Furin and Prohormone Convertase 1/3 Are Major Convertases in the Processing of Mouse Pro-Growth Hormone-Releasing Hormone

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We investigated the proteolytic processing of mouse pro-GHRH [84 amino acids (aa)] by furin, PC1/3, PC2, and PC5/6A. We created six point mutations in the N- and C-terminal cleavage sites, RXR \( \rightarrow \) and RXRXXR \( \rightarrow \), respectively. The following results were obtained after transient transfection/ cotransfection and metabolic pulse-chase labeling studies in several neuroendocrine cells. 1) Furin was the most efficient convertase in cleaving the N-terminal RXRX/RXRR site to generate intermediate I, 12–84aa, whereas PC1/3 was the most potent in processing the C-terminal RXRXXR site to yield mature GHRH, 12–53aa. 2) Both PC1/3 and PC5/6A also processed the N-terminal site but less efficiently than furin. 3) PC2 was much weaker in cleaving the C-terminal site relative to PC1/3 to generate mature GHRH. 4) The Q10R mutant was significantly more susceptible to furin cleavage at the N-terminal site than the wild-type pro-GHRH. And 5) the N- and C-terminal P1 Arg residues, R11 and R54, respectively, were essential for mature GHRH production. We also showed localization of the GHRH immunoreactive peptides in Golgi and secretory granules in neuroendocrine cells by an immunofluorescence assay. We conclude that the efficient production of mature GHRH from pro-GHRH is a stepwise process mediated predominantly by furin at the N-terminal cleavage site followed by PC1/3 at the C terminus. (Endocrinology 145: 1961–1971, 2004)

Pro-GHRH is processed in the hypothalamus to generate bioactive/mature GHRH that is released into the hypothalamic-pituitary portal vessels and ultimately to the pituitary gland to control the synthesis and secretion of GH from the anterior pituitary somatotrophs (1). After the cloning and characterization of human and rat pro-GHRH (2–4), mouse pro-GHRH was cloned and shown to have an open reading frame of 103 amino acids (aa) including a signal sequence of 19 aa (5, 6) (see Fig. 1). Mouse pro-GHRH shows 68 and 62% amino acid identity to the rat and human precursors, respectively. It is noteworthy that the C-terminal prosegment of mouse pro-GHRH has greater homology with rat than human, especially at the beginning of exon 5. Among other noteworthy differences are: 1) human GHRH has an amidated C terminus due to the presence of Gly at the P2 position in the C-terminal cleavage site, compared with the nonamidated mouse and rat GHRH, and 2) human GHRH has Tyr at its N terminus relative to His in mouse and rat. In mouse, the N-terminal processing site for generation of mature GHRH, RXRX \( \downarrow \), differs from the N-terminal cleavage site of rat and human, RXRR \( \downarrow \), whereas the C-terminal RXRXX \( \downarrow \) processing site is the same in all three. These are typical recognition sequences for prohormone convertase (PC) mediated processing. GHRH-containing neurons are shown to localize primarily in the arcuate nucleus of the hypothalamus, and only a few thousand such neurons have been identified (7, 8), rendering studies of the processing of pro-GHRH in vivo difficult.

After the discovery of the yeast convertase, Kex2 (9, 10), seven mammalian basic residue cleaving homologs have been identified during the past decade of which two members, PC1/3 and PC2, are abundantly expressed in neuroendocrine tissues (11–18), and among the rest, furin, PC5/6A, PC5/6B, PACE4, and PC7 are broadly distributed (19–33), whereas PC4 is expressed in testicular and ovarian tissues (34–37). Studies have shown that PC5/6A is present in secretory granules of neuroendocrine cells similar to PC1/3 and PC2 (38–43), whereas furin, apart from its trans-Golgi network (TGN)/endosomal localization, can also be sorted transiently into immature secretory granules (ISGs) of the regulated secretory pathway in neuroendocrine cells (44–46). Whereas the roles of PC1/3 and PC2 in processing various prohormones and proneuropeptides are well documented including in animal models (47–62), several studies have shown the abilities of PC5/6A and furin to process a growing number of prohormones and proneuropeptides such as pro-melanin-concentrating hormone (56), pro-neurotensin/neuromedin (63, 64), pro-cholecystokinin (65), pro-PTH (66), pro-TRH (67), proinsulin (68), chromogranin A (69), and 7B2 (70) in various systems having regulated and/or constitutive secretory pathway. Recently a PC1/3 inhibitory protein pro-SAA5, distributed mostly in neuroendocrine tissues, has been shown to be cleaved by furin both in vitro and in neuroendocrine cells (71, 72).

