF processing was inhibited *in vivo* and *in vitro* confirming the involvement of a vacuolar-type ATPase in prohormone processing. The ATP requirement for processing could be circumvented *in vitro* by incubating permeabilized cells at acidic pH in the presence of protonophores, indicating that an acidic pH rather than a $[H^+]$ gradient was necessary for processing; a pH of ~6.1 in the TGN was optimal for proSRIF cleavage. To investigate the mechanism of protein sorting to secretory granules, the budding of nascent secretory vesicles from the TGN was determined. No vesicle formation occurred at 20°C; in contrast, at 37°C the budding of nascent secretory vesicles was about 40% efficient and was dependent on ATP, GTP, and cytosol. Vesicle budding required cytosolic factors that were tightly membrane associated and could be removed only by treating the permeabilized cells with high salt. Following high salt treatment, vesicle formation was dependent on added cytosol or the dialyzed salt extract. The characterization of cytosolic factors that mediate formation of nascent secretory granules will be discussed.

Towards Defining the Three Dimensional Structures of Processing Enzymes

B7-028 PROTEIN INHIBITORS OF SUBTILISINS: A STRUCTURAL COMPARISON,

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There are many families of protein inhibitors of the serine proteinases (Laskowski, M. & Kato, I., *Annu. Rev. Biochem.* **49**, 593-626, 1980). Crystallographic complexes of subtilisin with structural data are available only for the members of the potato inhibitor I family, eglin-c and chymotrypsin inhibitor 2 (CI-2). The structures of the potent inhibitor, eglin-c complexed to a variety of subtilisin-like enzymes are known at high resolution. Comparisons of these structures with the structures of the native, uncomplexed enzymes and inhibitor comprising these complexes, show that there is a small inhibitor-induced-fit of inhibitors to subtilisins. There is also a reduction in the flexibility of both the enzyme and inhibitor in the region of contact upon forming the complex. Details of the binding interactions in several of these complexes will be presented and discussed.

Thermodynamic binding data have been measured for 25 P_1 variants of the third domain of the turkey ovomucoid (OMTKY3) with subtilisin (Laskowski *et al.*, personal communication). The variation in these binding constants shows that the S_1 site in subtilisin does not like charged residues, neither positive nor negative charges. The hypothetical complex between subtilisin Carlsberg and OMTKY3 has been modeled on the basis of the known complex with subtilisin Carlsberg and eglin-c (McPhalen, C.A. & James, M.N.G., *Biochemistry* **27**, 6582-6598, 1988). The potential enzyme inhibitor contacts in this complex will also be presented.

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B7-029 DESIGN OF INHIBITORS FOR SUBTILISIN-RELATED PROPROTEIN CONVERTASES BASED ON AVIAN OVOMUCOID THIRD DOMAINS,

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Workers in the field of serine proteinases enjoy having available strong and specific inhibitors for the enzymes they study. Therefore, the discovery of an especially important serine proteinase or a group of serine proteinases is almost always followed by a search for both natural and designed inhibitors. Such was the case for subtilisin-related proprotein convertases (see Steiner et al, 1992 for a review), where the description of the enzymes was quickly followed by the design of small molecule (Steinke-Gröber, 1992) and protein (Lu et al, 1993; Anderson et al, 1993) inhibitors and by the report of a natural protein or peptide inhibitor (Martens et al, 1994). We (Lu et al, 1993) based our design on the frame of avian ovomucoid third domain as these domains are frequently superb inhibitors of subtilisin-related and of chymotrypsin-related enzymes. Furthermore, we have already shown (eg Komiyama et al, 1991) that small changes in the reactive site sequence of these inhibitors lead to striking and predictable alternations in their specificity. We, therefore, introduced the consensus sequence Arg X LysArg↓ into turkey ovomucoid reactive site and found it to be a moderate inhibitor of SPC1 or furin. We have now extended these studies to several more ovomucoid variants and to several more SPC's. The results of these studies will be discussed.

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B7-030 HOMOLOGY MODELLING OF THE CATALYTIC DOMAIN OF HUMAN FURIN. A MODEL FOR THE EUKARYOTIC SUBTILISIN-LIKE PROPROTEIN CONVERTASES,

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Furin is a multi-purpose serine protease with a specificity for multi-basic sites. It cleaves precursors of bioactive proteins such as hormones, neuropeptides, growth factors, plasma proteins involved in blood clotting, receptors and viral-envelope glycoproteins. Furin is the prototype of the mammalian proprotein convertases, which include PC1/PC3, PC2, PC4, PACE4 and PC5/PC6. These mammalian proprotein convertases are multi-domain proteins with the serine protease (or catalytic) domain at the N-terminus. They all have a specificity for cleavage after di- or multi-basic residues and they constitute a subfamily of the subtilisin-like serine proteinases, also abbreviated as *subtilases* [1]. Corresponding proteases of this subfamily have now also been discovered in various lower eukaryotes, including yeasts. Over 100 members of the subtilase family are presently known, but only a limited number of 3-dimensional structures has been determined (e.g. subtilisins, thermitase, proteinase K), and none is to date available for the proprotein convertases. Homology modelling can be used to make predictions for the catalytic domain of subtilases concerning structure, stability, substrate specificity, etc.[1]. A model has been constructed for the 3-dimensional structure of the catalytic domain of human furin and its interaction with model substrates, and it also applies to other members of the eukaryotic proprotein convertases [2]. This homology model is based on the crystal structures of subtilisin BPN' and thermitase in complex with the inhibitor eglin. Predictions are made of the general protein fold, inserted loops, disulfide bonds, Ca^{2+} binding sites and salt bridges. A detailed prediction of the substrate-binding region attempts to explain the basis of specificity for multiple basic residues preceding the cleavage site. Negative charges are abundant in and around the substrate-binding region, which should be ideal for binding highly positively-charged substrate segments. Specific acidic residues in the S1, S2 and S4 subsites of the substrate-binding region of furin and other proprotein convertases are identified which appear to be of particular importance, while residues of the other subsites may also contribute to binding. Based on this model, protein engineering can be employed not only to test the predicted enzyme-substrate interactions, as demonstrated for human furin, but equally importantly to design proprotein convertases with a desired specificity, or to design novel substrates or inhibitors.

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Cellular Aspects of Processing Enzymes II, Inhibitors Design

B7-031 THE BIOSYNTHESIS AND PROCESSING OF PRO-PC2 and PRO-PC3, Kevin Docherty, Kathleen I J Shennan, Neil A Taylor, Glenn Matthews* and Joanne Jermany, Department of Molecular and Cell Biology, University of Aberdeen, Marischal College, Aberdeen, AB9 1AS, UK. *School of Biochemistry, University of Birmingham, Birmingham, B15 2TT,UK.

PC2 and PC3, which is also known as PC1, are subtilisin-like proteases that are involved in the intracellular processing of pro-hormones and pro-neuropeptides. Both enzymes are synthesized as propolypeptides that undergo proteolytic maturation within the secretory pathway. We have used an *in vitro* translation-translocation system from Xenopus egg extracts to investigate mechanisms in the processing of pro-PC3 and pro-PC2. Pro-PC3 underwent rapid ($t_{½}$<10min) processing of the 88kDa peptide at the sequence RSKR^{83} to generate the 80 kDA active form of the enzyme. This processing was blocked when the active side aspartate was changed to asparagine, suggesting that an autocatalytic mechanism was involved. Processing of Pro-PC3 was optimal at pH7.5 with very little activity observed below pH6.0. Processing was not calcium dependent. These results are consistent with pro-PC3 maturation occurring at an early stage in the secretory pathway, possibly within the endoplasmic reticulum, where the pH would be close to neutral and the calcium concentration less than that observed in later compartments. Processing of pro-PC2 in the Xenopus egg extract was much slower than that of pro-PC3 ($t_{½}$ = 8 hrs). It exhibited a pH optimum of 5.5-6.0 and was dependent on calcium ($K_{0.5}$=2-4mM). The enzymic properties of pro PC2 processing were similar to that of the mature enzyme. Further studies using mutant pro-PC2 constructs suggested that cleavage of pro-PC2 was catalysed by the mature 68kDa PC2 molecule. The results were consistent with pro-PC2 maturation occurring within a late compartment of the secretory pathway that contains a high calcium concentration and low pH.

B7-032 PROCESSING OF CHROMOGRANINS/SECRETOGRANIN TO FUNCTIONAL PEPTIDES, Reiner Fischer-Colbrie, Department of Pharmacology, University of Innsbruck, Peter Mayr Str. 1a, A-6020 Innsbruck, Austria.

The chromogranins comprise acidic secretory proteins which are widely expressed throughout the endocrine, neuroendocrine and nervous system. Established members of this class of proteins are chromogranin A, chromogranin B and secretogranin II. Within the cell chromogranins are stored in the large dense core vesicles and released from them after stimulation of cells by exocytosis. The function of the chromogranins is only partially understood yet. Recent data, however, suggest that chromogranins might be precursors of biologically active small peptides. We characterized the proteolytic processing of chromogranins in several tissues and analyzed the endopeptidases involved. In any of the tissues investigated chromogranin A is processed proteolytically, however to a different extent. In adrenal medulla and anterior pituitary chromogranin A exists mainly as the intact precursor. In contrast, in the brain, the posterior pituitary, the gastrointestinal tract and the endocrine pancreas chromogranin A is processed to a marked extent. From these tissues we characterized and identified a novel peptide (GE-25) generated from the chromogranin A precursor. This peptide is located in the conserved C-terminal part of chromogranin A and flanked by pairs of basic amino acids. The physiological role of this novel peptide is not known yet. Secretogranin II as shown by infection of GH_4C_1 cells with genetically modified vaccinia viruses is processed *in vivo* by endopeptidases PC1 and PC2 to several fragments of intermediate size. To generate secretoneurin, a neuropeptide derived from secretogranin II, PC 1 alone is sufficient. Secretoneurin is widely expressed in throughout the central nervous system and can induce dopamine release from striatal slices in a calcium dependent manner. In the sensory system secretoneurin is co-localized with tachykinins in primary afferent fibers. The potent chemotactic activity of secretoneurin towards monocytes *in vitro* and *in vivo* together with the regulation of its biosynthesis by histamine and prostaglandin E suggest an involvement of secretoneurin in neurogenic inflammation.

B7-033 PROINSULIN BIOSYNTHESIS AND PROCESSING IN THE PANCREATIC ß-CELL J. C. Hutton, J. Creemers, E. M. Bailyes, E. F. Usac, S. D. Arden, P.C. Guest, C-G. Östenson, S.M. Abdel-Halim, S. Efendic & A. Clark University of Cambridge, Department of Clinical Biochemistry, Addenbrookes Hospital, Hills Road, Cambridge, CB2 2QR, U.K.

The conversion of proinsulin to insulin in the pancreatic ß-cell occurs through the concerted action of the subtilisin-related endopeptidases PC1 and PC2. The enzymes demonstrate substrate sequence specificity which depends both on the primary sequence around a dibasic motif plus features of secondary structure. pH and Ca play an important role not only in the regulation of the activity of the mature enzymes but also in their conversion from inactive precursors as can be demonstrated by experimental depletion of intracellular Ca. It is also clear that their biosynthesis responds to secretory stimuli in a characteristic manner, a factor which may in turn affect the composition and bioactivity of the secreted peptides stored in the cell. Yet another factor which contributes to their regulation is the rate at which the enzymes transverse the secretory pathway and are sorted from the TGN into the regulated pathway. This seems to be largely determined by the intrinsic structural properties of the enzymes as illustrated by domain swapping experiments.

To determine whether hormone production and secretory granule biogenesis are affected in type 2 diabetes, we have investigated the biosynthesis and post-translational processing of proinsulin and the proinsulin-converting enzymes PC1 and PC2 in isolated rat islets of diabetic GK rats. Radiolabelling studies showed that proinsulin biosynthesis was stimulated to the same extent (approximately 20-fold) in both GK and control islets in response to glucose (2.8 vs. 16.7mM). The biosynthesis of PC1 responded normally in GK islets, with a glucose-induced stimulation of 15-fold. PC2 biosynthesis however appeared to be impaired at low glucose concentrations and resulted in a lower content of the enzyme in the islets. Such changes may account for the small increase in unprocessed forms of proinsulin in diabetic islets which in turn may account for substantial elevation in circulating forms of these molecules as they have a relatively long biological half-lives compared to insulin. Immunocytochemical studies showed that the cellular distribution of insulin, PC1 and PC2 immunoreactivity was more heterogeneous in the GK islets suggesting that secretion was confined to a sub-population of ß-cells. The turnover of the intracellular storage pool is presumably greater in the active cells, a factor which again would contribute to increased rates of release of partially processed forms of the prohormone.

B7-034 7B2 IS A NEUROENDOCRINE CHAPERONE AND NATURALLY OCCURRING INHIBITOR OF PROHORMONE CONVERTASE PC2,
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The neuroendocrine polypeptide 7B2 is a highly-conserved protein selectively present in prohormone-producing cells equipped with a regulated secretory pathway. The 7B2 protein is carboxy-terminally processed, and only the processed and not the precursor form is secreted. We found that the amino-terminal half of 7B2 displays a remarkable amino acid sequence similarity with a region in chaperonins, a subclass of molecular chaperones, while its carboxy-terminal portion is distantly related to protein proteinase inhibitors of the so-called potato inhibitor I family. When incubated *in vitro* with newly synthesized proteins produced by *Xenopus* intermediate pituitary cells, the precursor form of recombinant 7B2 specifically associated with both the proenzyme and mature forms of prohormone convertase PC2; the processed form of recombinant 7B2 associated with only the proenzyme and not the mature form. During this incubation the conversion of proPC2 to the mature enzyme was inhibited by the intact but not the processed form of recombinant 7B2. Using amino- and carboxy-terminal deletion mutants of recombinant 7B2, we found that this inhibitory action is caused by the carboxy-terminal half of the molecule. Metabolic cell labelling combined with coimmunoprecipitation studies showed that *in vivo* the precursor form of 7B2 interacts with the proform of PC2. Pulse-chase analysis of biosynthetic events in *Xenopus* and mouse intermediate pituitary revealed that this association is transient and commences early in the secretory pathway. Biosynthetic experiments with the drug brefeldin-A (blocking protein export from the endoplasmic reticulum) and the ionophore monensin (arresting protein transport in the Golgi) showed that cleavage of 7B2 and dissociation of the processed form of 7B2 from proPC2 occurs slightly earlier than the conversion of proPC2 to mature enzyme in the TGN/secretory granule compartments of the *Xenopus* cells. In contrast, in cultured tumor cells the processed form of 7B2 does not appear to dissociate from proPC2 and was found to remain associated with this convertase after secretion. As in mammalian cells, the conversion of proPC1 to mature enzyme clearly occurred earlier than proPC2 maturation in the secretory pathway of the amphibian intermediate pituitary cells. In *in vitro* enzyme assays, the precursor form but not the processed form of recombinant 7B2 was found to inhibit PC2 but not PC1. Using the deletion mutants and site-directed mutagenesis, this inhibitory activity could be localized to a distinct region in the carboxy-terminal portion of 7B2. Together, the results suggest that 7B2 is a novel type of molecular chaperone assisting transport and preventing premature activation of proPC2 in the secretory pathway of neurons and endocrine cells.

