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Processing of Prothyrotropin-Releasing Hormone (Pro-TRH) by Bovine Intermediate Lobe Secretory Vesicle Membrane PC1 and PC2 Enzymes

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ABSTRACT

TRH is synthesized from a larger 26-kilodalton (kDa) prohormone (pro-TRH). Rat pro-TRH contains five copies of the TRH progenitor sequence (Gln-His-Pro-Gly) and seven other cryptic peptides. Each of the five TRH progenitor sequences is flanked by pairs of basic amino acids. We used a bovine intermediate lobe secretory vesicle membrane preparation, which contains the prohormone convertases (PCs) PC1 and PC2, to study the *in vitro* processing of pro-TRH. Pro-TRH was radiolabeled using [^{3}H]Leu in AtT_{20} cells transfected with prepro-TRH complementary DNA, and the labeled 26-kDa pro-TRH was isolated from the cell extract by preparative sodium dodecyl sulfate-gel electrophoresis. Incubation of [^{3}H]pro-TRH with the intermediate lobe secretory vesicle membrane preparation was followed by immunoprecipitation with antibodies specific for various regions of the pro-TRH sequence, and the immunoprecipitates were analyzed by sodium dodecyl sulfate-gel electrophoresis. Immunoprecipitation of the reaction mixture with anti-pCC_{10} antibody (an antibody that recognizes the intact precursor and amino-terminal intermediate products of processing) showed a time-dependent appearance of a 15-kDa and a 6-kDa peptide and, at times, a 3.8-kDa peptide with diminution of the 26-kDa substrate. Immunoprecipitation of the incubate with the C-terminal-directed antibody, pYE_{17} (an antibody that recognizes the intact precursor and C-terminal intermediate products of processing), showed the generation of 16.5-, 10-, and 5.4-kDa products in a time-dependent manner, with disappearance of the substrate. Western blot analysis demonstrated that the secretory vesicle membrane preparation contains PC1 and PC2. Immunodepletion studies with antiserum specific for PC1 or PC2 demonstrated that PC1 and PC2 can process pro-TRH to these intermediate products. An initial site of cleavage appeared to be either at the 152–153 or the 158–159 pair of basic residues to yield a 15-kDa N-terminal fragment that was then processed to the 6-kDa [TRH-(25–74)] and 3.8-kDa [TRH-(83–112)] forms. The 10-kDa C-terminal peptide generated by this cleavage was then processed to a 5.4-kDa peptide [TRH-(208–255)]. Alternatively, an initial cleavage at the 107–108 or the 112–113 bonds was also observed, yielding a 16.5-kDa C-terminal product that was further processed to the 5.4-kDa peptide. The pH profile for the appearance of both C- and N-terminal products showed a bimodal distribution, with optima at both 5.5 and 7.5. The cleavage of pro-TRH was enhanced by Ca^{2+} and partially inhibited by Zn^{2+}. This study provides evidence for the first time that PC enzymes can process pro-TRH. (*Endocrinology* **136:** 4462–4472, 1995)

TRH (thyroliberin; pGlu-His-Pro-NH_2) is synthesized as part of a biologically inactive larger precursor (1). Pro-TRH, similar to other peptide hormone systems such as proenkephalin, contains multiple copies of the peptide product. Pro-TRH has five copies of the progenitor sequence of TRH (Gln-His-Pro-Gly) flanked by pairs of basic amino acids (Lys or Arg), and seven cryptic peptides within the rat 26-kilodalton (kDa) prohormone molecule. Cleavage of the precursor to generate biologically active TRH occurs at paired basic residues by an as yet unidentified endopeptidase, followed by the action of carboxypeptidase H activity to remove the basic residue(s) (2). Gln-His-Pro-Gly is then amidated by the action of peptidyl glycine α-amidating monooxygenase (3), and the Gln residue undergoes cyclization to a pGlu residue to yield TRH (4). TRH functions as both a hypothalamic releasing factor, stimulating the synthesis and release of TSH, PRL, and GH from the anterior pituitary (5–7), and as a neurotransmitter or neuromodulator within the central nervous system (8, 9).

The enzymatic activation and inactivation of TRH have been characterized, except for the initial cleavage at the paired basic residues. By means of pulse-chase studies in AtT_{20} cells, we have shown that a 26-kDa pro-TRH polypeptide is the biosynthetic precursor to all pro-TRH-derived cryptic peptides (10). In addition, in AtT_{20} cells, pro-TRH appears to be differentially processed from either or both of two initial cleavage sites to generate a 15-kDa N-terminal intermediate and a 16.5-kDa C-terminal intermediate. These two intermediate peptides are further processed to generate TRH and mature peptides (Nillni, E. A., T. O. Bruhn, S. S. Huang, and I. M. D. Jackson, unpublished data). However the enzyme(s) involved in this processing remained unelucidated (10).

Recently, a new family of subtilisin-like proteases has been identified. Kex2, a yeast serine protease capable of processing the α-mating factor precursor (11), was found to have homology to the human protein, furin (12). In the last few years,

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six additional mammalian prohormone convertase (PC) homologs have been cloned; PC1 (also referred to as PC3) (13–16), PC2 (13, 17), PACE4 (18), PC4 (19), PC5/6A (20, 21), and PC6B (22). PC1 and PC2 messenger RNAs (mRNAs) were found to be localized in endocrine and neural tissue (13, 14, 23, 24), and PC4 in testicular tissue (19), whereas furin, PACE4, PC5/6A, and PC6B were ubiquitously distributed (18, 20–22, 25). In the pituitary, PC1 was present in both the anterior and intermediate lobes, whereas PC2 was found predominantly in the intermediate lobe (14, 24).

The enzymes, PC1 and PC2, have been shown to cleave the POMC molecule at paired basic residues in cotransfection experiments (26, 27). Additionally, we recently demonstrated that recombinant PC1 cleaves POMC (28) and the protective antigen produced by *bacillus anthracis in vitro* (29). The processing of pro-TRH by the PC enzymes has not been examined. During the time these experiments were designed and performed, no recombinant PC2 enzyme was available. Therefore, we used bovine intermediate lobe secretory vesicle membranes, a rich source of endogenous PC1 and PC2 (30), to study the *in vitro* processing of pro-TRH. We showed that this membrane preparation contains enzymes that process pro-TRH. The inhibitor profile of the activities together with immunodepletion experiments allowed us to conclude that the PC1 and PC2 enzymes in the secretory vesicle membranes can process pro-TRH.

Materials and Methods

Tissue culture and radiolabeling

AtT_{20} cells transfected with the complementary DNA for rat prepro-TRH were grown in 75-cm^2 flasks at 37 C in 5% CO_2-95% air for 7 days in Dulbecco's Modified Eagle's Essential Medium (GIBCO-BRL, Gaithersburg, MD) containing 10% fetal bovine serum, as previously described (31). At the time of the studies, each flask contained between 25–30 $\times$ 10^6 cells. For radiolabeling of pro-TRH, the regular growth medium was replaced with leucine-deficient Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum and incubated with 500 μCi [^{3}H]leucine/flask for 7 h.

Purification of [^{3}H]pro-TRH and unlabeled pro-TRH

After radiolabeling of transfected AtT_{20} cells, culture medium was removed, and the cells were acid extracted with 2 M acetic acid-HCl, pH 2, as previously described (10). Unlabeled pro-TRH was prepared similarly. The 26-kDa pro-TRH precursor was purified from the radiolabeled cell extract by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31). After the electrophoresis, pro-TRH was removed from the gel slices by electroelution using 25 mM Tris-192 mM glycine-0.1% SDS buffer (pH 6.8) at 20 mA for 5 h. The electroeluted sample (~400 μl) was subjected to Centricon-10 (Amicon, Beverly, MA) filtration with at least 10 vol (4 ml) of either 0.1 M NH_4-acetate buffer (pH 5.5) or 0.1 M Tris-Cl buffer (pH 7.5) to remove the SDS and adjusted to a final volume.

Preparation of secretory vesicle membranes

Bovine pituitaries were obtained from a local slaughterhouse and transported to the laboratory on ice. Intermediate lobe secretory vesicles were prepared (32), and membranes from these lysed vesicles were isolated as previously described (33). The protein concentration of the membrane preparation was determined by the method of Bradford (34).

Enzymatic cleavage of pro-TRH by the secretory vesicle membrane preparation

Bovine intermediate lobe secretory vesicle membranes (~3 μg protein) were incubated with [^{3}H]Leu-pro-TRH (~4000–6000 cpm) in various buffers containing 2 mM $CaCl_2$ at 37 C for 16 h, except for the time-course studies, in which the incubation period was from 3–16 h. For the pH studies, buffers used were 0.1 M NH_4 acetate, pH 4.0–6.0, and 0.1 M Tris-Cl, pH 6.5–8.0. After the optima pH of 5.5 and 7.5 were obtained, all experiments were performed at those two pH values. For inhibitor studies, the inhibitor was preincubated with the enzyme preparation for 15 min at 37 C before the addition of substrate. Purified full-length human 7B2 was a generous gift from A. Martin van Horssen and Gerard J. M. Martens (University of Nijmegan, Toernooiveld, The Netherlands). All incubation volumes were 50 μl. Each enzyme assay was carried out in three independent experiments. To identify the pro-TRH-related peptides formed after incubation of [^{3}H]pro-TRH with the membrane preparation, the products were immunoprecipitated with specific antibodies that recognize different regions of pro-TRH (Fig. 1), followed by SDS-PAGE (see *Immunoprecipitation* section below).