In the current studies using several different neuroendo-

Abbreviations: aa, Amino acid; ECFP, enhanced cyan fluorescence protein; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; IP, immunoprecipitation; ISG, immature secretory granule; PC, prohormone convertase; pc1-PDX, pc1-antitrypsin Portland; TGN, trans-Golgi network.

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crine cells, we performed detailed analyses to understand the roles of the two classical neuroendocrine convertases, PC1/3 and PC2, as well as the two broadly distributed convertases, furin and PC5/6A, in processing mouse pro-GHRH.

**Materials and Methods**

The mouse pro-GHRH (84 aa) expression plasmid was a kind gift from Dr. Kelley E. Mayo (Northwestern University, Evanston, IL). The two rabbit polyclonal antibodies against the mouse GHRH, 4398 and 7802, and the purified rat GHRH peptide were kind gifts from Dr. Lawrence A. Frohman (University of Illinois, Chicago, IL). The furin inhibitor, α1-antitrypsin Portland (α1-PDX), and recombinant human furin expression plasmid were kind gifts from Dr. Gary Thomas (Vollum Institute, Oregon Health Sciences University, Portland, OR). The expression plasmids for mouse PC2 and PC1/3 were generated in our laboratory. Neuro-2A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). RPE.40 cells were kindly provided by Dr. Thomas Moehring (University of Vermont, Burlington, VT). All the cell culture and PCR reagents were purchased from Invitrogen/Life Technologies (Carlsbad, CA). The transfection reagent, Effectene, and the endotoxin-free plasmid DNA purification kit were supplied by Dr. Thomas Moehring (University of Vermont, Burlington, VT). Neuro-2A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). RPE.40 cells were kindly provided by Dr. Thomas Moehring (University of Vermont, Burlington, VT). Neuro-2A cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). RPE.40 cells were kindly provided by Dr. Thomas Moehring (University of Vermont, Burlington, VT).

**Construction of the recombinant plasmids expressing the N- and C-terminal point mutants of mouse pro-GHRH**

Altogether six point mutants, three at the N-terminal RXRR site and three at the C-terminal RXRRR site, were constructed (Fig. 1). For the N-terminal site, Arg11 (R11) and Arg8 (R8) at positions P1 and P4, respectively, were individually changed to Ala (A) and Gln10 (Q10) at position P2 was substituted by Arg (R) using the overlap extension PCR method. After PCR, both the R11A and R8A mutant CDNAs were ligated at the restriction sites HindIII (5′) and EcoRI (3′) in a mammalian expression vector, pcDNA3.1 (+) (Invitrogen), whereas the Q10R mutant was ligated at HindIII (5′) and XhoI (3′) in the same expression vector. The oligonucleotide primers used for the overlap extension PCR were the following: 1) The upstream and downstream primers for both the expression vector, pcDNA3.1(+)/H11001 and pcDNA3.1(+)/H11003 site-directed mutagenesis kit from Stratagene (La Jolla, CA) and the PCR temperature used for the overlap extension PCR was one hold at 95 °C for 30 sec followed by 16 cycles at 95 °C for 30 sec, 55 °C for 1 min, and 68 °C for 12 min. All of the mutant constructs were verified by sequencing using the PE Biosystems kit (Warrington, UK).

