Prohormone-Convertase 1 Processing Enhances Post-Golgi Sorting of Prothyrotropin-releasing Hormone-derived Peptides*

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Rat prothyrotropin-releasing hormone (pro-TRH) is endoproteolyzed within the regulated secretory pathway of neuroendocrine cells yielding five TRH peptides and seven to nine other unique peptides. Endoproteolysis is performed by two prohormone convertases, PC1 and PC2. Proteolysis of pro-TRH begins in the trans-Golgi network and forms two intermediates that are then differentially processed as they exit the Golgi and are packaged into immature secretory granules. We hypothesized that this initial endoproteolysis may be necessary for downstream sorting of pro-TRH-derived peptides as it occurs before Golgi exit and thus entry into the regulated secretory pathway. We now report that when pro-TRH is transiently expressed in GH4C1 cells, a neuroendocrine cell line lacking PC1, under pulse-chase conditions release is constitutive and composed of more immature processing intermediates. This is also observed by radioimmunoassay under steady-state conditions. When a mutant form of pro-TRH, which has the dibasic sites of initial processing mutated to glycines, is expressed in AtT20 cells, a neuroendocrine cell line endogenously expressing PC1, both steady-state and pulse-chase experiments revealed that peptides derived from this mutant precursor are secreted in a constitutive fashion. A constitutively secreted form of PC1 does not target pro-TRH peptides to the constitutive secretory pathway but results in sorting to the regulated secretory pathway. These results indicated that initial processing action of PC1 on pro-TRH in the trans-Golgi network, and not a cargo-receptor relationship, is important for the downstream sorting events that result in storage of pro-TRH-derived peptides in mature secretory granules.

All of the peptides derived from pro-TRH2 are targeted to the regulated secretory pathway of neuroendocrine cells (1, 2), but it is not completely clear how pro-TRH and other prohormones are targeted to secretory granules from the trans-Golgi network (TGN). Proteins and peptides targeted to the regulated secretory pathway are ultimately stored in secretory granules until the cell receives a stimulus signaling for granule content release (3). Proteins that are secreted without storage in a cellular compartment are said to transit through the constitutive or constitutive-like secretory pathway (4, 5). Scientific debate continues as to what targets prohormones and other proteins into the regulated secretory pathway with some favoring a receptor-mediated model (6–8), self-aggregation of regulated secretory pathway cargo (9–11), or binding of the prohormone to lipid rafts (12–14), but it is generally agreed that there are unique structural elements of prohormones, not necessarily universal, that result in their targeting to the regulated secretory pathway (15–22). It has yet to be reported which elements of pro-TRH might be important in the targeting of this important prohormone to the regulated secretory pathway.

Rat pro-TRH is a prohormone composed of 255 amino acids, and when fully processed by prohormone convertases 1 and 2 (PC1 and PC2) yields five molecules of TRH and up to nine other peptides (23–25). For a number of years TRH was the only peptide derived from pro-TRH known to behave as a hormone, but recent work has demonstrated that the other peptides derived from pro-TRH may have physiological function (26). Direct physiological evidence of these peptides having functions independent of TRH is currently lacking but is being studied.

Processing of pro-TRH by PC1 begins in the TGN generating processing intermediates of pro-TRH prior to packaging into immature secretory granules (27, 28). Because the rat pro-TRH molecule contains several structural elements that have been implicated in the regulated sorting of other prohormone molecules such as dibasic residues (20, 21), a disulfide bond (17, 19, 29), and two RGD sequences (30), we have hypothesized that the processing of pro-TRH before packaging into immature secretory granules is critical for its downstream sorting. The early and subsequent PC1 processing events may expose sorting signals, which then become accessible for targeting of the various pro-TRH-derived peptides.

To study if pro-TRH sorting to the regulated secretory pathway is affected by PC1 processing, three experimental approaches were utilized: the first one where PC1 itself was either present or absent, the second where the proTRH sequence was mutated to prevent the initial processing by PC1 at the level of the TGN, and third, when pro-TRH was co-expressed with a constitutively secreted PC1 construct (31). Under both steady-state and pulse-chase conditions in two neuroendocrine cell lines, we found that if PC1 is absent or if PC1 processing is blocked it results in pro-TRH sorting to the constitutive secretory pathway. We conclude that the effect of PC1 on pro-TRH sorting is not because of a cargo-receptor interaction like that observed between brain-derived neurotrophic factor and carboxypeptidase E (32), because the constitutively secreted PC1 acted to increase regulated sorting of pro-TRH as did the wild-type PC1, indicating that processing of
pro-TRH itself is important for downstream sorting of pro-TRH. In this study we present the first evidence that PC1 activity greatly enhances the sorting of pro-TRH-derived peptides to the regulated secretory pathway of two neuroendocrine cells lines.

EXPERIMENTAL PROCEDURES

Antibodies and Other Materials

Rabbit polyclonal antibodies against pro-TRH-derived peptides TRH, pYE26, pAV37, pYE17, and pFE22 were generated by BIO-SOURCE and protein G purified before use, except for pFE22 which was affinity-purified before use. These antibodies have been fully characterized by our laboratory, and TABLE ONE describes the epitopes used to create the antibodies and the moieties recognized by each antibody (24, 28, 33). PC1ΔC under control of the Rous sarcoma virus promoter was kindly provided by Dr. Nabil Seidah (34) of the Clinical Research Institute of Montreal. Protein-A/G-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). l-[3,4,5-3H]Leucine was obtained from PerkinElmer Life Sciences. Leucine-deficient Dulbecco’s modified Eagle’s media (DMEM) for pulse-chase experiments was obtained from Specialty Media (Phillipsburg, NJ). Fetal bovine calf serum was obtained from Atlanta Biologicals (Atlanta, GA). Unless otherwise specified, all other chemicals and reagents were obtained from Sigma.

