Processing of Prothyrotropin-releasing Hormone by the Family of Prohormone Convertases

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The post-translational processing of prothyrotropin-releasing hormone (pro-TRH25–255) has been extensively studied in our laboratory, and the processing pathway to mature TRH has been elucidated. We have also demonstrated that recombinant PC1 and PC2 process partially purified pro-TRH to cryptic peptides in vitro and that pro-TRH and PC1 mRNAs are coexpressed in primary cultures of hypothalamic neurons. To further define the role of each convertase, and particularly PC1 and PC2, in pro-TRH processing, recombinant vaccinia viruses were used to coexpress the prohormone convertases PC1, PC2, PACE4, PC5-B, furin, or control dynorphin together with rat prepro-TRH in constitutively secreting LoVo cells or in the regulated endocrine GH4C1 cell line.

Radioimmunoassays from LoVo-derived secreted products indicated that furin cleaves the precursor to generate both N- and C-terminal intermediates. PC1, PC2, and PACE4 only produced N-terminal intermediates, but less efficiently than furin. In GH4C1 cells, PC1, PC2, furin, PC5-B, and PACE4 produced both N-terminal and C-terminal forms. Significantly, TRH-Gly and TRH are mostly produced by PC1, PC2, and furin. Utilizing gel electrophoresis to further analyze the cleavage specificities of PC1 and PC2, we found that PC1 seems primarily responsible for cleavage to both intermediates and mature TRH, since it generated all products at significantly higher levels than PC2. The addition of 7B2 to the coinfection did not augment the ability of PC2 to cleave prepro-TRH to either N- or C-terminal forms.

Many neuropeptides are first synthesized as large prohormones, precursors that must be post-translationally proteolysed to elaborate smaller bioactive peptides. This processing occurs through limited endoproteolytic cleavage at paired basic residues, either Lys-Arg or Arg-Arg, with cleavage at monobasic sites occurring less frequently (1, 2). Through such post-translational processing, prothyrotropin-releasing hormone (pro-TRH) is cleaved to mature TRH.

Mature TRH is a modified 3-amino acid neuropeptide (pyroglutamate-histidine-proline-amide) that stimulates the synthesis and release of thyrotropin, prolactin, and growth hormone from the mammalian pituitary (3–6). In the rat, mature TRH is derived from a 231-amino acid prohormone, which contains five TRH progenitor sequences (Gln-His-Pro-Gly) (7, 8). Each of these sequences is flanked by dibasic residues, and another dibasic residue exists that does not flank a TRH progenitor sequence (7). Cleavage at all dibasic residues yields five copies of the TRH progenitor and seven cryptic peptides (7, 9). Mature TRH is achieved by exoproteolytic excision of the basic residues flanking the TRH progenitor by carboxypeptidase E, amidation of the carboxyl-terminal proline by peptidylglycine α-amidating monoxygenase, and cyclization of the glutamine (10–12).

Much of the characterization of pro-TRH and its post-translational processing and sorting have been done using transfected AtT-20 cells, which yield high levels of prohormone expression (7). Using this system, pro-TRH was definitively established as a 26-kDa protein TRH precursor (7). The processing of this precursor has also been partially elucidated in these cells (7, 13). There are two primary processing pathways, which yield different N- and C-terminal intermediate products. The two processing schemes yield identical end products, resulting from cleavage at all dibasic residues, but differ by the sites of initial cleavage of the 26-kDa precursor. If cleavage initially occurs at prepro-TRH Lys152–Arg153 or Arg158–Arg159, a 15-kDa N-terminal peptide (prepro-TRH25–151–151–152) and a 10-kDa C-terminal peptide (prepro-TRH154–255 or prepro-TRH160–255) are formed. Further processing of the 15-kDa peptide results in the formation of a 6-kDa peptide (prepro-TRH25–74) and a 3.8-kDa peptide (prepro-TRH77–106) fragment. The 6-kDa peptide is then proteolyzed to a 4-kDa (prepro-TRH77–106) fragment and a 3-kDa (prepro-TRH77–106) peptide. Further processing of the 10-kDa fragment yields a 5.4-kDa peptide (prepro-TRH108–253). If cleavage initially occurs at Lys107–Arg108 or Arg112–Arg113, a 16.5-kDa C-terminal peptide is formed (prepro-TRH109–255 or prepro-TRH115–255) along with a 9.5-kDa N-terminal peptide (prepro-TRH25–106 or prepro-TRH25–111). The overall processing scheme is presented in Fig. 1, which delineates the further intermediates resultant from initial cleavage at prepro-TRH Lys107–Arg108 or Arg112–Arg113. Primary hypothalamic culture studies confirmed that this processing pathway is physiologically relevant (14).

Our previous in vitro (15, 16) and recent (14) studies implicate two members of a family of prohormone convertases (PCs), PC1 and PC2, in the physiological processing of pro-TRH. The PCs are a family of seven subtilisin/kexin-like endoproteases designated furin (17, 18), PC1 (19) (also known as PC3; Ref. 20), PC2 (21, 22), PC4 (23, 24), PACE4 (25), PC5-A (26) (also known as PC6-B; Ref. 27) and its isoform PC5-B (also known as PC6-B; Ref. 28), and PC7 (29) (also known as LPC (30), PC8 (31), and SPC7 (32)). The structures of these serine proteinases resemble both bacterial subtilisins and yeast kexins (1, 2, 33).

