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Source: Zoological Science, 20(10) : 1183-1198

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.1183

ZOOLOGICAL SCIENCE **20**: 1183–1198 (2003)

[REVIEW]

Comparative Aspects of Intracellular Proteolytic Processing of Peptide Hormone Precursors: Studies of Proopiomelanocortin Processing

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ABSTRACT—In this review, the mechanisms underlying the intracellular processing of peptide hormone precursors, with a focus on proopiomelanocortin (POMC), were discussed on the basis of recent information. POMC as well as other prohormones is processed to active peptides through proteolytic cleavage by prohormone convertases PC1 and/or PC2. However, the cleavage-specificity of PC1 and PC2 in mammals is somewhat different from that in amphibians. From the comparative endocrinological point of view, expression and tissue distribution of PC1 and PC2 were discussed here. In mammals, proteolytic processing of POMC occurs coordinately with the maturation of secretory granules. Studies using immunoelectron microscopy with DAMP (3-[2,4-dinitroanilino]-3'-amino-N-methyldipropylamine) as a pH probe revealed that the acidic pH in the secretory granules, generated by vacular type-H^+-ATPase, provides a favorable environment for activating PC1 in AtT-20 cells, a mouse corticotrope tumor cell line. Recent data indicate that the 7B2 protein serves as a chaperone in the regulation of PC2 activation and to control the timing for activating the convertase. Together, secretory granules in endocrine and neuroendocrine cells provide proper sites for biosynthesizing hormones in addition to serving as storage sites and vehicles for the transport of peptide hormones.

Key words: proteolytic processing, proopiomelanocortin, PC1, PC2, secretory granules

INTRODUCTION

In endocrine and neuroendocrine cells, most peptide hormones and neuropeptides are produced from larger, inactive precursors through limited endoproteolysis at pairs of basic amino acid residues during their transport through the exocytic pathway. This process is known as intracellular processing. In general, because peptide hormones acquire physiological activities for the first time after the proteolytic cleavage of their prohormones, the intracellular processing by processing enzymes (prohormone convertases) is an important step not only as a part of the biosynthetic process, but also as a regulatory step in diverse physiological processes in a variety of organisms. Among the prohormones, some contain a segment in which the peptide hormone sequence is located in the N-terminal region [e.g., pro-atrial natriuretic peptide (ANP), pro-gonadotropin-releasing hormone (GnRH), pro-pancreatic polypeptide, and pro-vasopressin] or in the C-terminal region [e.g., pro-corticotropin-releasing hormone (CRH), pro-melanin-concentrating hormone, pro-parathyroid hormone, and pro-somatostatin]. Some prohormones contain many different potential hormones joined together [e.g., pro-opiomelanocortin (POMC), pro-enkephalin, and pro-glucagon] or many similar peptides in the form of repeating units (e.g., pro-thyrotropin-releasing hormone). Others are processed to their active forms by liberating segments from both N- and C-terminal regions [e.g., pro-calcitonin, pro-calcitonin gene-related peptide (CGRP), and pro-glucagon)] or from a central portion in the proprotein (e.g., pro-insulin).

In this review, the molecular mechanisms underlying the intracellular processing of peptide hormone precursors (with POMC as a prototype) is discussed on the basis of information obtained recently.

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Discovery of prohormone convertases

Prohormone convertase, which had been sought for a

long time since the discovery of proinsulin, has been characterized by molecular techniques. This progress is largely attributable to the research on KEX2, the gene responsible for the cleavage at paired basic residues of pro-α-mating factor and pro-killer toxin in yeast (Steiner, 1991). Yeast Kex2 enzyme was shown to cleave mammalian prohormones in a number of mammalian cell lines (Thomas *et al*., 1988), but initially mammalian prohormone convertases could not be identified. Later, however, a homology search on the KEX2 gene revealed that furin, a human gene product previously thought to be a proto-oncogene, was shown high homology with Kex2 protease, and thus it was identified as a mammalian processing enzyme (Fuller *et al*., 1989). Based on this important breakthrough, mammalian prohormone convertases PC1 (also called PC3) and PC2 were cloned from mammalian endocrine cells by using a PCR strategy with primers based on the highly conserved nucleotide sequences encoding the active site of Kex2 (Seidah *et al*., 1990; Smeekens and Steiner, 1990). Other proprotein convertases were subsequently identified as PC4, PACE (paired basic amino acid residue-cleaving enzyme) 4, PC5 (also called PC6), and PC7 (Rouille *et al*., 1995; Seidah and Chretien, 1999; Zhou *et al*., 1999). Among these convertases, only PC1 and PC2 are considered to be involved in the cleavage of peptide hormones and neuropeptides.

Tissue-specific processing

In mammals, adrenocorticotropin (ACTH)-related peptides in corticotropes in the pars distalis and α-melanocyte-stimulating hormone (α-MSH)-related peptides in melanotropes in the pars intermedia of the pituitary are known to be produced posttranslationally by intracellular proteolytic cleavage of the large precursor molecule known as POMC. Nevertheless, the processing of POMC differs between these 2 lobes: in corticotropes ACTH, β-lipotropic hormone (β-LPH), and a 16-K fragment are the major end products; whereas in melanotropes ACTH is processed further into α-MSH and corticotropin-like intermediate peptide (CLIP), and