**Results and Discussion**

**Processing of mouse pro-GHRH in neuroendocrine cells**

The rationale to analyze the roles of furin, PC1/3, PC2, and PC5/6A in pro-GHRH processing are the following: 1) im-
munochemical studies have shown that all of these convertases are expressed in the arcuate nucleus of the hypothalamus, the site of GHRH synthesis (7, 8, 17, 64); 2) The amino acid sequence, RXXR↓, located just before the N terminus of GHRH, is a minimal consensus site for furin cleavage (44, 75, 76) but also has been shown to be cleaved by PC1/3 in some instances (77, 78); 3) PC1/3 and PC2 are known to cleave the sequence RXXR↓ (78, 79) as is found at the C terminus of GHRH in mouse pro-GHRH; and 4) PC1/3 null mice are growth retarded and have been demonstrated to lack mature GHRH associated with a significant accumulation of unprocessed pro-GHRH-like material in hypothalamic extracts, compared with their wild-type littermates (48).

We studied the processing of wild-type mouse pro-GHRH in GH4C1 cells, derived from a rat pituitary tumor. These cells have been reported to contain considerable amounts of both furin and PC5/6A but lack PC1/3 and PC2 (70, 80), which has also been confirmed in our laboratory. Studies following transient transfection/cotransfection and pulse-chase labeling (see Materials and Methods for details) show that in absence of any exogenous PCs, wild-type pro-GHRH is processed into a single major peptide, intermediate I, 12–84aa (Fig. 2, A–C). It constitutes only 2% of the total levels of pro-GHRH after a 25-min pulse (Fig. 2A, lane 2), 25% in the cells after a 150-min chase (Fig. 2A, lane 3), and 45% of the GHRH immunoreactive peptides in the 150-min chase medium (Fig. 2, B and C, lane 2), indicating significant processing at the N-terminal RXXR site by endogenous furin in the absence of PC1/3 and PC2. We examined the processing of wild-type mouse pro-GHRH using a shorter chase period (30 min) and found very low levels of intermediate I (data not shown), indicating the effectiveness of a longer chase period (150 min) as used in the subsequent experiments.

Next, we examined the roles of PC2 or/and PC1/3 coexpression on pro-GHRH processing. In the presence of PC2, we did not find any significant increase in the levels of intermediate I in both cells and medium (Fig. 2, A, lanes 4 and 5, and B and C, lane 3), compared with wild-type alone. However, two other peptides were generated, intermediate II, 1–53aa, and mature GHRH, 12–53aa. These appeared in the 150-min chase medium (Fig. 2C, lane 3) after processing at the C-terminal RXXRXR site of pro-GHRH and intermediate I, respectively. These data suggest that PC2 is unable to process the N-terminal RXXR site but is moderately efficient in cleaving the C terminus (see Fig. 2C, which is a longer exposure of Fig. 2B). When PC1/3 was cotransfected with wild-type pro-GHRH, production of intermediate I was considerably increased, especially in the 150-min chase medium, with the ratio of 2:1 between the intermediate I and pro-GHRH (Fig. 2, B and C, lane 4). The results indicate a considerable ability of PC1/3 to cleave the N-terminal site of pro-GHRH. Also, PC1/3 very efficiently cleaved the C-terminal site especially of the predominant intermediate I liberating 64% mature GHRH from the total pool of GHRH immunoreactive peptides after 150-min chase in both cells and medium (Fig. 2, A, lane 7, and B, lane 4). The amounts of mature GHRH produced by PC1/3 relative to PC2 were 4-fold higher (Fig. 2C, lane 4 vs. 3).