*a The abbreviations used are: TRH, thyrotropin-releasing hormone; VV, vaccinia virus; RIA, radioimmune assay; PIPES, 1,4-piperazinedi-ethanesulfonic acid; ACTH, adrenocorticotropic hormone; PAM, peptidyl glycine α-amidating monoxygenase.
The selective expression of PC1 and PC2 in endocrine and neuroendocrine cells specifically suggests they may be significant in prohormone processing (1, 19, 22, 26, 34); accordingly, PC1 and PC2 have been shown to process proinsulin (35–37), proenkephalin (38), prosomatostatin (39, 40), and pro-opiomelanocortin (41, 42) to various intermediates and end products in coexpression experiments. The fact that PC1, PC2, and pro-TRH are endogenous constituents in rat hypothalamic neuronal cells suggested the involvement of these endopeptidases in pro-TRH processing (14). The strongest evidence implicating PC1 in the processing of pro-TRH is derived from our recent studies showing the coexpression of pro-TRH and PC1 by double in situ hybridization in rat hypothalamic neurons (14). Statistical counting of more than 100 fields suggested that more than 50% of the neurons coexpressed TRH and PC1, and all TRH-positive cells also expressed PC1. Interestingly, we did not find any single cell expressing only TRH mRNA.

Recombinant PC1 isolated from transfected fibroblast cells was found to be capable of cleaving pro-TRH to a number of predicted processing intermediates (15). However, the difference in the percentage of N- and C-terminal intermediate products formed suggests a differential sensitivity to PC1 processing during this sequential cleavage (15). The processing capabilities of PC1 and PC2 on pro-TRH have also been examined using a bovine intermediate lobe secretory vesicle membrane preparation (16). Importantly, full processing to TRH, TRH-Gly, and TRH-Gly-Lys was undetectable in both of these studies, indicating that under these in vitro conditions, PC1 and PC2 alone were not able to fully process pro-TRH to TRH (15, 16). This is expected, since the antisera used only recognize TRH cyclized forms (pGlu), which would not form in these in vitro conditions.

To investigate the ability of PC1, PC2, and the other PC enzymes (PC5-B, PACE4, and furin) to cleave pro-TRH, we performed coinfection studies using recombinant vaccinia virus vectors. By coexpressing both a given PC enzyme and pro-TRH in cell lines containing regulated and constitutive secretory pathways or solely a constitutive secretory pathway, differences in cleavage can be evaluated and compared. This system has been successfully utilized to study the ability of PC1 and PC2 to cleave pro-opiomelanocortin (41, 42), proenkephalin (38), prosomatostatin (39), prooprotatin II (40), and proinsulin (36).

In this study, vaccinia virus recombinants expressing PC enzymes (VV:PCs) were coinfected with a VV:prepro-TRH into GH4C1 and LoVo cells. The regulated cell line, GH4C1, was chosen because of the endogenous absence of PC1 and PC2 (1, 22); in contrast, AT2-20 cells endogenously synthesizes all PCs except for PC4 (1, 29). Furthermore, GH4C1 cell lines contain large secretory granules, a prerequisite to TRH maturation. Both GH4C1 and the constitutively secreting LoVo cell line (which is deficient of furin activity) have been well characterized for use with the vaccinia virus coinfection system (38, 40, 43, 44). In this paper we demonstrate for the first time that PC1 and PC2 are capable of generating TRH-Gly and mature TRH. We also show cleavage profiles for PC1 and PC2 and examine more grossly the capabilities of the other PC enzymes to cleave pro-TRH. The evidence presented here suggests that PC1 is primarily responsible for pro-TRH cleavage in GH4C1 cells and that PC2 plays an as yet undefined subsidiary role. Finally, to further support the role of PC1 and PC2 in the processing of pro-TRH, protein colocalization of pro-TRH and PC1 or PC2 were performed using our well defined primary cultures of hypothalamic neurons.

**MATERIALS AND METHODS**

*Infection of GH4C1 Cells by Vaccinia Virus Recombinants*—The coinfections of either GH4C1 or LoVo cells (50 × 10⁶ cells) with a recombinant vaccinia virus of each convertase or a combination thereof (VV: mPC1, VV:mPC2, VV:furin, VV:PACE4, and VV:PC5-B) and prepro-TRH were performed at one plaque-forming unit/cell as previously reported (40, 41, 44). Following infection, cells were washed and resuspended in serum-free media for 4 h, as previously reported (40, 43). Cell media were collected, lyophilized, washed, resuspended, and analyzed using SDS-polyacrylamide gel electrophoresis and radioimmun assay (RIA). Cell content was not utilized, since preliminary results indicated that most of the peptides were contained in the media fraction.