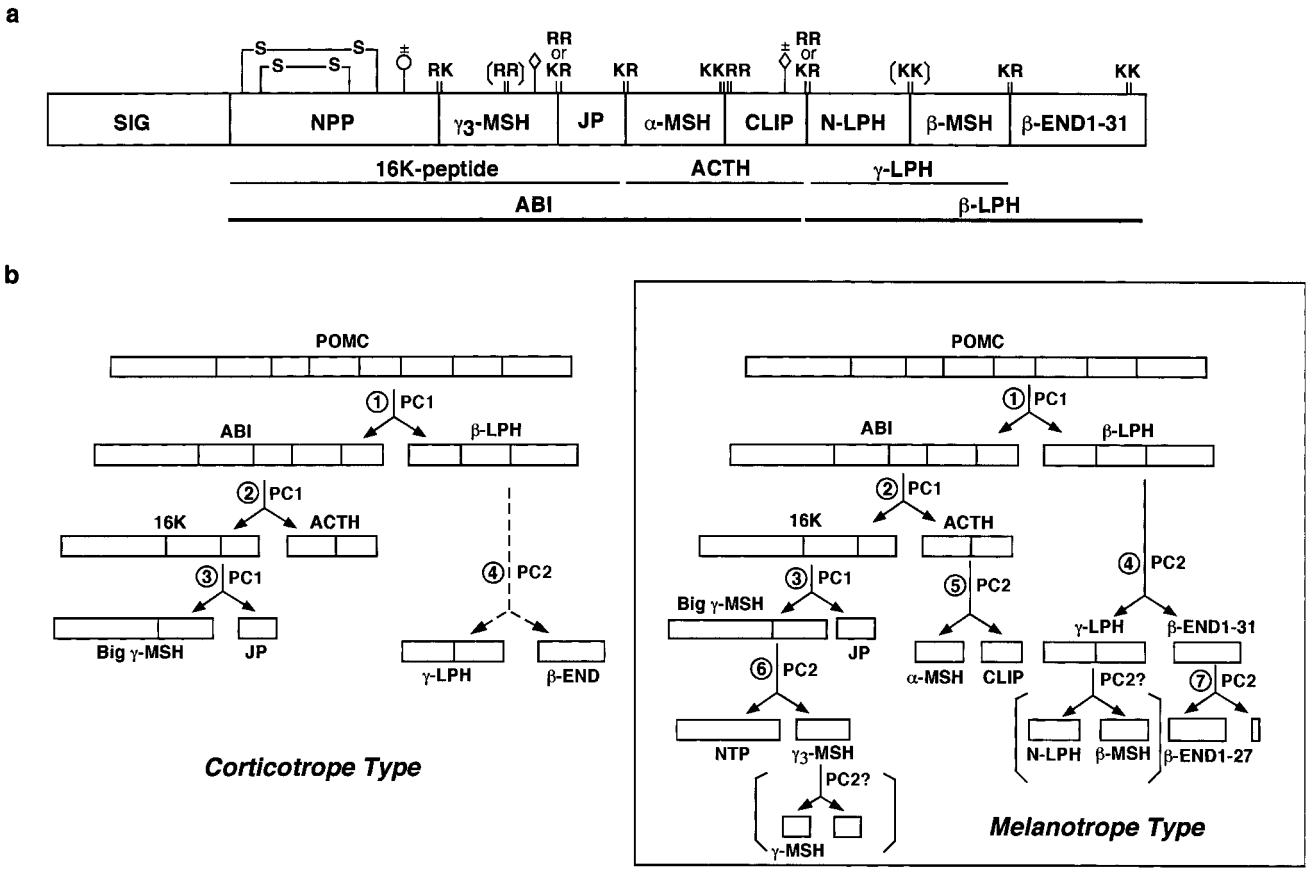

Fig. 1. (a) The molecular structure of POMC. Paired basic residues (e.g., KR) indicate cleavage sites where each peptide is released. In the N-terminal region, 2 disulfide bonds are present. Diamonds and circles indicate *N*-glycosylation and *O*-glycosylation sites, respectively. Paired basic residues in square brackets are missing in rat and mouse. K: lysine; R: arginine. SIG: signal peptide; NPP: N-terminal peptide of POMC; JP: joining peptide; CLIP: corticotropin-like intermediate peptide; LPH: lipotropic hormone; END: endorphin; ABI: ACTH-biosynthetic intermediate. (b) Tissue-specific processing of POMC by PC1 and PC2. The numbers indicate the sequence of the processing steps. Proteolytic processing of the corticotrope type or the melanotrope type is achieved by the cleavage-specificity of PC1 and PC2. Whether the processing steps indicated in square brackets occur or not depends on the animal species. These cleavages are presumed to be made by PC2, but this has not yet been determined.

β-LPH is processed almost completely into β-endorphin (Fig. 1) (Eipper and Mains 1980; Rosa *et al*., 1980; Chretien *et al*., 1989). In addition, POMC is produced in the arcuate nucleus of the hypothalamus, and the POMC products produced there are similar to those of the melanotropes. Thus there is tissue-specific processing of this common precursor. Tissue-specific processing also occurs in the case of the processing of proglucagon and prosomatostatin (Dickerson and Noel, 1991).

Both PC1 and PC2 are expressed in endocrine and neuroendocrine cells and cleave at paired basic residues of prohormones. The cleavage-specificity of PC1 and PC2 in POMC processing was revealed by cellular transfection experiments: PC1 cleaves POMC into ACTH and β-LPH, whereas PC2 subsequently cleaves ACTH and β-LPH to α-MSH and β-endorphin, respectively (Benjannet *et al*., 1991; Thomas *et al*., 1991). This finding was confirmed by another experiment (Zhou *et al*., 1993): when antisense PC1 cRNA was introduced into AtT-20 cells, a mouse corticotrope tumor cell line, the production of ACTH was inhibited. On the other hand, α-MSH was newly cleaved from ACTH when the cells were transfected with the PC2 gene. In addition to these data, histological evidence supports this differential processing: PC1 was expressed in corticotropes, whereas both PC1 and PC2 were found in melanotropes (Seidah *et al*., 1991; Day *et al*., 1992). The tissue-specific processing of POMC is thus due to the differential expression of PC1 and PC2 in these 2 cell types (Fig. 1). Such roles for these convertases were confirmed by the generation and analysis of PC1 or PC2 null mice (Laurent *et al*., 2002; Zhu *et al*., 2002).

In POMC cells of the amphibian pituitary gland, however, PC1 and PC2 do not always display the cell-specific expression observed in mammals (Kurabuchi and Tanaka, 1997). In the pars distalis, immunoreactivity of the convertases showed a different pattern among several anuran amphibians: both PC1 and PC2 were observed in the corticotropes in *Rana brevipoda porosa*, whereas either PC1 or PC2 was found in these cells in other species; although PC2-immunopositive cells did not express α-MSH (Kurabuchi and Tanaka, 1997). Moreover, a considerable amount of N-terminal peptide of POMC not containing γ-MSH, which is present only in the pars intermedia of mammals, was purified from the pars distalis of the adult bullfrogs (Iwamuro *et al*., 1992). If this peptide was generated as in the same way as in the pars intermedia (Ekman *et al*., 1981), PC2 would have to be present in the bullfrog pars distalis. It remains, accordingly, unsolved whether the cleavage-specificity of the prohormone convertases in amphibians is similar to that in mammals. This issue is of interest for the field of comparative endocrinology.

Comparative aspect of prohormone convertases

In a recent study, we created a cDNA library from bullfrog (*Rana cabesbeiana*) pituitary glands to clone and sequence 2 cDNAs encoding PC1 and PC2 (Yaoi *et al*., 2003). Both PCs were structurally characterized as having a signal peptide, a prosegment, a catalytic region, a P-domain, and a variable C-terminal region (Figs. 2 and 3). The predicted amino acid sequence of these PCs showed high homology with the corresponding sequences of various other species. The bullfrog PC1 cDNA was predicted to encode a 736-amino acid protein with a putative 26-residue signal peptide; and the PC2 one, a 638-amino acid protein with a putative 23-residue signal peptide. It has been shown that PCs are first synthesized as inactive precursor enzymes, which undergo autocatalytic excision or furin-dependent cleavage of their N-terminal prosegment via cleavage at a specific Arg-Arg-Ser-Arg-Arg and Arg-Ser-Lys-Arg in the PC1 protein, and at Lys-Arg-Arg-Arg and Arg-Lys-Lys-Arg in the PC2 protein (Muller and Lindberg, 1999). Although there are 2 possible cleavage sites in the prosegment of both bullfrog PCs, the actual sites are considered to be Arg-Thr-Lys-Arg^{110} in the PC1 protein and Arg-Lys-Lys-Arg^{109} in the PC2 protein, because these sites correspond to the cleavage sites of prosegment in mammalian PCs (Benjannet *et al*., 1992; Zhou and Lindberg, 1993). The mature PC1 protein contains 626 amino acids with 2 putative *N*-glycosylation sites, whereas the mature PC2 one contains 529 amino acids with 3 such sites. The catalytic domain is well conserved, especially in the regions surrounding the catalytic triad of PC1 (Asp^{168}, His^{209}, and Ser^{383}) and of PC2 (Asp^{167}, His^{208}, and Ser^{384}). In the P-domain of both PCs, the canonical integrin binding Arg-Gly-Asp sequence is also present, as found in all mammalian convertases except PC7 (Seidah and Cretien, 1992; Seidah *et al*., 1996) though this sequence is absent from the PC2 protein of *Rana ridibunda* (Vieau *et al*., 1998).

The amino acid sequence homology between the bullfrog PC1 and *R. ridibunda* (Gangnon *et al*., 1999), human (Creemers *et al*., 1992), mouse (Seidah *et al*., 1991), rat (Hakes *et al*., 1991), anglerfish (Roth *et al*., 1993), *Amphioxus* (Oliva *et al*., 1995), *Aplysia* (Gorham *et al*., 1990), and Hydra (Chan *et al*., 1992) PC1 was 95.6%, 62.7%, 67.2%, 66.8%, 68.1%, 56.1%, 53.5%, and 41.5%, respectively. Similarly, the amino acid sequence homology between the bullfrog PC2 and *R. ridibunda* (Vieau *et al*., 1998), *Xenopus* (Braks *et al*., 1992), human (Smeekens *et al*., 1990), mouse (Seidah *et al*., 1991), rat (Hakes *et al*., 1991), *Amphioxus* (Oliva *et al*., 1995), ascidian (AB086187), *Aplysia* (Chun *et al*., 1994), and snail (Smit *et al*., 1992) PC2 was 95.5%, 90.4%, 85.9%, 84.2%, 84.2%, 71.6%, 64.6%, 60.2%, and 61.0%, respectively.