Development Transgenics and Viral Infections

B7-035 GLYCOPROTEINS OF ENVELOPED VIRUSES AND THEIR PROCESSING ENZYMES.
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Surface proteins of many enveloped viruses such as influenza virus, paramyxoviruses, and HIV, are activated by host proteases. Cleavage is essential for infectivity of these viruses and in some cases, an important determinant for spread of infection and virus pathogenicity. Cleavage depends on the presence of the appropriate protease and on the susceptibility of the glycoproteins to these proteases. In general, the glycoproteins contain either a single arginine or multibasic residues with the consensus sequence R-X-K/R-R at their cleavage site. The available evidence indicates that proteases which recognize the multibasic motifs belong to the subtilisin-like eukaryotic endoproteases. Furin, the prototype of this protease family, is an ubiquitous enzyme, and therefore, a good candidate for virus activation. Furin is localized in the TGN by two independent sorting signals in the cytoplasmic domain of furin. TGN-localization is in good agreement with the concept that viral glycoproteins containing the motif R-X-K/R-R are activated in this cell compartment. Furin is inhibited by peptidylchloroalkyl ketones containing the correct cleavage site motif. By blocking glycoprotein activation, these inhibitors prevent spread of virus infection.

B7-036 ENGINEERING THE MAMMARY GLAND BY EXPRESSION OF PACE/FURIN,
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We hypothesized that the posttranslational protein processing capacity of specific animal organs may be modified by the expression of selected processing enzymes. We tested this hypothesis by targeting the expression of the human proprotein processing enzyme, paired basic amino acid-cleaving enzyme, PACE/furin, to the transgenic mouse mammary gland to increase endoproteolytic processing of heterologous vitamin K-dependent proteins. Endoproteolytic processing of the precursor of human Protein C (HPC) to its mature form requires cleavage of the propeptide after a pair of dibasic amino acids, Lys^{-2}-Arg^{-1}, as well as the removal of a Lys^{156}-Arg^{157} dipeptide which connects the light and heavy chains. This processing was inefficient when recombinant HPC (rHPC) was expressed in the mammary gland of transgenic mice and pigs. Coexpression of PACE with HPC resulted in the efficient conversion of the precursor to mature rHPC, in comparison to mice expressing rHPC alone. Cleavage was observed to occur at the appropriate sites, at determined by amino acid sequencing of purified rHPC. Double-transgenic mice expressing PACE, mutated in the catalytic domain, Ser^{261}Ala, did not demonstrate improved processing of rHPC, indicating that the observed processing in HPC/PACE mice was due to the active enzyme. The health of the animals was unaffected and two of the four HPC/PACE transgenic lines showed normal lactation and nursing, even at PACE expression levels an order of magnitude higher than that of the endogenous gene. These results suggest the involvement of PACE in the processing of the HPC precursor *in vivo* and represent the first example of the engineering of a selected animal organ into a bioreactor with enhanced protein processing capabilities.

B7-037 ONTOGENY OF PROCESSING ENZYME EXPRESSION DURING MAMMALIAN EMBRYOGENESIS, John E. Pintar[1], Min Zheng[1], Jiwen Zhang[1], Maureen E. Czick[1], Suzana Petanceska[2], Lakshmi Devi[2], James L. Roberts[3], Marc J. Glucksman[3], Adrian Pierotti[4], Nabil Seidah[5], and Betty A. Eipper[6]. UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854; [2]Dept. Pharmacology, NYU Medical School, [3]Cntr. for Neurobiology, Mt. Sinai Medical School, [4]Dept. Biological Sciences, Caldonian University, Glasgow, Scotland, [5]Clinical Research Institute of Montreal, Quebec, H2W1R7, Canada; [6]Dept. Neuroscience, Johns Hopkins University.

Cellular specificity of processing enzyme expression represents an important control mechanism in the production of bioactive peptides from inactive precursors. In order to assess the possible differential contributions that distinct sets of processing enzymes may have through prenatal rodent development, in situ hybridization has been used to delimit the expression pattern of mulitple processing enyzme mRNAs. Thus far, the mRNAs encoding multiple members of the prohormone convertase, cysteine protease, and metalloendoprotease families of proteolytic enzymes have been examined, in addition to variants of peptidyl-a-amidating (PAM) mRNA that arise by alternative splicing. The genes encoding these enyzmes all are activated by early post-implantation, exhibit striking spatial and temporal patterns of expression as development proceeds, and often are co-expressed with putative substrates. For example, furin mRNA is detected in the early post-implantation embryo in a pattern similar to IGF-II mRNA, which encodes a precursor protein having multiple furin consensus sites. PC1 and PC2 exhibit distinct spatial patterns of expression during nervous system development, and changes in expression of pituitary PC2 are generally correlated with developmental changes in the extent of POMC processing. Cathepsin S is expressed in discrete subpopulations of cells detected as early as e15 and, in the nervous system, is especially prominent in different groups of neurons in the spinal cord during late prenatal development. Cathepsins B and L, in contrast, are prominent in liver and placenta and various extraembryonic membranes. Within these tissues there is also heterogeneity in expresson; in the developing placenta, for example, cathepsin L is more prominent than cathepsin B in the labyrinth zone, while cathepsin B is particularly abundant in the junctional zone at e14. The metalloendoproteases NRD-convertase (structurally related to pitrilysin and insulinase) and E.C. 3.4.24.15 (abundant in adult rat testis) are also expressed prenatally. In addition to its high expression in the developing urogenital system, NRD is also expressed in the spinal cord and peripheral ganglia prenatally, while 24.15 is abundant in the ventricular zone of the developing nervous system. Finally, probes specific for multiple PAM isoforms have been used to show that exon B-containing PAM transcripts are the predominant PAM mRNAs at all prenatal ages examined. In addition, rat PAM-4 transcripts, which encode only the peptidylglycine-a-hydroxylating portion of PAM, are relatively enriched in the dorsal horn of the spinal cord, but are absent from the ependymal region, which instead is rich in exon B PAM transcripts. Taken together, these results demonstrate temporal and spatial changes in the expression pattern of established and processing processing enzyme genes may regulate changing patterns of prohormone and propeptide processing during embryogenesis.

Molecular Biology of Pro Hormone Convertases I & II

B7-100 mPC1 AND hFURIN ENZYMATIC ACTIVITIES ARE INHIBITED BY METAL IONS AND THIOL-REACTING COMPOUNDS.

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The effects of various metal ions on the *in vitro* enzymatic activity of the convertases mPC1 and hFurin were studied. We found that, at a level of μM concentration, some metal ions in the following order, $Hg^{++} > Cu^{++} > Zn^{++} > Cd^{++}$, were inhibitors of both enzymatic activities whereas some others such as Co^{++}, Ni^{++}, Mg^{++} and Li^{+} up to 10 mM had little or no effect on these activities. Using different chelators and thiol-containing compounds, we were able to demonstrate that this inhibition is reversible. However, we showed that Hg^{++} inhibition is prevented only by thiol-containing compounds but not by chelating compounds thus suggesting that this inhibition occurs probably through formation of a mercaptide bound with a free cysteine. On the other hand, the other metal ions inhibition is prevented by both chelating and thiol-containing reagents suggesting that it is effected by a chelating mechanism. Using p-chloromercuribenzoate (pCMB), a thiol labeling reagent able to inhibit either enzymes, we showed that a synthetic peptidyl substrate can effectively prevent its action suggesting that there is at least one free reactive cysteine located within the active site. According to computer modeling of the active site of these enzymes, we propose that the thiol moiety in the conserved cysteine located three residues away from the active site His residue in either mPC1 or hFurin remains free within the active site and is thus sensitive to the action of mercuric ions and pCMB. Accordingly, we propose that the inhibition by the other metal ions would be achieved through chelation of the metal ions by both this cysteine and the histidine of the catalytic triad.

This study was supported by the Medical research Council of Canada

B7-101 PROTEOLYTIC PROCESSING OF PROINSULIN-LIKE GROWTH FACTOR IA TO MATURE IGF-I,

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Insulin-like growth factor I is a 70 amino acid peptide that plays a critical role in the regulation of cell and tissue growth during embryonic development and post-natal growth. Mature IGF-I is generated from proIGF-IA by removal of the 35 amino acid carboxy-terminal E-domain. Proteolytic removal of the E-domain may be an important activation step, since proIGF-IA has 10-fold less affinity for the IGF-I receptor and binding proteins than does mature IGF-I. We have constructed a CMV expression vector containing human proIGF-IA with an epitope tag, the FLAG peptide, at the amino terminus. The FLAG peptide does not affect signal peptide removal or propeptide processing. HEK 293 cells transfected with this vector secrete mature IGF-I, proIGF-I and glycosylated proIGF-I into the culture medium. Cells lysates from HEK 293 cells also contain mature IGF-I, proIGF-I and glycosylated proIGF-I, indicating that processing occurs intracellularly. ProIGF-I contains an unusual cleavage site consisting of five basic residues each separated by two non-basic residues: $KxxKxxR^{71}xxRxxR$. Arg^{71} appears to be the natural processing site *in vivo*. However, transiently transfected HEK 293 cells process proIGF-I at arg^{71} and a downstream site, possibly arg^{77}. Interestingly, the furin-deficient LoVo cell line processess proIGF-I only at Arg^{71}. This suggests that mature IGF-I can be generated from proIGF-I by an enzyme distinct from furin. Site-directed mutagenesis studies are currently underway to determine which basic residues in the proIGF-I cleavage site are required for substrate recognition by the converting enzyme.

B7-102 IDENTIFICATION OF A NEW MEMBER OF THE PROHORMONE CONVERTING ENZYME FAMILY,

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Parathyroid hormone-related protein (PTHrP) is the mediator of humoral hypercalcemia of malignancy. PTHrP is expressed by many cancers, particularly those of squamous origin, and those which metastasize to bone. In addition to its production by cancers, PTHrP is expressed by a wide variety of tissues and has demonstrated actions on placental calcium transport, inhibition of osteoclastic bone resorption, smooth muscle contractility and on cellular growth and differentiation. These actions have been defined to different regions of PTHrP some of which are bound by mono- or dibasic amino acids, indicative that PTHrP may be post-translational cleaved by prohormone converting enzymes (PCEs) to liberate regions of distinct biological activities. We have characterized a variety of cell lines and tissues to determine whether particular members of the PCE family are co-expressed with PTHrP and to identify novel PCEs that may be involved with the processing of PTHrP.

RNA from rat and mouse tissues and several human cell lines was subjected to RT-PCR analyses using oligonucleotides specific to PTHrP and to members of the PCE family. Expression of furin and PACE4, like PTHrP, were ubiquitous, whilst PC1 and PC2 were not concordantly expressed with PTHrP. To identify novel members of the PCE family which might be involved in PTHrP processing, degenerate oligonucleotide primers to conserved regions within the catalytic domain of the PCEs were used in RT-PCR with RNA from the PTHrP-producing human squamous carcinoma cell line BEN. One clone, BC20, represented a 339 bp product and its predicted protein sequence displayed conserved sequence homology to the catalytic domain of other PCEs, indicative that it is a new member of this family. BC20 was found to be a close homolog of furin sharing 58% amino acid and 63% nucleotide sequence identity, suggesting that these PCEs may have arisen via gene duplication. By RT-PCR BC20, like furin, was ubiquitously expressed in a range of rat and murine tissues, including the adrenals, brain, heart, kidney, liver, lung, spleen, testis and thymus. We are presently defining the cDNA sequence of BC20 since its co-expression with PTHrP makes this a candidate enzme for processing of PTHrP, along with furin and PACE4.

B7-103 STUDIES ON THE SUBSTRATE SPECIFICITY OF THE SPCs BY MOLECULAR MODELLING AND SITE-DIRECTED MUTAGENESIS OF SUBTILISIN BPN',

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Recent studies in our laboratory have led to the identification of two subtilisin-like prohormone convertases (SPCs), PC2 and PC1/PC3, which participate in the processing of peptide precursors at dibasic sites. A model of the three-dimensional structure of these SPCs has been proposed, based on X-ray data on subtilisin-eglin complex (Lipkind *et al.* in preparation). SPCs have an increased number of negatively charged residues in the active site region compared with subtilisin and these might contribute to their specificity for substrates containing dibasic residues. Another significant structural difference was also found in a loop formed by residues 165-171 which contains a *cis* peptide bond between Tyr167-Pro168 in subtilisins and a *trans* peptide bond between Ala167-Thr168 in SPCs. In the present work, we attempted to change the specificity of subtilisin BPN' toward substrates with P1 Arg or Lys residues by site-directed mutagenesis of subtilisin BPN'. Residue Val165 was substituted with Asp. The corresponding Asp in SPCs was predicted to be located in the bottom of the S1 pocket and to enhance binding of Arg in the substrate P1 site. The mutant (Val165Asp) enzyme had decreased activity towards both s-AAPFpNA and s-AAPRpNA substrates as compared to the wild type. Western blot analysis of the culture medium of the mutant revealed the presence of mature processed subtilisin BPN'. Two other mutants, Pro168Thr and Gly169Asp, were prepared, but did not appear in the medium. These results suggest that although Asp165 is likely to be the most critical residue for binding P1 Arg, the residues surrounding Asp165 may also be important in determining the P1 specificity and also that the loop formed by residues 165-171 is an important determinant of the structural stability of these proteins.

B7-104 COMPARISON OF THE SPECIFICITY OF THE HEPATIC PROALBUMIN CONVERTASE AND FURIN FOR RECOMBINANT PROALBUMIN VARIANTS, Elizabeth C. Ledgerwood, Peter M. George and Stephen O. Brennan, Molecular Pathology Laboratory, Christchurch School of Medicine, Christchurch, New Zealand.

