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Review

Prohormone and Proneuropeptide Processing

Recent Progress and Future Challenges

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Our knowledge of prohormone and proneuropeptide processing and its relationship to the secretory pathway has advanced significantly in the last five years. The recent discovery of the prohormone convertase family of proteolytic enzymes has provided new candidates for the prohormone and proneuropeptide convertases. The increasing appreciation of the role of proteolysis in diverse cellular processes has also brought the prohormone processing field closer to the fields of growth factor processing, the role of host proteases in viral and bacterial pathogenesis and toxicity, control of the cell cycle, inflammation, and apoptosis.

The last five years have been very productive, but the most interesting questions are still unanswered. Which enzymes are actually responsible for prohormone cleavages in specific tissues? What structural features of the prohormones determine where it will be processed or how it is recognized as secretory material by the sorting machinery? How is tissue-specific processing determined and regulated? The availability of protease knockout mice and a more detailed understanding of the complex biosynthetic activation of these enzymes will provide at least some of the answers.

Key Words: PC1; PC2; PC3; PC4; PC5; PC6; PC7; PACE4; furin; peptide processing.

Peptide Discovery Is Still an Active Field

In the last 30 years, the discovery of over 50 biologically active peptides from mammals has occurred with new ones being discovered every year. The addition of peptide growth factors, antibiotic peptides, and cytokines to this list would probably double or triple it. Knowledge of the chemistry, biological activity, cDNA structure, and distribution of these peptides is much more complete than understanding of the temporal order of cleavages and which enzymes are responsible for their tissue-specific processing. Much valuable information has been gained about sites of prohormone processing from isolation and cloning of the prohormone cDNAs, as well as the purification and sequencing of peptide products isolated from tissues. Sensitive methods like matrix-assisted laser desorption mass spectrometry (MALDI-MS) have facilitated sequence determination *(1)* where sample quantity is lacking.

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The Context of Prohormone Processing

The recognition that prohormone processing takes place within the regulated secretory pathway has forced investigators in this field to think about processing in its correct cellular context. Questions of prohormone protein structure, folding, and sorting, the nature of the intracellular compartments where processing takes place, and the biochemistry of transport between endoplasmic reticulum (ER), Golgi, and the cell surface need to be considered.

In the last 10 years, there has been remarkable progress in the elucidation of the mechanism of vesicular transport within the secretory pathway in both endocrine and neuronal cells *(2,3)*. A large number of proteins have been identified in yeast and in mammalian cells which participate in this process.

The Major Events in Prohormone Processing

A typical prohormone is cotranslationally inserted into the ER based on the ability of the signal recognition particle to target the signal sequence of the growing peptide chain to the translocation complex in the ER membrane. The signal peptidase on the luminal side of the ER cleaves the signal peptide from the prohormone, releasing it into the lumen of the ER. There with or without the assistance of chaperones, it folds into its correct conformation. Incorrectly folded proteins are either degraded in the ER or exported from the ER (through the translocation complex) to the cytosol where they are ubiquinated and degraded by proteosomes *(4)*.

In the ER, *N*-linked glycosylation occurs. The prohormone is transported to the Golgi where *O*-linked glycosylation, serine phosphorylation, *N*-acetylation, and tyrosine sulfation are performed. The intracellular sites of processing have not been examined in detail for many prohormones, although some pro-TRH cleavages have been reported to occur in the Golgi before sorting into vesicles *(5)*. In the *trans*-Golgi network, most prohormones are thought to be sorted with the processing enzymes into regulated secretory vesicles. Within these granules, the contents are condensed, the calcium concentration is increased, and the pH is lowered. This is the specialized milieu where most of the prohormone cleavages are thought to take place.

Prohormone cleavages occur mainly at specific paired basic sites, although some single and tetrabasic sites are also cleaved. These cleavages appear to take place in a strict temporal order and may involve more than one endoprotease. Endoproteolysis is followed, in some cases, by the action of amino- and carboxypeptidases to remove residual arginine and lysine residues followed (in the case of amidated peptides) by conversion of the carboxyl-terminal glycine into an amide by the amidating enzyme *(6)*.