In Amphioxus, a primitive chordata, PC1 and PC2 mRNAs are distributed thought the body, but the expression ratio of PC1 and PC2 varied depending upon the body region: 1:1 in the cranial region, 5:1 in the mid-body region, and 1:5 in the caudal region (Olviva *et al*., 1995). Amphioxus PC1 and PC2 are considered to be involved in the processing of many neuropeptides including pro-insulin-like peptide (a homologue of mammalian insulin/insulin-like growth factor), but the substrate-specificity of these convertases and

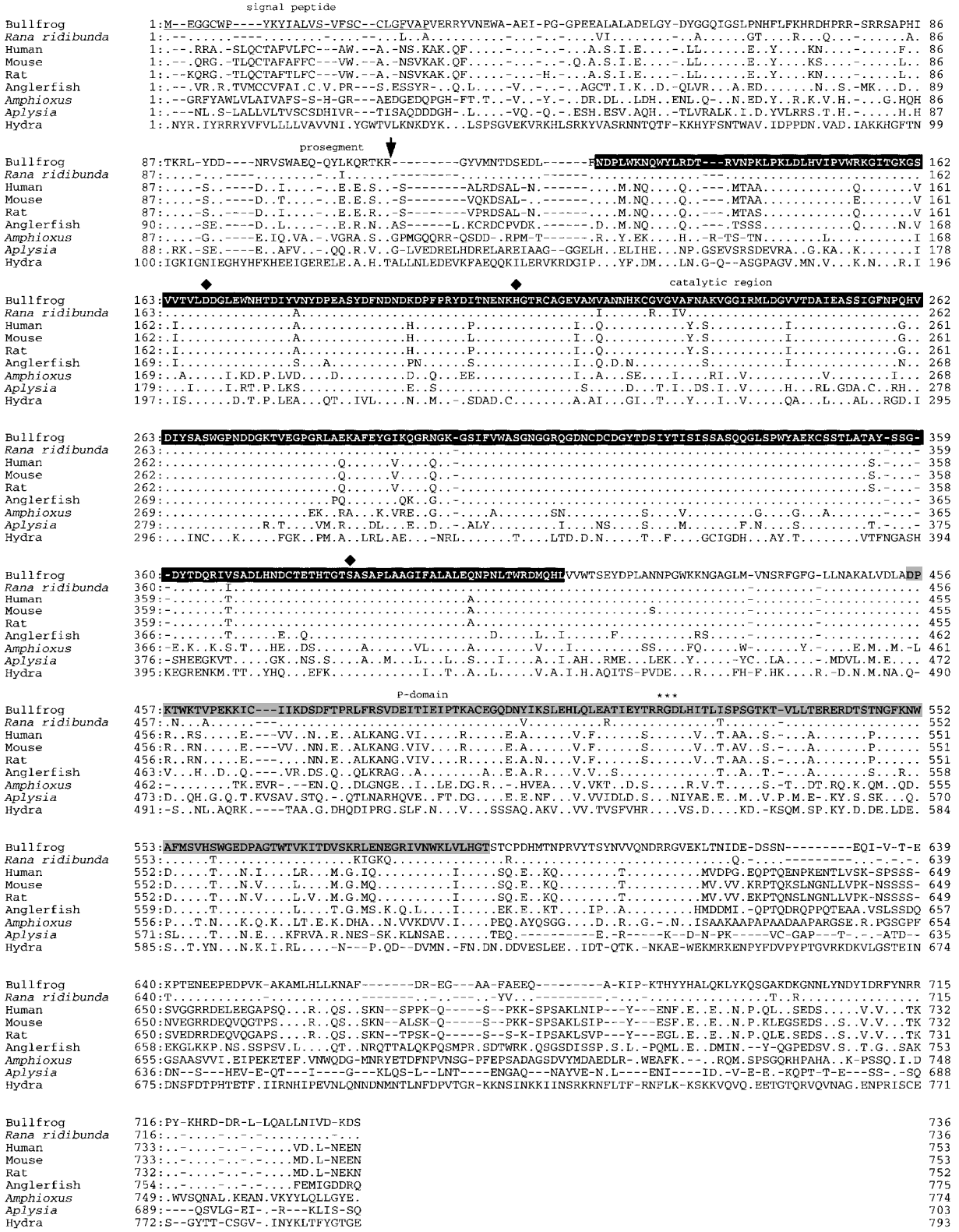

Fig. 2. Comparison of the predicted amino-acid sequence of the bullfrog (*Rana catesbeiana*) PC1 with those sequences of other vetebrates PC1s. The amino acid residues that match those of bullfrog PC1 are shown as dots. Gaps, indicated by dashed lines, have been introduced to obtain maximum homology. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed by black and gray boxes, respectively. Diamonds indicate Asp, His, and Ser of active site of catalytic region. Canonical integrin binding sequence indicates by asterisks. The sequence for *Rana ridibunda* (Gangnon *et al*., 1999), human (Creemers *et al*., 1992), mouse (Seidah *et al*., 1991), rat (Hakes *et al*., 1991), anglerfish (Roth *et al*., 1993), *Amphioxus* (Oliva *et al*., 1995), *Aplysia* (Gorham *et al*., 1990), and Hydra (Chan *et al*., 1992) PC1s are shown. From Yaoi *et al*. (2003).

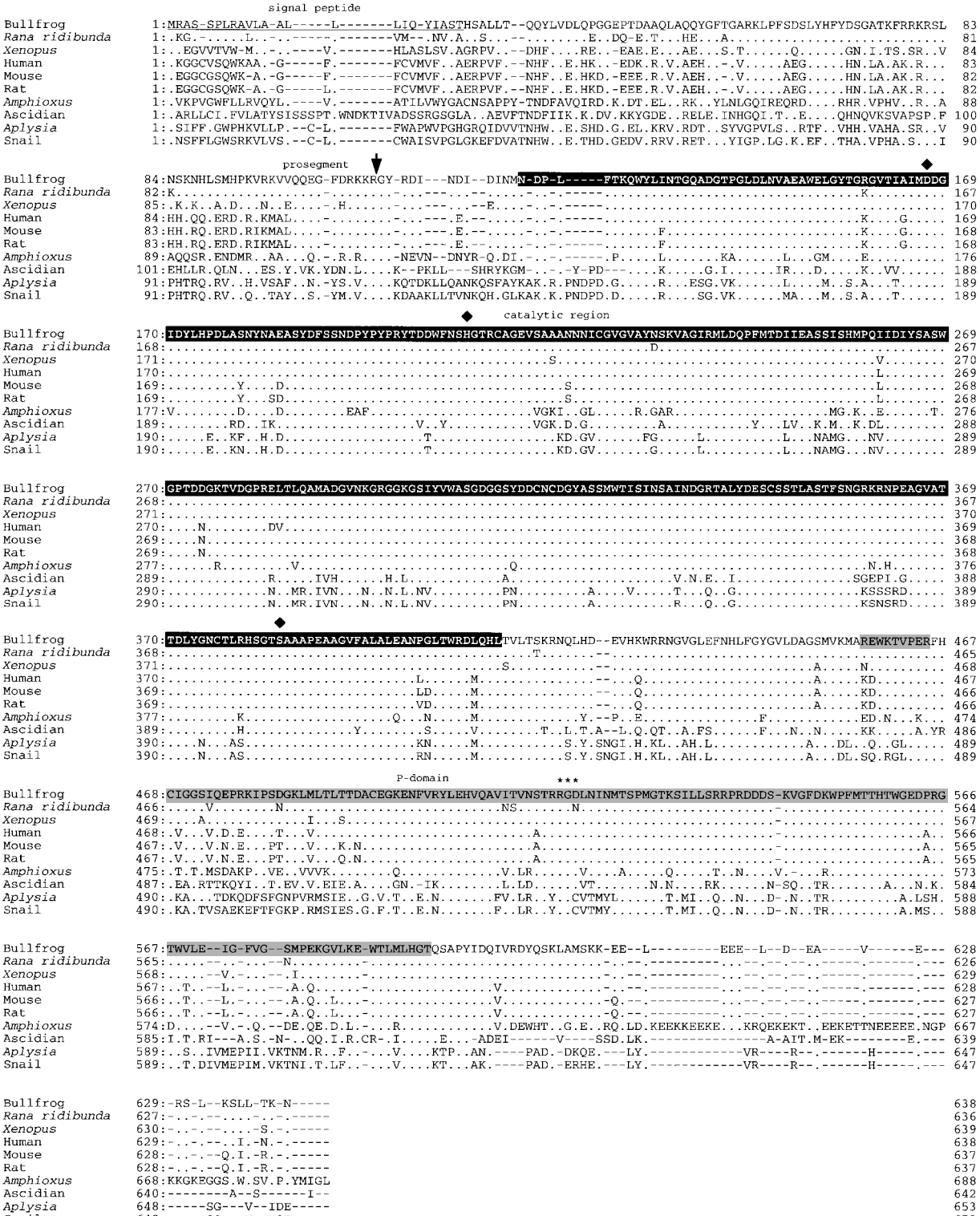

Fig. 3. Comparison of the predicted amino-acid sequence of the bullfrog (*Rana catesbeiana*) PC2 with those sequences of other vetebrates PC2s. The amino acid residues that match those of bullfrog PC2 are shown as dots. Gaps, indicated by dashed lines, have been introduced to obtain maximum homology. The underlined letters indicate the amino acids comprising the signal peptide sequence. The putative cleavage site of prosegment indicates by arrow. Catalytic region and P-domain are enclosed by black and gray boxes, respectively. Diamonds indicate Asp, His, and Ser of active site of catalytic region. Canonical integrin binding sequence indicates by asterisks. The sequence for *Rana ridibunda* (Vieau *et al*., 1998), *Xenopus* (Braks *et al*., 1992), human (Smeekens *et al*., 1990), mouse (Seidah *et al*., 1991), rat (Hakes *et al*., 1991), *Amphioxus* (Oliva *et al*., 1995), ascidian (AB086187), *Aplysia* (Chun *et al*., 1994), and snail (Smit *et al*., 1992) PC2s are shown. From Yaoi *et al*. (2003).

their exact expression sites are not fully documented. Because it is known that Amphioxus has Hatschek's pit organ, which is regarded as being an adenohypophyseal homologue, it is interesting to consider whether Hatschek's pit organ expresses prohormone convertases. This issue remains to be solved.