Albumin is the major secreted protein of the liver and prior to secretion the six residue propeptide Arg-Gly-Val-Phe-Arg-Arg is cleaved in the trans Golgi network or secretory vesicles. In 1988 we identified a proalbumin converting activity in hepatic Golgi membranes. This membrane bound convertase was Ca^{++} dependent, optimally active at pH5.5 and specifically inhibited by α_1-antitrypsin Pittsburgh. In addition this activity was very similar to the yeast Kex2 protease which is the prototype of the eukaryotic family of subtilisin-like serine proteases. Since 1990 at least seven mammalian cDNA homologues of Kex2 have been identified, primarily using PCR methodologies. The tissue distribution of mRNA for these putative convertases shows that furin and PACE4 are likely candidates for the hepatic proalbumin convertase. In order to investigate the relationship between furin and the hepatic convertase we have coexpressed normal human proalbumin and five variants in *kex2* deficient yeast and used the unprocessed proalbumins to compare the substrate specificities of these two endoproteases. Furin and the hepatic convertase broadly mirror each other's specificity with the Arg-Gly-Arg-Arg sequence being the optimal sequence, cleaved 40 fold faster than normal. The monobasic Val-Phe-His-Arg sequence is not a substrate and Arg-Phe-His-Arg was cleaved 8 fold slower than normal, due to the P2 histidine. Both enzymes cleaved the simple dibasic sequences Arg-Arg and Lys-Arg sequences equally well. The variant with a P1′ arginine was not processed implying structural constraints limit processing to the P1-P1′ bond, and these enzymes will not process sites with a P1′ basic residue.

B7-105 ACTIVATION OF PRO-REGION CHIMERAS OF FURIN ENDOPROTEASE, Richard Leduc, Normand Rivest and Nabil Seidah*, Department of Pharmacology, Faculty of Medecine, Université de Sherbrooke, Sherbrooke, Quebec J1H 5N4 and the *J.A. DeSève Laboratory, Clinical Research Institute of Montreal, Montreal, Quebec H2W 1R7 CANADA

Human furin endoprotease is a calcium-dependent serine endoprotease that catalyzes the proteolytic maturation of many precursor proteins at the consensus cleavage site Arg-X-X-Arg. The enzyme is initially synthesized as an inactive zymogen that undergoes intramolecular autocatalytic activation. It is though that the pro-region of the protein may play a critical role in guiding proper folding of the enzyme thereby permitting a correct conformation that would lead to the activation step. In order to investigate the role of the pro-region in furin activation we initially examined the pro-regions of rat and mouse furin and found 88% and 92% percent homologies at the amino acid level. To evaluate if the non-conserved residues from the pro-region of these species could directly contribute in the activation process of the human enzyme, we created furin chimeras using the prepro-region of mouse and rat furin fused to the human furin catalytic domain. We used the pCB6(+) plasmid (a generous gift from Dr. Yves Rouillé, University of Chicago) which has a CMV promotor driving the various constructs. We expressed the plasmids in COS-7 cells and evaluated expression using Western blots (recognizing the human catalytic domain) and fluorogenic assay (using the boc-Arg-Val-Arg-Arg-AMC) of secreted enzyme. The results show that neither constructs significantly affected the capacity of the zymogen to be converted into the active enzyme. This would mean that the differences in the pro-region occuring between species are not sufficient to selectively impede the intramolecular activation process. We have also constructed chimeras having a greater divergence in homology in the pro-region and we have created site-directed mutants replacing residues that we believe may play a crucial role in the activation of pro-furin. The data resulting from these constructs will be presented.

B7-106 DIFFERENTIAL BINDING OF NUCLEAR PROTEINS FROM NEUROENDOCRINE CELL LINES TO THE UPSTREAM REGULATORY REGIONS OF THE GENES ENCODING THE PROHORMONE CONVERTASES PC2 AND PC3 (PC1), Kaare Lund and Donald F. Steiner, Howard Hughes Medical Institute, The University of Chicago, Chicago, IL 60637

The two subtilisin-related prohormone processing enzymes PC2 and PC3 (PC1) are expressed in a tissue-specific manner, predominantly in tissues and cell lines of neuroendocrine origin, where they are believed to be involved in the processing of e.g. proinsulin and proopiomelanocortin. The organisation of the genes encoding the two proteases has recently been described and the promoter/enhancer regions been identified. Common to the regulatory regions of the PC2 and PC3 (PC1) genes is the lack of functional TATA and CCAAT boxes, but in both genes the presence of putative binding sites for other transcriptional regulatory factors has been predicted. In order to define the functional promoter/enhancer regions of the PC2 and PC3 (PC1) genes in more detail, we have mapped the actual sites of interaction of nuclear proteins from various cell lines with DNA fragments enclosing the proposed promoter/enhancer regions of the two genes by DNase I footprinting and electrophoretic mobility shift assays (EMSAs). In the case of the PC2 gene the cluster of predicted Sp1 binding sites at position 383 to 437 as well as the single Sp1 site between 518 and 524 bp upstream from the translation initiation site are protected from DNase I degradation by proteins present in nuclear extracts from α-TC1, β-TC3, AtT20 and HepG2 cells. One region of the PC2 gene enhancer, between 340 and 315 bp upstream from the start codon, that has not previously been recognised as a protein binding site, is strongly protected in the footprinting assay by a factor present only in nuclear extract from β-TC3 cells among the four cell lines tested. These results were corroborated by EMSA using oligonucleotides enclosing sequences corresponding to the protected regions. Preliminary experiments show that the PC3 (PC1) gene enhancer is protected in two regions by factors present in nuclear extracts from AtT20 cells, between 430 and 450 bp upstream from the initiator methionine, a region that contains no predicted protein binding site, and between 265 and 285 bp upstream from the start codon, a region containing a predicted cyclic AMP responsive element (CRE).

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B7-107 SITE-DIRECTED MUTAGENESIS OF THE MOUSE PRO-HORMONE CONVERTASE PC2: THE OXYANION HOLE AND THE RGD MOTIF, Juliette E. Lusson*, Suzanne Benjannet[+], Diane Savaria*, Michel Chrétien[+] and Nabil G. Seidah*, Laboratories of Biochemical* & Molecular[+] Neuroendocrinology, Clinical Research Institute of Montréal, Mtl, Qc, Canada H2W 1R7.

Pro-hormone convertases are serine proteases of the Subtilisin family which possess a conserved Asn residue in the oxyanion hole that stabilizes the tetrahedral transition state during hydrolysis of the peptide bond. They are synthesized as zymogens which must undergo processing of their N-terminal pro-segment at an ArgX(Lys/Arg)Arg↓ bond to produce the active form of the enzyme. While proPC1 is rapidly transformed into PC1 within the endoplasmic reticulum, proPC2 is slowly transported to the *trans* Golgi network (TGN) where it is processed into PC2. This spatial and temporal difference in the maturation of PC1 versus PC2, is likely responsible for the observed order of cleavage reactions of precursors whereupon PC1 acts first followed by PC2. Interestingly, PC2 is the only convertase that exhibits an Asp_{285} in place of the usual Asn found in all other subtilases. In order to substitute the Asp_{285} residue by Asn, we first subcloned mouse PC2 into the phage M13 and using a mutant primer and site-directed mutagenesis we isolated a vaccinia virus recombinant of the mutant $D_{285}N$. A comparative analysis of the biosynthesis, intracellular transport and processing of POMC by the wild type and mutant mPC2 was undertaken in both constitutive and regulated cells, the results of which will be presented. This mutant was also analyzed for its ability to bind intracellularly 7B2 which is a specific PC2 binding protein. All proprotein convertases contain a conserved R**RGD**$_{495}$L sequence in their P-domain. This sequence which could intracellularly bind integrins was tested for its importance in the cellular trafficking of PC2. Accordingly, we mutated it to RRGEL and compared the mutant and wild type for the rate of transport and activation of proPC2 in the TGN, and the ability of this enzyme to selectively process POMC to produce β-Endorphin and α-MSH as final products.

B7-108 *IN VITRO* FUNCTIONAL CHARACTERIZATION OF SOLUBLE PACE4 AND FURIN DERIVED FROM EXPRESSION SYSTEMS,

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Among the six known members of the convertase family of proteases, PACE4 and furin are two of the most widely distributed. Using cell culture systems, we have recently expressed these and other convertases *in vitro* in order to examine their structure and kinetic behavior. A vaccinia virus recombinant of human PACE4 was expressed in a monkey kidney epithelial cell line (BSC40). Incubation of the cell culture medium with a radiolabeled covalent inhibitor ([^{125}I]-Ac-Tyr-Glu-Lys-Glu-Arg-Ser-Lys-Arg-CH_2Cl) followed by SDS-PAGE revealed two proteins that were labeled in a Ca^{2+}-dependent manner. The first major species had an apparent molecular weight (>200 kDa) that is much larger than the size expected for even the full-length protein. The second species had an approximate molecular weight of 65 kDa, most likely representing the core N-terminus of the protein following a cleavage near the RGD domain. Soluble forms of human furin have also been expressed and studied with BSC40 cells. Two truncated forms of the enzyme in which the membrane spanning region was deleted and differing by the presence or absence of the cysteine-rich region are presently being used to investigate the functional role of the latter domain. Enzymatic activity assays using a variety of fluorogenic peptide substrates have been carried out for PACE 4 and furin to assess their substrate specificities as well as their susceptibilities to various synthetic inhibitors. In addition, a high-yield expression system employing baculovirus-infected Sf-9 insect cells has proven successful for the expression of enzymatically active soluble furin. This methodology is currently being optimized to enable the production and purification of large amounts of this protein for use in future structure-function studies.

B7-110 **STRUCTURAL ELEMENTS WHICH DIRECT SPECIFIC PROCESSING OF DIFFERENT MAMMALIAN SUBTILISIN-LIKE PROHORMONE CONVERTASES**

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When several members of the mammalian prohormone convertases (PC) family are expressed in AtT-20 cells (mouse corticotrope tumor cells), they undergo proregion cleavage at various rates (the proregion cleavage of PC1 in the ER; PC2 proregion, mostly in granules; PC1, also C-terminal cleavage). In parallel with PC1 and PC2 processing, POMC (proopiomelanocortin) is sequentially cleaved by PC1 and then PC2 into several smaller products. To investigate the structural elements directing the processing of different PCs, we constructed a series of mutant PC proteins and stably transfected AtT-20 cells. A C-terminally truncated PC1 protein (PC1 stop^{D616}) underwent proregion cleavage as efficiently as the endogenous PC1, whereas this processing step was completely blocked in the active site mutant PC1 S382A, indicating that the proregion cleavage of PC1 is intramolecular. When the proregions of PC1 and PC2 were substituted with one another, both fusion proteins failed to cleave the "foreign" prosequences. By contrast, a PACE4 protein with its proregion substituted with that of PC1 was able to perform proregion processing. The mutants which failed to cleave the proregions were also unable to undergo oligosaccharide maturation and remained in the ER. No changes in POMC processing were observed in AtT-20 cells expressing inactive PCs, indicating no block in endogenous PC1 function. We conclude that proregion cleavage of these PC proteins is a molecule-specific process, essential for routing and for endoproteolytic activity. Interestingly, C-terminally truncated PC1 exhibited accelerated basal secretion and strikingly increased processing of POMC, indicating a unique role of the carboxyl terminal of PC1.

B7-109 EXPRESSION OF PC2 AND PC3 IN COS7 CELLS; THE ROLE OF RESIDUE 310.

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The vector pSR was used to transiently express PC2 and PC3 in Cos-7 cells. The cells were pulse-labelled with ^{35}S-methionine for 30 minutes and chased for periods of time before the media and cell extracts were immunoprecipitated using a rabbit anti-PC2 or a sheep anti-PC3 antibody. Expression of PC2 in Cos 7 cells resulted in the slow release of inefficiently processed protein. PC2 appeared in the medium after a 2 hour chase period. Inside the cell the predominant form was the unprocessed 75 kDa precursor. In contrast expression of PC3 in Cos 7 cells led to the rapid secretion of a single 80 kDa protein, which first appeared in the medium after less than one hour. A possible explanation for this difference may lie in residues surrounding the active site. In the subtilisin family of proteases the amino acid corresponding to residue 310 plays a role in determining the pH optimum of the enzyme. In PC3 and other members of the prohormone convertase family residue 310 is an asparagine. PC2 has an Asn to Asp substitution in this position, which has been predicted to decrease the pH at which the enzyme will be active. We used site directed mutagenesis to generate Asn^{310}-PC2 and expressed it in Cos7 cells. The mutant PC2 was released into the medium more rapidly than wild type PC2, appearing after one hour. The material detected in the medium was also much more efficiently processed, with only one form present. The presence of an Asp instead of an Asn residue in the oxyanion hole of PC2 appears to delay maturation and secretion of PC2 relative to PC3 and suggests that one role for the Asn/Asp substitution might be to help maintain PC2 in an inactive state until late in the secretory apparatus.

Biochemical and Enzymatic Properties of the Mammalian Convertases; Evolutionary Models of Mammalian Convertases

B7-111 POST-TRANSLATIONAL PROCESSING OF PARATHYROID HORMONE-RELATED PROTEIN

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Parathyroid hormone-related protein (PTHrP) can stimulate placental calcium transport and is involved in epithelial cell growth and/or differentiation. Evidence suggests that, like other peptide hormones, prepro-PTHrP is cleaved post-translationally, into its constituent biologically-active fragments. It is likely that mammalian prohormone convertases, such as furin, are involved in this processing since potential cleavage sites for furin exist within the prepro-region and at the C-terminus of PTHrP. The aim of this study was to investigate the potential involvement of mammalian convertases, and in particular furin, in the processing of PTHrP.

Specific primer pairs made to the catalytic region of members of the convertase family, were used in RT-PCR analysis to identify convertase specific mRNA in three PTHrP producing human cell lines; BEN, COLO 16 and HaCaT cells. Furin and PACE4 mRNA were detected in all three cell lines, while PC1 and PC2 mRNA distribution was more restricted. All three cell lines secreted a C-terminally truncated PTHrP species representing PTHrP(1–108) in size and immunoreactivity. Little or no processing was observed when HEK 293 cells were transiently transfected with a cDNA expression construct for full-length PTHrP(-36–141). However, co-transfection of a furin expression construct resulted in a substantial increase in the observed bioactivity as measured by PTH bioassay suggesting that processing of the prepro-region had occurred. Some processing of the C-terminal region was also evident as shown by Western blot analysis and by an increase in C-terminal immunoreactivity.

This data suggests that furin, or an enzyme with similar substrate specificity, is involved in the N-terminal processing of prepro-PTHrP to its mature, bioactive form and may also be involved in C-terminal cleavage of PTHrP. The presence of appropriately located cleavage sites, characteristic of those cleaved by furin, further support this hypothesis. The role of a novel furin-related convertase, which we have recently identified, is currently being examined for its involvement in the processing of PTHrP.