Generation of Pro-TRH Mutants

Prepro-TRH was removed from the pCMV-TRH-SV40 vector (1) by EcoRI digestion and then subcloned into the pCNDA3.1/zeo+ plasmid (Invitrogen), which drives expression of cloned genes with the constitutively active cytomegalovirus promoter. This construct was used for in vitro expression of wild-type prepro-TRH and as a template for creation of the pro-TRH mutant, PC1-Block (Fig. 1). The mutations to pro-TRH were created with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s specifications. Successful mutation and cloning were confirmed by DNA sequencing.

Cell Culture

AtT20 cells (D16v-F2 subclone; ATCC, Manassas, VA) were cultured in DMEM supplemented with high glucose, 30 mM sodium bicarbonate, 1 mM sodium pyruvate, 10% fetal bovine calf serum, and 0.1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO2. GH4C1 cells were grown in the same media as AtT20 cells. GH4C1 cells stably expressing PC1 (GH4C1+PC1) were grown in the same media with 250 µg/ml G418 (Invitrogen) added to select for cells stably expressing PC1 (35). Dr. Iris Lindberg of the Louisiana State University Health Sciences Center kindly provided the GH4C1 lines.

Transfection

Plasmid DNA was transiently transfected into AtT20 or GH4C1 cells using Lipofectamine 2000 transfection reagents (Invitrogen). Transfection efficiency was evaluated by co-transfection with pEGFP1 (Clontech) and subsequent visualization of green fluorescent protein fluorescence efficiency was evaluated by co-transfection with pEGFP1 (Clontech) and subsequent visualization of green fluorescent protein fluorescence.

Selection of Stable Clones

1 × 107 AtT20 cells were transiently transfected with either wild-type prepro-TRH or PC1-Block. Forty eight hours after transfection, the cells were put under a selection pressure of 250 µg/ml of geneticin (Invitrogen). Clonal populations were then removed from each dish and cultured individually under continual selection pressure. Clones were evaluated for pro-TRH expression, fold stimulation, and by immunocytochemistry to ensure that all cells in each clonal line expressed the transfected constructs. The number of clones generated as well as their expression levels and fold stimulation of pro-TRH (average of duplicate samples) are described in TABLE THREE.

Radioimmunoassay (RIA) Analysis

The assays used for pro-TRH-derived peptides were developed in our laboratory by using custom-synthesized or commercially available peptides and primary antibodies (29). The trace was iodinated using the chloramine T oxidation-reduction method followed by high pressure liquid chromatography purification. The N-terminal antisera anti-prepro-TRH-(25–50) (pYE26), the C-terminal antisera anti-prepro-TRH-(240–255) (pYE17), and TRH, which were used to study pro-TRH processing and RIAs, were performed as standard in our laboratory (36). All RIAs were performed on the same volume of material, in duplicate. The intra- and inter-assay variabilities were 5–6 and 9–12% respectively.

Pulse-Chase Studies

Transfected AtT20 or GH4C1 cells were cultured in DMEM labeling media lacking leucine for 20 min to clear cells of endogenous leucine stores. The preclear media were removed and replaced with 5 ml of a mixture containing 90% DMEM labeling media, 10% complete DMEM, and 400 µCi of [3H]leucine. The pulse was continued for 2 h, which is the optimal time determined experimentally for labeling sufficient amounts of pro-TRH for later detection. This was followed by two washes with complete DMEM and a 4-h chase with complete DMEM containing a 4-fold excess of leucine. This 4-h chase was designed to allow for release of most or all of the recently synthesized peptide, which was traveling within the constitutive and or constitutive-like secretory pathways. The complete DMEM was then collected for immunoprecipitation of pro-TRH peptides and replaced with basal DMEM media lacking serum (basal media). After 1 h, basal medium was collected for immunoprecipitation and replaced with a defined secretion medium (basal media with 1 mM BaCl2 added as a secretagogue) designed to stimulate release of granule contents. This medium was collected for immunoprecipitation, and the cells were then scraped into cold PBS and pelleted by centrifugation. The cells were then lysed by addition of 500 µl of 2 n acetic acid with protease inhibitor mixture (Sigma) and heating to 95 °C for 10 min. Cell lysates were clarified by centrifuging at 16,000 × g for 30 min at 4 °C. Clarified lysates were then lyophilized to prepare them for immunoprecipitation.