In the ascidian *Halocynthia roretzi*, immunohistochemistry using mammalian PC1 and PC2 antisera demonstrated that both convertases are present in some nerve cells in cerebral ganglon and dorsal strand (Kawahara *et al*., 2003). Because POMC-derived peptides are also produced in the cerebral ganglon and dorsal strand (Kawahara *et al*., 2003), PC1 and PC2 may be involved in POMC processing as well as higher vertebrates. Recent study isolated a PC2 cDNA clone (AB086187) with its amino acid sequence showing approximately 65% homology with the sequences of vertebrate PC2 cDNAs, but PC1 cDNA has not yet cloned. Furthermore, two forms of GnRH, namely tGnRH-I and tGnRH-II, were isolated from the neural complex of the ascidian *Chelyosome productum* (Powell *et al*., 1996). In vertebrates, PC2 has been postulated as a candidate enzyme for pro-GnRH processing (Wetsel *et al*., 1995). Consequently, it is important to search localization of ascidian PC2 mRNA and tGnRHs in the neural complex for determining the substrate-specificity of PC2.

Hydra PC1 is expressed predominately in nerve cells of the body column, but some other nerve cells in the head and peduncle regions react with antisera against Arg-Phe-NH_2, cholecystokinin, substance P, neurokinin, and oxytocin (Chan *et al*., 1992). It seems likely that the Hydra head and peduncle regions contain a PC1-like molecule, but this molecule has not yet been identified in these regions.

In the Moluscus, *Aplysia* PC1 and PC2 have been identified using the cDNA cloning (Ouimet *et al*., 1993; Chun *et al*., 1994; Gorham *et al*., 1996). These convertases play an important role in the processing of the egg-laying hormone precursor that is synthesized in the neuroendocrine bag cells and in the processing of egg-laying hormone-related precursors in the atrial gland, an exocrine organ. In the snail, on the other hand, expression of PC2 mRNA is restricted to the nerve cells of the cerebral ganglia that express a variety of prohormones and neuropeptides including APGWamide and moluscan insulin-related peptides (Smit *et al*., 1992). The fact that many efforts to demonstrate the existence of snail PC1 have failed raises the possibility that the PC1 molecule is not present in the snail. Considering that *Aplyisa* and the snail belong to the same phylum, Moluscus, the possible lake of PC1 in the snail generates an interesting idea concerning their evolution: that the snail lost a PC1-like convertase as these two species evolved along separate paths (Spijker *et al*., 1999).

By the use of RT-PCR, both bullfrog PC1 and PC2 mRNAs were found to be expressed in the pars distalis, pars neurointermedia, brain, and pancreas of the bullfrog (Yaoi *et al*., 2003). This finding is mostly consistent with the reports concerning mammalian species (Seidah *et al*., 1990; Vieau *et al*., 1998; Gangnon *et al*., 1999). It is of interest that only PC1 mRNA was expressed in the bullfrog stomach and intestine, whereas PC2 mRNA was the only type in the testis. The expression of PC1 mRNA may be involved in the processing of the intestinal type of proglucagon (Dhanvantari *et al*., 1996) and in the processing of progastrin in the stomach (Macro *et al*., 1996), whereas the PC2 may have some effect on the posttranslational processing in the bullfrog testis. However, considering that only PC4 has been identified in the mammalian testis (Nakayama *et al*., 1992; Seidah *et al*., 1992; Mbikay *et al*., 1999; Li *et al*., 2000), it is possible that the PC related with PC4 is present in the bullfrog testis. Further studies are necessary to identify another type of PC in the frog testis, and to compare substrate-specificity between PC2 and PC4. Although PC2 mRNA has been detected in the pars distalis of the adult bullfrog by RT-PCR, it appears that PC2 mRNA was not translated there, because PC2 protein is not detected in the pars distalis by immunohistochemical staining (Kurabuchi and Tanaka, 1997) or by *in situ* hybridization (Yaoi *et al*., 2003).

Using *in situ* hybridization, Yaoi *et al*. (2003) found PC1 mRNA to be expressed in the pars distalis and the pars intermedia, but detected PC2 mRNA only in the pars intermedia, of the adult bullfrog. Similar results were obtained with *Rana ridibunda* (Vieau *et al*., 1998; Gangnon *et al*., 1999). An *in situ* hybridization experiment with the antisense RNA of *Xenopus* PC2 also revealed that PC2 mRNAs were predominantly expressed in the pars intermedia of the *Xenopus* pituitary (Braks *et al*., 1992). The expression pattern of PC1 and PC2 mRNAs in the bullfrog pituitary is also in good agreement with the immunohistochemical findings showing that the pars distalis expressed PC1 protein and the pars intermedia contains both PC1 and PC2 proteins (Kurabuchi and Tanaka, 1997). In addition, in the latter the degree of expression of PC2 was higher than that of PC1, being consistent with data from mammals (Day *et al*., 1992). Interestingly, PC2 mRNA was expressed in the α-MSH-positive corticotropes of the bullfrog tadpole. In mammals, it is known that, during development, PC2 mRNA is also expressed in corticotropes in the pars distalis until neonatal week 3, thereby producing α-MSH (Marcinkiewicz *et al*., 1993). The α-MSH is considered to have stimulatory effects on intrauterine growth (Swaab *et al*., 1976) and growth-stimulating effects on the adrenal zona glomerulosa (Robba *et al*., 1986). However, the exact role of α-MSH during development remains unclarified. Similarly, in the urodele amphibian *Ambystoma*, corticotropes produce α-MSH during the larval period (Dores *et al*., 1989, 1990, 1993). The production of α-MSH during the neonatal period or larval period is considered to be a general phenomenon, and to vary with fluctuation in PC2 expression. However, no PC2 mRNA was detected by *in situ* hybridization in corticotropes of the adult bullfrogs, although α-MSH-immunoreactivity was detected in these cells (Yaoi *et al*., 2003). This may imply that very little PC2 mRNA is translated or that α-MSH, which had been produced at larval stages, remained stored in the

secretory granules. In this connection, α-MSH was found in some of the ACTH-positive secretory granules in corticotropes of the adult rat (Tanaka and Kurosumi, 1986), implying that PC2 was sorted to certain secretory granules.

An *in situ* hybridization study showed that PC1 mRNA was expressed in the gonadotropes of the bullfrog (Yaoi *et al*., 2003). It is conceivable that proprotein processing does not take place in these cells. However, it is possible that other proteins such as granin family proteins with proteolytic cleavage sites are contained in the secretory granules and that these proteins are cleaved by PC1. Indeed, Uehara *et al*. (2001) reported that both PC1 and PC2 were expressed in the rat pituitary gonadotrope, suggesting that PC2 may be involved in the proteolytic processing of secretogranin II and that both PC1 and PC2 may be necessary to process chromogranin A (CgA).