To determine whether secretory vesicle membranes could generate TRH or TRH progenitor forms from pro-TRH, unlabeled pro-TRH was purified from transfected AtT_{20} cells as described above. Unlabeled pro-TRH (10 ng) was incubated with bovine intermediate lobe secretory vesicle membranes (~3 μg protein) in 0.1 M NH_4 acetate buffer (pH 5.5) and 0.1 M Tris-Cl (pH 7.5) containing 2 mM $CaCl_2$ at 37 C for 16 h. The reaction mixture was stopped by placing the incubate at 4 C and adding an inhibitor cocktail composed of phenylmethylsulfonylfluoride (PMSF; 5.7×10^{-4} M), pepstatin A (1.4×10^{-4} M), bestatin (2.9×10^{-4} M), aprotinin (0.5 trypsin inhibitory units/ml), bacitracin (7.0×10^{-5} M) and $ZnCl_2$ (10 mM). The reaction mixture was analyzed either directly by RIA (see below) or subjected to HPLC analysis (see below) followed by RIA.

Analysis of TRH, TRH-Gly, and TRH-Gly-Lys by HPLC

A Rainin HPLC system coupled to a Microsorb C_{18} column (Rainin Instrument Co., Woburn, MA; 5 $\times$ 250 mm) was used to analyze reaction mixtures for pGlu-His-Pro-NH_2 (TRH), pGlu-His-Pro-Gly (TRH-Gly), and pGlu-His-Pro-Gly-Lys (TRH-Gly-Lys). Solvent A was 0.1% trifluoroacetic acid, and solvent B was 80% acetonitrile in 0.1% trifluoroacetic acid. A linear gradient from 2.5–40% solvent B over 26 min at a flow rate of 1 ml/min was used. One-milliliter samples of the eluate were collected, lyophilized, and analyzed by RIA. The following standards were run: TRH, TRH-Gly, and TRH-Gly-Lys (all from Peninsula Laboratories, Belmont, CA).

Peptide RIAs

The RIAs for TRH, TRH-Gly, and TRH-Gly-Lys were performed as described previously (35). Each antiserum did not cross-react with the other peptides or with the noncyclized form of TRH.

Immunoprecipitation (IP)

IP was performed as outlined previously (10). Briefly, after enzymatic incubation of [^{3}H]pro-TRH with the membrane preparation, the reaction was stopped by adding the above inhibitor cocktail followed by boiling for 4 min. The pH was adjusted to pH 7.2, and the peptides were resuspended in 200 μl buffer A [10 mM $NaPO_4$ (pH 7.2), 1 mM EDTA, 0.1% Triton X-100, and 0.2% BSA] and incubated with 20 μl protein G-purified antibodies (31) against specific cryptic peptides for 1 h at 37 C and for 16 h at 4 C. Next, 25 μl goat antirabbit immunoglobulin G (IgG) together with 150 μl buffer B (50 mM NaH_2PO_4, pH 7.4, containing 500 mM KCl, 5 mM sodium EDTA, and 0.25% Triton X-100) were added to the solution. Samples were further incubated for 4 h at 4 C and centrifuged for 10 min at 12,000 $\times$ g. Immunoprecipitates were washed once with buffer B and once with buffer C (10 mM NaH_2PO_4, pH 7.2, containing 15 mM NaCl). The washed immunoprecipitates were then dissolved in sample buffer. The following antibodies were used for the IP: the N-terminal antibody, anti-pCC_{10}, which recognizes prepro-TRH-(25–255) (26 kDa), prepro-TRH-(25–151) (15 kDa), and prepro-TRH-(25–74) (6 kDa); the C-terminal antibody, anti-pYE_{17}, which recognizes pre-

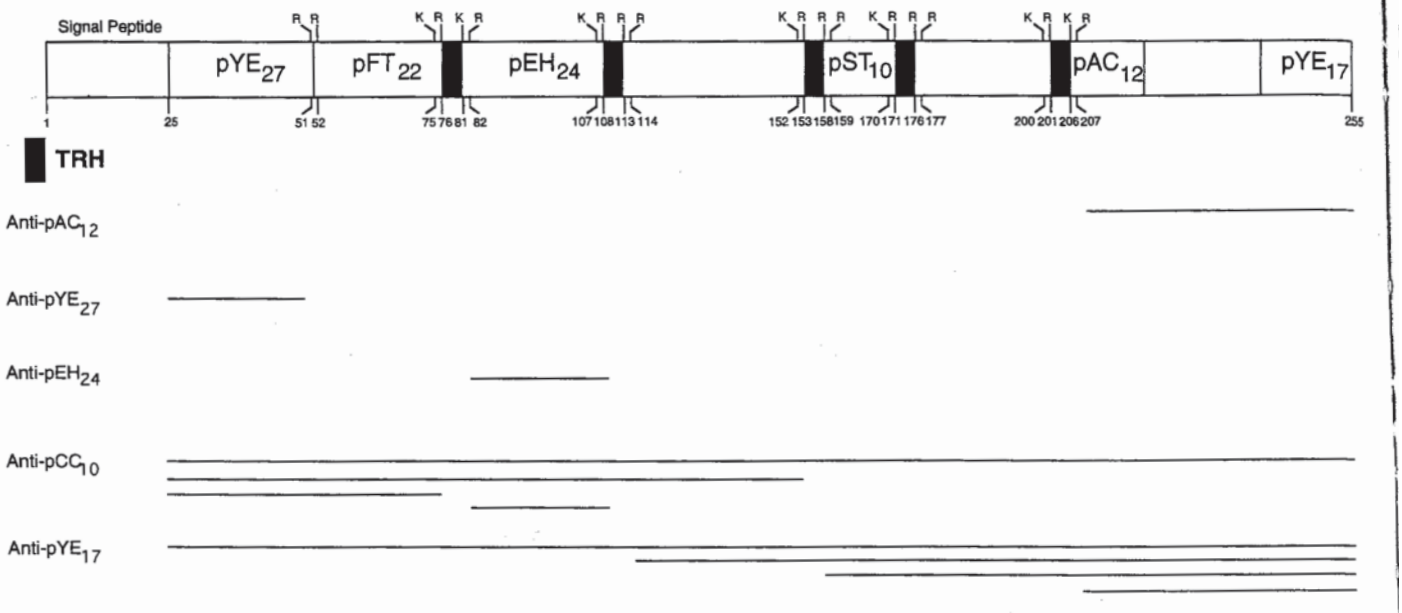

FIG. 1. Diagrammatic representation of rat prepro-TRH and the TRH-related cryptic peptides recognized by the various polyclonal antibodies used in this study. The positions of paired basic residues are indicated by numbers.

pro-TRH-(25–255) (26 kDa), prepro-TRH-(115–255) (16.5 kDa), prepro-TRH-(160–255) (10 kDa), and prepro-TRH-(208–255) (5.4 kDa); antibody anti-pAC_{12}, which recognizes prepro-TRH-(208–255) (5.4 kDa); antibody anti-pYE_{27}, which recognizes prepro-TRH-(25–50) (4 kDa); and antibody anti-pEH_{24}, which recognizes prepro-TRH-(83–106) (2.8 kDa). Figure 1 depicts the cryptic peptides within the pro-TRH molecule that are recognized by the various polyclonal antibodies.

The immunoprecipitates were loaded onto a 6% (1.5-mm thick) discontinuous polyacrylamide gel. After electrophoresis, gels were cut into 2-mm slices in a gel slicer (Hoeffer Scientific Instruments, San Francisco, CA), and prepared for counting. Immunoprecipitated peptides were extracted from the gel slices by incubation in 0.5 ml 2 N acetic acid for 18 h at 4 C. Scintillation fluid (Bio Safe II, RPI, Mount Prospect, IL) was added, and the samples were counted in a scintillation counter. The following M_r markers were used: BSA, 80.0 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; and lysozyme, 18.5 kDa (from Bio-Rad Laboratories, Richmond, CA), and trypsin inhibitor, 20.4 kDa; myoglobin, 16.9 kDa; myoglobin fragment IV, 14.4 kDa; myoglobin fragment III, 8.2 kDa; myoglobin fragment II, 6.2 kDa; and myoglobin fragment I, 2.5 kDa (from Diversified Biotech, Newton, MA).