Immunoprecipitation

Media—Conditioned media were first preclarified with normal rabbit serum (3 µg/5 ml of media; normal rabbit serum was from BIO-SOURCE) and 50 µl of 50% proteinA/G-plus resin for 1 h at 4 °C. The preclarified media were then placed in a fresh tube containing 30 µl of proteina/G-plus-agarose preconjugated to 5 µg of either α-pFE22 or α-pAV37. The immunoprecipitation was allowed to continue overnight at 4 °C on a rotator. The protein A/G-agarose–antibody conjugates were collected by centrifugation and then washed three times in wash buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA) followed by one wash in PBS. 2× SDS-PAGE Sample buffer was then added to the beads, and the sample was boiled for 10 min. The tubes were centrifuged for 30 s, and the supernatants were fractionated with Tris/Tritcine gels as described previously (37). Gels were then cut into 2-mm slices with a gel slicer (Hoefer Scientific Instruments, San Francisco) and placed individually into scintillation vials. Peptides were...
extracted from the gel slices with a 16-h incubation at room temperature in 500 µl of 2 N acetic acid. Radioactivity was determined by addition of 5 ml of Bio-Safe II scintillation fluid (Research Products International, Mount Prospect, IL) followed by 10 min of counting per tube in a beta counter (Beckman Coulter, Fullerton, CA). Counts/min were determined automatically.

Cell Content—Lyophilized cell content was solubilized in 500 µl of resuspension buffer (0.5% BSA, 17 mM NaH2PO4, 150 mM Na2PO4, pH 7.4) and cleared of insoluble material by centrifuging at 16,000 × g for 20 min at 4 °C. Cell lysate was then precleared, immunoprecipitated, and analyzed as described for the media samples.

Calculations

To correct for the fact that counts/min measurements only determine amounts of [3H]leucine and not relative amounts of peptide recovered, the counts/min of lower molecular weight peaks recovered by immunoprecipitation were multiplied by the following formula: corrected cpm = (determined cpm) × (number of leucines expected in highest molecular weight peak recovered/number of leucines expected in other recovered peak/s). By utilizing this formula, all cpm results are presented as the theoretical value that would have been obtained had each peptide recovered contained the same number of leucines thus indicating the relative abundance of each prepro-TRH-derived peptide recovered.

Immunocytochemistry

Immunocytochemistry experiments were conducted as described previously (2). Briefly, AtT20 or GH4C1 cells were fixed for 1 h with 4% paraformaldehyde in phosphate sucrose buffer (PSB: 120 mM sucrose, 100 mM PIPES, 5 mM EGTA, 1 mM MgCl2, pH 6.8). Cells were then permeabilized with 0.2% Triton X-100 in PSB for 7 min. Cells were then washed with 1% BSA in PSB, followed by three 10-min washes with 100 mM NH4Cl in PBS (buffer A). The cells were then incubated in buffer A with 5% BSA for 1 h followed by three washes with 100 mM NH4Cl in PBS. Antibody was added to the cells at a 1:1,000 dilution in buffer A with 0.2% normal goat serum (buffer B) and incubated overnight at 4 °C. The next day cells were washed three times with buffer B and then incubated with goat anti-rabbit conjugated to fluorescein isothiocyanate (FITC; Vector Labs Burlingame, CA) for 2 h in buffer B. Cells were then washed three times in buffer B and then covered with mounting media containing 4,6-diamidino-2-phenylindole (Vector Laboratories). This mounting medium is used to stain cell nuclei and to protect against photobleaching.

Confocal Imaging

Confocal images were acquired with a Nikon PCM 2000 digital camera (Nikon Inc. Melville, NY) using the argon (488) laser. Serial optical sections were performed with Simple 32, C-imaging computer software (Compix Inc., Cranberry Township, PA). Z series sections were collected at 0.7 µm with a ×60 PlanApo lens and a scan zoom of ×1. Images were processed and reconstructed in NIH Image shareware (National Institutes of Health, Springfield, VA).

RESULTS

PC1 Increases Regulated Sorting of Pro-TRH in GH4C1 Cells—To test if the presence of active PC1 enzyme was necessary for proper sorting of pro-TRH-derived peptides, we transiently transfected either GH4C1 or GH4C1 cells stably expressing PC1 (GH4C1-PC1). GH4C1 cells are known to lack PC1 and PC2, possess secretory granules, and possess the ability to sort and store endogenously and exogenously expressed pro-teins to the regulated secretory pathway with modest efficiency (35), making them an ideal neuroendocrine cell line to test the importance of PC1 processing on pro-TRH sorting. For these experiments we had the use of one clonal GH4C1-PC1 cell line. This line was described to express PC1, sort PC1 to the regulated secretory pathway, and to increase the regulated storage and release of exogenously expressed proinsulin (35). To follow the processing and sorting of pro-TRH, we conducted pulse-chase experiments. It is a well reported fact that although tumoral cell lines serve as a good model to study protein trafficking to the regulated secretory pathway, the majority of their newly synthesized material is released in an unregulated fashion (38). For this reason the pulse-chase experiment is designed with a long initial chase of 4 h to allow material that has not entered the regulated secretory pathway to exit constitutively. The long chase is then followed by two 1-h chases that allow us to study protein release from secretory granules under basal and stimulated conditions. Under these experiments we determined that protein is sorted to the regulated secretory pathway if there is an increase in labeled peptide release in the presence of secretagogue. If release is absent or continues at a similar rate under the last two chase conditions, we know that protein is being secreted via the constitutive and/or constitutive-like secretory pathways. Expression levels were double-checked by RIA (data not shown).