Further processing of peptide hormones for expression of biological activity

Following processing by PC1 and/or PC2, peptide hormones have 2 basic residues at their C-terminus. These paired basic residues are removed by carboxylpeptidase E (CPE), which thereby produces the active forms of some peptides (Fig. 4) (Fricker *et al*., 1991). Some other peptide hormones are activated by further modification such as amidation at the C-terminus or acetylation at the N-terminus. The modification of peptide hormones by these enzymes is an important step for activating them. For example, α-MSH, which is involved in background-adaptation by causing a change in skin-color, occurs in 2 forms: acetylated and non-acetylated forms. In *Xenopus laevis*, skin-color is mainly regulated by the biologically active form, acetylated α-MSH; whereas in the lizard *Anolis carolinensis*, the skin-color is regulated by a large amount of non-acetylated α-MSH having low potency and a short half-life. By utilizing non-acetylated α-MSH, *Anolis* seems to respond rapidly for the background-light (Dores *et al*., 1994). In contrast, when β-endorphin is acetylated, the opiate activity of this peptide is completely eliminated (Smyth *et al*., 1979; Deakin *et al*., 1980). Interestingly, neuronal cells secrete non-acetylated β-endorphin, whereas cells in the pars intermedia secrete acetylated β-endorphin (Jenks *et al*., 1988).

Recently, it was reported that a point mutation in the CPE gene causes the genetic disorder of obesity in *fat/fat* mice (Naggert *et al*., 1995). Because in the *fat/fat* mouse Ser substitutes for Pro at residue 202 in CPE, the mutant CPE does not have normal enzyme activity, thereby producing insulin intermediates retaining their paired basic residues. As a result, the mouse secretes inactive insulin and develops an obesity/diabetes. Song and Fricker (1995) noticed that carboxylpeptidase-like activity did not completely disappear in the *fat/fat* mouse, and then identified a new carboxylpepidase, carboxylpeptidase D, as being responsible for the residual activity. In the *fat/fat* mouse, this enzyme presumably compensates for the inactive CPE.

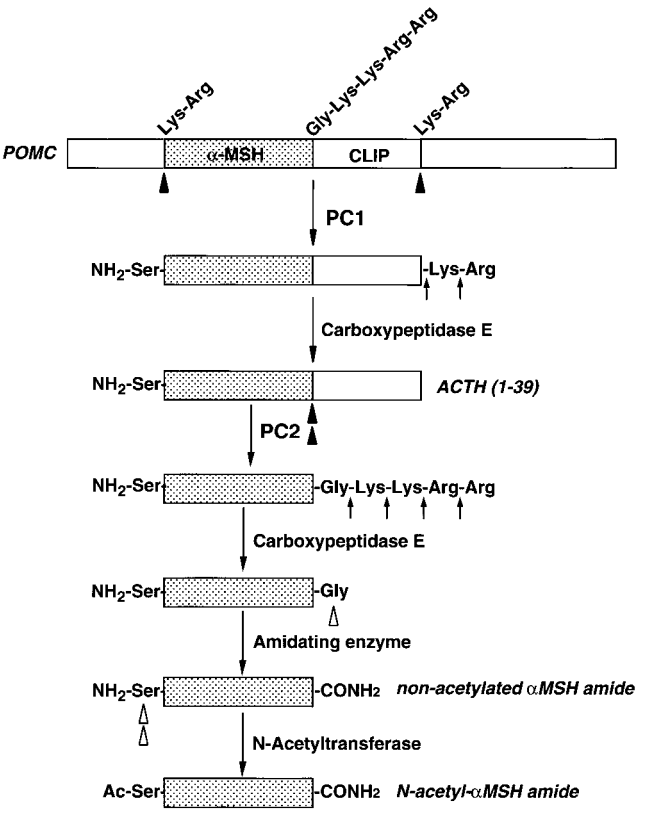

Fig. 4. Proteolytic cleavage stages of POMC to α-MSH.

In which intracellular sites does the proteolytic cleavage occur?

The biosynthesis and processing of peptide hormones take place along the secretory route of the prohormone from the rough-endoplasmic reticulum, the site of synthesis, to the trans-Golgi network (TGN), similar to the case of other secretory proteins. At the TGN, peptide hormones are segregated from the other proteins, such as lysosomal enzymes and constitutive secretory proteins including plasma membrane proteins. The immunocytochemical identification of start and end sites of the cleavage on the same cell sections is of considerable importance for a proper understanding of the mechanism of processing. In POMC processing, Tooze *et al*. (1987) showed, by using an antibody that was able to specifically recognize a cleavage site of POMC, that some POMC was packaged into secretory granules prior to its cleavage. On the other hand, Schnabel *et al*. (1989) concluded that processing of POMC began in the trans-most cisterna of the Golgi apparatus based on their detection of processed products in this compartment. However, neither study examined whether or not both the precursor and mature peptides were present in the same compartments in POMC cells. Addressing this point by using antibodies specific for the cleavage site of POMC and for a mature peptide [amidated joining peptide (JP)], we detected both the precursor POMC and amidated JP in the same secretory granule in POMC cells (Fig. 5) (Tanaka and Kurosumi, 1992;

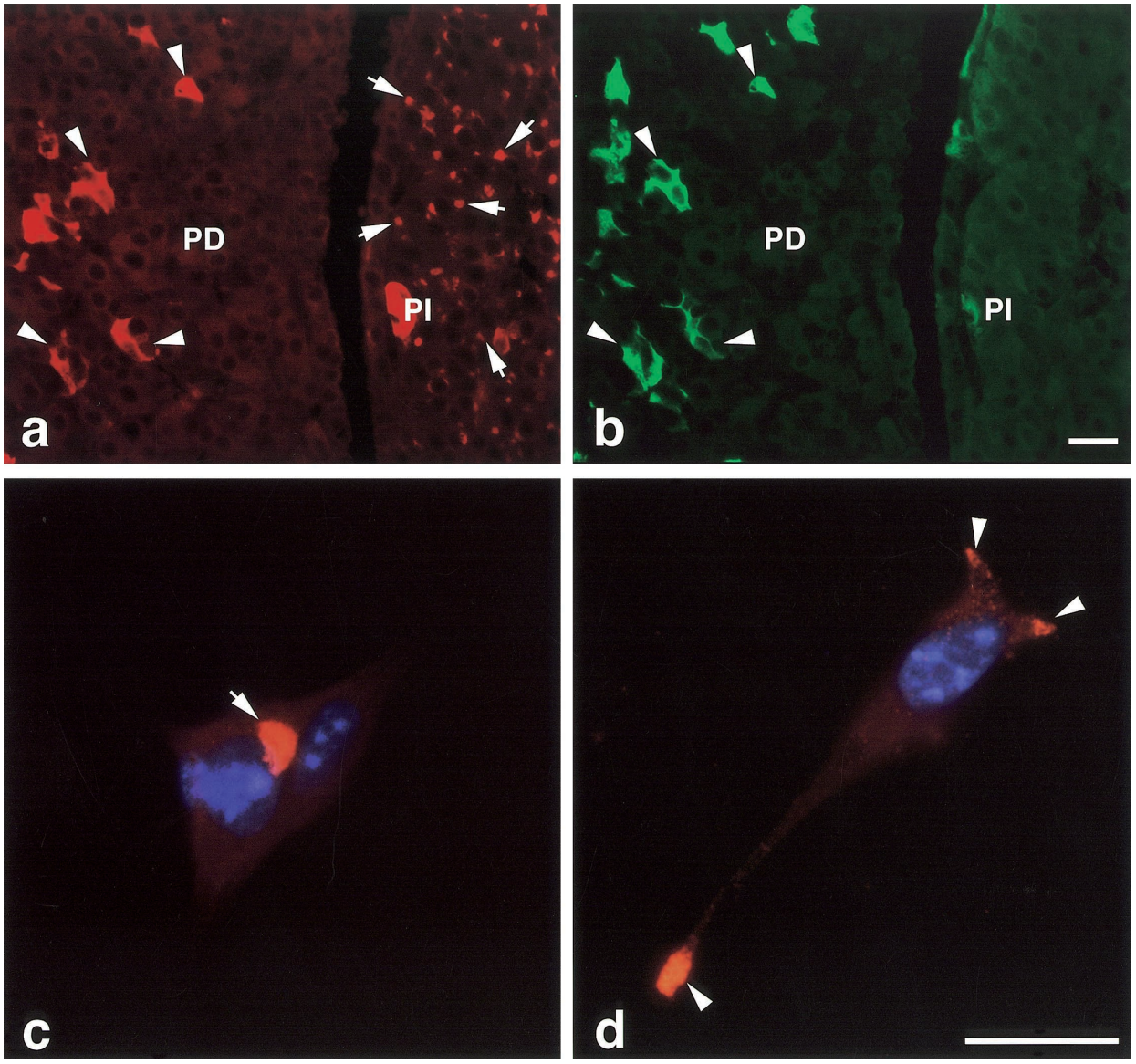

Fig. 5. Immunofluorescence images showing localization of POMC and amidated joining peptide (JP) in the pituitary gland (a, b) and AtT-20 cells (c, d). Both POMC and amidated JP (arrowheads) are visible throughout the cytoplasm in several corticotropes (a, b), whereas POMC (arrows) is visible as a strong spot in the perinuclear region in melanotropes (a). PD: the pars distalis; PI: the pars intermedia. In AtT-20 cells, the intense staining for POMC (arrow) is found exclusively in the Golgi region (c), and the punctate staining for amidated JP (arrowheads) is distributed at the tips of cell processes (d). The blue color is nuclear DNA stained with DAPI. Bar: a, b=20 μm, c, d=20 μm. c, d from Tanaka *et al*. (1997).