B7-112 PROCESSING OF AMYLOID PRECURSOR PROTEIN BY CATHEPSIN S.

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Cysteine proteases play a vital role in normal cellular protein metabolism; they have been implicated in a number of disease states including cancer, arthritis, and Alzheimer's disease. In an effort to identify novel cysteine proteases, we used polymerase chain reaction and primers directed against the catalytic sites of previously cloned cysteine proteases. From rat brain mRNA, a 600 basepair band was amplified; cloning and partial sequence analysis of this band resulted in the identification of cathepsins B and L, and five novel sequences. The insert with highest homology was used to screen a rat brain cDNA library; a 1334 basepair cDNA was isolated and the nucleotide sequence determined. This sequence encodes an open reading frame of 330 amino acids which is highly homologous to human cathepsin S suggesting that this sequence represents rat cathepsin S. Cathepsin S is a cysteine lysosomal protease resistant to neutral pH. Our studies with *in situ* hybridization histochemistry show that in rat brain, the cathepsin S transcript is preferrentialy expressed in microglia.

Microglia is a cell type that is considered relevant for the processing of the amyloid precursor protein (APP) and in establishing the Alzheimer's disease pathology. This makes cathepsin S a strong candidate for a protease involved in the generating of amyloid beta peptide, and/or potentially amyloidogenic fragments from the amyloid precursor protein. In order to address this possibility, we examined the ability of purified cathepsin S to process APP; the products of cleavage were characterized using domain specific antisera. In the system used, cathepsin S is capable of producing amyloidogenic fragments of 23-28 kDa from the APP. Its potential role in the metabolism of the APP *in vivo* and significance for the Alzheimer's pathology remain to be elucidated.

B7-113 FURIN-MEDIATED CLEAVAGE OF PSEUDOMONAS EXOTOXIN, DIPHTHERIA TOXIN AND HYBRID TOXINS.

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Pseudomonas exotoxin (PE) is cleaved within mammalian cells between Arg279 and Gly280 to generate an enzymatically active C-terminal fragment of 37 kD which translocates to the cytosol and ADPribosylates EF-2. Most likely, furin or a furin-like enzyme is responsible for this cleavage. In biochemical experiments, furin cleaved PE with an apparent Km of 7μM; cleavage was on the C-terminal side of the sequence of RQPR (amino acids 276-279) producing the same fragments as those generated within cells. Cleavage had a pH optimum of 5.0-5.5 and was inhibited by EDTA. Mutational analysis of the amino acids located near the site of cleavage, confirmed the importance of arginines at P1 and P4. Unicked diphtheria toxin (DT) was also cleaved by furin; cleavage was on the C-terminal side of the sequence RVRR (aa-190-193) and generated the toxin's A chain. Cleavage was seen at pH values ranging from 5.5 to 8.5 and had an optimum at pH 8.0. Furin cleaved DT with a 100-fold faster turnover rate than PE. To investigate possible reasons for this, the amino acid composition near the cleavage site of PE was altered to resemble more closely the arginine-rich sequence from DT. Four PE-DT mutants were generated, whereby 1,5,6 or 8 amino acids at the PE cleavage site were changed to amino acids found at the DT cleavage site. The rate of furin-mediated cleavage improved dramatically. However, this increase did not change the pH optimum for furin-mediated cleavage of PE toxins, which remained at 5.0-5.5. When radioactive versions of selected PE-DT proteins were added to intact cells, enhanced cleavage relative to that of native PE was also seen. Since all the PE-DT proteins were less toxic than native PE, we conclude that changes that favor increased proteolysis also interfere with other important toxin functions.

B7-114 IDENTIFICATION OF A NOVEL PROPROTEIN PROCESSING ASPARTYL PROTEASE, MKC7, INVOLVED IN POLARIZED GROWTH IN YEAST,

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Kex2 protease cleaves pro-α-factor and other secretory proproteins at pairs of basic residues in a late Golgi compartment in yeast. A *kex2* null mutation results in reversible cold-sensitivity -- failure to grow below 20°C -- characterized by a defect in maintenance of polarized growth. After a shift from 30°C to 16°C, *kex2* null cells arrest after about three doublings with one or two small buds. The mother cell continues to increase in size, exhibiting delocalized chitin deposition and intense staining of actin cortical dots. Three multicopy suppressors of *kex2* cold sensitivity were isolated. One of these, *MKC7*, encodes an aspartyl protease homologue most closely related to the product of the *YAP3* gene, previously isolated as a multi-copy suppressor of the pro-α-factor cleavage defect of a *kex2* null (M. Egel-Mitani, et al. (1990)*Yeast* **6**, 127-137). Multi-copy *MKC7* and *YAP3* suppressed the α-specific mating defect of a *kex2* null to the same degree but multi-copy *MKC7* was a superior suppressor of cold-sensitivity. Whereas disruption of *MKC7* or *YAP3* alone resulted in no observable phenotype, disruption either of both genes or of one in combination with a *kex2* null resulted in slow growth phenotype at 37°C. Disruption of both *MKC7* and *YAP3* along with *KEX2* resulted in stronger temperature-sensitivity and exacerbated the cold-sensitivity due to the *kex2* null mutation. The synergism of null mutations in *MKC7* and *YAP3* with a null mutation in *KEX2*, which encodes a well-characterized processing enzyme, argues strongly that Mkc7p and Yap3p represent a new class of aspartyl proteases involved in proprotein processing. We conclude that Kex2, Mkc7 and Yap3 proteases together act on substrate polypeptides whose processed products (i) are required for normal growth at both low and high temperatures and (ii) function either in a late secretory compartment or at the cell surface.

B7-115 INTERACTION OF SUBTILISINS WITH SERPINS,
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Our laboratory is interested in the inhibitory mechanism of serpins. Generally, serine proteinases of the chymotrypsin family are used to probe serpin interactions, and the interactions with subtilisins have been rarely studied. The subtilisin family members have a peptide chain fold that is unrelated to that of the chymotrypsin family. However, the two families have closely related catalytic mechanisms and substrate binding orientations. We set out to determine whether serpins inhibit subtilisins, and whether the mechanism is the same as the inhibition of chymotrypsins.

Here we report on the interaction of subtilisin *Carlsberg*, subtilisin BPN' and proteinase K with the serpins $\alpha_1$proteinase inhibitor (α_1PI) and $\alpha_1$antichymotrypsin (α_1ACT). α_1PI inhibits subtilisins with temperature dependent partition ratios, and forms SDS-stable complexes. α_1ACT inhibits proteinase K with a higher partition ratio than α_1PI toward the enzyme, but does not significantly inhibit other two subtilisins. We provide kinetic data of the interaction between subtilisins and serpins. Our data on the interaction are models of interactions for some mammalian proteinases of the subtilisin family, and would be applicable to explain inhibition of α_1PI mutants toward furin.

B7-116 THE ROLES OF THE PRO-SEQUENCE AND *N*-GLYCOSYLATION IN EUKARYOTIC SUBTILISIN PROTEASE ACTIVITY,
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Subtilisin proteases characteristically contain an N-terminal pro-sequence which is autocatalytically cleaved upon folding to an active conformation. The presence of this sequence has previously been shown to be a strict requirement for appropriate folding of prokaryotic subtilisins *in vivo* and for *in vitro* refolding. I have investigated the pro-sequence requirement of a eukaryotic subtilisin, krp from S.pombe, by expression in a cell-free translation system and fluorogenic peptide cleavage assays. The protein produced *in vitro* has equivalent activity to the *in vivo* product, but can be quantitated more directly and the translation conditions can be manipulated using reagents such as the tripeptide N-Y-T, which competitively inhibits N-glycosylation.

Unexpectedly, significant (up to 10% of wild type) activity was obtained from the translation product of a krp cDNA lacking the entire prosequence, although this was increased when the prosequence was supplied in *trans* by co-translation. The resulting K_m was similar in both cases to that of the wild type enzyme, indicating that the activity seen was due to a proportion of the enzyme which has become correctly folded, rather than all molecules adopting a partially active conformation.

Previous experiments have shown that subtilisin proteases can fold to an active conformation, as demonstrated by autocatalytic prosequence cleavage, in the absence of N-glycosylation. The activity of the wild-type enzyme was therefore measured after translation in the presence of excess N-Y-T tripeptide to inhibit N-glycosylation. Although K_{cat} was unaffected, the K_m of the non-glycosylated enzyme was increased approximately three-fold, indicating that one or more of the N-glycosylation sites must be co-translationally modified for a native conformation to be attained.

B7-117 KEX2 CLEAVES PARATHYROID HORMONE-RELATED PROTEIN (1-141) PREFERENTIALLY AT 19RRR21.
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Parathyroid hormone-related protein (PTHrP) contains several sites characteristic of targets for the action of convertases, but little is known of processing events. In order to identify which cleavage sites might be of physiological significance, studies were carried out *in vitro* using purified recombinant Kex2 and pure recombinant PTHrP(1-141) and PTHrP(1-141) with mutations at potential cleavage sites. Products were identified by resolution on HPLC, N-terminal sequencing and Western blotting analysis.

The preferred cleavage site in PTHrP(1-141) was carboxyl to 19RRR21 with a Km of 3.3$\pm$1.7 μM and K_{cat} of 6$\pm$1.2 sec^{-1}. The Kcat/Km was within 6 fold of the specificity constant for the best synthetic substrate described for Kex2. Substitutions of A for R at each position indicated that all three arginines were essential. Furthermore, cleavage of PTHrP(1-34) at that site was reduced to 50% of that of PTHrP(1-141). Four smaller products (<10% of 19RRR21 product) were resolved by HPLC and identified to represent cleavages carboxyl to residues KR97, KR105, RR106 and TR108. The preferred C-terminal target site was KR105 with a Km 8.4$\pm$2.74 μM and K_{cat} 0.8$\pm$0.2 sec^{-1} which suggested that this could also be a target for a more specific convertase *in vivo*. Very little change in the degree of conversion at the C-terminal sites was seen using PTHrP(1-141)20A to eliminate 19RRR21 cleavage. However the relative degree of each of the cleavages was altered when the substrate was PTHrP with substitutions at R-A106, R-A108 or both R-A106&R-A108.

These data indicate that 19RRR21 and to a lesser degree KR105 are the preferred sites for KEX2 cleavage and that the tertiary structure contributes to enzyme action. These sites are likely to be targets for more specific convertases *in vivo* which could act in a tissue-specific manner to elaborate multiple paracrine actions of PTHrP.

B7-118 CHARACTERIZATION OF cDNAs ENCODING APLYSIA CALIFORNICA FURIN AND PC2,
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The neuroendocrine bag cells and exocrine atrial gland of *Aplysia californica* express genes belonging to the egg-laying hormone (ELH) family and process the resulting precursor at mono-, di-, tri-, and tetrabasic sequences. Some of these cleavages may result from proteolysis by a furin-like or PC2-like enzyme; these enzymes have been identified in organisms as diverse as yeast and humans. The atrial gland is particularly interesting because the organ produces large (milligram) amounts of ELH-related peptides and may be a relatively rich source of prohormone processing enzymes. An atrial gland cDNA library was constructed and screened using a furin-related PCR probe and a clone encoding a furin-like protein was isolated. The insert is identical to a furin-related PCR product from the bag cells. The library was also screened using a *Lymnaea* PC2 probe (provided by A.B. Smit and W.P.M. Geraerts, Holland) and a clone encoding a PC2-like protein was isolated. Both clones have relatively long 3′ untranslated regions (UTR's). The 3′-UTR's contain multiple polyadenylation sites which could be used to generate multiple mRNAs. Furthermore, the 3′-UTR's also contain relatively long microsatellite repeat sequences $(CA)_n$ and $(TG)_n$. The eventual goal of these studies is to elucidate the potential role of these enzymes in prohormone processing in *Aplysia*. Supported by NIH Grant NS 29261.

B7-119 CHARACTERIZATION OF A SUBTILISIN-LIKE PROPROTEIN CONVERTASE FROM DIROFILARIA IMMITIS: A CANDIDATE PROTEASE FOR PROCESSING THE "LADDER" PROTEIN OF FILARIAL NEMATODES, Catherine Poole, Dave Landry and Larry McReynolds, New England Biolabs, Beverly, MA 01915

Filarial parasites are a major cause of disease in animal and human populations worldwide. They cause a range of debilitating symptoms in humans including blindness and elephantiasis. Animal infections are equally grievous: dogs infected with D.immitis (dog heartworm) suffer severe damage to the heart and other vital organs. As a potential target for chemotheraputic intervention in filariasis, we are cloning and characterizing subtilisin-like proprotein convertases (SPC) from D.immitis which might be responsible for processing the filarial "ladder" protein [1]. Di5, the D.immitis ladder protein gene, is composed of 25-50 direct tandem repeats of a 399 bp monomer. Metabolic labeling studies of adult parasites showed that Di5 is synthesized as a large precursor that is subsequently cleaved creating a protein "ladder" from 14 to >200 kDa with steps about 15 kDa apart. N-terminal sequencing of the Di5 protein monomer revealed the tetrabasic site, RRKR, a common processing motif of SPCs. Using degenerate oligonucleotide probes based on the catalytic domains of Furin, Kexin and Blisterin, a fragment was amplified from adult D.immitis mRNA and used to screen a D.immitis cDNA library. The predicted amino acid sequence of the largest clone (699 bp) identified by screening exhibited 72%, 51% and 60% identity with the catalytic domains of Furin, Kexin and Blisterin respectively. This 699 bp insert was fused in frame to maltose-binding protein and expressed for antiserum production. Anti-D.immitis SPC serum reacts with 4 bands of approximately 25, 37, 60 and 120 kDa on Western blots of adult D.immitis protein extracts. The 60 kDa protein was purified using the specific antibody in conjunction with Western blots to moniter the protein through multiple purification steps. Preliminary data indicate that this highly enriched 60kDa preparation is proteolytically active. Presently, we are determining the pH optimum, substrate requirements and inhibitor sensitivities of this protease activity.