For these experiments we chose to use α-pFE22 for immunoprecipitation because it recognizes intact pro-TRH and several processing intermediates (Fig. 1 and TABLE ONE) and is affinity-purified, increasing its specificity. Under these conditions we observed that in the absence of PC1 and PC2 pro-TRH was still processed into mature peptides (prepro-TRH-(178–199), pFE22), but these mature forms and intact pro-TRH were recovered during the 4-h chase only and thus not stored in compartments for stimulated release indicating mis-sorting (Fig. 2A). Processing of pro-TRH observed in GH4C1 cells is most likely mediated by furin, which can process pro-TRH very efficiently, generating TRH and all non-TRH peptides (33). Prosecretin and TRH and a peak expected to be mature pFE22 were recovered from GH4C1-PC1 cells during the 4-h chase indicating that much of the newly synthesized pro-TRH is released in a constitutive and constitutive-like fashion as expected from a tumoral cell line. However, no pro-TRH was recovered during the basal period, and a significant amount of pFE22 was released during the subsequent 1-h incubation with secretagogue (26.2 ± 3.2% of total recovered peptide, n = 3; Fig. 2B). In contrast GH4C1 cells did not exhibit any significant stimulated release of pro-TRH peptides in the presence of secretagogue (11.9 ± 11% of total recovered peptide, n = 3; Fig. 2A). This indicates that pro-TRH was sorted to secretory granules where release was dependent on the secretagogue in GH4C1-PC1 cells. Notably, only mature pFE22 was recovered from the cells during incubation with the secretagogue indicating that pro-TRH was also more efficiently processed when targeted to the regulated secretory pathway. Only when PC1 was present was regulated release observed, indicating that PC1 processing, which occurs at the TGN, is important for downstream trafficking. Experiments done with PC1 transiently transfected with pro-TRH (Fig. 7) indicate that these results are not because of clonal effects of the GH4C1-PC1 cell line.

Immunolocalization of Pro-TRH-derived Peptides Does Not Differ between GH4C1 and GH4C1-PC1 Cells—Given that under pulse-chase conditions GH4C1 cells did not store pro-TRH-derived peptides in the regulated secretory pathway, we were interested to know what the steady-state distribution of pro-TRH-derived peptides was in these cells and in GH4C1-PC1 cells. Fig. 3 demonstrates that there was no difference in these distributions. In both cell lines, α-pFE22-reactive peptides appear in a punctate pattern indicative of storage in secretory granules
This indicates that even in the absence of PC1, a small portion of newly synthesized pro-TRH-derived peptides was targeted to granules, but given the results in Fig. 2, they are not readily stored in these compartments. Because these images are taken under steady-state conditions, they reveal the localization of pro-TRH that has been stored since the beginning of its expression. Therefore, even if only a small amount of pro-TRH is targeted to the regulated secretory pathway in GH4C1 cells over time, this will enrich the pool of secretory granules produced with pro-TRH peptides. Thus it is not completely unexpected that the steady-state localization of pro-TRH peptides does not differ between GH4C1 and GH4C1-PC1 cells even though the efficiency with which they sort pro-TRH to the regulated secretory pathway does (Fig. 2). To test if the immunocytochemistry data are quantitatively misleading, we assayed the cellular extracts of both GH4C1 and GH4C1-PC1 cells

| Antibodies  | Epitopes            | Moieties recognized          |
|-------------|---------------------|------------------------------|
| α-pYE26     | Prepro-TRH-(25–50)  | Prepro-TRH-(25–50) (4 kDa)  |
| α-pAV37     | Prepro-TRH-(115–151)| Prepro-TRH-(25–50) (26 kDa) |
|             |                     | Prepro-TRH-(115–151) (5.0 kDa) |
| α-pFE22     | Prepro-TRH-(178–199)| Prepro-TRH-(25–255) (26 kDa) |
|             |                     | Prepro-TRH-(25–255) (10 kDa) |
|             |                     | Prepro-TRH-(208–255) (5.4 kDa) |
| α-pYE17     | Prepro-TRH-(240–255)| Prepro-TRH-(25–255) (26 kDa) |
|             |                     | Prepro-TRH-(25–255) (10 kDa) |
|             |                     | Prepro-TRH-(208–255) (5.4 kDa) |

(39).
expressing prepro-TRH for both TRH and pYE26 by radioimmunoassay (TABLE TWO). GH4C1-PC1 cells contain 7.4 times more TRH than GH4C1 cells and 5.9 times more pYE26 than GH4C1 cells at steady-state. This indicates that PC1 processing increases pro-TRH storage and that the immunocytochemistry data shown in Fig. 3 are quantitatively misleading.

Blockade of Initial PC1 Processing through Mutation Results in Sorting of Pro-TRH-derived Peptides — By utilizing GH4C1 and GH4C1-PC1 cells, we were able to test if the presence of PC1 enzyme activity was necessary for sorting of pro-TRH and its derived peptides. However, from these experiments we were unable to determine whether PC1 acts as a sorting receptor for pro-TRH or if the initial processing of pro-TRH by PC1 was necessary for proper pro-TRH trafficking to the regulated secretory pathway. To address this question, we generated a pro-TRH mutant in which both sites of initial PC1 processing that occurs in the TGN (28) were altered to glycines (PC1-Block; Fig. 1). We designed this construct with the intent that it would not be processed at the mutated sites but that the other residues surrounding those sites, which may be important for conferring any potential binding to PC1, would not be altered. For these experiments we used AtT20 cells because they are a model neuroendocrine cell line that endogenously expresses PC1, and they have been shown to process pro-TRH and sort its peptides to the regulated secretory pathway in an in vivo like fashion (1, 23, 27, 28). The results of sorting studies on some prohormones have been found to be dependent on the cell line in which the protein is exogenously expressed (29), so we