Tanaka *et al*., 1997). The corticotropes of the adult rat pituitary have 2 distinct types of secretory granules, i.e., small electron-dense and large electron-lucent ones. The former was found not only near the Golgi apparatus, but also among the peripheral secretory granules arranged in a single row along the cell membrane. The latter was observed mainly among the peripheral secretory granules. POMC was localized in the electron-dense secretory granules, whereas amidated JP was found in the electron-lucent secretory granules in the same ultrathin cryosections (Fig. 6). Colocalization of POMC and its cleaved peptides in the same secretory granules was rarely observed. Only POMC immunolabeling was detected in the Golgi cisternae. In AtT-20 cells, POMC was detected predominantly in the secretory granules around the Golgi apparatus and in condensing secretory materials on the rims of the *trans*-Golgi cisternae, whereas amidated JP was predominant in the secretory granules at the tips of the cell processes. Actually, both PC1 and PC2 were found in secretory granules of pituitary POMC cells (Kurabuchi and Tanaka, 1997; Tanaka *et al*., 1997) and pancreatic endocrine cells (Malide *et al*., 1995; Tanaka *et al*., 1996). In addition, POMC and α-MSH appear in separate secretory granules in melanotropes of the adult rat (Tanaka *et al*., 1991), and POMC is therefore thought to be cleaved mainly in secretory granules coordinately with their maturation.

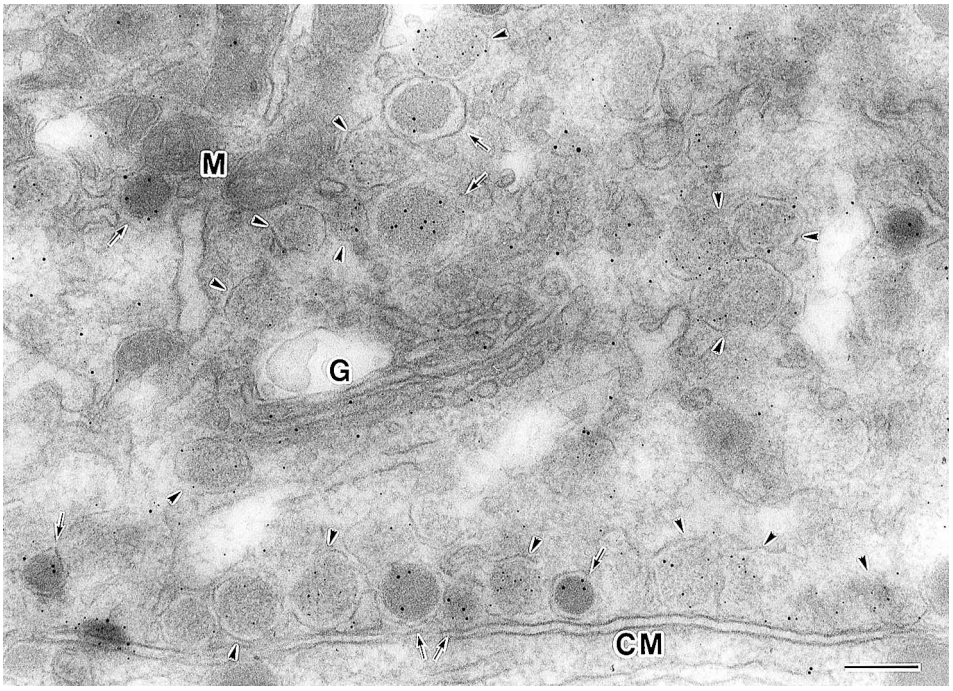

Fig. 6. Immunoelectron micrograph showing localization of POMC and amidated joining peptide (JP) in the rat corticotrope cells. Two kinds of secretory granules, dense (arrowheads) and lucent ones (arrows), are visible. Large immunogold particles indicating the presence of POMC are visible in both the Gogli cisternae and the dense granules, while small particles indicating the presence of amidated JP are predominately in the lucent granules. G: the Golgi apparatus, M: mitochondria, CM: cell membrane. Bar=0.2 μm. From Tanaka *et al*. (1997).

Acidified secretory granules provide the proper environment for activating PC1 and PC2

Enzymological studies have shown that the prohormone convertases PC1 and PC2 are most active at a relatively acidic pH, i.e., pH 5.0 - 6.5 (Davidson *et al*., 1988; Rufaut *et al*., 1993; Zhou and Lindberg, 1993; Lamango *et al*., 1996); whereas furin, which is localized mainly in the TGN and is involved in the cleavage of precursors for constitutively secreted proteins and plasma membrane proteins rather than prohormones, has a neutral pH optimum (Hatsuzawa *et al*., 1992; Molloy *et al*., 1992, 1994). These facts indicate that there may be a close relationship between the optimum pH of the enzymes and their subcellular localization. It is therefore important to know whether or not the pH of secretory granules is adequate for the action of prohormone convertases. Elegant studies by Orci *et al*. (1986, 1987, 1994) using DAMP [3-(2,4-dinitroanillino)-3'amino-N-methyldipropylamine] as a pH probe showed that in pancreatic β-cells, conversion of proinsulin to insulin occurred coordinately with the acidification of maturing secretory granules. Similarly, Tanaka *et al*. (1997) demonstrated, also using the DAMP method, that in AtT-20 cells the granular pH varied from one granule to another over a range of 5.2 to 7.0. These granular pH values are in good agreement with those for granules in pancreatic β-cells reported by Orci *et al*. (1986, 1987, 1994). Moreover, there was a tendency that the labeling density for DAMP correlated negatively with that for POMC, and positively with that for amidated JP (Figs. 7 and 8) (Tanaka *et al*., 1997). Taken together with the observations of the colocalization of POMC and amidated JP in the same granule noted above, POMC processing in AtT-20 cells is presumed to occur mainly in acidifying secretory granules.

Nelson and Taiz (1989) discovered that the internal acidic environment in the secretory pathways was generated by a vacuolar-type-H^+-ATPase (V-ATPase). V-ATPase was later purified from the Golgi apparatus (Moriyama and Nelson, 1989) and secretory granules (Moriyama and Futai, 1990) and characterized. From various experiments, V-ATPase was found to play an important role in intracellular processes such as trafficking, endocytosis, recycling, hydrolysis and secretion of protein in addition to the processing of proteins. The V-ATPase molecule is organized into 2 main parts (Schoonderwoert and Martens, 2001): a cytoplasmic ball-like structure (V_1) and a membrane sector (V_0) bridged by a cytoplasmic stalk. The peripheral catalytic V_1 sector (molecular mass of ~840 kDa) is responsible for the hydrolysis of ATP, whereas the 260-kDa integral V_0 sector is responsible for proton translocation across the membrane. The V_1 sector consists of 8 different subunits (A–H). The V_0 sector is formed by 5 different subunits, namely, subunits a and d, and proteolipids c, c', and c''. Because V-ATPase is made of multi-components, proper assembly of these subunits is required to drive the proton pump. Consequently, it will be a challenging issue to elucidate the regulatory mechanism of its assembly.