**SDS-Polyacrylamide Gel Electrophoresis**—Cell media were analyzed using the Protein 16-cm cell apparatus (Bio-Rad Laboratories, Richmond, CA), a 1.5-mm thick polyacrylamide gel-Tricine-SDS system. Prestained molecular mass markers were used as follows: 106, 80.0, 49.5, 32.5, 27.5, and 18.5 kDa from Bio-Rad; 29, 20.4, 14.4, 6.5, and 2.8 kDa from Diversified Biotech (Newton, MA). After electrophoresis, gels were cut into 2-mm slices in a gel slicer (Hoeffer Scientific Instruments, San Francisco, CA). Each slice was placed in 2 N acetic acid for protein extraction prior to RIA and incubated for 4 days, and the gel slices were removed. An almost 90% recovery of peptides from the gel slices was achieved by using RIA before and after gel electrophoresis (7).

**Antibodies**—The following antibodies were used in the RIAAs: anti-pCC10, recognizing prepro-TRH35–125 (26 kDa), prepro-TRH35–125 (26 kDa), prepro-TRH35–125 (26 kDa), anti-pYE17, recognizing prepro-TRH35–125 (26 kDa), prepro-TRH115–255 (16.5 kDa), prepro-TRH150–255 (10 kDa), prepro-TRH208–255 (5.4 kDa), prepro-TRH25–50 (4.6 kDa), and anti-pEH24, recognizing prepro-TRH25–50 (4.4 kDa). Table 1 depicts the peptides within the pro-TRH molecule that are recognized by the various polyclonal antibodies (7). For immunocytochemistry purposes, the following antibodies were used. The antibodies used in our previous studies (14) against different regions of PC1 and PC2 sequence were kindly donated by Dr. Nigel Birch from University of New Zealand. For double staining immunocytochemistry, affinity-purified anti-prepro-TRH115–151 (pAV37) was directly conjugated with Texas Red (see below).

**Peptide RIAAs**—The RIAAs for pCC10, pYE17, pEH24, pYE17 (7), TRH-Gly, and TRH (15) were performed as standard in our laboratory, as
described elsewhere. All RIAs were performed on the same volume of material in duplicate.

Hypothalamic Cultures—Cultures were undertaken as described previously (14). In brief, diencephalic tissue was dissociated to single cells by neutral protease digestion (1 unit/tissue; Sigma). The cells were cultured for up to 14 days on four-chamber glass LabTek (Nunc Inc., Naperville, IL) slides (10^6 cells/ml) with L-15-Dulbecco's modified Eagle's medium (14) containing 10% fetal calf serum (Life Technologies, Inc.). Prior to plating, all wells were coated with poly-D-lysine (20 μg/ml, Sigma). To induce differentiation of neuronal cells and pro-TRH biosynthesis, the cells were cultured with 50 μM BrdU during the first 4 days as described previously (14).

Double Staining Immunocytochemistry—Hypothalamic neurons (3 × 10^5) from 14-day-old cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline as described previously (14). After fixation, three washes with PIPES/sucrose buffer (0.1M PIPES, pH 6.8, 0.12 M sucrose), the cells were incubated in 0.2% Triton X-100 in the same buffer. After blocking with 5% albumin, the cells were washed with phosphate-buffered saline containing 0.1 M NH₄Cl and 1% normal goat serum. Immunoreaction with primary antibody was performed at 4 °C for 24 h. Goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate was used as the fluorescence marker. A wide range of dilutions for the primary and secondary antibodies was tested. The optimal dilutions were found to be 1:1,500 for the primary antibody and 1:2,000 for the secondary, with an incubation time of 24 h at 4 °C for the primary antibody and 2 h at room temperature for the secondary antibody. Control experiments, including the incubation of cells without primary antibody or preimmune sera and the blocking of the primary antibody with the synthetic cryptic peptide for which the antibody was generated, were performed and did not show any positive staining. For the pro-TRH antibodies, Texas Red-X-succinimidylester was directly conjugated to affinity-purified anti-pAV37 antibodies as instructed by the manufacturer for conjugation of immunoglobulins with amine-reactive probes (Molecular Probes Inc., Eugene, OR) with the following modifications; binding reaction of Texas Red with antibody was performed for 1 h at room temperature, and the separation of the conjugate from unreacted labeling reagent was performed by Centricon 30 filtration instead of gel filtration chromatography.

RESULTS

Multiple RIAs directed against different epitopes of pro-TRH were performed on fractions derived from infected GH4C1 and LoVo cells. The cleavage pattern of pro-TRH processing achieved by each PC enzyme was thereby evaluated. In Fig. 1, the structure of pro-TRH and the different intermediates and cryptic peptides are presented, as determined from previous studies (7, 8, 13). The immunoreactivity and size of the peptides evident in this study correspond to those previously determined in pulse-chase, size fractionation, and radiolabeling experiments (7, 8, 13).