Immunodepletion of TRH-cleaving activity of PC1 and PC2 by anti-PC1(fus)- and anti-PC2(fus)-specific polyclonal antisera

The specificity of the anti-PC1(fus) and anti-PC2(fus) fusion antisera used was confirmed by demonstrating that the anti-PC1(fus) antiserum only recognizes the PC1 fusion protein, and the anti-PC2(fus) antiserum only recognizes the PC2 fusion protein. PC1 and PC2 glutathione-*S*-transferase (GST) fusion protein antisera (raised in rabbits against a PC1- or PC2-glutathione *S*-transferase fusion protein) (30) were depleted of antibodies that recognized GST by passing the serum down a GST-Sepharose affinity column. For the anti-PC1(fus) antiserum, 10 ml antiserum were brought to 50% saturation with ammonium sulfate, and the precipitate was sedimented by centrifugation. The pellet was resuspended in 10 ml PBS and dialyzed overnight against two changes of PBS. Insoluble material was pelleted by centrifugation, and the soluble supernatant was passed over a GST-Sepharose 4B affinity matrix three times. This affinity column was prepared by coupling purified GST to cyanogen bromide-activated Sepharose 4B at a concentration of 4 mg/ml Sepharose 4B, according to the manufacturer's instructions (Pharmacia LKB Biotechnology, Piscataway, NJ). For the anti-PC2(fus) antiserum, 100 μl antiserum were diluted to 1 ml in PBS and passed directly over a 0.5-ml GST-Sepharose column.

The anti-GST-depleted antisera were then used to probe Western blots. GST and the PC1 and PC2 fusion proteins were purified using a glutathione-Sepharose affinity matrix (Pharmacia, Piscataway, NJ). Equal amounts (50 ng) of GST, GST-PC2, and GST-PC1 were separated on SDS-polyacrylamide gels (12.5% acrylamide and 0.3% *N,N'*-methylene-bisacrylamide; 10 × 8 cm) under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes for immunodetection. The final dilution of the primary antibodies was 1:1000. A peroxidase-linked goat antirabbit Ig secondary antibody (1:2000 final dilution) was used, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL), as described by the manufacturer (Amersham International, Aylesbury, UK).

The presence of PC1 and PC2 in the membrane preparation was determined by separation on a 12% SDS-Tris-glycine gel (1-mm thickness; Novex, San Diego, CA) under reducing conditions and electroblotting onto nitrocellulose membranes. After blocking in 3% BSA in PBS-0.1% Tween-20 for 1 h, the blots were probed overnight at 4 C with anti-PC1(fus) polyclonal antiserum or anti-PC2(fus) polyclonal antiserum at dilutions of 1:10,000 and 1:5,000, respectively. The antigen was visualized by the ECL procedure (Amersham Life Sciences, Arlington Heights, IL) using the peroxidase-linked donkey antirabbit IgG at a dilution of 1:20,000 as the secondary antibody.

To determine the specificity of immunodepletion experiments, intermediate lobe secretory vesicle membranes (~70 μg protein) were incubated with either anti-PC1(fus) antiserum or anti-PC2(fus) antiserum (final dilution, 1:66) for 16 h at 4 C in a total of 100 μl buffer (10 mM Tris-Cl, pH 7.5). To this mixture, 50 μl protein A-Sepharose CL-4B (Pharmacia; 0.15 g/ml PBS, pH 7.4, containing 3% BSA and 0.1% NaN_3) were added, and the mixture was further incubated for 45 min at room temperature while mixing by rotation. The beads were sedimented by centrifugation at 300 × *g* for 5 min, and the supernatants were removed and subjected to a second immunoprecipitation with both anti-PC1(fus) and anti-PC2(fus) antisera (both at a dilution of 1:66). This was performed to remove the large amounts of rabbit serum albumin that would have interfered with the gel run. After this second incubation and binding by the protein A-Sepharose beads, the supernatants were removed, and the beads were boiled in SDS-PAGE loading buffer for 5 min to release the bound proteins. The released proteins were analyzed by Western blot for the presence of PC2 as described above.

Secretory vesicle membranes (~10 μg protein) were immunoprecipitated with nonimmune serum, anti-PC1(fus) antiserum, anti-PC2(fus) antiserum, or both anti-PC1(fus) and anti-PC2(fus) antisera (final dilu-

tion, 1:66) as described above, except that all steps were performed at 4 C to preserve enzymatic activity. After a single immunodepletion, the supernatant was removed and used for incubation studies with pro-TRH as described above.

Results

pH profile of the cleavage of pro-TRH by bovine intermediate lobe secretory vesicle membranes

[^{3}H]Leu-pro-TRH was incubated with bovine intermediate lobe secretory vesicle membranes at pH values ranging from 4.0–8.0 to determine the pH optima. The reaction mixture was incubated for 16 h, at which time there was no remaining substrate, and smaller pro-TRH intermediates and cryptic peptides were formed (see below). Using antibodies that only recognize the pro-TRH cryptic peptides (35), pYE_{27} [prepro-TRH-(25–50); 4 kDa], pEH_{24} [prepro-TRH-(83–106); 2.8 kDa], and pAC_{12} [prepro-TRH-(208–255); 5.4 kDa], we were able to determine the optimal pH of the reaction. After SDS-PAGE, the counts of the peptides immunoprecipitated with these antibodies were summed and plotted against the pH (Fig. 2). As shown in Fig. 2, A–C, the formation of all three peptide products occurred at two pH optima, 5.5 and 7.5. The C-terminal product, prepro-TRH-(208–255), had the greatest accumulation at pH 7.5 (Fig. 2A), whereas the N-terminal products, prepro-TRH-(25–50) (Fig. 2B) and prepro-TRH-(83–106) (Fig. 2C) showed maximal formation at pH 5.5. Thus, we concluded that there were two enzymatic activities in the membrane preparation capable of processing pro-TRH, one an acidic activity (pH 5.5) and the other a neutral activity (pH 7.5). We proceeded to further characterize these two activities.

Time course of the cleavage of pro-TRH by bovine intermediate lobe secretory vesicle membranes

Figure 3 shows the time course for the appearance of N-terminal peptides (immunoprecipitated with anti-pCC_{10} antibody; Fig. 3A) and C-terminal peptides (immunoprecipi-

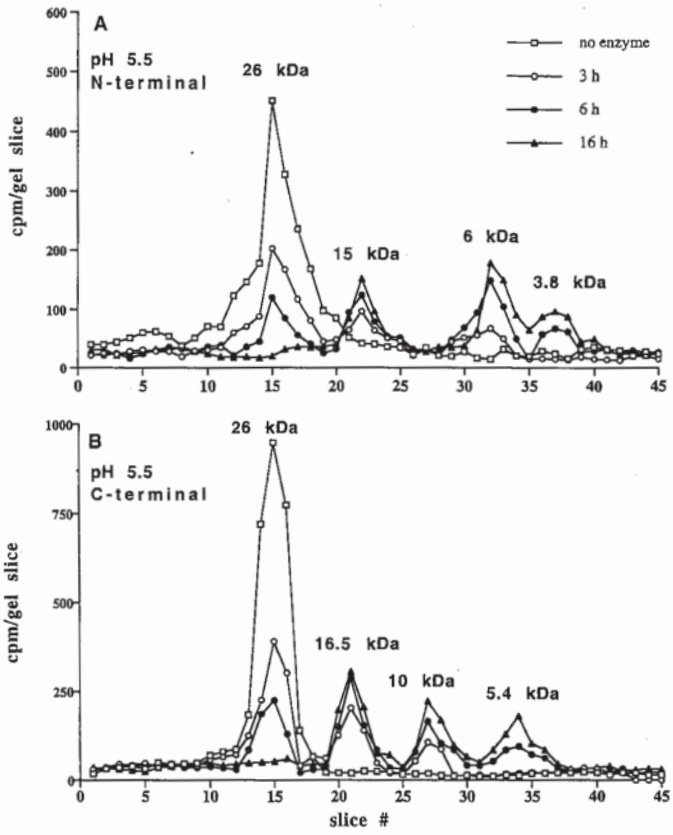

FIG. 3. Time course of the formation of pro-TRH-derived peptides by bovine intermediate lobe secretory vesicle membranes at pH 5.5. [^{3}H]Pro-TRH was incubated with the membrane preparation at pH 5.5, and the reaction mixture was immunoprecipitated with anti-pCC_{10} (A), an antibody that recognizes N-terminal peptides, and anti-pYE_{17} (B), an antibody that recognizes C-terminal peptides. A control tube without enzyme was incubated for 16 h. The immunoprecipitates were electrophoresed on a SDS-polyacrylamide gel, and the counts were plotted against gel slice. The mol wt of the peaks is indicated based on the migration of mol wt standards.