These findings indicate the significantly greater potency of PC1/3 in generating mature GHRH. After cotransfection of PC1/3 and PC2 together, we did not find any synergistic effects on the production of mature GHRH (data not shown). It is noteworthy that mouse pro-GHRH (84aa) and intermediate I (12–84aa) contain five and four Met residues, respec-
tively, whereas mature GHRH (12–53aa) has only one. Because we used 35S-Met for all of our pulse-chase labeling studies, the net differences in the Met content with reference to band intensities between pro-GHRH and intermediate I and between pro/intermediate I and mature GHRH in cell extracts, and media were taken into consideration for quantitative estimation.

Next, we analyzed the importance of the P2 Arg in the N-terminal cleavage site by creating a mutant pro-GHRH, Q10R (see Fig. 1), corresponding to the rat and human sequences (described in detail in Materials and Methods). The presence of a basic residue, Arg/Lys, at the P2 position significantly increased the ability of furin to cleave the site, RXRR, compared with the site lacking the P2 basic residue, RXXR (44, 81), as demonstrated in Fig 3, A and B. We found that 45% of the Q10R pro-GHRH was converted into intermediate I after a 25-min pulse (Fig. 3A, lane 2). After a 30-min chase in cells, 70% of the mutant pro-GHRH was processed into intermediate I (Fig. 3A, lane 3), which ultimately comprised more than 95% of the total levels of GHRH immunoreactive peptides secreted into the 150-min chase medium (Fig. 3B, lane 3). This significant early increase in the N-terminal processing of the Q10R mutant confirms that furin is a potent convertase in producing intermediate I and also implies the existence of a similar processing event for both the rat and human precursors in which both have this P2 Arg residue. When we analyzed the individual efficiencies of PC1/3 or PC2 in processing this Q10R mutant, we found, especially in the chase medium, a 30% lower accumulation of intermediate I in the presence of PC1/3, compared with PC2 and, correspondingly, 6-fold higher levels of the mature GHRH relative to PC2 (Fig. 3F, lane 3 vs. 3D, lane 3). The total amount of mature GHRH liberated by PC1/3 in both cells and medium after 150 min of chase was 67% of the total levels of GHRH immunoreactive peptides (Fig. 3, E, lane 4, and F, lane 3). Mature GHRH, liberated due to coexpression of PC1/3 and the Q10R mutant, comprised 45% of the total secreted anti-GHRH reactive peptides at 150 min relative to 36% from the wild-type prohormone (Fig. 3 vs. Fig. 2). Increased accumulation of mature GHRH by PC1/3 as opposed to PC2 was also observed in the cells after a 150-min chase (Fig. 3E, lane 4 vs. Fig. 3C, lane 4). These data confirm the greater ability of PC1/3 to cleave the C-terminal RXRXXR site to generate mature GHRH.

To further examine the role of furin in processing the N-terminal RXR site of wild-type pro-GHRH, we employed a furin null Chinese hamster ovary cell line, RPE.40 (82). As shown in Fig. 4, after transfection of wild-type pro-GHRH, no processing was observed even after a long chase (5 h) in both cell extracts and medium (Fig. 4, A, lanes 2–5, and B, lanes 2–4). When furin was cotransfected, 50% of pro-GHRH was processed into intermediate I during the chase period in