Cleavage of Pro-TRH in GH4C1 Cells Co-injected with Control Prodynorphin—Coinfection of GH4C1 cells using vaccinia virus as a cellular coexpression system for prodynorphin and pro-TRH revealed no processing of pro-TRH. The control prodynorphin is not an enzyme but rather the precursor of three
leucine-enkephalin opioid peptides (45). This construct thus serves as a control for the PC enzyme constructs. Uniformly, using antibodies to both C-terminal and N-terminal epitopes, no pro-TRH cleavage was evident (data not shown).

General Cleavage Capabilities of the PC Enzymes—Coinfection of LoVo cells with individual PCs and pro-TRH yielded different cleavage capabilities for each enzyme. Fig. 2, A and B, depict RIA results from LoVo cell media as monitored by anti-pYE17 and anti-pYE27, respectively. With anti-pYE17, furin-mediated cleavage to C-terminal intermediates was 6 times greater than that of PC1, PC2, PACE4, PC2, and PC5-B exhibited minor cleavage capabilities. With anti-pYE27, PC1, PC2, and PACE4 cleavage was one-third that of furin, while PC5-B did not cause cleavage above that of the control dynorphin. Fig. 2, A and B, thus suggests that furin is significantly capable of pro-TRH cleavage to intermediates in the absence of a regulated secretory pathway, while PC1, PC2, PACE4, and PC5-B exhibit much lower cleavage capabilities in these constitutively secreting cells, which are devoid of endogenous furin activity (46).

Coexpression of pro-TRH and the PCs in GH4C1 (containing a regulated secretory pathway) demonstrated that PC1- and PACE4-mediated cleavage is augmented to levels achieved by furin. Fig. 2D demonstrates that PC1 and furin comparably cleave to N-terminal products and that PC2 achieves product formation of about 30% as compared with PC1. On the other hand, with C-terminally directed antibodies (anti-pYE17), the products generated by PC2 increased only slightly over the control dynorphin (Fig. 2C). PACE4 and PC5-B also demonstrated significant cleavage capability for both N- and C-terminal products.

Cleavage of the Pro-TRH Precursor to Immunoreactive TRH-Gly and TRH—We now wanted to determine if each PC enzyme could cleave pro-TRH to the extended TRH form, TRH-Gly. Full processing to TRH-Gly was determined using the anti-TRH-Gly RIA, performed on GH4C1 (Fig. 3A) and LoVo (Fig. 3C) cell media. In GH4C1 cells, PC1 coinfection resulted in the largest amount of TRH-Gly formation, while furin and PACE4 demonstrated lower quantities. PC2 and PC5-B resulted in TRH-Gly formation at levels only slightly higher than controls. This is the first demonstration that PC1, PC2, and furin are capable of producing the TRH progenitor sequence from prepro-TRH. With LoVo cells, which only contain the constitutive secretory pathway, furin coinfection resulted in a large quantity of TRH-Gly production relative to PC1, PC2, PC5-B, and PACE4, which were comparably low. Full analysis of the processing to TRH was determined using the anti-TRH RIA on the GH4C1 cell medium. LoVo cell medium was not analyzed, since this cell line does not contain secretory granules and peptidyl glycine 6-amidating monoxygenase (PAM) and thus is incapable of forming mature immunoreactive TRH. As shown in Fig. 3B, both furin and PC1 produced comparable levels of TRH, while PACE4, PC2, and PC5-B produced progressively lower levels of TRH. This is the first demonstrated production of immunoreactive TRH generated by any of the PC enzymes.

Because both PC1 and PC2 expression is confined to neu-

![Fig. 2. Cleavage of pro-TRH as determined using anti-pYE17 RIA in LoVo cells (A) and GH4C1 cells (C) and using anti-pYE27 RIA in LoVo cells (B) and GH4C1 cells (D). Coinfections of pro-TRH were done with PC1, PC2, Dyn, PACE4, PC5-B, and furin in GH4C1 and LoVo cells. RIAs were performed against the resuspended serum-free media. Cell means of recognized products in picograms are plotted against the indicated coinfected construct. Data are the mean values of six identical wells/condition, with p < 0.05. Data were subjected to ANOVA program (Abacus Concepts, Inc., Berkeley, CA) followed by the Tukey-Kramer test for multiple comparisons.]
roendocrine and endocrine cells, studies have focused on these proconvertases as potentially significant enzymes in prohormone maturation. Consequently, in vitro experiments with pro-TRH have also focused on these enzymes (15). Therefore, we further sought to characterize PC1 and PC2 intermediate cleavage profiles using SDS-polyacrylamide gel electrophoresis and RIA.