### TABLE TWO

| Cells          | TRH* | pYE26  |
|---------------|------|--------|
|               | pg/mg| pg/mg  |
| GH4C1         | 0.22 ± 0.12 | 0.86 ± 0.12 |
| GH4C1 + PC1   | 1.62 ± 0.25<sup>b</sup> | 5.05 ± 0.33<sup>b</sup> |

* Values are picograms of pro-TRH peptide per mg of cellular protein.
<sup>b</sup> p < 0.05 compared with GH4C1. Analysis of variance was followed by a multiple comparison using a Tukey-Kramer test.
Processing Affects Sorting of Pro-TRH-derived Peptides

| Expression level and fold stimulation of pro-TRH peptides from stable AtT20 clones |
|---------------------------------|--------------------|-----------------|---------------------|---------------------|
| Clone                          | Peptide expression level (ng of peptide/µg of extracted protein) | Fold stimulation (stimulated release/[basal release])<sup>a</sup> |
|                                | TRH               | pYE26           | pYE17              | pYE26               | pYE17              |
| WT.1                           | 0.9               | 26.3            | 20.0               | 1.9                 | 1.7                |
| WT.2                           | 1.6               | ND<sup>b</sup>  | ND                 | 1.9                 | 1.9                |
| WT.3<sup>c</sup>               | 2.0               | 36.3            | 33.3               | 1.6                 | 2.0                |
| WT.4                           | 1.1               | 28.1            | 30.7               | 1.7                 | 1.5                |
| PC1-Block.1                    | 1.8               | 7.2             | 4.9                | 1.1                 | 1.2                |
| PC1-Block.2                    | 3.1               | 16.7            | 11.9               | 1.0                 | 0.7                |
| PC1-Block.3                    | 3.0               | 31.8            | 28.1               | 1.1                 | 1.0                |
| PC1-Block.4                    | 1.3               | 13.3            | 7.2                | 1.4                 | 1.1                |
| PC1-Block.5                    | 1.4               | 17.1            | 11.1               | ND                  | 1.3                |

<sup>a</sup> Fold stimulation = concentration of peptide recovered during 1 h of stimulated release/concentration of peptide recovered during 1 h of basal release.

<sup>b</sup> ND indicates the value was not determined.

<sup>c</sup> Clones used in further studies due to robust and similar expression levels are highlighted in boldface.

also wanted to test how interfering with pro-TRH processing would effect sorting in another neuroendocrine cell line. The PC1-Block construct was stably transfected into AtT20 cells, and several clones were tested to rule out clonal effects of the results. We compared this with AtT20 cells that had been stably transfected with wild-type pro-TRH.

The expression levels of pro-TRH and PC1-Block were similar between the two cell lines as determined by RIA (TABLE THREE). Each stable cell line was placed in basal and then secretion medium for 1 h. Direct RIA of conditioned media detected peptide release from these cells. Under these steady-state conditions we expected that regulated sorting and thus storage in secretory granules would be indicated by an increase in peptide release during incubation in secretion medium. We observed that PC1-Block-derived peptides are secreted via the constitutive secretory pathway compared with wild-type pro-TRH-derived peptides, which were secreted in a regulated fashion in response to secretagogue (Fig. 4). Two of the peptides tested for by RIA, TRH and pYE26, are only recognized in their completely processed form. This indicates that mutations made in PC1-Block did not dramatically alter the structure of pro-TRH such that it could not be processed. Also, because we detected peptides in the conditioned media of AtT20 cells expressing PC1-Block, we know that this construct was not degraded early in the secretory pathway indicating that it was most likely not misfolded. This indicates that pro-TRH was secreted via constitutive and constitutive-like pathways when the initial TGN processing of pro-TRH was prevented.

Because our RIA experiments do not fully test for processing, we also followed the fate of PC1-Block-derived peptides in AtT20 cells by pulse-chase analysis using the same methodology outlined for the results presented in Fig. 2. For these experiments we chose to use α-pAV37 antisum for immunoprecipitation because this peptide is liberated from the precursor by processing at the sites mutated in PC1-Block. Immunoprecipitating this peptide allowed us to test if the PC1-Block mutations made in PC1-Block did not affect the structure of pro-TRH such that it could not be processed. Also, because we detected peptides in the conditioned media of AtT20 cells expressing PC1-Block, we know that this construct was not degraded early in the secretory pathway indicating that it was most likely not misfolded. This indicates that pro-TRH was secreted via constitutive and constitutive-like pathways when the initial TGN processing of pro-TRH was prevented.