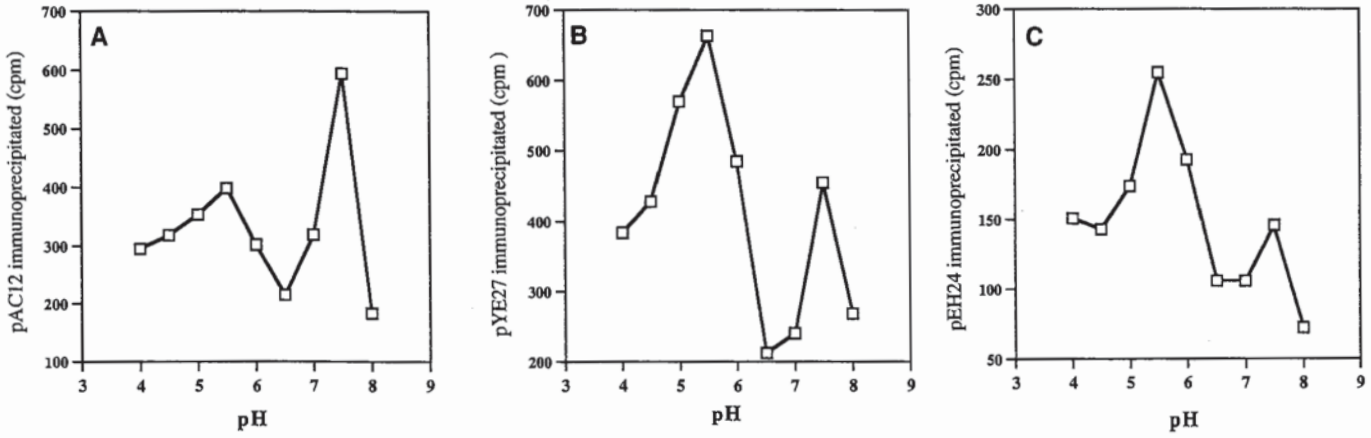

FIG. 2. pH profile of generated cryptic peptides from [^{3}H]pro-TRH by bovine intermediate lobe secretory vesicle membranes. Radiolabeled pro-TRH was incubated with the membrane preparation at various pH values, and the reaction mixture was immunoprecipitated with anti-pAC_{12}, a C-terminal antibody that recognizes prepro-TRH-(208–220) (A); anti-pYE_{27}, an N-terminal antibody that recognizes prepro-TRH-(25–50) (B); or anti-pEH_{24}, an N-terminal antibody that recognizes prepro-TRH-(83–106) (C). The immunoprecipitates were electrophoresed on a SDS-polyacrylamide gel, and the counts corresponding to the 5.4-kDa product prepro-TRH-(208–255) (A), the 4-kDa product prepro-TRH-(25–50) (B), and the 2.8-kDa product prepro-TRH-(83–106) (C) were summed and plotted against pH.

tated with anti-pYE_{17} antibody; Fig. 3B) after incubation of pro-TRH with bovine intermediate lobe secretory vesicle membranes at pH 5.5. Figure 3A shows that in the absence of membranes, only the 26-kDa form, representing intact precursor, was present. At 3 h, there was formation of the 15- and 6-kDa intermediates, with diminution of the 26-kDa precursor. By 6 h, there was formation of the 3.8-kDa intermediate, and by 16 h, there was increased formation of the 15-, 6-, and 3.8-kDa intermediates with disappearance of the substrate. It is noteworthy that the 15- and 6-kDa forms appeared simultaneously, whereas the appearance of the 3.8-kDa form was delayed.

As shown in Fig. 3B, incubation of pro-TRH with bovine intermediate lobe secretory vesicle membranes generated 16.5-, 10-, and 5.4-kDa C-terminal peptides in a time-dependent manner with a concomitant diminution of the substrate peak. The 16.5- and 10-kDa products appeared to be formed simultaneously, whereas the appearance of the 5.4-kDa product was delayed.

The time course of pro-TRH processing by bovine intermediate lobe secretory vesicle membranes to N-terminal (Fig. 4A) and C-terminal products (Fig. 4B) at pH 7.5 was also examined. For the N-terminal-derived peptides, in the absence of membranes, intact 26-kDa pro-TRH was present. In the presence of membranes, this peak diminished with time of incubation, whereas there was a concomitant formation of the 15-kDa intermediate. A small amount of the 6-kDa form was detected.

Formation of the C-terminal-derived peptides was similar at pH 7.5 and pH 5.5, with 16-, 10-, and 5.4-kDa peptides appearing in a time-dependent manner (Fig. 4B).

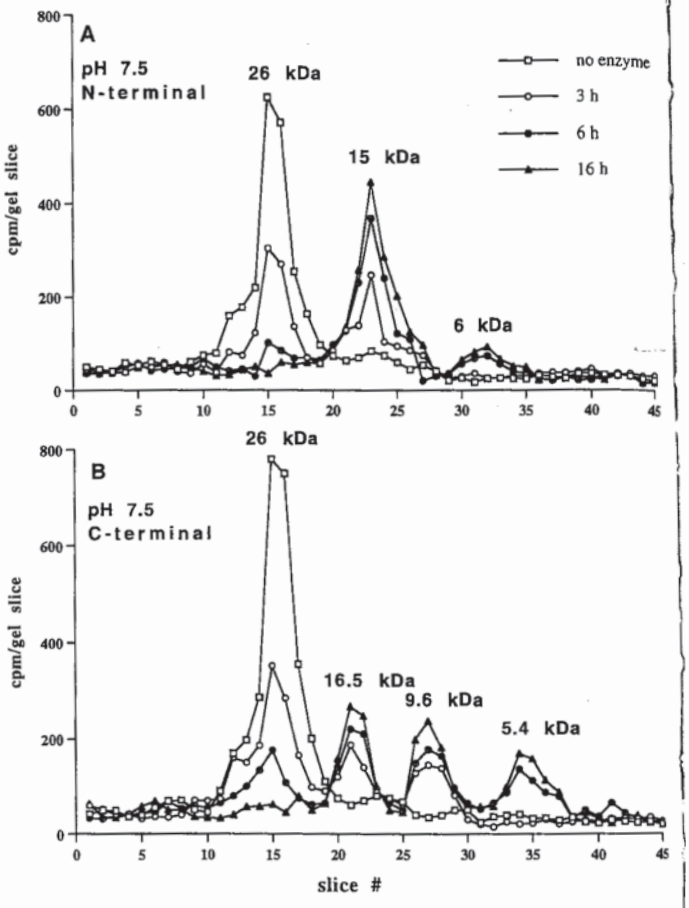

FIG. 4. Time course of the formation of pro-TRH-derived peptides by bovine intermediate lobe secretory vesicle membranes at pH 7.5. [^{3}H]Pro-TRH was incubated with the membrane preparation at pH 7.5, and the reaction mixture was immunoprecipitated with anti-pCC_{10} (A), an antibody that recognizes N-terminal peptides, and anti-pYE_{17} (B), an antibody that recognizes C-terminal peptides. A control tube without enzyme was incubated for 16 h. The immunoprecipitates were electrophoresed on a SDS-polyacrylamide gel, and the counts were plotted against gel slice. The mol wt of the peaks is indicated based on the migration of mol wt standards.

Effects of protease inhibitors on the cleavage of pro-TRH by bovine intermediate lobe secretory vesicle membranes

Table 1 demonstrates the effects of protease inhibitors on the formation of prepro-TRH-(25–50) (recognized by the antibody against pYE_{27}) from pro-TRH incubated with bovine intermediate lobe secretory vesicle membranes. $ZnCl_2$ inhibited 73% and 55% of the activity at pH 5.5 and 7.5, respectively, whereas EGTA inhibited 55% and 42% of the activity at pH 5.5 and 7.5, respectively, and EDTA inhibited 78% and 77% of the activity at pH 5.5 and 7.5, respectively. Purified 7B2 (400 nM) inhibited 55% and 71% of the activity at pH 5.5 and 7.5, respectively.

Incubation of unlabeled pro-TRH by bovine intermediate lobe secretory vesicle membranes to generate TRH, TRH-Gly, and TRH-Gly-Lys

RIAs for TRH, TRH-Gly, and TRH-Gly-Lys were performed directly on the incubate of pro-TRH and bovine intermediate lobe secretory vesicle membranes at both pH 5.5 and 7.5. No radioimmunoassayable products were generated under these reaction conditions. Additionally, when the reaction mixture was analyzed by HPLC and the eluate was assayed by RIAs for TRH, TRH-Gly, and TRH-Gly-Lys, no radioimmunoassayable products were detected.