**PC1- and PC2-mediated Cleavage of Pro-TRH—**Coinfection of GH4C1 cells with pro-TRH/PC1 resulted in a number of different cleavage products, as monitored by RIA for N- and C-terminal products. Fig. 4A depicts RIA results from cell extracts using anti-pCC10, which detects the 26-kDa precursor and N-terminal intermediates. Immunoreactive peaks occurred at 26, 9.5, and 3.8 kDa, with a diffuse peak around 15 kDa. This peptide profile indicates major cleavage at prepro-TRH Lys152-Arg153 or Arg158-Arg159 and cleavage at prepro-TRH Lys75-Arg76 or Lys81-Arg82. Cleavage at prepro-TRH Lys107-Arg108 or Lys113-Arg114 is also indicated by a broad peak at 9.5 kDa determined using anti-pYE27 (Fig. 4B). The 4-kDa peak was evident as a “tail” with this antiserum, resultant from overlapping peaks. C-terminal cleavage intermediates were monitored using the anti-pYE17 RIA, which detects the 26-kDa precursor and 16.5-, 10-, and 5.4-kDa peptides. As evident in Fig. 4C, coexpression of prepro-TRH with PC1 in GH4C1 cells yielded a peak in the 5.4-kDa range with no 26-kDa precursor or 10-kDa or 16.5-kDa peptides, suggesting a complete conversion of the TRH precursor to the 5.4-kDa form. This profile indicates significant proteolysis at prepro-TRH Lys200-Arg201 or Arg206-Arg207. GH4C1 cells infected with PC2 contained similar N-terminal peptide profiles as those infected with PC1. In the anti-pCC10 RIA (Fig. 5A), a precursor peak was evident, as well as small 15- and 9.5-kDa peaks. This peptide profile indicates cleavage at prepro-TRH Lys152-Arg153 or Arg158-Arg159 and cleavage at prepro-TRH Lys107-Arg108 or Lys113-Arg114. The 3.8-kDa peptide was large relative to the other moieties, suggesting efficient proteolysis at prepro-TRH Lys75, Arg80, or Lys81-Arg82. Both the 9.5- and 4-kDa peptides were evident in the anti-pYE27 RIA (Fig. 5C), indicating as with PC1 initial cleavage at prepro-TRH Lys107,Arg108 or Lys113-Arg114. A modest C-terminal cleavage beyond the precursor was evident in the anti-pYE27 RIA (Fig. 5C), probably indicating that PC2 inefficiently cleaves the C-terminal portion of pro-TRH.

**PC1- and PC2-mediated Cleavage of Prepro-TRH77–106 to Prepro-TRH83–106—**Using the N-terminal anti-pEH24 serum, we next characterized the ability of PC1 and PC2 to produce pEH24 (prepro-TRH83–106) from its TRH-extended form (prepro-TRH77–106). Electrophoretic separation of extracted peptides from GH4C1 cells coinjected with pro-TRH/PC1 (Fig. 6A) had shown a further cleavage of the 3.8-kDa peptide to the 2.8-kDa peptide (prepro-TRH52–107), a conversion probably resultant from the C-terminal excision of the TRH progenitor sequence from the 3.8-kDa peptide. The 3.8-

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**Fig. 3.** Cleavage of pro-TRH to TRH-Gly and TRH as determined using the anti-TRH-Gly and anti-TRH RIAs. Coinfections of pro-TRH were done with PC1, PC2, Dyn, PACE4, PAC5-B, and furin in GH4C1 (A and B) and in LoVo cells (C). RIAs were performed against the resuspended serum-free media. Panel B shows the cleavage of pro-TRH to TRH in GH4C1 cells using anti-TRH RIA. Cell means of TRH-Gly and TRH in picograms are plotted against the indicated coinfected construct. Data are the mean values of six identical wells/condition, with p < 0.05. Data were subjected to ANOVA program (Abacus Concepts, Inc., Berkeley, CA) followed by the Tukey-Kramer test for multiple comparisons.

**Fig. 4.** Cleavage of pro-TRH after coinfection with PC1 and pro-TRH in GH4C cells, as determined by anti-pCC10, anti-pYE27, and anti-pYE17 RIAs. Panel B shows the cleavage of pro-TRH to TRH in GH4C1 cells using anti-TRH RIA. Cell means of TRH-Gly and TRH in picograms are plotted against the indicated coinfected construct. Data are the mean values of six identical wells/condition, with p < 0.05. Data were subjected to ANOVA program (Abacus Concepts, Inc., Berkeley, CA) followed by the Tukey-Kramer test for multiple comparisons.
kDa peptide peak is larger than the 2.8-kDa peak, suggesting efficient conversion to the 3.8-kDa but not the 2.8-kDa peptide. This "efficiency of proteolysis" is suggested in that within a given assay a larger peak may indicate a greater conversion to that product. A 9.5-kDa peptide was also seen with this antibody, which is similar in size to the 9.5-kDa peak seen with anti-pCC10. If this 9.5-kDa peak is the same, this should indicate cleavage at prepro-TRH Lys107-Arg108 or Lys113-Arg114. No peak of the size of the TRH precursor was observed because this antiserum does not detect the 26-kDa form. When GH4C1 cells were coinfectected with pro-TRH/PC2 (Fig. 6B), the 9.5-kDa peak was again evident as well as the doublet of 3.8 and 2.8 kDa; in the doublet, the 2.8-kDa peak is larger than the 3.8-kDa peak, suggesting that PC2 is more efficient than PC1 at cleaving the 3.8-kDa form to generate the 2.8-kDa peptide. This excision indicates that PC2-mediated release of TRH from the pEH24 peptide, possibly at the prepro-TRH Lys81-Arg82 site. This is the first possible evidence that PC2 may play a role in generating TRH from TRH-extended forms later in the secretory pathway. We still have to investigate the formation of all pro-TRH intermediate forms in the presence of PC1 and/or PC2 to pinpoint the precise cleavage pathways.