The Prohormone Convertase Family of Processing Enzymes

A major advance in the prohormone processing field was the discovery of the protein convertase (PC) family of enzymes, which now numbers seven *(7,8)*. These mammalian enzymes include PC1 (also known as PC3) *(9)*, PC2 *(10)*, PC4 *(11)*, PACE 4 *(12)*, PC5/6 *(13,14)*, PC7/8 *(15)*, and furin (also known as SPC1 or PACE, paired basic amino acid cleaving enzyme) *(16,17)*. The somewhat confusing nomenclature has arisen from the discovery of the same enzymes by different groups. Some of these enzymes like PC5/6 also have multiple splice variants (A and B), further complicating the nomenclature.

The discovery of the *kex 2* yeast enzyme, which processes the α mating factor and killer toxin precursor, inspired the search for mammalian equivalents, which could process mammalian precursors. PC2 *(10)* was cloned by PCR based on the similarity of its catalytic domain to *kex 2*. A database search with the PC2 sequence uncovered the partial sequence of a related human protein *fur*, found in *c-fes/fps* proto-oncogene. Cloning of this protein, which was later called furin, provided another member of the family *(16,17)*. The other family members PC1, PC4, PC5/6, PC7/8, and PACE4 were subsequently cloned by PCR based on the highly homologous catalytic domain.

Some of these enzymes are sorted and activated in the regulated secretory pathway (PC1, PC2, PC4, PC5/6) and thus are candidates for processing prohormones whose secretion is regulated.

The others, like furin, PACE4, PC5/6B, and PC7/8, have a transmembrane-spanning domain, cycle to the cell surface, and are involved in the processing of prohormones and precursors in the constitutive pathway.

These calcium-activated serine proteases have a signal sequence, an amino terminal pro-peptide of about 80–90 amino acids, which is removed at a cleavage site with the motif RXK/RR. They are glycosylated and sulfated. They all have a highly conserved catalytic domain with active site aspartic, histidine, and serine residues, which is very similar to subtilisn from *Bacillus subtilis*. They also have a conserved P or Homo B domain of about 150 residues with an integrin recognition motif RDG.

These enzymes undergo autoactivation with the removal or the pro-peptide. PC1 activation is thought to occur in the ER with a pH of about 7–8 with no additional calcium, whereas PC2 is activated much more slowly in the *trans*-Golgi network with a pH of about 5.5–6.0 and a millimolar calcium concentration *(18)*. This difference in the time-course of activation may explain why PC1 frequently cleaves prohormones before PC2.

Detailed examination of the life cycle, intracellular localization, catalytic activity, and tissue distribution of these PCs has yielded a gold mine of information. Production of active PC2 requires an additional protein called 7B2 *(19)*. It serves as a chaperone, helping it to reach its correct cellular destination. The amino-terminal 21-kDa protein is responsible for this chaperone action and enhances transport of PC2, whereas the 31 amino acid peptide is an inhibitor of PC2 activity, which is active at a nanomolar concentration. Activation of PC2 in the *trans*-Golgi network is accompanied by the cleavage of this inhibitory domain of 7B2 by PC2.

Most of these enzymes cleave at dibasic pairs,such as Arg-Lys, some monobasic sites are also cleaved *(20,21)*, whereas furin cleaves single basic sites preferentially with an upstream arginine with a consensus sequence Arg-X-X-Arg *(22)*.

Precursor Processing in the Regulated Secretory Pathway

PC1 and PC2 are widely distributed in neural and endocrine tissues and cell lines, and have been shown to be involved in the processing of prohormones, like POMC (pro-opiomelanocortin) *(23,24)*, insulin *(25)*, glucagon *(26)*, CCK *(21,27,28)*, gastrin (*29)*, dynorphin *(20)*, enkephalin *(30)*, TRH *(31)*, neurotensin *(32)*, and somatostatin *(33)* in the regulated pathway. The newly discovered PC5/6 is a good candidate for prohormone processing in the brain and intestine *(13,13,34)*. Other members like PC4 have a unique distribution, are restricted to the testis, and may be involved in processing of prohormones in this tissue.