Effects of anti-PC1(fus) and anti-PC2(fus) antisera on the cleavage of pro-TRH by bovine intermediate lobe secretory vesicle membranes

The involvement of the PC family of enzymes in the cleavage of pro-TRH was implicated, but not proven, by the inhibitor profile that showed inhibition by Zn^{2+}, *p*-chloromercuriphenylsulfonic acid, and metal chelators, but not PMSF. To ascertain whether PC1 and PC2 are responsible for pro-TRH processing, the presence of these enzymes in the membrane preparation was demonstrated by Western blot, and the specificity of these enzymes in pro-TRH processing was assessed by immunodepletion studies using antisera specific for PC1 and PC2. The specificity of the anti-PC1(fus) and anti-PC2(fus) antisera was first confirmed by Western blot. Both antisera were initially depleted of anti-GST antibodies to allow comparison of their specificity toward the PC1-GST and PC2-GST fusion proteins. Successful depletion was confirmed by their failure to recognize GST by Western blot analysis (Fig. 5, A and B). Anti-PC1(fus) antiserum recog-

TABLE 1. Effect of protease inhibitors on the formation of prepro-TRH-(25–50) from pro-TRH by secretory vesicle membranes

Inhibitor	% Inhibition
pH 5.5	
Phenylmethanesulfonylfluoride (1 mM)	17 ± 2.8[a]
Pepstatin (1 mM)	0
$ZnCl_2$ (1 mM)	73 ± 1.6[b]
Bacitracin (1 mM)	0
Bestatin (1 mM)	0
Aprotinin (1 mM)	0
p-Chloromercuriphenylsulfonic acid (1 mM)	51 ± 4.6[a]
N^a-*p*Tosyl-L-lysine chloromethyl ketone (1 mM)	1 ± 0.6
E64 (1 mM)	5 ± 0.9
7B2 (400 mM)	55 ± 3.0[b]
EGTA (10 mM)	55 ± 3.3[a]
EDTA (10 mM)	78 ± 3.4[b]
No added $CaCl_2$	39 ± 2.0[b]
pH 7.5	
$ZnCl_2$ (1 mM)	55 ± 1.6[b]
p-Chloromercuriphenylsulfonic acid (1 mM)	44 ± 3.6[a]
N^a-*p*-Tosyl-L-lysine chloromethyl ketone (1 mM)	15 ± 2.5
E64 (1mM)	5 ± 1.1
7B2 (400 mM)	71 ± 2.0[b]
EGTA (10 mM)	42 ± 3.3[a]
EDTA (10 mM)	77 ± 1.5[b]

[^{3}H]Pro-TRH was incubated with secretory vesicle membranes in the presence of several protease inhibitors at 37 C, pH 5.5 or 7.5, for 16 h in the presence of 1 mM Ca^{2+}. The inhibitor was preincubated with the enzyme preparation for 30 min at 37 C before addition of the substrate. The prepro-TRH-(25–50) peptide formed after incubation was detected with anti-pYE_{27} antibody after SDS-PAGE analysis. The values shown are expressed as the percent inhibition ± SEM compared to the control value and are the mean of three experiments.

[a] $P < 0.05$.

[b] $P < 0.005$.

nized the 31.6-kDa PC1-GST fusion protein, but failed to recognize PC2-GST (Fig. 5A). Anti-PC2(fus) antiserum recognized the 34.6-kDa PC2-GST fusion protein, but failed to recognize PC1-GST (Fig. 5B). These results confirm the specificity of each antiserum for its respective fusion protein under denaturing conditions.

Western blot analysis demonstrated that secretory vesicle membranes contain a band at 66 kDa when probed with the anti-PC1(fus) antibody and a band at 64 kDa when probed with the anti-PC2(fus) antibody (Fig. 5C; lanes 1 and 2). When secretory vesicle membranes were immunodepleted with anti-PC1(fus) antiserum, the resultant supernatant contained a 64-kDa (PC2) band when probed with anti-PC2(fus) antiserum (Fig. 5C, lane 3). The presence of PC2 in lane 3 indicates that anti-PC1(fus) antiserum did not immunodeplete PC2 from the IL membranes. That the PC1 antiserum was specific was further supported by the presence of PC1, but not PC2, when immunoprecipitated by anti-PC1(fus) antiserum (data not shown). When the same membrane preparation was immunodepleted by anti-PC2(fus) antiserum, the resultant supernatant showed the absence of a band corresponding to PC2 when probed with anti-PC2(fus) antiserum (Fig. 5C, lane 4). This absence of PC2 in lane 4 demonstrates that PC2 was specifically immunodepleted from the intermediate lobe membranes by anti-PC2(fus) antiserum.

The membrane preparation was then immunodepleted with antisera specific to PC1 and PC2, and the activity in the supernatant was compared with that of the membrane preparation exposed to nonimmune serum. When pro-TRH was incubated at pH 5.5 with the membrane preparation immunodepleted with nonimmune serum and analyzed for N-terminal (Fig. 6A) and C-terminal products (Fig. 6B), processing occurred in a manner similar to that seen in the nonimmunodepleted membrane preparations (Fig. 3). However, when the membrane preparation was immunodepleted of PC1 activity by preincubation with anti-PC1(fus) antiserum, and the subsequent reaction mixture was analyzed for N-terminal products, most of the processing was blocked with a diminution of the 15-, 6-, and 3.8-kDa product peaks and a restoration of the 26-kDa substrate peak (Fig. 6A). Immunodepletion restored approximately 70% of the counts of the substrate peak. These findings suggest that PC1 can effectively cleave pro-TRH at pH 5.5 to generate N-terminal products.

Figure 6B shows the effect of immunodepletion of the membrane preparation with nonimmune, anti-PC1(fus), anti-PC2(fus), or both anti-PC1(fus) and anti-PC2(fus) antisera on the C-terminal processing of pro-TRH at pH 5.5. As shown in Fig. 3B, incubation of the membrane preparation pretreated with nonimmune antiserum generated 16.5-, 10-, and 5.4-kDa intermediates (Fig. 6B). With incubation of the membranes pretreated with anti-PC1(fus) or anti-PC2(fus) antiserum, there was a partial increase in the substrate peak (34% for PC1 and 45% for PC2) with an appropriate decrease in the product peaks. It is noteworthy that formation of the 5.4-kDa peak was completely blocked by the addition of either antiserum, whereas the 16.5- and 10-kDa peaks were only partially blocked. When the membranes were pretreated with both anti-PC1(fus) and anti-PC2(fus) antisera, there was a restoration of the substrate peak and a diminution of the product peaks in a similar manner to that with anti-PC1(fus) antiserum alone.

The effect of immunodepletion of the membrane preparation with nonimmune, anti-PC1(fus), anti-PC2(fus), or both anti-PC1(fus) and anti-PC2(fus) antisera on the N-terminal processing of pro-TRH at pH 7.5 is shown in Fig. 6C. Incubation of the membrane preparation pretreated with nonimmune antiserum generated 15-kDa intermediates, with disappearance of the 26-kDa substrate peak (Fig. 6C). With incubation of the membranes pretreated with anti-PC1(fus), the chromatogram was similar to that for the nonimmune treatment, indicating that PC1 does not contribute to the cleavage at pH 7.5. On the other hand, preincubation of the membranes with anti-PC2(fus) antiserum caused a restoration of the 26-kDa substrate peak (29%), with a decrease in the 15-kDa product peak. Preincubation of the membranes with both anti-PC1(fus) and anti-PC2(fus) antisera gave results similar to that with PC1 alone. These results indicate that PC2, but not PC1, can process pro-TRH at pH 7.5.

As Fig. 6C demonstrated that PC2 processes pro-TRH at pH 7.5, we conducted immunodepletion experiments with anti-PC2(fus) antiserum to examine the C-terminal processing at that pH (Fig. 6D). Membranes immunodepleted with nonimmune antiserum showed a large decrease in the substrate peak, with the appearance of 16.5-, 10-, and 5.4-kDa peaks. When the membrane preparation was preincubated with anti-PC2(fus) antiserum, there was a 50% restoration of