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

B7-120 IN VITRO PROCESSING OF MOUSE PROGLUCAGON BY RECOMBINANT PC1 AND PC2, *Mark E.Rothenberg, *Carmen D. Eilertson, †Yi Zhou, †Iris Lindberg, *John K. McDonald, *Bryan D. Noe, *Department of Anatomy and Cell Biology, Emory University, Atlanta, GA 30322, †Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, LA 70112

We are examining the role of PCs in processing proglucagon (proGlu) in the mouse tumor cell line, αTC1-6. To date, neither the nucleic acid nor the protein sequences for mouse proGlu have been published. Utilizing RT-PCR, a 668 bp product was amplified from αTC1-6 mRNA. Sequencing of the mouse preproGlu cDNA revealed nucleic acid homologies between mouse and either rat or human of 89.1% and 84% respectively, and at the predicted amino acid level homologies of 94% and 89.4% respectively. In the αTC1-6 cell line proGlu is proteolytically processed to release the following products: major proglucagon fragment (MPGF), glicentin (gli), oxyntomodulin (oxy), glucagon (glu), and glucagon-like peptide-I (GLP-I). Mouse glucagon-related peptides were metabolically labeled with ^{3}H-tryptophan and separated by reverse phase HPLC. The identity of proGlu and peptides having retention times identical to known proGlu cleavage products was verified by RIA and peptide mapping. Results from pulse-chase experiments revealed that the release of MPGF from proGlu is the first processing event mediated by the PCs, followed by gli and oxy; glucagon appears concomitantly with gli and oxy, followed by GLP-I. Using glu- and PC2-directed antisera, αTC1-6 cells were stained to determine immunolocalization within secretory granules. Cells stained with either glu or PC2 antisera displayed numerous punctate granules. Co-labeled slides revealed glu and PC2-like immunoreactivity to be present within the same granules. To determine the potential role of PC1 or PC2 in proGlu processing, we incubated HPLC purified ^{3}H-tryptophan labeled proGlu with purified recombinant PC1 and/or PC2 *in vitro* and separated the cleavage products by HPLC. Under these conditions, either PC1 or PC2 proteolytically cleaved proGlu to yield MPGF, gli, and oxy, while PC1 alone was capable of releasing GLP-1 from MPGF. Neither PC1 nor PC2 processed glu from proGlu *in vitro*. These results suggest a potential role for PC1 and/or PC2 in cleaving MPGF, gli, oxy, and GLP-I from proGlu, but that another PC(s), such as PC5/6 (known to be expressed in αTC1-6 cells), may be involved in releasing glu from its precursor.

B7-121 PROGLUCAGON IS PROCESSED TO GLUCAGON-LIKE PEPTIDE 1 IN AtT-20 CELLS, Yves Rouillé and Donald F. Steiner, Howard Hughes Medical Institute and the Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637

Proglucagon is processed differentially in the pancreatic α cells and in the intestinal L cells to yield either glucagon or glucagon-like peptide 1 (GLP-1), respectively, structurally related hormones with opposing metabolic actions. We have previously identified the prohormone convertase PC2 as the key processing endopeptidase responsible for processing of proglucagon to glucagon in cells with the α-cell phenotype. In this study proglucagon was expressed in AtT-20, a mouse anterior pituitary tumor cell line expressing negligible levels of PC2, but large amounts of the related endoprotease PC3. We have found, that these cells process proglucagon into GLP-1 (7-36amide), glicentin and oxyntomodulin, but only small amounts of glucagon, a pattern reminiscent of the L-cell phenotype. This result suggests, that PC3 might be involved in the processing of proglucagon in the cells with the L-cell phenotype. In addition to dibasic processing sites, the release of GLP-1 (7-36amide) from the precursor involves a cleavage step at a single arginine. We have shown that PC3 is unable to perform this reaction *in vitro*. This result is consistent with the known dibasic specificity of this enzyme and implies that a second endopeptidase is present in the regulated secretory pathway of AtT-20 cells. Taken together, these observations suggest that AtT-20 cells require both the prohormone convertase PC3 and an as yet unidentified protease to process proglucagon into a set of peptides the pattern of which is very similar to the one observed in intestinal L cells.

B7-122 MUTANT AND FUNCTIONAL ANALYSIS OF THE THE ESSENTIAL KEX2-LIKE GENE *BLI-4* IN THE NEMATODE *CAENORHABDITIS ELEGANS*, Colin Thacker, Martin Srayko, and Ann M. Rose, Department of Medical Genetics, University of British Columbia, Vancouver, BC V6T 1Z3, Canada.

The *bli-4* gene of the nematode *Caenorhabditis elegans* encodes four structurally distinct proteins with sequence similarity to members of the kex2/subtilisin-like family of proprotein convertases. Each gene product is composed of twelve common exons, which include the protease domain, alternatively spliced to one or more specific 3' exon(s). Identification of mutations in 3 independent *bli-4* lethal mutants which arrest in late embryogenesis indicated that these molecular lesions resided within the first twelve common exons. We have rescued the mutant phenotypes of lethal alleles *q508* and *s90*, and the viable blistered mutant *e937* by DNA transformation. Rescue has been achieved using a series of 'mini-genes' that encode only one or combinations of the four gene products. The *e937* phenotype is the result of deletion of one gene product designated blisterase A. This suggests that this isoform is required only for production or maintenance of the adult cuticle. Surprisingly, all tested mutants are rescued by a mini-gene which encodes only blisterase A. In contrast, a construct encoding a second isoform, called blisterase B, did not rescue the lethal mutants. However, transformation of *e937* animals with the blisterase B mini-gene decreased blistering. Both of the *bli-4* isoforms are similar in structure and differ only by the addition of alternative carboxy-terminal ends. Taken together, these results imply that the first twelve exons are not sufficient for rescue of the lethal phenotype, and that the unique carboxy-termini provide essential information for functional activity. Furthermore, the rescue experiments indicate that a functional redundancy exists between the various *bli-4* gene products. This partial compensatory activity between blisterase A and B may explain the incomplete penetrance associated with the *e937* mutation.

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B7-200 PURIFICATION AND CHARACTERISTICS OF CANDIDATE PROHORMONE CONVERTASES, PC1/3, PC2, and 70 kDa ASPARTIC PROTEINASE FROM CHROMAFFIN GRANULES,

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Three candidate prohormone convertases were purified to apparent homogeneity from bovine adrenal medulla chromaffin granules (CG) by chromatography on con A-Sepharose, Sephacryl S200, pepstatin A agarose, and immunoaffinity columns. Two of the enzymes, PC1/3 and PC2, belong to the family of subtilisin-related proprotein convertases, while the third one is an aspartic proteinase. The activity of the PCs were assayed with ^{35}S-preproenkephalin (^{35}S-PPE) and Z-Arg-Val-Arg-Arg-MCA (pH optimum 6.5), while that of the aspartic proteinase with ^{35}S-PPE (pH optimum of 5.0-5.5). All three proteinases are glycoproteins with molecular weight of ~70 kDa. Endogenous PC1/3 and PC2 showed similar substrate specificity and proteinase inhibitor profiles. Z-Arg-Val-Arg-Arg-MCA was the best substrate among fluogenic tri- and tetrapeptides tested, with K_m values of 40 and 66 mM, and V_{max} values of 352 and 255 nmol/h//mg, for PC2 and PC1/3, respectively. Specificity of PC1/3 and PC2 for dibasic sites was confirmed by potent inhibition with active site-directed inhibitors (D-Tyr)-Glu-Phe-Lys-Arg-CH_2Cl and Ac-Arg-Arg-CH_2Cl. CG PC1/3 and PC2 were activated by DTT and inhibited by thiol-blocking reagents. PC1/3 and PC2 are calcium-dependent proteinases with their activity being blocked by EGTA and stimulated with addition of calcium. PC activities were reduced by several serpin inhibitors -- α_1-antitrypsin, α_1-antichymotrypsin (ACT), and ACT-like proteins from CG. The 70 kDa aspartic proteinase from CG is sensitive to pepstatin A, a group specific inhibitor of aspartic proteinases, but not to inhibitors of serine, cysteine, and metallo-proteinases, and is activated by DTT. The aspartyl proteinase cleaved recombinant proenkephalin between Lys_{173}-Arg_{174}, located at the COOH-terminus of (Met)enkephalin-Arg-Gly-Leu. The similarity of this CG aspartic proteinase to other paired basic residue specific aspartic proteinases -- pituitary POMC-converting enzyme and yeast aspartic proteinase 3 -- indicates CG 70 kDa aspartic proteinase as a member of the family of the candidate prohormone processing aspartic proteinases.

B7-201 MOLECULAR STRUCTURE AND BIOSYNTHESIS OF ENDOPEPTIDASE-24.18: PROTEOLYTIC PROCESSING OF RAT ALPHA SUBUNIT BY A FURIN-LIKE ENZYME,

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Endopeptidase-24.18 (EC 3.4.24.18; meprin) is a multisubunit metallopeptidase of the astacin family. It is found in brush-border membranes of rodent kidney and human intestine. The membrane-bound enzyme is composed of α/ß dimers. Molecular cloning has shown that both subunits have a similar structural domain organization. Soluble α_2 dimers have also been observed *in vivo* and in transfected cells. The structure of all known α subunits contains, upstream from the transmembrane domain, the sequence RXKR which corresponds to the RXK/RR consensus sequence for specific cleavage by furin. In order to investigate the involvement of this putative cleavage site in the secretion process of endopeptidase-24.18 α subunit, we expressed in COS-1 cells rat α subunits in which residues R_{655} or S_{656} (within the sequence $_{652}$RPKRS$_{656}$) were mutated to valine or leucine, respectively. In contrast to the wild-type protein, the αR655V and αS656L mutants were not secreted in the culture media. Moreover, when cells expressing the α subunit were infected with a furin-encoding vaccinia virus, immunoblotting showed an increase in the amounts of secreted α subunit and a shift of the major cell-associated form of endopeptidase-24.18 α subunit from 98 kDa to 88 kDa. This decrease of 10 kDa was consistent with cleavage at the furin site and was not observed with the mutant α subunits. As observed for the 98 kDa species, the 88 kDa cell-associated protein was sensitive to endoglycosidase H treatment suggesting that the proteolytic cleavage occured in the endoplasmic reticulum or in an early Golgi compartment. Similar experiments using PACE4 and PC5 instead of furin showed that these enzymes were not able to generate the 88 kDa species. We conclude that furin is most probably the cellular enzyme involved in the proteolysis resulting in secretion of rat endopeptidase-24.18 α subunit, confirming the role of the furin site in the secretion process.

B7-202 BOVINE BRAIN PYROGLUTAMYL AMINOPEPTIDASE TYPE-1: A SOLUBLE NEUROPEPTIDE-PROCESSING ENZYME,

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Pyroglutamyl aminopeptidase or PAP is capable of removing the N-terminal pyroglutamic acid residue from a number of physiologically significant neuropeptides, thereby resulting in the inactivation or potentiation of their neurological functions. This enzyme is widely distributed throughout mammalian tissues, including brain, and is believed to play an important role in the regulation of specific neuropeptide-mediated processes. To date, two distinct forms of this enzyme have been identified. A soluble or cytosolic form, type-1 (or PAP-I), and a particulate or membrane-bound form, type-2 (or PAP-II).

PAP-I has been purified from a range of different mammalian tissues including guinea-pig [1], rat [2] and human [3] brain. This enzyme has been found to be a low molecular weight thiol protease with a broad substrate specificity which includes neurotransmitters such as thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LH-RH), neurotensin, and bombesin. PAP-II has also been purified from guinea-pig [4] and rat [5] brain but has been found to be a high molecular weight metalloprotease which will only recognize and inactivate TRH or closely related peptide analogs.

This study reports the purification of a PAP-I activity from the cytosolic fraction of bovine brain. The enzyme can be specifically assayed for using the fluorimetric substrate pyroglutamate - 7-amino-4-methyl-coumarin (or pGlu-MCA). Like other reported PAP-I activities, the enzyme in this study has a low molecular weight (approx. 23,000 daltons), appears to be a thiol protease, and has a typically broad substrate specificity which includes a range of neuropeptides. Further study of this enzyme will focus on its involvement in thyrotropin-releasing hormone (TRH) regulation.

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B7-203 ISOLATION AND CHARACTERISATION OF KRP, A DIBASIC ENDOPEPTIDASE REQUIRED FOR CELL VIABILITY IN FISSION YEAST.

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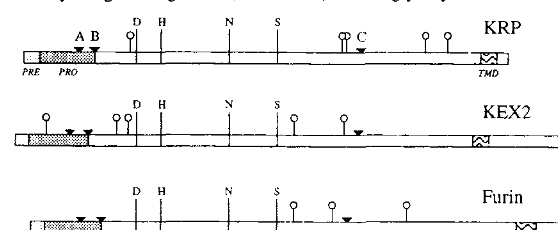
I have isolated and characterised the first member of the subtilisin-like family of enzymes from the fission yeast *Schizosaccharomyces pombe*. The enzyme, which has been named krp for KEX2-related-protease, is a type I membrane-bound endopeptidase that cleaves substrates after pairs of dibasic residues. It is synthesised as a pre-pro-protein that is likely to undergo processing following translocation into the endoplasmic reticulum. Processing has been characterised in a translation/translocation system prepared from *Xenopus* eggs. Krp is N-glycosylated at 5 sites and both the pre-sequence and the pro-sequence are quickly removed following translocation, the latter probably by autocatalytic cleavage. The inhibitor profile of krp reflects the known properties of the eukaryotic subtilisin proteases while its pH and Ca^{2+} dependence are consistent with activity in the secretory pathway. One of its physiological substrates is the pheromone precursor pro-P-factor which it processes in an *in vitro* system but identification of other substrates is complicated because, unlike other members of this family, krp is essential for cell viability.

Krp compared to KEX2 (*S.cerevisiae*) and furin (human). PRE = pre-region, PRO = pro-region, TMD = transmembrane domain. D, H, N and S = catalytic amino acids.
▼ = Lys-Arg cleavage sites (A, B and C). ° = N-glycosylation sites.



B7-204 PROHORMONE CONVERTASES FROM HUMAN BRAIN AND THEIR COEXPRESSION WITH POTENTIAL SUBSTRATES IN A BACULOVIRUS SYSTEM.

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Mammalian proteases of the subtilisin-related proprotein convertase family are involved in the processing of precursors for peptide hormones and neuropeptides at dibasic amino acids. Several members of the family have been shown to be expressed in the CNS in mouse and rat, and a number of precursor substrates have been identified in tissues such as the pituitary. We are studying human prohormone convertase expression patterns and their enzymatic activity on potential proprotein substrates in the human brain.

We have determined the relative levels of expression of the human prohormone convertases in different regions of human brain. Furin, PC1, and PC2 are differentially expressed in human cortex and hippocampus. Using a baculovirus-insect cell system, we have coexpressed several human prohormone convertases with precursors of CNS proteins in order to test the processing activity of the human prohormone convertases. The human prohormone convertases exhibit differing abilities to process the members of the NGF family of neurotrophic factors. Thus, different prohormone convertases may be the physiologically important proteases for maturation of neurotrophic factor precursors in specific regions of human brain.