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Processing Affects Sorting of Pro-TRH-derived Peptides

| MW (kDa) | 29 | 20.4 | 14.4 | 6.5 | 2.9 |
|----------|----|------|------|-----|-----|
| Gel Slice Number | 1 | 3 | 5 | 7 | 11 |

A. WT proTRH

| MW (kDa) | preproTRH3-169 | Undefined Peptide |
|----------|----------------|------------------|
| Gel Slice Number | 1 | 3 | 5 | 7 | 11 | 13 | 15 | 17 | 19 | 21 | 23 |

B. PC1-Block

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**FIGURE 5.** Pro-TRH-derived peptides are secreted constitutively when initial PC1 processing is blocked. Approximately 1 × 10^7 AtT20 cells stably expressing wild-type (WT) prepro-TRH (A) or PC1-Block (B) were pulse-labeled for 2 h with 400 μCi of [3,4,5-3H]leucine, followed by a 4-h chase, a 1-h chase in basal secretion media, and a 1-h chase in stimulation media (see “Experimental Procedures”). α-pAV37 reactive peptides (indicated on the figure; see also Fig. 1) were immunoprecipitated from the media, and cell contents at the end of chase followed by SDS-PAGE fractionation and gel slicing. Peptides were then extracted from gel slices, and the counts/min of each slice were determined and corrected to account for the lesser leucine content of mature peptides and to allow for a comparison of relative peptide levels (see “Experimental Procedures”). The position in the gel of each molecular weight standard is indicated at the top of each figure. The left panels in A and B show a cartogram of the corrected counts/min results obtained from a representative experiment, while the right panels represent average percentage of each peptide recovered from the various media and cell samples in chronological order (n = 3, ± S.E.).

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**FIGURE 6.** Immunolocalization of pro-TRH and PC1-Block-derived peptides does not differ in AtT20 cells. AtT20 cells stably expressing either wild-type prepro-TRH (A) or PC1-Block (B) were fixed and stained with α-pAV37 followed by goat α-rabbit IgG-FITC. Each image is a projection of seven optical sections, collected at 0.7 μm. In all panels punctate staining indicative of secretory granule localization can be seen. Arrows point to tips and arrowheads to cell bodies of cells with secretory granules stained positively for α-pAV37.

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**Processing and Constitutive-like Secretion of Peptides Derived from PC1-Block.**

**Immunolocalization of Pro-TRH and PC1-Block-derived Peptides Does Not Differ in AtT20 Cells**—As observed in the experiments using GH4C1 and GH4C1-PC1 cells expressing pro-TRH (Fig. 2), the localization of pro-TRH and PC1-Block-derived peptides did not differ in AtT20 cells (Fig. 6). Staining was observed in the cell bodies and tips of AtT20 cells in a punctate pattern when either construct was expressed and stained with α-pAV37 (Fig. 6), indicative of targeting to the regulated secretory pathway (26, 40). This indicates that the mutations made in PC1-Block were effective in preventing the initial processing of pro-TRH but did not totally mis-sort all newly synthesized material away from granules. This is similar to our observations with GH4C1 and GH4C1-PC1 cells expressing pro-TRH shown in Fig. 3. The clones used in this experiment, WT.3 and PC1-Block.3, had similar levels of TRH, pYE26, and pYE17 peptides, indicating that the immunocytochemistry data were not quantitatively misleading at least for these clones that had similar steady-state levels of pro-TRH-derived peptides (TABLE THREE). However, this may be an effect of using clones that stably express pro-TRH and PC1-Block as opposed to transiently expressing prepro-TRH as was done in the GH4C1 experiments.

**PC1 Processing of Pro-TRH Increases Sorting of Pro-TRH-derived Peptides to the Regulated Secretory Pathway**—Our results observed when PC1 was absent or unable to process a mutated pro-TRH indicate that without an initial PC1 processing event pro-TRH-derived peptides are diverted from the regulated secretory pathway to the constitutive secretory pathway. However, our prior experiments did not exclude the possibility that it is the interaction of PC1 with pro-TRH that pulls pro-TRH into the regulated secretory pathway where PC1 would then presumably begin processing pro-TRH. To test this possibility, we utilized a PC1 construct that has 137 amino acids deleted from its C terminus (PC1ΔC) and results in constitutive secretion of PC1 in both AtT20 and GH4C1 cells (31, 41). GH4C1 cells were transiently transfected with prepro-TRH, prepro-TRH and PC1, or prepro-TRH and PC1ΔC, and secretion from the transfected cells was monitored under steady-state conditions of basal and regulated release using RIA (pro-TRH expression levels were similar based on RIA; results not shown.

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PC1ΔC resulted in increased sorting of pro-TRH-derived peptides to the regulated secretory pathway in GH4C1 cells as indicated by the increased fold stimulations in comparison to GH4C1 cells only expressing pro-TRH and no PC1 construct (Fig. 7). Because both PC1 and PC1ΔC increase the regulated secretion of pro-TRH-derived peptides in GH4C1 cells while being sorted to different secretory pathways themselves, we conclude that it is the processing of pro-TRH that is important for increasing peptide delivery to the regulated secretory pathway and not a cargo-receptor type relationship between PC1 and pro-TRH. In addition these steady-state experiments using nonclonal cell lines yield results similar to those obtained in the pulse-chase studies with GH4C1 and GH4C1-PC1 cells, indicating to us that the results shown in Fig. 2 are not because of clonal effects.

**DISCUSSION**

In this study we have found that the processing of pro-TRH by PC1 is important for the downstream targeting and storage of the peptides derived from this precursor. In either the absence of PC1 enzyme or by using a pro-TRH mutant construct (PC1-Block) that cannot be processed by PC1, we found that the pro-TRH precursor still underwent processing but that the peptides derived from pro-TRH were not efficiently sorted to the regulated secretory pathway (Figs. 2, 4, and 5). Additionally, the PC1ΔC construct, which results in constitutive secretion of PC1, was able to increase sorting of pro-TRH to the regulated secretory pathway similarly to the effect of wild-type PC1 on pro-TRH sorting (Fig. 7). Combined, this indicates to us that PC1 processing of pro-TRH and not a PC1-pro-TRH receptor cargo interaction is important for the downstream sorting of pro-TRH-derived peptides.