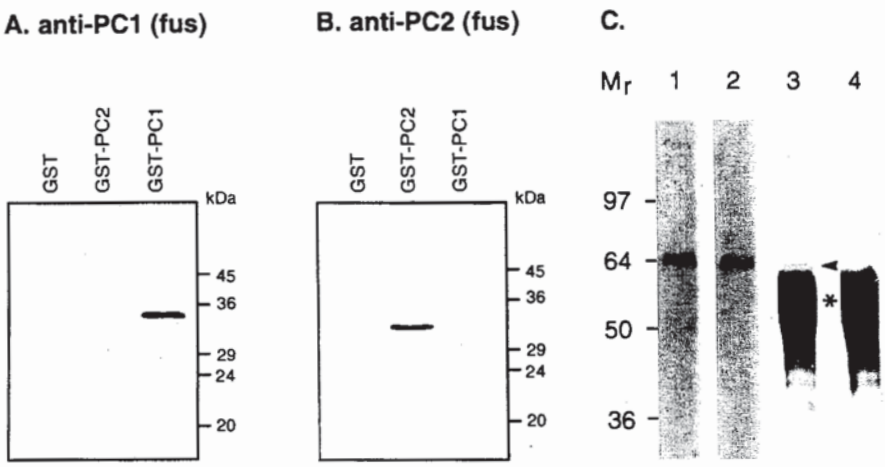

FIG. 5. Western blot analyses demonstrating the specificity of the anti-PC1(fus) and anti-PC2(fus) antisera. Purified GST, GST-PC2 fusion protein, and GST-PC1 fusion protein were run on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-PC1(fus) fusion antiserum (A) or anti-PC2 fusion antiserum (B). The antisera only recognize their specific proteins. Intermediate lobe secretory vesicle membranes were run on a 12% SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-PC1(fus) fusion antiserum (C, lane 1) or anti-PC2 fusion antiserum (C, lane 2). Single bands of 66 and 64 kDa, corresponding to PC1 and PC2, respectively, are seen. Intermediate lobe secretory vesicle membranes were immunodepleted with either anti-PC1(fus) antiserum (C, lane 3) or anti-PC2(fus) antiserum (C, lane 4), and the supernatants were removed and subjected to a second immunoprecipitation with both anti-PC1(fus) and anti-PC2(fus) antisera. The resulting immunoprecipitate was run on a 12% SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-PC2 fusion antiserum. A 64-kDa band corresponding to PC2 (C, lane 3; indicated by the *arrowhead*) was seen in the supernatant that had first been immunodepleted with anti-PC1(fus) antiserum, whereas this band was absent in the supernatant that had been first immunodepleted with anti-PC2(fus) antiserum (C, lane 4). The mol wt of standards are given for all gels. *, Immunostaining of the rabbit IgG due to binding by the secondary antibody (donkey antirabbit IgG).

the 26-kDa substrate peak, a partial decrease in the 16.5- and 10-kDa peaks, and a complete blockade of formation of the 5.4-kDa peak. When pro-TRH was incubated at pH 7.5 with nonimmune antiserum alone (no membrane preparation), there were no products formed (data not shown), indicating that the serum does not contain nonspecific proteases capable of degrading pro-TRH.

Discussion

Processing of pro-TRH by the secretory membrane preparation occurred at two pH optima, 5.5 and 7.5. In our previous studies, the processing of pro-TRH was found to begin in the Golgi complex (36), but we have not determined in which Golgi compartment. The neutral pH optimum, 7.5, is consistent with that found in the early parts of the Golgi complex. The acidic pH optimum, 5.5, is similar to that found in the secretory granules, a site where the later stages of processing of pro-TRH occurs (36). Immunodepletion studies with anti-PC1(fus) and anti-PC2(fus) antisera suggest that cleavage of pro-TRH by these two enzymatic activities in the membranes can function at these two pH values. The pH optima for each of the PC enzymes appears to be substrate dependent. We previously described a PC2-like activity in the same secretory vesicle membrane preparation that was capable of cleaving ACTH at its tetrabasic site (33). This activity had a pH optimum of 8.0 with ACTH-(1–39) as the substrate and 7.5 with ACTH-(1–24) as substrate (33). On the other hand, both PC1 (type I endopeptidase) and PC2 (type II endopeptidase) have a pH optimum of 5.5 with proinsulin as the substrate (37). Similarly, PC2, expressed by microinjection into *Xenopus* oocytes, cleaved the fluorogenic substrate, Boc-Gln-Arg-Arg-AMC, most efficiently at pH 5.5 (38). We showed that the pH optima for cleavage by recombinant PC1 with POMC (28) and anthrax toxin-protective antigen (29) is 6.0. Additionally, recombinant PC1 was found to cleave the synthetic substrates, Cbz-Arg-Ser-Lys-Arg-AMC (39) and Boc-Arg-Val-Arg-Arg-AMC (40), with a pH optima of 6.0. Thus, it appears that PC1 functions at an acidic pH, whereas PC2 may be able to function at either a neutral or acidic pH, depending on the substrate. As PC2 exists in three forms (75, 70, and 65 kDa) (41), the different pH optima may reflect which form predominates.

Experiments performed in the presence of different enzyme inhibitors confirmed that the activities at pH 5.5 and 7.5 in the intermediate lobe secretory vesicle membranes were most likely due to a protease in the PC family. $ZnCl_2$ and PCMS, inhibitors of the PC class of enzymes (42, 43); EGTA and EDTA, chelators of Ca^{2+} and divalent cations, respectively; and elimination of calcium all substantially inhibited the activities. 7B2, a specific inhibitor of PC2 (44), inhibited 71% of the activity at pH 7.5 and 55% of the activity at pH 5.5. The fact that 29% of the activity remained at pH 7.5 in the presence of 7B2 is probably due to the relatively low dose (400 nM) of the inhibitor used. The serine protease inhibitor, PMSF, inhibited only a small percentage of the cleavage of pro-TRH at pH 5.5. PMSF does not inhibit PC2 isolated from rat liver (45) or rat insulinoma secretory granules (43), but partially inhibits the 74/66-kDa (but not the 87-kDa) forms of recombinant PC1 (46). Pepstatin A, an aspartic protease inhibitor capable of inhibiting POMC-converting enzyme (EC 3.4.23.17) (47) present in these membranes; the thiol protease inhibitors, bestatin and E64; the serine protease inhibitors, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone and aprotinin; and the general protease inhibitor, bacitracin, were without effect. This inhibitor profile is consistent with the

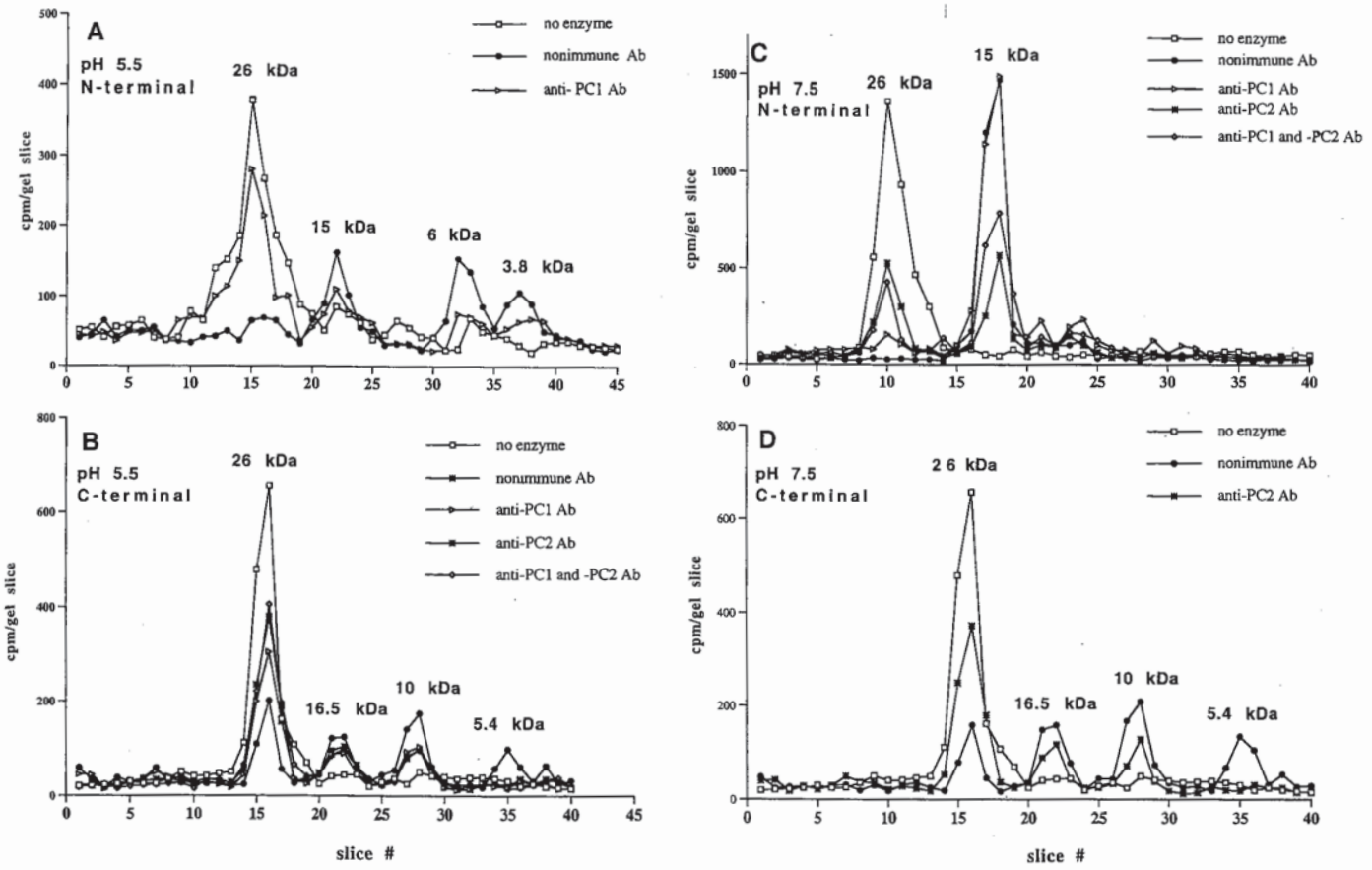

FIG. 6. Immunodepletion of pro-TRH-cleaving activity of bovine intermediate lobe secretory vesicle membranes with anti-PC1(fus) and anti-PC2(fus) antisera. The membrane preparation was incubated with nonimmune, anti-PC1(fus), anti-PC2(fus), or both anti-PC1(fus) and anti-PC2(fus) antisera, and the resultant enzyme-antibody complex was removed by incubation with protein A-Sepharose CL-4B beads followed by centrifugation. The supernatant was incubated with [^{3}H]pro-TRH at pH 5.5 (A and B) or pH 7.5 (C and D), and the reaction mixture was immunoprecipitated with anti-pCC_{10} (A and C), an antibody that recognizes N-terminal peptides, and anti-pYE_{17} (B and D), an antibody that recognizes C-terminal peptides. A control tube without enzyme was incubated for 16 h. The immunoprecipitates were electrophoresed on a SDS-polyacrylamide gel, and the counts were plotted against gel slice number. The mol wt of the peaks is indicated based on the migration of mol wt standards.