![Fig. 3. Processing of N-terminal Q10R mutant with the RXRR cleavage site in GH4C1 cells. The Q10R mutant (1 μg) was transiently transfected or cotransfected without or with PC2 (1 μg) or PC1/3 (1 μg). Cells were pulse labeled for 25 min and chased for two time points, 30 and 150 min. Both the cell extracts (CE) and media (MED) were IP with the rabbit anti-GHRH antibody followed by separation in tricine-SDS-PAGE. Lane 1 for all the panels indicates the position of the iodinated authentic GHRH peptide as well as the locations of the prestained protein molecular-weight markers. A, C, and E and B, D, and F were exposed for 3 and 9 d, respectively.](https://academic.oup.com/endo/article-abstract/145/4/1961/2878837)
cells beginning at early time points (Fig. 4C, lanes 3 and 4) after a 25-min pulse (Fig. 4C, lane 2). In the chase medium, intermediate I constituted more than 95% of total secreted peptides (Fig. 4D, lanes 3 and 4). However, we found only low levels of mature GHRH in the presence of exogenous furin (Fig. 4, C and D), indicating the very limited ability of this convertase to cleave the C terminus of both pro-GHRH and intermediate I in RPE.40 cells. In similar experiments, using another furin defective cell line, LoVo, derived from a human colon carcinoma (83), we found significant generation of intermediate I from pro-GHRH by exogenous furin (data not shown). Employing a furin inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethylketone (Bachem, Torrance, CA) (44), we observed a significant inhibition of the N-terminal processing of wild-type pro-GHRH in GH4C1 cells in absence of any exogenous PCs (data not shown). All these results strongly suggest that furin can be a key convertase in cleaving the N-terminal RXRR/RXRR site of pro-GHRH.

Next, we examined the role of PC5/6A on pro-GHRH processing with the help of a mouse neuroblastoma cell line, Neuro-2A, having undetectable or very low levels of PC5/6A, PC2, and PC1/3 but considerable amounts of furin (80). We used the Q10R mutant because of its much greater susceptibility to furin-mediated cleavage at the N terminus in generating significantly higher levels of intermediate I (Fig. 3) relative to the wild-type pro-GHRH (Fig. 2). We showed that the mutant pro-GHRH, when transfected alone, was significantly cleaved at its N terminus after a 25-min pulse producing high levels of intermediate I, and it constituted 40% of the total amounts of proform (Fig. 5A, lane 2). After the 150-min chase, the levels of intermediate I in cells were considerably augmented, reaching a ratio of 1:1 (Fig. 5A, lane 3). In the 150-min chase medium, the predominant peptide was intermediate I (Fig. 5A, lane 4), suggesting that the N-terminal RXRR site of the Q10R mutant was efficiently cleaved by endogenous furin in Neuro-2A cells. After cotransfection with PC5/6A, we found a modest increase in N-terminal processing after the 25-min pulse amounting to 50% conversion of pro-GHRH into intermediate I (Fig. 5A, lane 5). This N-terminal cleavage was further enhanced during the chase, especially within the cells in which the ratio of intermediate I to pro-GHRH rose to 2:1 (Fig. 5A, lane 6). We did not find any detectable levels of mature GHRH under these conditions (Fig. 5A, lanes 5–7). The results indicate a modest but significant role of PC5/6A in cleaving at the N-terminal RXRR site but no evidence for cleavage at the C-terminal site of either intermediate I or pro-GHRH by this convertase. When PC5/6A was coexpressed with wild-type pro-GHRH in GH4C1 cells, the levels of intermediate I were clearly augmented in both cells and medium, especially after 150 min chase, compared with pro-GHRH alone (Fig. 5B, lanes 6 and 7 vs. 3 and 4). After cotransfection of a furin inhibitor, a1-PDX (44), processing of pro-GHRH into intermediate I was significantly blocked (Fig. 5C, lanes 2–4). To examine the ability of PC5/6A in counteracting the furin-inhibitory action of a1-PDX, we coexpressed PC5/6A with both a1-PDX and wild-type pro-GHRH and showed that PC5/6A was able to generate significant amounts of intermediate I, confirming its ability to process the N-terminal RXRR/RXRR cleavage site (Fig. 5C, lanes 6 and 7).