Previous work on the relationship of processing and sorting has been somewhat limited. An earlier study indicated that the prosegment of prorenin and its subsequent processing were required for sorting of renin to the regulated secretory pathway and that this cleavable prosegment could be added to constitutively secreted proteins and would subsequently reroute these proteins to the regulated secretory pathway (42). However, it was not made clear in that paper whether the processing of prorenin was occurring intracellularly or post-secretion, and the chase conditions used in the experiments were exceptionally long. There are several other studies that contradict the findings of these authors (1, 43–47). Another case is that of proinsulin sorting. One group has presented the idea that proteolytic processing of this precursor enhances its sorting when expressed exogenously in neuroendocrine cell lines (35). Another group has demonstrated that a processing-deficient proinsulin mutant is sorted normally when expressed in primary beta cells from rat (48). This debate over proinsulin processing and targeting was recently reviewed by the leaders of the two respective groups working on proinsulin trafficking (49). Thus previous studies have yielded conflicting results on the importance of processing on sorting.

Other studies have suggested that the dibasic residues where prohormones are endoproteolytically cleaved could serve as sorting signals (20, 21, 50, 51). This is an attractive hypothesis as dibasic residues are found in all prohormones that undergo processing within the secretory pathway, making them the only primary structural element that is conserved between various prohormones and across species. We designed our experiments with pro-TRH to test the relevance of processing and the possibility of dibasics serving as sorting signals. Because wild-type pro-TRH was not sorted to the regulated secretory pathway in the absence of PC1 (Fig. 2B) and because PC1-Block was not sorted to the regulated secretory pathway (Fig. 5B) despite the fact that the precursor was processed in both experiments, we feel that dibasic residues alone do not act as sorting signals for pro-TRH. In both cases numerous dibasic residues were present pro-TRH and this did not prevent increased constitutive trafficking in the absence of PC1 processing, indicating that dibasic residues are not sufficient for pro-TRH targeting.

An important question raised by our data is as follows. If furin processes pro-TRH in the absence of PC1, why is this processing not sufficient to direct pro-TRH peptides into the regulated secretory pathway as we have observed for PC1? One potential reason is pro-TRH may be clipped by furin in a post-Golgi compartment after pro-TRH is missorted to the constitutive pathway in GH4C1 cells. In this case pro-TRH may be sequestered in the TGN with other regulated secretory pathway proteins like PC1 by some unknown mechanism, at which point processing allows for proper entry into and/or retention by immature secretory granules. If processing does not occur, pro-TRH would enter the constitutive pathway directly or through the constitutive-like pathway where its processing would be mediated by furin, which is known to act on constitutively secreted proteins (52). Another possibility is that although pro-TRH can be processed completely by furin, it may not happen rapidly enough to ensure proper downstream sorting of pro-TRH-derived peptides. Pro-TRH does not contain any Arg-(Lys/Arg)-Arg furin consensus sites, and a P4 Arg residue has been reported to be important for efficient furin processing (53, 54).

Immunocytochemistry of pro-TRH in the presence or absence of PC1 (Fig. 4) and of wild-type pro-TRH and PC1-Block in AtT20 cells (Fig. 7) revealed similar pro-TRH peptide localizations. This result was unexpected because of our biochemical results showing increased constitutive release of pro-TRH when initial processing is prevented (Figs. 2, 4, 5, and 7). One potential explanation for this is that the increased misrouting of peptides observed during pulse-chase experiments is not occurring with 100% of all newly synthesized peptide. If even a small percentage of newly synthesized pro-TRH is targeted to granules, over time granule contents will be enriched with pro-TRH-derived peptides. This can explain why under the steady-state conditions immunocytochemistry reveals peptide localized to granules, whereas biochemical
results indicate a lack of storage. By analyzing the peptide levels in these cells under steady-state conditions, we found that at least in the GH4C1 and GH4C1+PC1 cells the immunocytochemistry results are quantitatively misleading with GH4C1+PC1 cells storing 7.4 times more TRH than GH4C1 cells and 5.9 times more pYE26 than GH4C1 cells at steady-state (TABLE TWO). However, the staining does indicate that the missorting is not 100% without PC1 present or with PC1 processing blocked by mutation.

Our results strongly suggest that the initial processing of pro-TRH by PC1 at the level of the TGN enhances the post-Golgi sorting of pro-TRH peptides to the regulated secretory pathway. This sorting was due solely to processing and not a receptor-cargo relationship between PC1 and pro-TRH as evidenced by the fact that coexpression of PC1ΔC and pro-prepro-TRH in GH4C1 cells resulted in regulated release of pro-TRH peptides (Fig. 7). If a receptor cargo interaction occurred between pro-TRH and PC1, we would have expected that the constitutively secreted PC1ΔC would have resulted in increased sorting of pro-TRH to the constitutive secretory pathway, but instead it resulted in increased sorting of pro-TRH to the regulated secretory pathway. Processing could effect the trafficking of pro-TRH by exposing potential sorting signals on derived peptides after processing or perhaps through a change in protein structure or the chemical properties of smaller peptides as opposed to the intact prohormone.