pro-TRH activities at both pH 5.5 and 7.5 being due to the PCs, and the 7B2 data provide further evidence that the neutral activity is due to PC2, whereas the acidic activity is due to both PC1 and PC2.

The pH profiles and immunodepletion data confirm that PC1 cleaves pro-TRH most effectively at pH 5.5 and that PC2 has two pH optima for pro-TRH. The neutral activity (optimum at pH 7.5) is immunodepleted to a large extent by anti-PC2(fus) antiserum. However, the activity at pH 5.5 is also immunodepleted by anti-PC2(fus) antiserum, suggesting that PC2 can function at that acidic pH.

Time-course and immunodepletion experiments demonstrate that the initial cleavage of pro-TRH to generate N-terminal 15-, 6-, and 3.8-kDa peptides was at pH 5.5. This cleavage was mainly due to PC1, with only a small contribution by PC2 at this pH. The cleavage at pH 7.5 generated the 15-kDa peptide, and immunodepletion experiments suggest that this cleavage was due to PC2. The cleavage from the N-terminal 15-kDa fragment to the 6- and 3.8-kDa peptides and the cleavage of the 6-kDa form to the 4-kDa form occurred primarily at pH 5.5 and appears to be mainly due to PC1. However the pH profiles do show a small amount of accumulation of pYE_{27} and pEH_{24} at pH 7.5, demonstrating that the neutral form of PC2 can also generate the 4-kDa form of pYE_{27} and pEH_{24} (3.8 kDa). Thus, the majority of the generation of cryptic peptides derived from the N-terminal intermediates occurs at acidic pH later in the secretory pathway (*trans*-Golgi network or in the immature secretory granules), with only a small amount of cleavage occurring in the Golgi complex (neutral pH). In support of these results, our recent immunocytochemistry and immunoelectromicroscopy studies on transfected AtT20 cells using anti-pCC10 antiserum showed positive staining in three specific compartments, *cis*- and medium-Golgi, *trans*-Golgi network, and immature secretory granules budding from the *trans*-Golgi network. As this antibody recognizes the intact precursor as well as the 15- and 6-kDa intermediates derived from the N-terminal end, we cannot precisely determine which of those peptides are in each compartment. Nevertheless, like other secretory proteins, it is expected that during the transport to the storage granules, the prohormone will be subjected to endoproteolytic processing. Therefore, smaller intermediate forms will be found in the *trans*-Golgi network and immature secretory granules, which is, in fact, what we

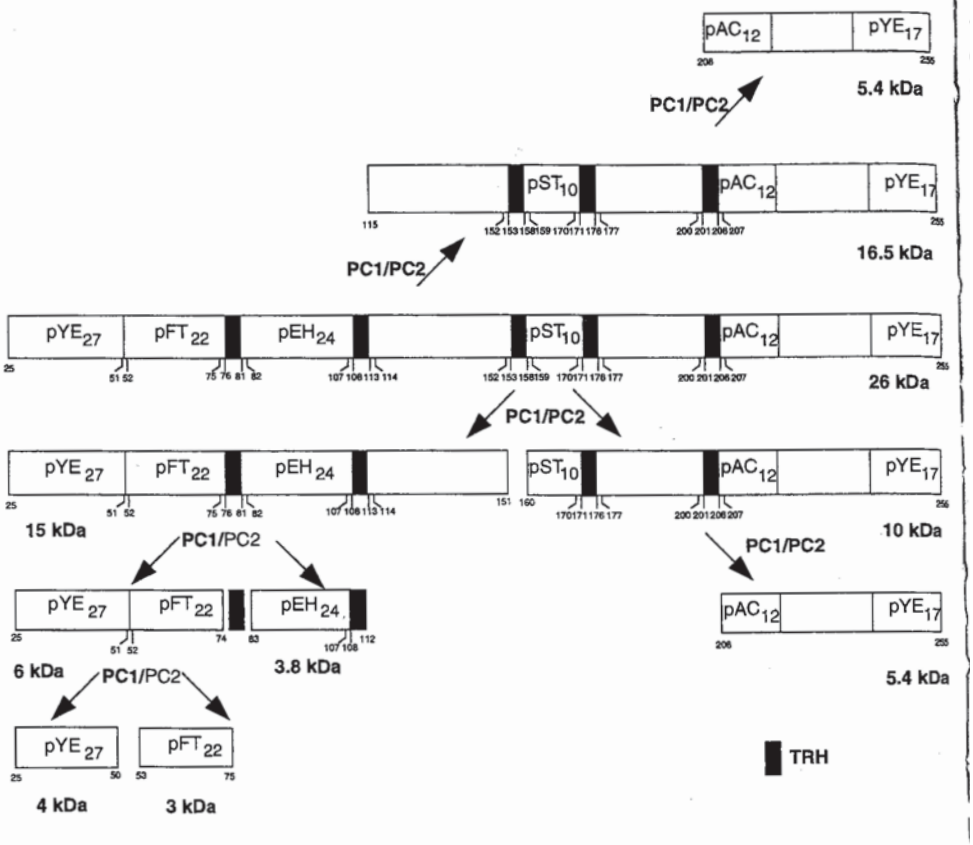

FIG. 7. Diagrammatic representation of the postulated processing of rat pro-TRH to TRH-related cryptic peptides by PC1 and PC2, as determined by incubation with bovine intermediate lobe secretory vesicle membranes combined with immunodepletion experiments with anti-PC1(fus) and anti-PC2(fus) antisera. Major cleavage by a particular enzyme ia indicated in *boldface*. The cleavages by PC1 occurred at pH 5.5, whereas the cleavages by PC2 occurred at both pH 5.5 and 7.5. As residual activity was still present after immunodepletion, it is possible that another pro-TRH-processing enzyme(s) was also present. The positions of paired basic residues are indicated by numbers.

demonstrated in an early work (36). However, although N-terminal intermediate products of pro-TRH were seen in these compartments, only end products (cryptic peptides) of processing were detected in mature secretory granules (48). Therefore, further processing of N-terminal intermediate peptides takes place in the immature secretory granules, and cryptic peptides are already formed by the time secretory granules mature.

The incubation of pro-TRH with the membrane preparation initially generated two C-terminal peptides, one 16.5 kDa and the other 10 kDa. Thus, it appears that there is simultaneous cleavage after either prepro-TRH-108 or prepro-TRH-114, and after either prepro-TRH-153 or prepro-TRH-159. Cleavage at the former would generate the 16.5-kDa peptide, whereas cleavage at the latter would generate the 10-kDa peptide. In transfected AtT_{20} cells, pro-TRH was also processed at these two sites (10); however, the 10-kDa peptide was not identified and was believed to be unstable. After initial cleavage by the membrane preparation to generate the 16.5- and 10-kDa peptides, further cleavage of these peptides occurred to generate a 5.4-kDa C-terminal peptide. Generation of this peptide was readily immunodepleted by either anti-PC1(fus) or anti-PC2(fus) antiserum, suggesting that both enzymes contribute to the generation of this product. Although we found the C-terminal peptides generated by cleavage of pro-TRH after either prepro-TRH-(107–108) or prepro-TRH-(113–114), the N-terminal 9-kDa product [prepro-TRH-(25–106) or prepro-TRH-112] expected was not seen, as this peptide is rapidly metabolized to the 6- and 3.8-kDa peptides (10). The 6-kDa fragment generated at pH 7.5 is most likely from the 9-kDa unstable intermediate.