Bafilomycin A_1 is known to be a specific inhibitor of V-ATPase in chromaffin granules, lysosomes and the Golgi

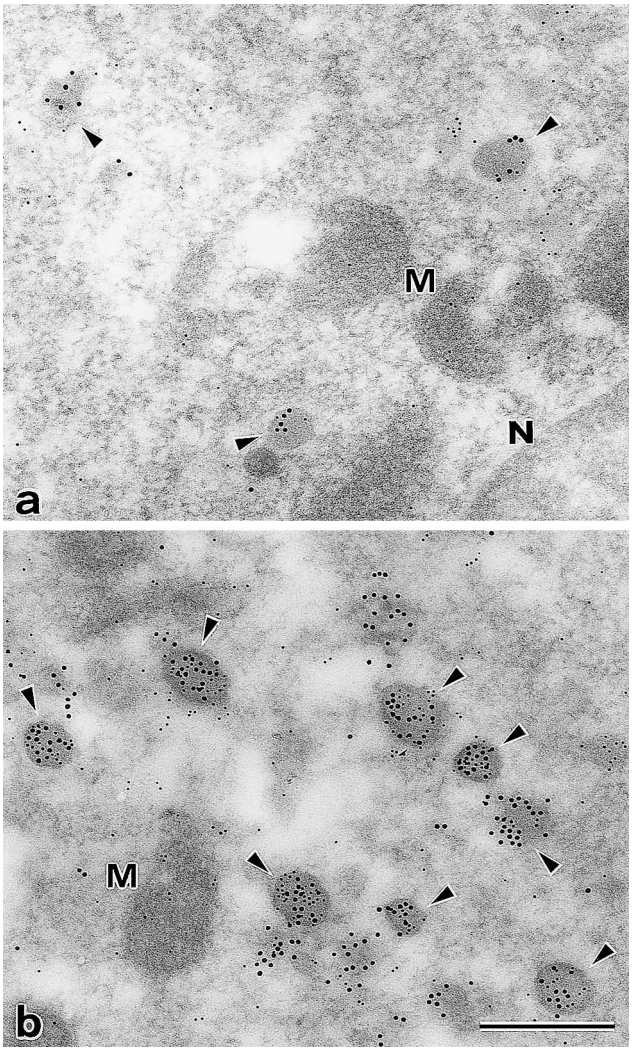

Fig. 7. Localization of DAMP (small gold particles) and POMC (large gold particles) (a), and of DAMP (small gold particles) and amidated JP (large gold particles) (b) on the same ultrathin sections of AtT-20 cells. The labeling density of DAMP was lower in POMC-positive granules than in amidated JP-positive granules. Arrowheads: secretory granules, M: mitochondria, N: nucleus. Bar=0.25 μm. From Tanaka *et al.* (1997).

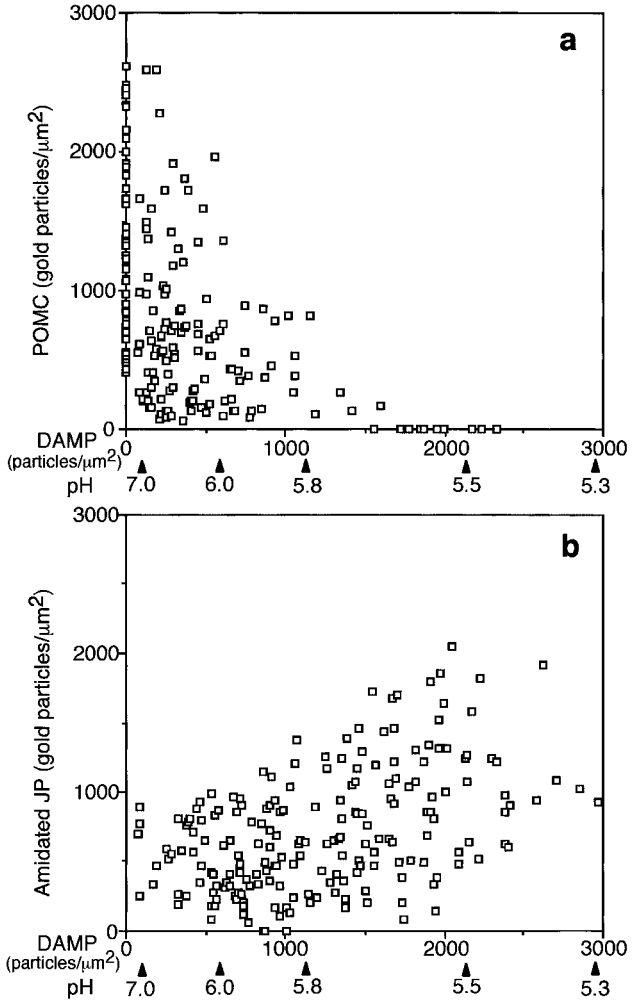

Fig. 8. The relationship between DAMP accumulation and proteolytic cleavage of POMC. There is (a) a slight negative correlation between DAMP and POMC labeling and (b) a slight positive correlation between DAMP and amidated JP labeling. The rate of proteolytic cleavage of POMC to amidated JP increases in correlation with the acidification of secretory granules. The pH values are estimated from the incorporation of DAMP. Modified after Tanaka *et al.* (1997).

apparatus (Bowman *et al.*, 1988; Moriyama and Nelson, 1989), and the application of bafilomycin A_1 would therefore be expected to define the role of V-ATPase-mediated acidification in the secretory pathways. Pulse-chase experiments have shown that bafilomycin A_1 inhibited POMC processing, and electron microscopic analysis detected POMC, but not amidated JP, in bafilomycin A_1-treated cells (Tanaka *et al.*, 1997). Since in the drug-treated cells the accumulation of DAMP in granules was blocked to background levels, it appears that the V-ATPase inhibitor neutralized the granules, thereby inhibiting POMC processing. On the other hand, Mains and May (1988) reported that chloroquine, which also dissipates intracellular acidic environments, but by a mechanism different from that of bafilomycin A_1, did not inhibit POMC processing. These data indicate that a acidic environment is not essential for the processing of POMC by PC1. This view is indirectly supported by enzymological studies showing that although PC1 has an acidic pH optimum, it still exhibited some activity around neutral pH (Davidson *et al.*, 1988; Rufaut *et al.*, 1993; Zhou and Lindberg, 1993), and is in line with the data of Schnabel *et al.* (1989) showing that POMC processing began in the TGN. Certainly, the data described above clearly demonstrate that proteolytic processing of POMC in AtT-20 cells occurs coordinately with the maturation of secretory granules.

Regulation of the activation of prohormone convertase

Because the precursors of PC1 and PC2 are present and become active forms after the cleavage of their prosegment, this process is considered to be involved in the regu-

lation of prohormone processing (Muller and Lindberg, 1999). Pro-PC1 proteins are transformed into their active forms within several min, whereas the transformation of pro-PC2 proteins into their active forms requires 1–2 hr (Zhou and Mains, 1994). The 7B2 protein was found to participate as a chaperone in the regulation of PC2 activation and to control the timing for activating the convertase (Martens *et al*., 1994). 7B2 is a member of the granin family of acidic, neuroendocrine-specific secretory granule proteins that include chromogranin A and secretogranin II (Huttner *et al*., 1991; Mbikay *et al*., 2001). Because 7B2 was expressed in parallel with the expression of POMC in *Xenopus* melanotrope cells of the pars intermedia, this protein was proposed to act in some way in the regulation of the secretory pathway (Ayoubi *et al*., 1991).

First, it was found that human 27kD-7B2, produced by the use of recombinant DNA techniques, inhibited PC2 activity but that the 21-kD form of 7B2 did not have such an activity (Braks and Martens, 1994; Martens *et al*., 1994). Second, 27-kD 7B2 makes molecular complexes by specific binding to pro-PC2 (75 kD) in the endoplasmic reticulum, which complexes are then transferred to the Golgi apparatus (Braks and Martens, 1994; Benjannet *et al*., 1995). These molecular complexes, cleaved by furin or PACE4 in the TGN, are partially dissociated after the 27-kD 7B2 form is split into 21-kD 7B2 and a C-terminal domain known as the CT peptide (Paquet *et al*., 1994). The N-terminal 21-kD portion then dissociates from the pro-PC2, whereas the CT peptide remains bound to it. In the proper intracellular sites for PC2 activity, probably immature secretory granules, the CT peptide is further processed and then dissociates from pro-PC2 (Zhu *et al*., 1996; Muller and Lindberg, 1999). At this time, pro-PC2 is cleaved by autolysis, and then converted to the active PC2 form (Fig. 9).

On the other hand, PC1 is transformed to its active form autocatalytically in the endoplasmic reticulum after production as a 92–94 kD pro-form (Benjannet *et al*., 1993; Lindberg, 1994; Milgram and Mains, 1994). Consequently, association of the 27-kD 7B2 protein with pro-PC2 is considered to be a safety system for inhibiting the precocious activation of PC2. This delay in activating PC2 is thus an ingenious system with respect to the order of proteolytic cleavage of prohormones such as POMC and proinsulin. In addition, recent studies suggest that proSAAS, a granin family protein that is broadly distributed in neuroendocrine tissues, is a natural inhibitor of PC1, similar to the situation of 7B2 and PC2 (Fricker *et al*., 2000; Basak *et al*., 2001).