B7-205 PROPERTIES OF PLASMA CARBOXYPEPTIDASES SPECIFIC FOR C-TERMINAL ARGININES AND LYSINES

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There have been numerous reports of carboxypeptidase activity in human plasma and serum. Until recently it has been assumed that the only carboxypeptidase capable of releasing C-terminal Arg and Lys is carboxypeptidase N (CPN). However, recently a distinct carboxypeptidase with a similar activity was discovered, plasma carboxypeptidase B (pCPB).

Carboxypeptidases can participate in diverse biological processes. The Arg and Lys releasing carboxypeptidases are known to be important inactivators of several potent peptides, including kinins, anaphylatoxins, fibrinopeptides, enkephalin hexapeptides, and creatine kinase. Many pro-proteins are processed at basic residues, and thus, Arg and Lys specific carboxypeptidases are likely to act as maturation proteases. In addition it could be speculated, that the basic carboxypeptidases are involved in the fibrinolysis, where it is well documented that Lys binding sites are essential for regulation.

We have purified and characterized pro-pCPB, following a procedure from literature. We have studied the activation process and the protein binding properties of activated as well as proform. The activated form has been characterized kinetically in a comparative study with CPN, the other basic carboxypeptidase found in plasma, and the pancreatic, basic carboxypeptidase B from rat.

B7-206 EVIDENCE FOR ISOFORMS OF ALPHA$_1$-ANTICHYMOTRYPSIN AS ENDOGENOUS INHIBITORS OF THE PROHORMONE PROCESSING ENZYMES PTP, PC2, AND PC1/3,

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Regulation of prohormone processing enzymes by endogenous protease inhibitors may provide a mechanism for observed differences in the extent of prohormone processing. We have previously reported potent inhibition of the 'prohormone thiol protease' (PTP) by endogenous pituitary ACT-like protein (Hook et al., *J. Biol. Chem.* 268, 20570-20577). Inhibition involves complex formation of the cysteine protease PTP and the serpin ACT based on parallel P_1 specificities. Further studies in this report indicate that ACT-like proteins are colocalized with PTP in secretory vesicles of bovine adrenal medulla (chromaffin granules) and pituitary. In chromaffin granules, two forms of ACT with molecular weights of 56 and 70 kDa were identified immunologically by anti-ACT western blotting. Sucrose density gradient analyses of catecholamine-containing chromaffin granules indicated the colocalization of ACT in the secretory vesicle fraction. The ACT-like proteins were purified from chromaffin granule lysate by affinity chromatography on concanavalin A-agarose and by ion-exchange FPLC on a Mono-Q column. These 70 and 56 kDA ACT-like proteins were tested for inhibition of the processing enzymes purified from chromaffin granules -- 'prohormone thiol protease' (PTP), PC1/3, and PC2 (PC=prohormone convertase). PTP was inhibited by both 56 and 70 kDa ACTs at µM concentrations. When the 56 kDa ACT and 70 kDa ACTs were tested at the same concentration (µM) with the PC enzymes, the 56 kDa ACT inhibited PC1 and PC2 but the 70 kDa ACT had no effect. The presence of endogenous protease inhibitors of processing enzymes is consistent with the limited processing of proenkephalin in chromaffin granules in which approximately 90% of (Met)enkephalin exists as incompletely processed high molecular weight intermediates. Future definition of each isoform of ACT in regulating prohormone processing enzymes will be important for understanding control mechanisms of peptide hormone formation.

B7-207 CHARACTERIZATION OF A NOVEL METALLO-PROTEASE FROM *Fusarium oxysporum.*

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A novel metalloprotease purified from *Fusarium oxysporum* (p45 maturase) has been shown to process the zymogen form of a *F. oxysporum* tryptic protease which results in active fungal trypsin. Cleavage of the pro-region can occur using a number of neutral or alkaline metalloproteases, however, the *Fusarium* p45 maturase is the most efficient.

The p45 maturase has a molecular weight of approximately 45 kDa (based on SDS-PAGE), a pI of approximately 8.5, and an optimum pH of 9.5. Loss of p45 maturase proteolytic activity was accomplished by removal of the zinc center with 1,10-phenanthroline (1mM) followed by titration with Zn^{+2} to restore full activity. Protein sequence of the p45 maturase bears little homology to sequences of other thermolysins, except for a metalloprotease from *Aspergillus fumigatus.*

A synthetic pentapeptide representing the pro-sequence of the pro-trypsin does not inhibit the normal processing of the *Fusarium* pro-trypsin to active trypsin by the p45 maturase. Therefore, the pro-sequence alone is not important for recognition by the p45 maturase.

B7-208 THE PROHORMONE CONVERTASE PC2 PROCESSES PRONEUROPEPTIDE Y IN SUPERIOR CERVICAL GANGLION, Luc Paquet* and Richard E. Mains. Department of Neuroscience. The Johns Hopkins University, Baltimore MD 21205 *Fellow from the Medical Research Council of Canada.

Recent studies have identified a family of mammalian proprotein processing enzymes highly homologous to the yeast convertase Kex2. Of these, two (PC1 and PC2) are primarily expressed in the nervous and endocrine systems. In the superior cervical ganglion (SCG), proneuropeptide Y (proNPY) is very efficiently converted to its biologically active form through the sequential action of an unknown convertase, carboxypeptidase E and peptidylglycine α-amidating monooxygenase. SCGs were shown to express PC2 mRNA at a level comparable to the amount found in the anterior pituitary while PC1 mRNA was barely detectable. The ubiquitous convertase furin was found in comparable amount in SCGs and anterior pituitary. While expressed in relatively high amount in the anterior pituitary, PACE4 transcript was undetectable in SCGs. The amount of PAM mRNA was significantly less in the SCGs compared to the anterior pituitary. The major form of PAM produced by the SCGs was found to be PAM-2 by PCR analysis. In SCG primary culture, PC2 immunoreactivity was found in the cell body and in the axon where it colocalized with NPY immunoreactivity. Experiments were undertaken to compare the kinetics of endoproteolytic processing of proNPY in SCGs (which express mostly PC2) to proNPY transfected AtT-20 (which express mostly PC1). Cells were pulse labeled for 15 minutes and chased for various amounts of time. After 60 minutes of chase, only 40% of proNPY was converted to smaller forms in SCGs while almost 90% of it was already converted in AtT-20. Taken together, these results suggest that PC2 is the endoprotease responsible for proNPY processing in SCGs. Experiments are in progress to modulate the level of PC1 and PC2 in SCGs using recombinant adenoviruses.

B7-209 THE NRD CONVERTASE AND AN AMINOPEPTIDASE-B : TWO METALLOPEPTIDASES WHICH MAY BE INVOLVED IN PRO-PROTEIN PROCESSING.

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A 110-140 kDa metalloendopetidase isolated from rat brain cortex and testis was recently cloned. Its cleavage specificity, when tested on peptides like somatostatine-28, dynorphin A or ANF(1-28), suggests its implication in pro-protein processing. It cleaves these peptides on the N-terminus of Arg (R) residues in dibasic stretches and was thus named NRD convertase. The protein sequence presents the zinc binding motif HXXEH and shows similarity with the members of the M16 family of metalloendopeptidases (48% with the insulin-degrading enzyme). A characteristic feature of the NRD convertase is the presence of a 71 amino acid long acidic domain composed of 80% of Glu and Asp residues. NRD convertase is highly expressed in testis where it shows a burst of expression in the late stages of the maturation of spermatids into spermatozoa. An immuno-cytochemical study in this tissue revealed a remarkable accumulation of the enzyme in the vicinity of the flagella, suggesting its possible association with the axonem structure. Although the NRD convertase was found secreted in primary cultures of isolated spermatids, its cytoplasmic localisation in spermatids together with the inability of its putative signal peptide to direct the protein to microsomes in *in vitro* experiments suggest that, at least in this tissue, the enzyme may not be present in the secretory pathway. Its subcellular localisation in neuronal cells is presently under investigation.

An aminopeptidase of the B-type, also purified from rat brain cortex and testis, has recently been characterized in our laboratory. It removes Arg or Lys residues from the N-terminus of several biological peptides except in the case of Arg-Pro bonds and presents a metalloenzyme character. Whether the NRD convertase and the aminopeptidase activites are associated *in vivo* still remains to be determined.

B7-210 EXPRESSION AND PROCESSING OF BARLEY SERINE CARBOXYPEPTIDASES IN BARLEY AND YEAST, Anne Rocher, Florence Dal Degan, Verena Cameron-Mills and Diter von Wettstein, Department of Physiology, Gl. Carlsbergvej 10, DK-2500 Copenhagen Valby, Denmark.

During maturation and germination of the barley grain, a family of at least six serine carboxypeptidases (Ser-CPs) is expressed (Dal Degan, F., Rocher, A., Cameron-Mills, V., and von Wettstein, D., 1994, Proc. Natl. Acad. Sci. US, in press) . Five of these (CP-MI, II, II.1, II.2 and II.3) appear to be synthesized as precursors with a signal peptide, propeptide and linker peptide. During enzyme maturation, the linker peptide would be excised and the two resulting polypeptide chains become linked by disulfide bonds. CP-MI and II exist in a mature form as dimers, each subunit consisting of two disulfide bonded polypeptide chains. In contrast, CP-MIII is a single chain monomer sharing close homology to CP-Y from yeast.

Northern, Western and RNA PCR analysis have shown CP-MII to be the only Ser-CP to accumulate in the endosperm during grain maturation and to be present in the mature grain in an active form. All six Ser-CPs are expressed *de novo* in the germinating grain, in the scutellum and/or in the aleurone and at least CP-MI, II and III are secreted into the endosperm. This supports the participation of Ser-CPs, in concert with cysteine endoproteases, in the mobilization of storage proteins to supply small peptides and amino acids to the germinating embryo. With the exception of CP-MI, all Ser-CPs are also expressed in the roots and shoots of the growing seedling, which suggest that they play additional roles, possibly in the processing of other enzymes.

To study the processing of the Ser-CPs precursors, a full-length cDNA clone for CP-MI isolated by Drs F. L. Olsen and R. Leah (Carlsberg Research Center) has been subcloned in yeast expression vectors with or without the original signal- and/or propeptide sequence. It is examined whether yeast endo- and exopeptidases can process the pre-, pro- and linker peptides of the barley precursors and if the barley Ser-CP can substitute for CP-Y, trimming the peptide chains to their mature form.

B7-211 EXPRESSION OF PROENKEPHALIN AND PRO-NEUROPEPTIDE Y IN *E. COLI*, AND IN VITRO PROCESSING BY 'PROHORMONE THIOL PROTEASE' (PTP), M.R. Schiller, L. Mende-Mueller#, K. Miller+, and V.Y.H. Hook*, Dept. of Neuroscience, Johns Hopkins University, Baltimore, MD., #Dept. of Biochemistry, Medical College of Wisconsin, Milwaukee, WI., +Dept. of Molecular Biology, Univ. of Wyoming, Laramie, Wyoming, and *Dept. of Medicine, Univ. of Calif., San Diego

Secretory vesicles of the adrenal medulla (chromaffin granules) contain enkephalin and neuropeptide Y that are synthesized by proteolytic processing of corresponding neuropeptide precursors. Previous studies have identified the 'prohormone thiol protease' as a candidatte proenkephalin (PE) processing enzyme in chromaffin granules. To assess PTP processing of PE and pro-neuropeptide Y (pro-NPY), these prohormones were obtained by high level expression in *E. coli* with the pET3c expression vector. PE was purified from cells by DEAE-Sepharose, preparative gel electrophoresis, and reverse phase HPLC. Pro-NPY was purified from cells using DEAE-Sepharose and Superose-12 FPLC chromatography. PTP cleaved PE more readily than pro-NPY, suggesting preference of PTP for PE over pro-NPY. Incubation of PTP with PE near in vivo levels of PE (10 μM) resulted in the rapid production of 22.5, 21.7, 12.5, and11.0 kDa intermediates that contain the NH_2-terminal region of PE, as assessed by peptide microsequencing. Based on the known specificity of PTP for dibasic and monobasic cleavage sites (Krieger and Hook, *J. Biol. Chem.* 266, 8376; Krieger et al., *J. Neurochem.* 59, 26; Azaryan and Hook, *FEBS Lett.*341, 197) the 22.5, 21.7, 12.5 and 11.0 kDa products are likely to represent PTP processing of PE from the COOH-terminal region of the precursor. This is consistent with in vivo processing of PE in adrenal medulla. Production of 12.5, 11.0, and 8.5 kDa products represented PTP cleavage between Lys-Arg at the COOH-terminus of (Met)enkephalin-Arg-Gly-Leu and processing of PE near its COOH-terminal region. Kinetic studies indicated PTP cleavage of PE with a $K_{m(app)}$ of 18.6 μM and $V_{max(app)}$ of 1.98 mmol/hr/mg. These kinetic values are consistent with estimated in vivo levels in adrenal medulla of PE, intermediates, and enkephalin peptides. These results demonstrate a role for PTP in prohormone processing.

B7-212 CLEAVAGE-PROCESSING OF ANGIOTENSIN CONVERTING ENZYME, Indira Sen, R. Ramchandran, Kunio Misono and Ganes C. Sen, Department of Molecular Cardiology and Molecular Biology, Cleveland Clinic Foundation Research Institute, Cleveland, OH 44195

Angiotensin-converting enzyme (ACE) is an ectoprotein anchored in the plasma membrane through a hydrophobic domain near its carboxyl-terminal region. Mouse epithelial cells transfected with rabbit testicular ACE cDNA, synthesize, glycosylate, and secrete ACE by cleavage processing of its membrane-anchoring carboxyl-terminal region. Because the cleavage-secretion process is slow, the enzyme accumulates on the cell surface. We show that this process can be enhanced by treatment of cells with tumor-promoting phorbol esters leading to depletion of the cell surface enzyme. The cleavage processing occurs only after the protein has reached the cell surface and is not affected by disruption of the Golgi apparatus or the lysosomal compartments. The exact peptide bond cleaved has been identified by sequencing the amino-terminal residues of the purified COOH-terminal tail left in the cells after ACE is secreted and the carboxyl-terminal residues of secreted ACE. The cleavage occurs at a monobasic site between Arg-663 and Ser-664 generating the soluble enzyme and leaving a cell-bound protein of 74 residues. These results demonstrate the existence of cellular mechanisms that regulate the conversion of cell-bound ACE to a soluble enzyme. Supported in part by NIH Grant HL48258 and a Grant-in-Aid from the American Heart Association.