**Quantitative Cleavage Capabilities of PC1, PC2, and PC2 plus 7B2 to Produce Pro-TRH Intermediates**—We now wanted to determine the contribution of PC1 and PC2 to pro-TRH cleavage and to determine whether the peptide 7B2 altered the ability of PC2 to achieve cleavage. 7B2 (47) is a peptide shown to be important for the maturation and regulation of pro-PC2.
activity (47, 48) and, thus, is a necessary component in confirming the cleavage capability of PC2. Consequently, as shown in Fig. 7, we coinfected PC1 and PC2 (or PC1, PC2, and 7B2) to achieve a total cleavage level and then individually coinfected PC1, PC2, and PC2 plus 7B2 to determine the relative cleavage capabilities of each convertase. As shown in Fig. 7, using anti-pYE17, the C-terminal 10- and 5.4-kDa intermediates were produced primarily by PC1. The addition of 7B2 did not alter the profile significantly, with the exception that in the presence of 7B2 PC2 completely cleaves pro-TRH to the 5.4-kDa intermediate, suggesting that 7B2 improved the capability of PC2 to cleave the Arg206-Arg207. Thus, PC2 seems to quantitatively contribute to a lesser degree than PC1 in the overall production of the TRH intermediates. Results using anti-pEH24 and anti-pYE17 indicated a similar conclusion (data not shown).

Quantitative Cleavage Capabilities of PC1, PC2, and PC2 plus 7B2 to Produce TRH-Gly and TRH—As shown in Fig. 8A, PC1 cleavage of pro-TRH produces the majority of immunoreactive TRH-Gly, while PC2 alone and in conjunction with 7B2 does not significantly contribute to TRH-Gly production. Similarly, in Fig. 8B, it is evident that PC1 produces the majority of immunoreactive TRH and that PC2 alone and in conjunction with 7B2 does not significantly contribute to TRH production. The lower levels of TRH-related peptides in the presence of triple infections have been noted before (38, 41, 43) and are probably due to the virus taking over the machinery of the cell and hence lowering the overall translation of endogenous proteins, such as carboxypeptidase E and PAM, which are necessary for the production of TRH-Gly and TRH, respectively.

To further clarify whether PC2 has a better ability to cleave the pro-TRH N-terminal side over the C-terminal one, we analyzed the processing products using the same coinfections depicted in Fig. 8, but monitoring the peptides obtained using antibodies against the N- (anti-pYE27) and C-terminal (anti-pYE17) ends of the TRH precursor. Fig. 9A shows that PC2 was able to generate almost 37% of the total pYE27 produced by PC1, and only 19% of the total pYE17 produced by PC1. These results are in agreement with the data presented in Fig. 2, C and D, and Fig. 5C, which supports the hypothesis that PC2 has higher capabilities to cleave the N-terminal side of pro-TRH.

Protein Colocalization of pro-TRH with PC1 and PC2 in Primary Cultures of Hypothalamic Neurons—Immunostaining of hypothalamic neurons with anti-pAV37 revealed that between 50 and 60% of the cells were positively stained with this antibody. A similar percentage of positive staining was found when the pro-TRH mRNA expression was evaluated (14). In contrast, almost 100% of the neurons were positively stained with anti-PC1, and less than 80% were positively stained with PC2. Fig. 10A shows positive immunostaining for pro-TRH peptides, and panel B shows positive immunostaining for PC1. Panel C shows the protein colocalization of pro-TRH and PC1 as well as some neuronal cells containing only PC1. While the intensity of fluorescence for PC1 in most cells was constant, for PC2 fluorescence intensity varied (panel E), suggesting a differential level of biosynthesis for PC2 enzyme in some subpopulation of cells. As depicted in panel F, from the two PC2 and pro-TRH colocalized cells shown, one was indicated to have less PC2 produced.

**DISCUSSION**

Post-translational processing of neuropeptides and prohormones, usually at dibasic residues, is essential for maturation of bioactive peptides contained within a given propeptide (33, 35). In this study, we used a vaccinia virus coinfection system to determine the processing pattern of pro-TRH by the PC family of endoproteases, in particular by PC1 and PC2. Evidence for a physiological role of PC1 and PC2 in the proteolytic conversion of pro-TRH came from our in vitro studies (15, 16).
and double in situ hybridization in primary cultures of hypothalamic neurons (14).

Because pro-TRH contains five copies of the TRH sequence and is predicted to produce diverse end products, it is one of the most complicated proprotein precursors for processing analysis. The intermediates of processing have been well described (7, 13), although the exact cleavage sites (i.e. N- or C-terminal to a given TRH progenitor sequence) are indeterminate without peptide sequencing analyses. Consequently, in most cases cleavage cannot be mapped to specific dibasic sequences, and the possibility of cleavage at either site must be allowed; only in pEH24 has immunological characterization confirmed that the C-terminal TRH of the 3.8-kDa form is excised at Lys107-Arg108 to obtain the 2.8-kDa peptide (7).