To understand the importance of each of the Arg residues in both the N- and C-terminal cleavage sites, we individually mutated the arginines at P1 and P4 of the N terminus and P1, P4, and P6 of the C terminus to Ala (see Fig. 1 and Materials and Methods). We demonstrated in cotransfection assays in GH4C1 cells that for both the P1 mutants, R11A and R54A, processing was significantly blocked at the N and C terminus, respectively, leading to total loss of mature GHRH synthesis in the presence of PC1/3 (see Figs. 6B and 7, A and B, lanes 8–10). We found considerable enhancement of intermediate II levels when R11A was coexpressed with PC1/3 (Fig. 6B, lanes 5–7 vs. lanes 2–4), indicating no apparent impairment of the C-terminal processing of this mutant by PC1/3. Generation of very low levels of intermediate II from
R11A in the absence of PC1/3 confirms that the C-terminal site is a poor substrate for furin. No other intermediate cleavage products were detected. When the N-terminal P4 mutant, R8A, was cotransfected with PC1/3, both intermediate I and mature GHRH were generated and appeared in the 150-min chase medium after processing at the N-terminal P1 Arg and the C-terminal site, respectively, along with a very low level of unprocessed pro-GHRH (Fig. 6A, lane 7). The diminished levels of both intermediate I and mature GHRH indicate the requirement of a P4 Arg for efficient processing of the N terminus. Processing at a single Arg residue by PC1/3, as observed for the R8A mutant, has been shown before (47, 60, 78). Similar to R11A, very low levels of intermediate II were generated from R8A in absence of exogenous PC1/3 (6A, lanes 2–4), confirming again the very limited ability of endogenous furin to process the C-terminal site of both the N-terminal mutants in GH4C1 cells. We did not find any detectable cleavage of either of the N-terminal mutants, R11A or R8A, in presence of exogenous furin (data not shown), confirming a requirement of the minimal consensus RXXR sequence for furin action (44, 75).

In GH4C1 cells, the C-terminal P1 mutant, R54A, when transfected alone, was considerably processed at its N terminus to generate intermediate I (Fig. 7A, lanes 9 and 10). When coexpressed with PC1/3, increased amounts of intermediate I were generated from R54A and secreted into the chase medium along with a very low level of unprocessed proform (Fig. 7B, lanes 9 and 10). The results suggest that the C-terminal P1 Arg to Ala mutation did not significantly affect the N-terminal processing. Similar to R54A, generation of intermediate I was not significantly affected for either of the other two C-terminal mutants, R51A or R49A, when expressed alone (Fig. 7A), indicating again a lack of influence of C-terminal site structure on processing at the N-terminal RXXR site. We showed in cotransfection assays using either of the mutants, R51A or R49A, that either of the Arg residues, P4 and P6, was required for complete processing of the C-terminal site of pro-GHRH.
terminus to generate maximum levels of mature GHRH in the presence of PC1/3 (Fig. 7B, lanes 3 and 4 and 6 and 7).

Subcellular localization of GHRH immunoreactive peptides

We used βTC-3 cells, derived from mouse pancreatic islets, to study the localization of anti-GHRH antibody reactive peptides after transient transfection/cotransfection of wild-type pro-GHRH without or with Golgi and secretory granule markers, pECFP-Golgi and pEGFP-PC1/3, respectively (see details in Materials and Methods). Our immunocytochemical analyses showed the distribution of GHRH or its precursors in the Golgi as indicated by Cy3 fluorescence alone (Fig. 8A, panel a) and combined fluorescence of ECFP + Cy3 (Fig. 8A, panel c). Both of these localization signals were comparable with pECFP-Golgi-generated fluorescence (Fig. 8A, panel b), showing the likely presence of GHRH precursor peptides in the Golgi after synthesis in the endoplasmic reticulum. We also showed the localization of GHRH immunoreactive peptides in the secretory granules as indicated by Cy3 alone (Fig. 8B, panel a) and the combined Cy3 and EGFP fluorescence (Fig. 8B, panel c), both of which were comparable with the staining generated by EGFP alone (Fig. 8B, panel b).

A model for pro-GHRH processing

We propose (see Fig. 9) that pro-GHRH, during its transit through Golgi → TGN → ISGs in the regulated secretory pathway is first cleaved at its N-terminal site by the combined actions of furin, PC1/3, and PC5/6A (furfurin → PC1/3 → TGN → PC5/6A).