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REFERENCES
1. Sevarino, K. A., Goodman, R. H., Spiess, J., Jackson, I. M., and Wu, P. (1989) J. Biol. Chem. 264, 21529–21535
2. Nillni, E. A., Luo, L. G., Jackson, I. M., and McMillan, P. (1996) Endocrinology 137, 5651–5661
3. Arvan, P., and Castle, D. (1998) Biochem. J. 332, 593–610
4. Paczkowski, J., and Loh, Y. P. (2000) J. Biol. Chem. 275, 27032–27036
5. Feng, L., and Arvan, P. (2003) J. Biol. Chem. 278, 31486–31494
6. Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Cell 88, 73–83
7. Normant, E., and Loh, Y. P. (1998) Endocrinology 139, 2137–2145
8. Cool, D. R., and Loh, Y. P. (1998) Mol. Cell. Endocrinol. 139, 7–13
9. Yoo, S. H. (1996) J. Biol. Chem. 271, 1558–1565
10. Jain, R. K., Joyce, P. B., and Gorr, S. U. (2000) J. Biol. Chem. 275, 27032–27036
11. Kleeer, C., Hockanson, M. E., and Dannies, P. S. (2004) J. Mol. Neurosci. 22, 43–50
12. Dhanvantari, S., and Loh, Y. P. (2000) J. Biol. Chem. 275, 29887–29893
13. Blazquez, M., Thiele, C., Huttner, W. R., Docherty, K., and Sherman, K. I. (2000) Biochem. J. 349, 843–852
14. Jacob, R., Alfalah, M., Grunberg, J., Obendorf, M., and Naim, H. Y. (2000) J. Biol. Chem. 275, 6566–6572
15. Moore, H. H., and Kelly, R. B. (1986) Nature 321, 443–446
16. Roy, P., Chevrier, D., Fournier, H., Racine, C., Zollinger, M., Crine, P., and Boileau, G. (1991) Mol. Cell. Endocrinol. 82, 237–250
17. Chanat, E., Weiss, U., Huttner, W. B., and Tootoo, S. A. (1993) EMBO J. 12, 2159–2168
18. Kromer, A., Glombik, M. M., Huttner, W. B., and Gerdes, H. H. (1998) J. Cell Biol. 140, 1331–1346
19. Glombik, M. M., Kromer, A., Salm, T., Huttner, W. B., and Gerdes, H. H. (1999) EMBO J. 18, 1059–1070
20. Feliciangeli, S., Kitabgi, P., and Bidard, J. N. (2001) J. Biol. Chem. 276, 6140–6150
21. Feliciangeli, S., and Kitabgi, P. (2002) Biochim. Biophys. Acta. 1530, 191–196
22. Tauner, L., Harper, K. L., Mahapatra, N. R., Parner, R. J., Mahata, S. K., and O’Connor, D. T. (2002) J. Cell Sci. 115, 4827–4841
23. Nillni, E. A., Sevarino, K. A., and Jackson, I. M. (1993) Endocrinology 132, 1260–1270
24. Nillni, E. A., Aird, F., Seidah, N. G., Todd, R. B., and Koenig, J. I. (2001) Endocrinology 142, 896–906
25. Friedman, T. C., Loh, Y. P., Cawley, N. X., Birch, N. P., Huang, S. S., Jackson, I. M., and Nillni, E. A. (1995) Endocrinology 136, 4462–4472
26. Nillni, E. A., and Sevarino, K. A. (1999) Endocr. Rev. 20, 599–648
27. Nillni, E. A., Sevarino, K. A., and Jackson, I. M. (1993) Endocrinology 132, 1271–1277
28. Cruz, I. P., and Nillni, E. A. (1996) J. Biol. Chem. 271, 22736–22745
29. Gorr, S. U., Huang, X. F., Cowley, D. J., Kuliawat, R., and Arvan, P. (1999) Am. J. Physiol. 277, C121–C131
30. Roreve, C., Lus, J., Bittesky, J. C., Basak, A., Marvaldi, J., Chretien, M., and Seidah, N. G. (1999) J. Biol. Chem. 274, 12461–12467
31. Jaturas, I., Seidah, N. G., and Reudelhuber, T. L. (2000) J. Biol. Chem. 275, 40337–40343
32. Lou, H., Kim, S. K., Zaisan, E., Snell, C. R., Lu, B., and Loh, Y. P. (2005) Neurog 45, 245–255
33. Schaner, P., Todd, R. B., Seidah, N. G., and Nillni, E. A. (1997) J. Biol. Chem. 272, 15184–15188
34. Jaturas, I., Seidah, N. G., Reudelhuber, T. L., and Brechler, V. (1997) J. Biol. Chem. 272, 896–906
35. Kuliawat, R., Prabakaran, D., and Arvan, P. (2000) Mol. Biol. Cell 11, 1959–1972
36. Nillni, E. A., Friedman, T. C., Todd, R. B., Birch, N. P., Loh, Y. P., and Jackson, I. M. (1995) J. Neurochem. 65, 2462–2472
37. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
38. Moore, H. P., Andresen, J. M., Eaton, B. A., Grabe, M., Haugwitz, M., Wu, M. M., and Machen, T. E. (2002) Arch. Physiol. Biochem. 110, 16–25
39. Varlamov, O., Eng, F. J., Novikova, E. G., and Fricker, L. D. (1999) J. Biol. Chem. 274, 14759–14767
40. Eaton, B. A., Haugwitz, M., Lau, D., and Moore, H. P. (2000) J. Neurosci. 20, 7334–7344