Our results suggest that a number of the enzymes of PC family are capable of cleaving pro-TRH to intermediates, TRH-Gly, and TRH (Figs. 2 and 3); furthermore, in a quantitative comparison of the ability of PC1 and PC2 to cleave to both intermediates and end products, PC1 seems primarily responsible for most if not all cleavage events (Figs. 7 and 8). These results are further supported by our previous studies in primary cultures of hypothalamic neurons and by the protein colocalization images presented in Fig. 10. Fig. 11 shows a diagrammatic representation of rat pro-TRH and its cleavage by PC1 and PC2 as proposed in this study as compared with the previous in vitro studies (15, 16).

As shown in Fig. 4, PC1 is capable of initially cleaving pro-TRH at both prepro-TRH Lys152-Arg153 or Arg158-Arg159.

FIG. 8. Cleavage of pro-TRH as determined using anti-TRH (A) RIA and anti-TRH-Gly (B) RIA. Coinfections of pro-TRH were done with Dyn, PC1, PC2, PC1-PC2, PC1-PC2-7B2, and PC2-7B2 in GH4C1 cells. RIAs were performed against resuspended serum-free media. Cell means of recognized products in picograms are plotted against the indicated coinfectected construct. Data are the mean values of six identical wells/condition, with p < 0.05. Data were subjected to ANOVA program (Abacus Concepts, Inc., Berkeley, CA) followed by the Tukey-Kramer test for multiple comparisons.

FIG. 9. Cleavage of pro-TRH as determined using anti-pYE27 RIA (A) and anti-pYE17 RIA (B). Coinfections of pro-TRH were done with Dyn, PC1, PC2, PC1-PC2, PC1-PC2-7B2, and PC2-7B2 in GH4C1 cells. RIAs were performed against resuspended serum-free media. Cell means of recognized products in picograms are plotted against the indicated coinfectected construct. Data are the mean values of six identical wells/condition, with p < 0.05. Data were subjected to ANOVA program (Abacus Concepts, Inc., Berkeley, CA) followed by the Tukey-Kramer test for multiple comparisons.
forming the 15/10-kDa pair, and prepro-TRH Ly$^{107}$-Arg$^{108}$ or Lys$^{113}$-Arg$^{114}$, forming the 9.5/16.5-kDa pair. This dual processing capability is significant in that differential processing and localization of pro-TRH intermediates may be an important regulatory step in producing different bioactive peptides. Besides TRH, a number of flanking peptides will also be produced that could have their own biological function. Examples include the putative prepro-TRH$^{160}$-$^{169}$ (49) and the controversial evidence that prepro-TRH$^{172}$-$^{199}$ is the corticotrophin-releasing inhibiting factor (50, 51). Despite the controversy around the in vitro ACTH inhibiting effects of prepro-TRH$^{178}$-$^{199}$, recent data show that the chemically intact peptide inhibits plasma ACTH and prolactin secretion in response to stress (52).

Tissue-specific cleavage of pro-TRH to either fully processed TRH and cryptic peptides or N-/C-terminal extended forms generates further diversity in secreted products. The concept of differential processing associated with differential subcellular localization and specific cell type is further reinforced by the observation that certain regions in the brain can give rise to several different pro-TRH-derived peptides in addition to, or instead of, TRH. For example, the reticular nucleus of the thalamus contains abundant pro-TRH mRNA and several pro-TRH-derived peptides but does not contain mature TRH (53, 54). Moreover, the extended forms of TRH, prepro-TRH$^{154}$-$^{169}$ and prepro-TRH$^{154}$-$^{169}$, have been found to be the major end products of pro-TRH processing in the olfactory lobe (in which pro-TRH is coexpressed only with PC2 as judged by double in situ hybridization (55)) (56–58) but not in the hypothalamus (which contains high concentrations of PC1 and PC2), where pro-TRH is completely processed to cryptic peptides and TRH (56). Thus, in the central nervous system, pro-TRH appears to be processed in a region-specific manner. Furthermore, in our recent in vivo studies, we found that opiate withdrawal produced an increase in the level of prepro-TRH mRNA and the N-terminal prepro-TRH$^{13}$-$^{74}$ peptide in the rat pariaqueductal gray, whereas the level of TRH remained unaltered (59). The pariaqueductal gray has a higher concentration of PC2 compared with PC1. These data demonstrate that levels of various products derived from pro-TRH can be post-translationally regulated in an independent fashion under altered physiological conditions. The fact that full processing of pro-TRH was observed in tissues with high levels of PC1 supports the findings of this current work, which propose that PC1 is the primary enzyme involved in the processing of pro-TRH.