B7-213 MAST CELL PROCARBOXYPEPTIDASE A: CHARACTERIZATION OF ITS PROCESSING WITHIN SECRETORY GRANULES. Eric B. Springman, Michael M. Dikov and William E. Serafin, Vanderbilt University School of Medicine, Nashville, TN 37232-0111.

We previously investigated the activation mechanisms for mast cell prochymases, protryptases, and procarboxypeptidase A (MC-proCPA), finding that each protease class required a distinct activating enzyme, with prochymases being activated by dipeptidylpeptidase I by removal of the propeptide Glu-Glu (*J. Biol. Chem.*, in press). We have found that murine MC-proCPA is an inactive zymogen. To investigate the mechanisms for this lack of enzymatic activity and the processing of the zymogen to the active form, we now have performed molecular modeling of the tertiary structure of murine MC-proCPA based on the x-ray crystallographic structures of porcine pancreatic procarboxypeptidases A and B. Our model predicts that MC-proCPA retains a high degree of structural similarity to its pancreatic counterparts. The globular propeptide physically blocks access to the fully-formed active site of the catalytic domain and contains a salt bridge to the substrate-binding region that precludes docking of even small substrates. Based on consideration of the predicted tertiary structure and charge-field characteristics of the model, the activation site (Glu-A94:Ile-1) appears to be highly exposed even after MC-proCPA binds to secretory granule proteoglycans. Based on the steady-state levels of MC-proCPA versus MC-CPA, cycloheximide inhibition of protein synthesis, and brefeldin A blockage of protein sorting, we show that MC-proCPA is processed rapidly in murine mast cell line KiSV-MC14 with a half-life of 26 ± 5 min, (mean $\pm$ S.D., n = 3), and the processing occurs within the secretory granules. The enzyme responsible for this processing may be a thiol protease, since treatment of the KiSV-MC14 with 200 μM E-64d, a selective thiol-protease inhibitor, increases MC-proCPA by 2.7 ± 0.2 fold (mean $\pm$ S.D., n = 3) within 6 hrs of application. We show that MC-proCPA ionically binds to intracellular proteoglycans, in low M.W. complexes (~250 kD), where the large majority is activated. Subsequently, the low M.W. complexes combine into large complexes (>20,000 kD) where any remaining unprocessed MC-proCPA becomes inaccessible to further processing. Thus, the distribution of the active form versus the zymogen defines two distinct subcompartments within the secretory granules and underscores the regulatory role of proteoglycans in protease processing.

B7-214 CHARACTERIZATION OF PROCARBOXYPEPTIDASE B FROM HUMAN PLASMA

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Recently we isolated and cloned a novel basic carboxypeptidase from human plasma. This plasma carboxypeptidase is structurally similar to tissue carboxypeptidase (tCPB) and is now known as human plasma carboxypeptidase B(pCPB). The physiological function of pCPB is still unclear. Initial characterization of pCPB has been hampered by our inability to generate active pCPB following trypsin treatment. After releasing the amino terminal activation peptide at Arg-92, a subsequent tryptic cleavage at Arg-330 inactivates pCPB. We have found that 6-amino-hexanoic acid (εACA), a compeptitive inhibitor of basic carboxypeptidases, selectively limits trypsin cleavage of pro-pCPB. In the presence of εACA, trypsin cleavage at Arg-330 is significantly limited while the activation cleavage at Arg-92 is unaffected. Using this approach active pCPB can now be obtained. Kinetic charactierization with natural peptides and synthetic substrates shows that pCPB behaves similar to carboxypeptidase N, another known basic carboxypeptidase found in plasma. However, unlike carboxypeptidase N which is more effective in hydrolyzing C-terminal lysine residue, pCPB is more specific for substrates with a C-terminal arginine. pCPB also hydrolyzes the synthetic ester substrate (hippuryl-L-argininic acid) more effeciently than the synthetic peptide substrate (hippuryl-L-arginine),especially at high pH. The active site Zn^{+2} atom can be replaced with other metals with change in substrate specificity. Binding studies using either Lys-plasminogen or Glu-plasminogen with pro-pCPB and activated pCPB show that pro-pCPB has a 10 fold higher affinity to both forms of plasminogen than active pCPB. These results suggest that the glycosylated activation peptide mediates the high affinity binding of pCPB to plasminogen. The ligand blot binding studies show that the binding site for pCPB to plasminogen is via the lysine binding site.

B7-215 CHARACTERIZATION OF A MICROSOMAL METALLOENDOPEPTIDASE THAT PROCESSES TUMOR NECROSIS FACTOR-α, J. David Becherer, Marcia L. Moss, D. Mark Bickett, Janet Warner, Jeremy Roth, Stephen Frye, Inder Patel, and Gerard M. McGeehan, Glaxo Inc. Research Institute, Research Triangle Park, N.C. 27709

The multifunctional cytokine TNF-α is a 17 kDa protein that is secreted after proteolytic processing of a 26 kDa precursor. Previously, we have demonstrated that a metalloproteinase inhibitor, GI 129471, blocks secretion of TNF at a post-translational step, presumably by inhibiting proteolytic processing of precursor TNF (McGeehan et. al. (1994) *Nature* **370**:555). We now report the characterization of a microsomal activity that specifically cleaves a synthetic peptide substrate spanning the cleavage sequence of proTNF, and is inhibited by GI 129471 in a dose-dependent manner. This same microsomal activity processes recombinant proTNF, yielding a correctly processed 17 kDa fragment. The endopeptidase is inhibited by metal chelators but not by protease inhibitors specific for serine, cysteine or aspartic proteases and appears to be an integral membrane protein. Purification by ion-exchange and photoaffinity chromatography, utilizing a photoreactive crosslinking derivative of GI 129471, has identified a putative TNF convertase.

B7-216 **RAS AND a-FACTOR CONVERTING ENZYME**

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Proteins that contain a C-terminal CAAX motif (where C is cysteine, A is usually aliphatic and X is any amino acid) undergo a number of sequential post-translational modifications. Such processing includes prenylation of the cysteine residue, proteolytic removal of the terminal three residues and methylesterification of the newly exposed carboxyl group of the prenylated cysteine. In the budding yeast *Saccharomyces cerevisiae,* proteins that undergo CAAX processing include, but are not limited to, Ras, **a**-factor mating pheromone and the γ-subunit of the pheromone response heterotrimeric G-protein.

Our lab is interested in the CAAX C-terminal protease from yeast which we have termed RACE (Ras and **a**-factor Converting Enzyme). This enzyme is a novel, prenylation-dependent, integral membrane endoproteinase. *In vitro* assays identified an analogous specific proteolytic activity in mammalian and amphibian extracts.

We have taken several genetic approaches to isolate mutants defective in CAAX processing. These mutants will enable us to isolate the RACE gene and understand the functional consequences of C-terminal proteolysis. One of the screens involves alteration of the CAAX sequence of **a**-factor, rendering the peptide a poor substrate of RACE *in vitro* and *in vivo.* To isolate the RACE gene we are currently investigating several genomic clones that, when maintained at high-copy, suppress the associated mating defect. In addition, we are utilizing a novel selection for mutants defective in the processing and secretion of **a**-factor. This selection involves heterologous expression of the **a**-factor receptor in **a** cells. Wild-type cells that are able to fully process and secrete mature **a**-factor undergo autocrine arrest while mutants defective in processing grow and form colonies. A preliminary selection yielded mutants with defects in CAAX-processing enzymes including the farnesyltransferase (*RAM1*/ *RAM2*) and methyltransferase (*STE14*) and in the **a**-factor transporter (*STE6*). We scaled up the autocrine arrest selection and currently are characterizing over 100 mutants defective in **a**-factor production.

B7-217 MOLECULAR CLONING OF BOVINE MAST CELL TRYPTASE: CORRELATION BETWEEN AMINOACID SEQUENCE AND BIOCHEMICAL PROPERTIES, Laura Fiorucci, Alessandra Gambacurta, Michele Pallaoro, Fulvio Erba, Giuseppina Mignogna, Donatella Barra and Franca Ascoli, Department of Experimental Medicine and Biochemcial Sciences, Univ. 'Tor Vergata' and Department of Biochemical Sciences, Univ. 'la Sapienza', Rome, Italy

Mast cell tryptases are secretory granule-associated serine proteases with trypsin-like specificity. Using synthetic substrates, bovine tryptase, as human tryptases, was shown to have a particular affinity for tripeptide substrates with adjacent basic aminoacid residues in P_1 and P_2 positions. At variance with human tryptases (J.A. Cromlish *et al.*, J. Biol. Chem. 262, 1363-1373 (1987), the bovine enzyme is inhibited *in vitro* by BPTI (bovine pancreatic trypsin inhibitor), a protein inhibitor immunodetected in bovine mast cells and recently colocalized with tryptase within the granules of bovine liver capsule mast cells (L. Fiorucci *et al.*, Biochim. Biophys. Acta, in press). The aminoacid sequence of bovine tryptase was determined by cDNA cloning and sequencing. The cDNA was obtained by reverse trancription of total RNA isolated from bovine liver capsule mast cells and PCR using degenerate oligonucleotide primers deduced from sequencing of fragmentated protein.

Comparison between bovine tryptase, other known tryptases and trypsin will be made to elucidate the structure-function relationships and to provide explanations for its peculiar biochemical features.

B7-218 ROLE OF RAF AND MAP KINASE IN DIFFERENTIATION OF NEURONAL CELLS. Wen-Liang Kuo, Tsung-Shu Oliver Chao, Martin McMahon* and Marsha Rich Rosner. University of Chicago, Chicago, IL 60637 and *DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304.

In order to study signal transduction pathways leading to cellular differentiation, we have generated conditionally-immortalized neuronal cell lines from rat hippocampal neurons by transduction of cells with a retrovirus expressing a temperature-sensitive SV40 large T antigen. Cell lines derived by these procedures proliferate in culture in response to EGF at the permissive temperature, and differentiate in response to bFGF or PDBu at the nonpermissive temperature. Treatment of one cell line, H19-7, with the differentiating agent bFGF resulted in the activation of c-Ras, c-Raf-1 and MAP kinase. To evaluate the role of Raf-1 in neuronal cell differentiation, we stably transfected H19-7 with v-raf and an oncogenic human raf-1/estrogen receptor fusion gene (Δraf-1:ER). Activation of Raf kinase in the transfectants was confirmed by both the band shift of Raf-1 protein in gels and *in vitro* phosphorylation of MEK, a RAF-1 substrate. Several of the v-raf transfectants became differentiated at both permissive and nonpermissive temperatures, although at the nonpermissive temperature the cells differentiated more rapidly and completely. In the presence of estradiol at the nonpermissive temperature, Δraf:hER transfectants expressed a differentiation phenotype similar to that of the v-Raf transfectants. At the permissive temperature, MAP kinase activities in the Δraf-1:ER cells were elevated constitutively from 15 min to 35 hours of estradiol treatment. However, at the nonpermissive temperature, MAP kinases were activated transiently and to a lower degree. Taken together, these results suggest that constitutive activation of MAP kinases (erks 1 and 2) is neither required nor sufficient for differentiation of H19-7 neuronal cells.

B7-219 THE CHARACTERIZATION OF THE INHIBITION OF MATURE CYSTEINE PROTEASES BY THEIR PROREGIONS, Kathleen Okamoto and Dieter Brömme, Khepri Pharmaceuticals, Inc., South San Francisco, CA 94080

The proenzyme is the inactive form of an enzyme, which is processed, thus forming the active enzyme. Evidence exists that propeptides also function as chaperones for correct folding of the mature enzyme. It has been shown in all four classes of proteases that the proregion serves as a specific inhibitor of its mature enzyme.

This specific inhibition of the parent enzyme by its propart could be especially important for enzymes involved in extracellular matrix degradation such as cathepsins S, L, B and O. Lysosomal cysteine proteases have been shown to be involved in tissue destructive disease processes that occur in arthritis, osteoarthritis, osteoporosis and during tumor invasion. Because of the destructive nature of these diseases, specific inhibitors, such as proparts of target enzymes could play an important role in understanding and controlling these diseases.

In this study, the proparts of cathepsins S and O have been expressed and their inhibition of mature cathepsins S, L, O and B have been characterized. Interestingly, these two proparts differ in their specificity of inhibition towards their respective mature enzyme and the other cysteine proteases. The cathepsin S propart specifically inhibits its parent enzyme, while the cathepsin O propart has a broader specificity and inhibits cathepsins O, S and L but not cathepsin B. These differences in specificity could help determine important regions of interactions between proparts and mature enzymes.

B7-220 THE SLA1 GENE OF *S. CEREVISIAE* IS REQUIRED FOR GOLGI PROTEIN SORTING, Véronique Patry[1], Doris Germain[1], David Y. Thomas[2] and Guy Boileau[1], [1]Department of Biochemistry, University of Montreal and [2] Genetic Group, Biotechnology Research Institute, National Research Council of Canada, Montreal, Canada.

Kex2p (KEX2 gene product) is a serine protease of the subtilisin family located in yeast late Golgi and involved in the proteolytic activation of the α-mating pheromone precursor and K1 and K2 toxins. The correct sorting of the yeast Kex2p to the Golgi apparatus depends on its cytoplasmic domain and also requires the presence of a functional clathrin heavy chain suggesting that Kex2p cycles between the late Golgi and an endosome-like compartment. The yeast two hybrid system was used to identify in *S. cerevisiae* gene products interacting with Kex2p cytosolic tail. The SLA1 gene was isolated. Disruption of this gene results in a defect in α-pheromone production and in mislocation of Kex2p to the vacuole. The SLA1 gene product appears to be a modular protein with at least three structural domains (SH3, charged and bindin domains). Using the two hybrid system and *in vitro* affinity assay we show that the central charged domain of Sla1p interacts with Kex2p cytosolic domain.

B7-221 SPLICING OF PROTEIN PRECURSORS IN VITRO: A DEFINITION OF THE REACTION AND ITS PROPOSED MECHANISM, Francine B. Perler, Ming-Qun Xu, Maurice W. Southworth, Eric Adam, Fana B. Mersha and Donald G. Comb. New England Biolabs, Inc. 32 Tozer Road, Beverly, MA 01915.