Recently, 7B2 null mice have been generated by using a novel transposon-based technique (Westphal *et al*., 1999). In the pituitary of these mice, inactive PC2 fails to generate α-MSH in the pars intermedia, resulting in a very high level of intact ACTH in this lobe that resembles pituitary Cushing's disease. In the neurointermediate lobe, secretion of POMC-related peptides is negatively controlled by dopamine. Interestingly, the 7B2 null mice contain only a quarter of the normal levels of dopamine, but adrenalectomy of 3-week-old mice induces normal levels of dopamine, rescuing these animals from the lethal phenotype. It is presumed that a diminished pool of dopamine in the nerve endings in the neurointermediate lobe in the 7B2 null might be involved in the enhanced secretion of ACTH (Laurent *et al*., 2002).

Prohormone sorting and secretory granule formation

Because prohormones are sorted to the regulated pathway at the TGN and packaged into secretory granules, the sorting event is closely linked to the formation of secretory granules. Two models have been proposed for the mechanism underlying this process (Halban and Irminger, 1994; Cawley *et al*., 1998; Teter and Moore, 1998). One is an aggregation passive sorting model in which the proteins, by forming a condensing aggregate, are trapped and packaged into the granules. The other is the sorting signal ligand-receptor model in which a sorting signal in the prohormone binds to a receptor located in the lumen of the TGN, which binding is followed by budding of the TGN and formation of an immature granule encapsulating the proteins destined for the regulated secretory pathway. In mammalian cells, evidence has accumulated that various hormones and granin proteins aggregate at the TGN under conditions of mildly acidic pH and high Ca^{2+} (Gerdes *et al*., 1989; Yoo *et al*., 1995; Colmer *et al*., 1996; Yoo and Lewis, 1996; Jain *et al*., 2000). If regulated secretory proteins are condensed at the TGN and constitutive secretory proteins remain dissolved as single molecules, self-aggregation may induce the sorting of the hormones to the regulated secretory pathway. On the other hand, Cool *et al*. (1995) found that the hairpin loop formed by disulfide bridges is important for the sorting to the regulated pathway and demonstrated that POMC was diverted to the constitutive pathway when this disulfide bridge was broken. Using radio-labeled N-POMC fragments including the N-terminal hairpin loop in POMC, Cool *et al*. (1997) identified a protein showing specific binding to N-POMC peptides from membranes of the TGN or secretory granules, and then identified this protein to be CPE. They provided evidence that when antisense CPE RNA was introduced into POMC-expressing Neuro-2a cells, POMC-derived peptides were sorted to the constitutive pathway, but not to the regulated pathway. This finding was confirmed by evidence that POMC-derived peptides are sorted to the constitute pathway in the *fat/fat* mouse having a mutation in CPE. These data suggest that CPE plays a role as a sorting receptor at the TGN. However, because not all prohormones have a hairpin loop structure similar to POMC, it remains to be determined whether this loop structure-CPE receptor hypothesis is generally applicable.

Recently, Kim *et al*. (2001) suggested that CgA, which is an acidic sulfated glycoprotein, could act as an on/off switch in endocrine cells to trigger or block secretory granule biogenesis. Using the antisense RNA strategy, they examined the effect of specific depletion of CgA on dense-core secretory granule formation in PC12 cells, a model neuroendocrine cell line. As a result, CgA-deficient PC12 cells

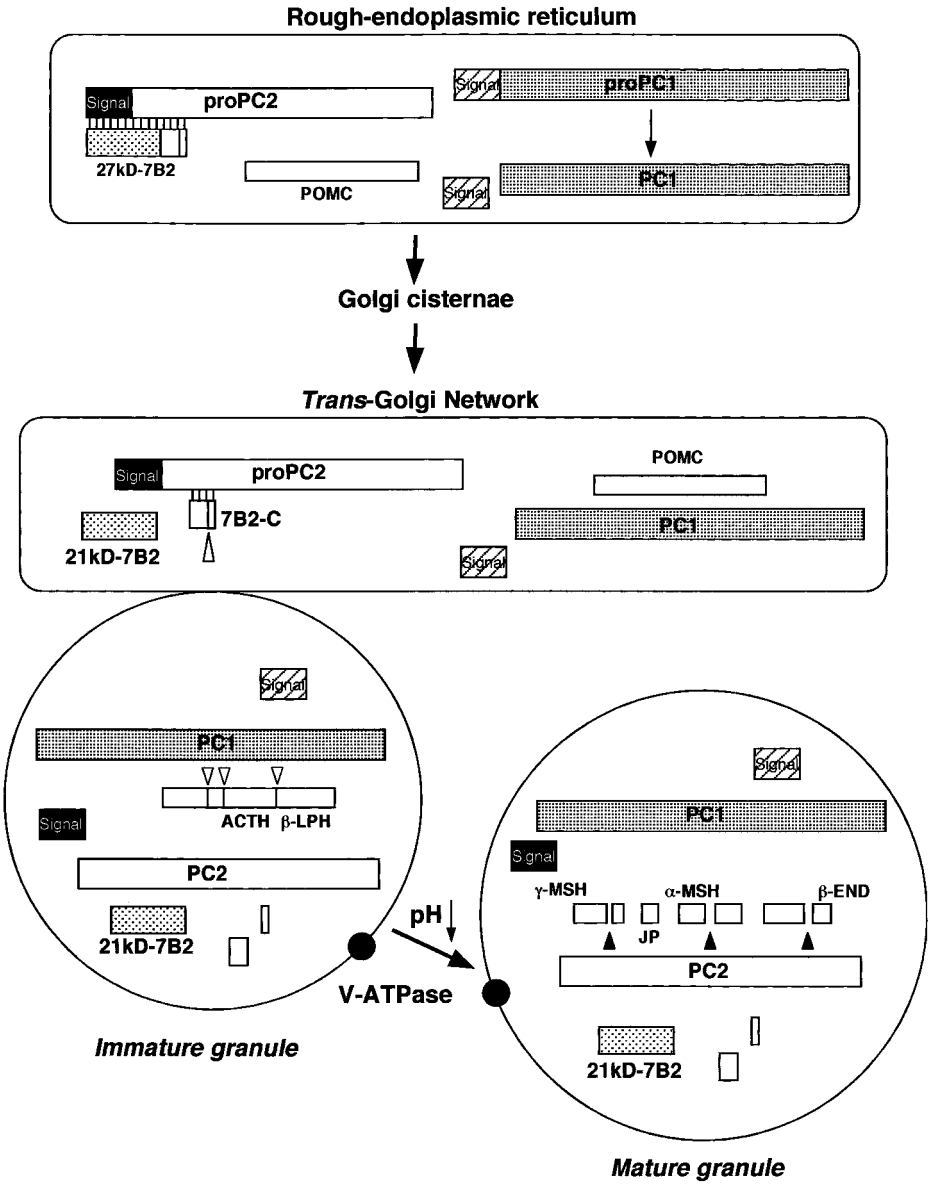

Fig. 9. Activation mechanisms of PC1 and PC2 and proteolytic processing of POMC. Refer to the text for details.

showed a decreased number of dense-core secretory granules; whereas transfection of a CgA-deficient pituitary cell line, 6T3, with CgA mRNA restored regulated secretion. However, this proposed function is not consistent with evidence showing that secretory granules exist in the absence of CgA and that cells can synthesize CgA in the absence of secretory granules (Day and Gorr, 2003). Day and Gorr (2003) suppose that CgA might act as an assembly factor in this process.

CONCLUSION

During the last decade, considerable progress has been made concerning the molecular identity of prohormone convertases, as well as elucidation of the molecular mechanism underlying intracellular processing. Secretory granules in endocrine and neuroendocrine cells have been found to function as proper sites for biosynthesis of hormones in addition to serving as storage sites and vehicles for the transport of peptide hormones. There yet remain unanswered questions about the mechanism of secretory granule formation. However, in the near future these issues are expected to be resolved.

ACKNOWLEDGMENTS

We thank Prof. Gerard J.M. Martens of the Department of Molecular Animal Physiology, University of Nijmegen for very thorough reading of the manuscript and many helpful suggestions and Dr. Masakazu Suzuki of our laboratory for helpful discussions. This study was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan.

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(Accepted July 23, 2003 / Invited Review)