As important as the PC enzymes appear to be, it is possible that proteases other than the PCs are involved in

prohormone processing *(35)*. A thiol protease has been identified and characterized from the adrenal that may be responsible for enkephalin processing *(36)*. Another thiol protease has been identified that cleaves dynorphin at single basic sites *(37)*. A metalloprotease called NRD convertase has been isolated and cloned, which is a candidate for prohormone processing, particularly in the testis *(38)*. Yeast aspartyl protease 3 cleaves a number of monobasic and dibasic substrates *(39)*. Its mammalian equivalent has not been identified, but immunostaining with YAP3 antisera has revealed its presence in a number of peptidergic cell populations in the brain *(40)*.

Precursor Processing in the Constitutive Pathway

Furin, PACE4, PC5/6B, and PC7/8 differ from the other members in having a transmembrane-spanning domain, which routes them to the cell surface. Furin is known to cycle between the cell surface, endosomes, the *trans*-Golgi network, and immature secretory granules. It has a casein kinase II phosphorylation site in its carboxyl-terminal and it appears that the phosphorylation–dephosphorylation of this site regulates its intracellular transport *(22)*.

Furin has been shown to be able to process at least 25 different precursors including nerve growth factor *(41)*, transforming growth factor β, neurotrophin 3 *(42)*, as well as plasma proteins like proalbumin, and pro-von Willebrand factor *(22)*. It is also a good candidate for the cellular enzyme responsible for the host proteolytic processing of bacterial toxins and viral proteins required for pathogenesis, including measles, HIV, CMV, Newcastle disease virus, *Shigella,* and *Psuedomonas aeruginosa*. *Psuedomonas* and *Shigella* toxins are taken up by endocytosis, move to endosomes, and eventually to the *trans*-Golgi network, and are transported backward to the ER where they are exported to the cytosol.

Because furin cycles to the cell surface and back it can pick up inhibitors like decanoyl-Arg-Val-Lys-Arg-chloromethylketone from the extracellular space. They work at micromolar concentration in cell culture *(43,44)*. Another more potent inhibitor, a modified serpin called α1-antitrypsin Portland works in the nanomolar range and has potential as an antiviral therapy *(45)*.

Experimental Systems to Study Processing

Much of the experimental work on the temporal order of cleavages and elucidation of the enzymes responsible for these cleavages has relied on COS-7 or other fibroblastic cells, or neural and endocrine tumor cells in culture. Some of these cell lines like At-T20, RIN5F, and PC12 normally express peptide mRNAs and some of the processing enzymes, and secrete processed peptides in response to secretagogs like cAMP *(46–49)*. If these cells do not express the peptide mRNA to be studied, it is transfected either transiently or stably. Specific processing enzymes can also be expressed in these cells with the prohormone with similar techniques or by infection with vaccinia *(23)* or adenovirus *(50)* vectors. As a first approximation, these endocrine cells appear to be a good model of peptide processing.

The question of which of these many enzymes are responsible for individual prohormone cleavages in tissues is difficult to answer. The possibility that there is considerable redundancy in their activities and that different enzymes are active on the same prohormone in different tissues cannot be excluded. In terms of CNS peptides, there are at least three enzymes (PC1, PC2, PC5/6) that have a distribution *(51–53)* that is consistent with a role in processing a number of proneuropeptides. Oxytocin (as well as CCK) is colocalized with PC1, PC2, and PC5/6 in supraoptic and paraventricular nuclei of the hypothalamus. The colocalization of CCK with these three enzymes appears to hold throughout the rat CNS.