Protein splicing is defined as the synthesis of a precursor protein followed by the precise excision of an internal polypeptide (the intein) and the ligation of the flanking protein sequences (the exteins) to form a native peptide bond. Inteins are not only the product of protein splicing, but are also the mediators of the splicing reaction. Ten inteins have been identified since the first report of the Sce VMA intein in 1990. Intein similarity is limited to homing endonuclease motifs and to conserved splice junction residues which have been implicated in catalysis (S/C at the N-terminus and His-Asn-(S/C/T) at the C-terminal junction). Direct examination of the mechanism of protein splicing was made possible by purification of a protein precursor consisting of an intein inserted into a foreign context (MBP-paramyosin). Protein splicing of archaeal inteins proceeds via a branched intermediate and is controllable by pH or temperature. We will discuss mutation data, branch lability and the presence of a succinimide ring at the intein C-terminus. These results are consistent with several mechanisms, one of which is similar to that of serine proteases. Moreover, controllable protein splicing opens up novel approaches to protein engineering.

B7-222 KINETIC CHARACTERIZATION OF NATURAL AND SYNTHETIC SUBSTRATES FOR INTERLEUKIN 1-β CONVERTASE (ICE), Scott A. Raybuck, Nara Margolin, Robert A. Aldape, Maureen T. DeCenzo, Yu-Ping Luong, and David J. Livingston, Vertex Pharmaceuticals, 40 Allston Street, Cambridge, MA 02139

Human interleukin 1-β converting enzyme (ICE) is a cysteine protease which processes the 33 kDa inactive precursor of IL-1β to the active proinflammatory cytokine IL-1β. The X-ray structure of ICE revealed it to have a new tertiary and quaternary structure among previously known proteases. ICE was the first member identified of a new family of cysteine proteases selective for Asp-X sequences, with 28% overall sequence identity with CED-3, a *C. elegans* protein identified genetically to be in the programmed cell death pathway.

ICE catalyzed processing of pro IL-1β occurs at two sites. Cleavage at Asp^{27}-Gly^{28} generates an intermediate 28 kDa fragment, additional proteolysis at Asp^{116}-Ala^{117} results in the formation of the 17 kDa mature interleukin 1-β. The role of the upstream cleavage site has not been well understood. We have determined the kinetic parameters for ICE-catalyzed proteolysis of pro IL-1β for both cleavage sites using native pro IL-1β and two point mutants that remove either processing site (D27E and D116E). Kinetic parameters were determined from densitometry of cleavage reactions on SDS-PAGE. These values and the relative catalytic efficiencies have been compared to the synthetic spectrophotometric substrates for ICE. The results suggest that the upstream processing site is more labile to proteolysis and that the resulting 28 kDa fragment may be more easily processed to the 17 kDa form than the 33 kDa precursor.

B7-223 COMMUNICATION OF POST-GOLGI ELEMENTS WITH EARLY ENDOCYTIC PATHWAY: REGULATION OF ENDOPROTEOLYTIC CLEAVAGE OF SEMLIKI FOREST VIRUS p62 PRECURSOR, Merja Sariola*, Jaakko Saraste$ and Esa Kuismanen*, *Department of Biochemistry, University of Helsinki, P.O.Box 5, 00014 University of Helsinki, FINLAND, $Department of Biochemistry, University of Bergen, Årstadveien 19, 5009 Bergen, NORWAY
Besides cellular proteins, a number of viral spike glycoproteins are cleaved at a basic recognition sequence. To characterize the membrane traffic step at which this proteolysis occurs we have studied the processing of Semliki Forest virus (SFV) spike glycoprotein precursor p62 in BHK21 cells. The p62, which is endoproteolytically cleaved at a tetrabasic Arg-His-Arg-Arg recognition sequence, has previosly been shown to remain uncleaved when accumulated at the TGN/20°C block site. Proteolytic processing is inhibited when the 20°C transport block is released in the presence of brefeldin A, energy inhibitors and AlF_4. We show that a membrane traffic step from the trans-Golgi is required for the cleavage of p62 indicating that the cleaving enzyme resides in membranes other than the TGN/20°C block site. Endocytosed antibodies against the SFV spike glycoproteins or against a peptide representing the enzymatically active motif of furin (PACE) inhibited the cleavage of p62. The results demonstrate a post TGN communication step between exocytic and endocytic elements. Kinetic experiments suggest that an early phase of endocytosis is involved in this communication.

B7-224 DIFFERENCES IN THE pH AND CALCIUM REQUIREMENTS FOR MATURATION OF PC2 AND PC3 INDICATE DIFFERENT CELLULAR LOCATIONS FOR THESE EVENTS. Kathleen I.J. Shennan, Neil A. Taylor, Joanne L. Jermany, Glenn Matthews and Kevin Docherty. Department of Molecular and Cell Biology, University of Aberdeen and Department of Biochemistry, University of Birmingham, U.K.
PC2 and PC3 are subtilisin-like proteases involved in the processing of pro-hormones and proneuropeptides. Both enzymes are synthesised as pro-polypeptides that undergo maturation within the secretory pathway. An *in vitro* translation/translocation system from Xenopus eggs was used to investigate mechanisms in the maturation of pro-PC2 and pro-PC3. Site-directed mutagenesis was used to modify the potential processing sites and the Asp residues in the catalytic site. mRNAs were transcribed *in vitro* from cDNAs inserted in the vector pSP64T.To study the maturation of pro-PC2 and pro-PC3, mRNAs were translated in the egg extract and the reactions chased for periods of time at different pH and calcium concentrations. Pro-PC3 underwent rapid ($t_{1/2}$<10min) processing of the 88kDa precursor at the sequence $RSKS^{83}$ to generate the 80kDa active form of the enzyme. This processing was blocked when the active site Asp was changed to Asn, suggesting that processing was autocatalytic. Over the pH range 5.5-7.0, processing of pro-PC3 was maximal at pH7.0 with very little activity observed below pH6.0. Processing was not dependent on additional calcium. These results are consistent with pro-PC3 maturation occuring at an early stage in the secretory pathway, possibly within the E.R. Processing of pro-PC2 in the egg extract was much slower than that of pro-PC3 ($t_{1/2}$=8 hours). It exhibited a pH optimum of 5.5-6.0 and was dependent on additional calcium ($K_{0.5}$=2-4mM). The enzymatic properties of pro-PC2 processing were similar to that of the mature enzyme. Further studies using mutant pro-PC2 constructs suggested that cleavage of pro-PC2 was catalysed by the mature 68kDa PC2 protein. The results were consistent with pro-PC2 maturation occuring late in the secretory pathway, in a compartment that contains high calcium concentrations and low pH.

B7-225 ELEVATION OF 'PROHORMONE THIOL PROTEASE' (PTP) AND (MET)ENKEPHALIN BY FORSKOLIN IN CHROMAFFIN CELLS: BLOCKADE OF INCREASED (MET)ENKEPHALIN BY A POTENT CYSTEINE PROTEASE INHIBITOR OF PTP, N. Tezapsidis*, and S. Noctor*, and V.Y.H. Hook, *Dept. of Biochemistry, Uniformed Services Univ., Bethesda, MD, Dept. of Medicine, Univ. of Calif., San Diego, CA. 92103
A novel 'prohormone thiol protease' (PTP) from bovine chromaffin granules has been demonstrated to be involved in the production of (Met)enkephalin from proenkephalin. In this study, regulation of PTP and proenkephalin proteolytic processing by forskolin, a direct aactivator of adenylate cyclase, was investigated in primary cultures of chromaffin cells. Forskolin treatment of chromaffin cells is known to increase intracellullar (Met)enkephalin by stimulating proenkephalin gene expression followed by an increase in proenkephalin synthesis and processing. Cells were treated with forskolin for 72 hours, and proteolytic activity in chromaffin granules was assessed by the production of TCA soluble products generated from ^{35}S-(Met)-enkephalin precursor (^{35}S-preproenkephalin, ^{35}S-PPE) as substrate. Forskolin treatment increased chromaffin granule ^{35}S-PPE cleaving activity to 170-180% of that measured in controls. The increased proteolytic activity was associated with the chromaffin granule membranes. Among different classes of protease inhibitors tested E-64c (μM), a potent inhibitor of PTP and cysteine proteases, was most effective at diminishing the observed increase in proteolytic activity. More specifically, the increased activity in solubilized membranes was immunoprecipitated with affinity-purified anti-PTP polyclonal antibodies. The data revealed that forskolin treatment of chromaffin cells increased the synthesis of PTP itself, as seen in ^{35}S-Met metabolic labeling studies and immunoprecipitation of ^{35}S-(Met)-PTP. This was also confirmed by detection of increased levels of PTP by Western blots of chromaffin granules from forskolin-treated cells. Furthermore, when cells were pretreated with Ep453, a lipophilic analogue of E-64c that potently inhibits PTP and cysteine proteases, the forskolin-stimulated increase in intracellular (Met)enkephalin was completely blocked. These results demonstrate that PTP is required for cellular proenkephalin processing, and that both PTP and (Met)enkephalin biosynthesis are regulated via a cyclic-AMP pathway.

B7-226 **ENGINEERED α_1-ANTITRYPSIN VARIANT INHIBITS PRODUCTION OF INFECTIOUS MEASLES VIRUS**, Timothy C. Wong [1], Michiko Watanabe [1], Akiko Hirano [1], and Gary Thomas [2], (1) Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195, (2) The Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, Oregon 97201.
Measles virus (MV), a leading cause of morbidity and mortality in the world population, produces a fusion (F) glycoprotein essential for virus infection. The F protein of MV is synthesized as an inactive precursor F_0, which is cleaved by a cellular endoprotease into the F_1 and F_2 subunits which dimerize to form the functional F protein. We have identified furin as the cellular enzyme responsible for cleavage of the F protein precursor of MV. An α_1-antitrypsin variant (α_1-PDX) has been engineered to specifically inhibit furin activity. When expressed transiently from a recombinant vaccinia virus vector in primate cells infected by MV, α_1-PDX inhibited cleavage of the MV F precursor without affecting the synthesis of other MV proteins. In human glioma cells stably expressing α_1-PDX, MV produced all the viral proteins but F_0 was not cleaved efficiently. These MV-infected cells did not form multinucleated giant cells (syncytia). The virions produced in the presence of α_1-PDX contained the uncleaved F_0, and infectious virus titers were reduced by 3 to 4 orders of magnitude. These developments open a new strategy against measles virus.

B7-227 SECRETION OF PACE/FURIN INTO THE MILK OF TRANSGENIC MICE, Roman Drews, Timothy K. Lee, Rekha K. Paleyanda, William N. Drohan and Henryk Luboń, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855.

The expression of the human proprotein processing enzyme, paired basic amino acid-cleaving enzyme, PACE/furin, was targeted to the mouse mammary gland to improve the endoproteolytic processing of precursors of heterologous vitamin K-dependent proteins, such as human Protein C (HPC). A 4.1 kb mouse whey acidic protein (WAP) promoter was used to direct the expression of a 2.47 kb human PACE cDNA located upstream of 1.6 kb of 3' WAP gene sequences. A similar construct containing an active site mutant of PACE, PACEM, in which *Ser*^{261} *is mutated to Ala was also prepared.* Double-transgenic mice were generated by the coinjection of these transgenes with a WAP/HPC gene construct. PACE transcripts were detected during pregnancy and lactation. The developmental regulation of transgene expression differed from that of the endogenous mouse furin gene, but was similar to that of the endogenous WAP gene, in that induction of expression was observed between days 14 and 17 of pregnancy. Western blotting of milk proteins with antibodies recognizing several PACE epitopes detected a protein with a molecular weight of approximately 83 KDa, indicating that PACE was secreted into milk. PACE activity was demonstrated by the cleavage of excess recombinant HPC (rHPC) precursor present in the milk of HPC transgenic mice. HPC/PACEM mice did not demonstrate improved processing of rHPC. Despite the secretion of considerable amounts of PACE in the milk of HPC/PACE mice, the mice appeared healthy and did not develop tumors. These results demonstrate the secretion of a functional, predominantly Golgi-localized enzyme into milk without deleterious effects on the animals.

Late Abstracts

SECRETION OF PACE/FURIN INTO THE MILK OF TRANSGENIC MICE, Roman Drews, Timothy K. Lee, Rekha K. Paleyanda, William N. Drohan and Henryk Luboń, Holland Laboratory, American Red Cross, 15601 Crabbs Branch Way, Rockville, MD 20855.

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SNAKE VENOMS AS MIXTURES OF PROCESSING ENZYMES AND THEIR INHIBITORS: ANALYSIS USING SETS OF COMPETING SUBSTRATES (ANSA-ANALYSIS), Andrey A. Nedospasov[1], Elena V. Rodina[1], Iraida G. Scharina[1], Alexander V. Cherkasov[2], Institute of Molecular Genetics[1], Russian Academy of Sciences; Russian Research Centre "Kurchatov Institute"[2], Moscow, 123182, Russia.

ANSA-analysis [1] was used to study proteases and their inhibitors in snake venoms. The method is based on proteolytic cleavage of sets of competing substrates containing substituted aminonaphthalenesulfonamide (ANSA) chromogenic detection groups. To reveal processing enzymes in venoms, we used sets of peptide substrates cleavable at R-ANSA or K-ANSA bonds. Hydrolysis of these substrate mixtures catalysed by the venoms of some *Crotalidae* and *Viperidae* species gave characteristic chromatograms (ANSA spectra) for each test sample. The kininogenase substrates PFR-ANSAs were the best for all investigated venoms. The other substrates with R-ANSA or RR-ANSA fragments were cleaved less effectively. A mixture of six such substrates was proposed for protease-based identification of venoms. Peptide-K-ANSAs in mixtures with R-ANSA analogs were poor substrates though GGLL-ANSA was cleaved by *A. halys* venom. A small aminopeptidase activity in snake venoms was found. Aminoacyl-ANSAs added to reaction mixtures confirmed negligible cleavage of peptide substrates due to this activity. A method for quantitative estimation of ANSA spectra's differences was proposed. Each ANSA spectrum can be represented by an n-dimensional vector, the degree of similarity between two ANSA spectra thus being characterised by an angle between the vectors. Ca^{2+} is a potent activator of protease activity in some venom enzymes. Accordingly, ANSA-spectra of venoms essentially depended on the presence of Ca^{2+}. Protease inhibitors (I) of snake venoms are known to form complexes both with venom (E^v) and exogenous proteases (E^e). The ANSA spectra of such mixtures expected to be a sum of E^v and E^e spectra in the absence of mutual proteolysis, inhibition or activation were shown not to be always additive. The most attractive explanation for activation of venom convertases is that an equilibrium $E^vI + E^e \leftrightarrow E^eI + E^v$ can exist in the enzyme mixtures.

References: 1. Nedospasov et al., Biochemistry (Moscow), 1994, 59, N 10