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Fig. 7. Processing of C-terminal mutants of mouse pro-GHRH in GH4C1 cells. Cells were either transfected with 1 μg of each of the C-terminal mutants, R49A, R51A, and R54A (A) or cotransfected also with PC1/3 (1 μg) (B). Cells were pulsed for 25 min and chased for 150 min followed by IP of both the cell extracts (CE) and media (M) with the rabbit anti-GHRH antibody. Lane 1 shows the locations of the iodinated authentic GHRH peptide and the pre-stained markers.

Fig. 8. Immunocytochemical localization of WT pro-GHRH in Golgi and secretory granules in βTC-3 cells. Cells were transiently transfected with either 500 ng of WT pro-GHRH (A and B, panel a) or 500 ng of pECFP-Golgi (A, panel b) or 1 μg of pEGFP-PC1/3 (B, panel b). WT pro-GHRH was cotransfected either with pECFP-Golgi (A, panel c) or with pEGFP-PC1/3 (B, panel c). The white arrows in panels a to c of A and B indicate the Golgi and secretory granules, respectively. (Magnifications: A, ×400; B, ×800.)
Processing of Pro-GHRH by PCs

PC1/3 > PC5/6A) to generate significant amounts of intermediate I, 12-84aa indicated by the blue and bold black arrows. We designated intermediate I as the major intermediate due to its high abundance. Processing then occurs at the C-terminal cleavage site catalyzed most efficiently by PC1/3 and weakly by PC2 to produce mature GHRH, 12-53aa (indicated by the green and bold black arrows). Our model also shows that PC1/3 acting alone can mediate processing of the N and C termini of pro-GHRH to generate substantial levels of mature GHRH (marked by a lighter black arrow) and small amounts of intermediate II, 1-53aa (the minor intermediate). Also, the model depicts that low levels of intermediate II can be generated by PC2 due to processing at the C-terminal site of pro-GHRH (indicated by a lighter black arrow). In studies with αTC1–6 cells having high levels of endogenous PC2 and undetectable amounts of PC1/3 (60), we found low levels of both the intermediate II and mature GHRH generated from pro-GHRH (data not shown). Using a C-terminally truncated cDNA generating intermediate II, we found in βTC-3 cells with high levels of both PC2 and PC1/3 (80) that it was efficiently converted to mature GHRH (data not shown).

The results altogether have indicated that PC1/3 is the most efficient convertase to liberate mature GHRH via two pathways: major, processing of the C-terminal site of intermediate I, which is generated from pro-GHRH via N-terminal cleavage mediated most efficiently by furin and partly by PC5/6A (furin >> PC5/6A); and minor, processing by PC1/3 of both sites in pro-GHRH. The results also indicate that PC1/3 may have a greater preference for intermediate I as a substrate than pro-GHRH probably because of a more favorable conformation of intermediate I after the removal of the N-terminal 11aa segment from pro-GHRH by the earlier action of furin. Our data suggest not only an overlapping function of these three convertases in targeting the same N-terminal cleavage site but also a mechanism to ensure the efficient processing of pro-GHRH at its N terminus during its transit through Golgi → TGN → ISGs in the regulated secretory pathway after its synthesis in endoplasmic reticulum. Recent studies have shown that PC5/6A is able to cleave proneurotensin/neuromedin at a dibasic Lys-Arg site and pro-cholecystokinin at a monobasic Arg/Lys site in the early compartments of the regulated secretory pathway (63, 65, 84). PC5/6 has also been shown to cleave the RXXR site of prorenin in neuroendocrine cells (24). Our data on pro-GHRH processing indicate for the first time that PC5/6A is able to process an RXXR/RXRR site of a hypothalamic prohormone in the regulated secretory pathway.