In order to address which of these enzymes is responsible for pro-neuropeptide cleavage in specific endocrine cells, an antisense strategy has been used, because there are no specific, nontoxic inhibitors of these enzymes. This strategy, in which PC1 or PC2 cDNAs are expressed stably in endocrine cells in the antisense orientation, has provided evidence that PC1 and PC2 are involved in POMC *(54)*, pro CCK *(27,28,55)* pro-enkephalin *(56)*, and glucagon processing *(26)*. These results have been extended using an inducible promoter system and adenovirus vectors to express the PC antisense mRNA *(57,58)*. This is a successful strategy in general, but complete inhibition of PC expression with antisense methods has been difficult to achieve. It is a technique limited to cells that can be transfected or infected.

The most promising approach to understanding the physiological relevance of these enzymes is the production of transgenic mice in which these enzymes are specifically deleted. PC2, furin, and PC4 knockout mice have been produced, but the details of their interesting phenotypes have not yet been published. Likewise a human patient with defective PC1 enzyme has been identified, but the details of her complex endocrine and metabolic defects have not been reported. The ability to use human monocyte-derived macrophage RNA for diagnostic analysis of human PC1 should greatly simplify genetic screening *(59)*.

The importance of carboxypeptidase activity for production of amidated peptides was made very clear with the discovery of the gene responsible for the adult-onset obesity, which characterizes the *fat/fat* mouse phenotype. The *fat/fat* mouse, which has a mutation in carboxypeptidase E *(60,61)*, has defective processing of a number of amidated peptides, including gastrin *(62)*, LHRH, CCK *(63)*, and substance P. By 8 wk, when the obese phenotype is well established, the levels of CCK 8 amide are decreased by at least 90% in most brain regions in *fat/fat* mice as compared to controls, with a concomitant rise in immunoreactive CCK 8 Gly Arg Arg levels.

The ability to produce recombinant prohormones and putative processing enzymes in quantity in bacteria *(36)*, mammalian cells *(19,24)* and insect cells with *baculovirus* vectors *(64)* has led to numerous studies of prohormone processing in vitro *(19,21,24,65–67)*. These experiments have provided many new insights, but in some cases there have been discrepancies between cleavages observed in transfection experiments in endocrine cells and in vitro incubations. These differences can perhaps be attributed to the difficulty in reproducing the milieu of the condensing secretory vesicles in vitro.

Prohormone Processing in the "Golden Age" of Proteolysis

The prohormone processing field has always been small. In the last five years there have been several international meetings in France (Jacques Monod) and in the United States (Gordon and Keystone), which have helped to provide a focus and have fostered active exchange of ideas. There is considerable overlap between the fields of processing, protein folding, and degradation, and the cell biology of secretion. The increasing appreciation of the role of proteolysis in diverse cellular processes has also brought the prohormone processing field closer to the fields of growth factor processing, the role of host proteases including proteosomes in viral and bacterial pathogenesis and toxicity, control of the cell cycle, regulation of gene expression, inflammation, and apoptosis. The stunning success of HIV proteases inhibitors is just one example of the therapeutic application of protease research. Furin inhibitors could provide novel antiviral and antibacterial therapies.

Future Challenges

Knowledge of the actual enzymes responsible for prohormone cleavages in specific tissues lags behind progress in other areas of the field owing to the technical difficulty of working with intact tissues and whole animals. It is clear that the subtilisin-like PC enzymes are major players, but that there are additional enzymes that are not in this family, which are also required for specific cleavages.

Progress in our ability to develop specific, bioavailable inhibitors for these enzymes as well as to develop conditional, tissue-specific knockouts will allow us to address more directly the question of which enzymes are physiologically relevant. Detailed analysis of the recently produced knockout mice should provide much insight into the physiological role of these enzymes.

The most interesting questions are still unanswered, although work is in progress to address them. What structural features of the prohormone determine where it will be processed and how it will be recognized as secretory material by the sorting machinery? How is tissue-specific processing determined and regulated? What is the physiological and clinical significance of this regulation?

Detailed investigation of the regulation of these processing enzymes and the complex process of their biosynthetic activation is likely to provide many answers. The possibility that tissue-specific differences in their activation and the presence of tissue specific inhibitory substances that play a role cannot be excluded.

In summary, there has been significant progress in the last five years, but many of the most interesting questions remain to be answered.

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