It has been demonstrated in AtT-20 cells that while the 15- and 9.5-kDa (N-terminal) forms are processed to intermediates only after sorting to the immature SG, the 16.5-kDa (C-terminal) form is processed in the trans-Golgi network to the 5.4-kDa peptide (60). Furthermore, N- and C-terminal intermediates seem to have partially different subcellular localizations in hypothalamic neurons (18) and transfected AtT-20 cells (61). These results suggest that differential production of different bioactive peptides can be achieved through differential cleavage of intermediates. PC1’s ability to cleave at both sites, as presented here, suggests it can generate the two intermediates required for this hypothesized differential regulation.

In contrast with the in vitro evidence (15), PC1 was able to release the C-terminal TRH from the 3.8-kDa peptide to form the 2.8-kDa peptide. The absence of the 2.8-kDa peptide in the in vitro evidence may be due to the artificiality of the in vitro system, which may not provide the conditions necessary for optimal PC1 recognition and cleavage of prepro-TRH Ly$^{107}$-Arg$^{108}$. Furthermore, the pro-TRH quantities obtained from AtT-20 cells were around 100-fold smaller than that generated in the vaccinia virus system; this probably contributed to the absence of the 2.8-kDa peak. The data presented in Fig. 6B, indicating cleavage to the 2.8-kDa peak, suggest that PC1 is able to form TRH from pro-TRH. The anti-TRH-Gly and anti-TRH RIA (Fig. 3, A and B) results confirm this hypothesis, demonstrating for the first time that PC1 is capable of cleavage to mature TRH.

Fig. 3A also demonstrates that in GH4C1 cells, furin and PACE4 produced TRH-Gly, while PC2 and PC5-B resulted in TRH-Gly formation at a level only slightly higher than the control. Similar results for TRH production in GH4C1 cells are evident (Fig. 3B). Although GH4C1 cells endogenously produce furin and PACE4, the control dynorphin coinfections indi-
cate that these endogenous enzymes did not significantly contribute to pro-TRH proteolysis. Interestingly, in LoVo cells furin produced TRH-Gly at a much greater level than any of the other PC enzymes (Fig. 3C). Recent data indicate that furin, which is ubiquitously expressed in all tissues, may serve a role in processing of prosomatostatin within the constitutive pathway (39, 40); since LoVo cells only contain the constitutive secretory pathway, the results presented here suggest that pro-TRH can be processed to a certain extent without entry into the regulated secretory pathway. The processing products obtained by furin and PC1 are similar, in agreement with their similar specificity observed in a number of cellular co-expression experiments (38, 40, 62) and in vitro data (63, 64). Fig. 6B demonstrates that PC2 coinfection resulted in a greater quantity of the 2.8-kDa relative to the 3.8-kDa peptide, while the converse was true for PC1, suggesting that PC2 may be important in generating TRH from this intermediate.

While the zymogen cleavage of pro-PC1 to PC1 occurs in the endoplasmic reticulum (65–67), pro-PC2 to PC2 conversion occurs late in the secretory pathway within the trans-Golgi network or immature secretory granules (65–70). PC2 was thus generally thought to play a role in producing smaller peptides from intermediates initially cleaved by PC1 (38, 41, 42). In contrast, PC1 has been implicated in early cleavage steps of pro-opiomelanocortin (41, 42, 71) and proinsulin (72) processing. The evidence presented here seems not to follow that model, suggesting that for pro-TRH, PC1 is involved in both early and late stages of processing, while PC2, although capable of processing, does not significantly contribute to peptide production.

To confirm and further understand the cooperative role between PC1 and PC2 in their ability to cleave pro-TRH to its predicted products of processing, we are currently conducting stable transfection experiments with pro-TRH, PC1, and PC2 in the PC12 cell line, which only endogenously produces furin (1). Our initial pulse-chase studies with transfected PC12 cells encoding either pro-TRH/PC1 or pro-TRH/PC2 indicated that both enzymes were capable of processing pro-TRH to its C-terminal intermediate forms, showing a processing profile similar to that previously demonstrated in transfected AtT20 cells (7). However, the main difference we have found so far between the colonies transfected with PC1/pro-TRH and PC2/pro-TRH is that processing by PC2 happens 30 min later than that performed by PC1. While at 30 min of chase PC1 was able to produce the first intermediate products of processing, at the same time for PC2 not much processing was evident. The generation of intermediate products of processing was observed at 60 min of chase in colonies transfected with PC2 (unpublished results). These results suggest that by lacking active PC1 to produce the first cleavage within the trans-Golgi network (14, 60), the intact prohormone is sorted to the secretory granules where then PC2 will initiate the processing cleavages. We have also initiated peptide sequencing studies that are necessary to confirm the cleavage points at which the various intermediates are generated; this knowledge should improve our understanding of the specificity of each PC enzyme for a given dibasic site.

In conclusion, we have established for the first time that TRH and TRH-Gly are produced from pro-TRH by a number of precursor convertases. Cleavage profiles for PC1 and PC2 are presented here, which suggest that to a certain extent both can endoproteolytically cleave pro-TRH. However, multiple coinfection experiments suggest that PC1 is primarily responsible for all cleavage events, and the role of PC2, if any, remains to be resolved. Further studies are needed to confirm these profiles and achieve a more extensive knowledge of the specificity of cleavage of the PC enzymes for pro-TRH.

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