To determine that pro-GHRH processing is a stepwise event rather than simultaneous, we showed in GH4C1 cells that high levels of intermediate I were generated after 25-min pulse labeling of the N-terminal mutant, Q10R (Fig. 3A, lane 3, and 3B, lane 2). The levels of intermediate I were significantly enhanced because the mutant was chased for a period of 30 min without any exogenous PCs (Fig. 3A, lane 3, and 3B, lane 2). These results corroborate with the previous findings on furin action in the early compartments (44, 81). On the other hand, we found low levels of mature GHRH after a 30-min chase in the presence of PC1/3 (Fig. 3E). As the chase time was extended, 6-fold more mature GHRH were generated from the Q10R mutant by PC1/3, compared with PC2 (PC1/3 >> PC2) (see Fig. 3, D, lane 3, and F, lane 3), confirming actions of both PC1/3 and PC2 later than furin, probably predominantly in secretory granules within the regulated secretory pathway (18, 40, 78). Similarly, for the wild-type pro-GHRH, we showed the late actions of both PC1/3 and PC2 (PC1/3 >> PC2) in generating mature GHRH (Fig. 2, A, lanes 5 and 7, and B and C, lanes 3 and 4).

In AtT20 cells (endogenous PC1/3 >> PC2) (60, 80), as expected, we found significant generation of mature GHRH from pro-GHRH. It is noteworthy that furin level was reported low in AtT20 cells (80) as we also found in our laboratory (data not shown). Also, our immunocytochemical studies showing localization of GHRH immunoreactive peptides in Golgi and secretory granules in βTC-3 cells (Fig. 8, A and B) corroborate with the subcellular sites for the actions of these convertases. All these results, using several neuroendocrine cells, along with those reported by others in a recent preliminary report (85), confirm the probable biological relevance of our studies.
On the basis of our current findings, we believe that the high-molecular-weight forms, present in the PC1/3 knock-out mouse hypothalamic extracts, may represent both unprocessed pro-GHRH, 84aa, and intermediate I, 12–84aa, possibly generated by furin. Western blot analysis of hypothalamic extracts to identify GHRH immunoreactive peptides has so far been unsuccessful (our unpublished data). To determine the exact role of furin in hypothalamic pro-GHRH processing, targeted disruption of furin expression in hypothalamus will be required because both furin and PC1/3 are present in the arcuate nucleus of the hypothalamus (17), the site of GHRH synthesis (7, 8).

The underlying causes of the major phenotypic differences between mice (48) and a human subject (86) lacking active PC1/3 are currently not well understood. Possible reasons include species differences in convertase expression or a greater vulnerability of the N-terminal RXRR site of human pro-GHRH to furin-mediated cleavage due to the presence of the P2 Arg in the human precursor as shown here. On the basis of our current results, we can propose two hypotheses to account for the normal somatic growth of the human subject lacking active PC1/3: 1) PC2 or some other PCs such as PC5/6A may be able to generate sufficient mature GHRH to account for the normal somatic growth of the human subject lacking active PC1/3; 2) a free N terminus of GHRH might be required for the absence of mature GHRH.

Because a free N terminus of GHRH might be required for intermediate I alone at high levels might be able to compensate for the absence of mature GHRH.

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References

28. Smeekens SP, Steiner DF 1991 Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. Proc Natl Acad Sci USA 3449–3453
32. Seidah NG, Hamelin J, Mamhardt M, Dong W, Tardos H, Mbikay M, Chretien M, Day R 1996 cDNA structure, tissue distribution, and chromo-
somal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kinin-like proteases. Proc Natl Acad Sci USA 93:3388–3393


44. Cain BM, Vishnuvardhan D, Reinfeld MC 2001 Neuronal cell lines expressing PC5, but not PC1 or PC2, process Pro-CCK into glycinextended CCK 12 and 22. Peptides 22:1217–1227


52. Schagger H, von Jagow G 1987 Tricine-sodium dodeyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 10 kDa. Anal Biochem 166:368–379


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