The initial cleavage of pro-TRH to generate the 16.5- and 10-kDa C-terminal peptides occurred at both pH 7.5 and 5.5. The cleavage at pH 5.5 was probably due to both PC1 and PC2 and was immunodepleted by both of those antisera. Most of the cleavage at pH 7.5 was probably due to PC2, as it was substantially immunodepleted by anti-PC2(fus) antiserum only. The fact that we did not obtain complete immunodepletion of pro-TRH cleavage when both antisera were used may be due to either inefficient immunodepletion or the existence of another pro-TRH-processing enzyme(s). The redundancy of the initial cleavage of pro-TRH suggests that this step is highly regulated. The *in vivo* specificity of cleavage of pro-TRH is probably determined by subcellular localization and intracellular factors, such as Ca^{2+} concentration and pH.

We did not find TRH, TRH-Gly, or TRH-Gly-Lys after incubation of the membrane preparation with pro-TRH at either neutral or acidic pH. It is likely that PC1 and PC2 generated a form of TRH not recognized by the three antisera (anti-TRH, anti-TRH-Gly, and anti-TRH-Gly-Lys) used in this study. For example, the antibodies require a pGlu residue in the first position, and as the enzyme required for cyclization of the Gln residue (glutaminyl cyclase) is a soluble enzyme and unlikely to be present in the membranes, no radioimmunoassayable products would be generated. The detection of TRH, TRH-Gly, and TRH-Gly-Lys requires the action of carboxypeptidase H if cleavage by PC1 and PC2 occurs on the carboxyl side of the pair of basic residues. Although this carboxypeptidase B-like enzyme has been shown to be present in intermediate lobe secretory vesicles (49), it is possible that the *in vitro* conditions were not fa-

vorable in the reaction mixture. Additionally, the amidation of TRH requires the presence of peptidyl glycine α-amidating monooxygenase (3). It is also noteworthy that three of the five progenitor sequences in the rat prohormone are TRH-Gly-Arg, which would not be recognized by the TRH-Gly-Lys antiserum. Because of these reasons, we cannot conclude that PC1 or PC2 is incapable of generating TRH.

The *in vitro* cleavage of pro-TRH deduced in this study by immunodepletion experiments is summarized in Fig. 7. By performing Western blot analyses, we demonstrated that the anti-PC1(fus) and anti-PC2(fus) antisera used for immunodepletion are specific for their respective peptides. The sites of cleavage have been inferred from the products formed, which were identified by immunoprecipitation with antisera specific for various peptides and their apparent mol wt. We listed the cleavages on the carboxy side of the pair of basic residues, a site expected, but not proven from this study. Other pro-TRH-processing enzymes may also exist. These cleavages should be confirmed by cotransfection experiments as well as with purified PC1 and PC2. We have recently shown coexpression of pro-TRH mRNA with PC1 mRNA in primary cultures of hypothalamic neurons and pituitary cells and their protein colocalization in the same tissues (50) as well as coexpression of pro-TRH mRNA with PC1 and PC2 mRNA in rat brain (51). The biochemical study presented here together with the morphological findings indicate that PC1 and PC2 are potential enzyme candidates for the processing of pro-TRH *in vivo*.

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References

1. **Lechan RM, Wu P, Jackson IMD, Wolf H, Cooperman S, Mandel G, Goodman RH** 1986 Thyrotropin-releasing hormone precursor: characterization in rat brain. Science 231:159–161
2. **Scott AP, Ratcliffe JG, Rees LH, Landon J, Bennett HPJ, Lowry PJ, McMartin C** 1973 Pituitary peptide. Nature [New Biol] 244:65–67
3. **Eipper BA, Stoffers DA, Mains RE** 1992 The biosysnthesis of neuropeptides: peptide α-amidation. Annu Rev Neurosci 15:57–85
4. **Jackson IMD** 1989 Controversies in TRH biosynthesis and strategies toward the identification of a TRH precursor. Ann NY Acad Sci 553:7–13
5. **Bowers CY, Friesen HG, Hwang P, Guyda HJ, Folkers K** 1971 Prolactin and thyrotropin release in man by synthetic pyroglutamyl-histidyl prolinamide. Biochem Biophys Res Commun 45:1033–1041
6. **Wilber JF, Utiger RD** 1967 In vitro studies on mechanism of action of thyrotropin releasing factor. Proc Soc Exp Biol Med 127:488–490
7. **Takahara J, Arimura A, Schally AV** 1974 Stimulation of prolactin and growth hormone release by TRH infused into a hypophyisial portal vessel. Proc Soc Exp Biol Med 146:831–835
8. **Metcalf G** 1974 TRH: a possible mediator of thermoregulation. Nature 252:310–311
9. **Hedner J, Hedner T, Jonason J, Lundberg D** 1981 Central respiratory stimulant effect by thyrotropin releasing hormone in the rat. Neurosci Lett 24:317–320
10. **Nillni EA, Sevarino KA, Jackson IMD** 1993 Identification of the thyrotropin-releasing hormone-prohormone and its posttranslational processing in a transfected AtT20 tumoral cell line. Endocrinology 132:1260–1270
11. **Fuller RS, Brake A, Thorner J** 1989 Yeast prohormone processing enzyme (KEX2 gene product) is a Ca^{2+}-dependent serine protease. Proc Natl Acad Sci USA 86:1434–1438
12. **van den Ouweland AMW, van Duijnhoven HLP, Keizer GD, Dorssers LCJ, Van de Ven WJM** 1990 Structural homology between the human fur gene product and the subtilisin-like protease encoded by yeast KEX2. Nucleic Acids Res 18:664
13. **Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M, Chretien M** 1990 cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. DNA Cell Biol 9:415–424
14. **Seidah NG, Marcinkiewicz M, Benjannet S, Gaspar L, Beaubien G, Mattei MG, Lazure C, Mbikay M, Chretien M** 1991 Cloning and primary sequence of a mouse candidate prohormone convertase PC1 homologous to PC2, furin, and Kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. Mol Endocrinol 5:111–122
15. **Smeekens SP, Avruch AS, LaMendola J, Chan SJ, 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 88:340–344
16. **Nakayama K, Hosaka M, Hatsuzawa K, Murakami K** 1991 Cloning and functional expression of a novel endoprotease involved in prohormone processing at dibasic sites. J Biochem (Tokyo) 109:803–806
17. **Smeekens SP, Steiner DF** 1990 Identification of a human insulinoma cDNA encoding a novel mammalian protein structurally related to the yeast dibasic processing protease Kex2. J Biol Chem 265:2997–3000
18. **Kiefer MC, Tucker JE, Joh R, Landsberg KE, Saltman D, Barr PJ** 1991 Identification of a second human subtilisin-like protease gene in the fes/fps region of chromosome 15. DNA Cell Biol 10:757–769
19. **Seidah NG, Day R, Hamelin J, Gaspar A, Collard MW, Chretien M** 1992 Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific Kex2/subtilisin-like proprotein convertase. Mol Endocrinol 6:1559–1570
20. **Lusson J, Vieau D, Hamelin J, Day R, Chretien M, Seidah NG** 1993 cDNA structure of the mouse and rat subtilisin/kexin-like PC5: a candidate proprotein convertase expressed in endocrine and non-endocrine cells. Proc Natl Acad Sci USA 90:6691–6695
21. **Nakagawa T, Hosaka M, Torii S, Watanabe T, Murakami K, Nakayama K** 1993 Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. J Biochem (Tokyo) 113:132–135
22. **Nakagawa T, Murakami K, Nakayama K** 1993 Identification of an isoform with an extremely large Cys-rich region of PC6, a Kex2-like processing endoprotease. FEBS Lett 327:165–171
23. **Day R, Schafer MK-H, Watson SJ, Chretien M, Seidah NG** 1992 Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary. Mol Endocrinol 6:485–497
24. **Hakes DJ, Birch NP, Mezey E, Dixon JE** 1991 Isolation of two complementary deoxyribonucleic acid clones from a rat insulinoma cell line based on similarities to Kex2 and furin sequences and the specific localization of each transcript to endocrine and neuroendocrine tissues in rats. Endocrinology 129:3053–3063
25. **Hatsuzawa K, Hosaka M, Nakagawa T, Nagase M, Shoda A, Murakami K, Nakayama K** 1990 Structure and expression of mouse furin, a yeast Kex2-related protease. J Biol Chem 265:22075–22078
26. **Benjannet S, Rondeau N, Day R, Chretien M, Seidah NG** 1991 PC1 and PC2 are proprotein convertases capable of cleaving proopiomelanocortin at distinct pairs of basic residues. Proc Natl Acad Sci USA 88:3564–3568
27. **Thomas L, Leduc R, Thorne BA, Smeekens SP, Steiner DF, Thomas G** 1991 Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells: evidence for a common core of neuroendocrine processing enzymes. Proc Natl Acad Sci USA 88:5297–5301
28. **Friedman TC, Loh YP, Birch NP** 1994 In vitro processing of proopiomelanocortin by recombinant PC1. Endocrinology 135:854–862
29. **Friedman TC, Gordon VM, Leppla SH, Klimpel KR, Birch NP, Loh**

YP 1995 In vitro processing of anthrax toxin protective antigen by recombinant PC1(SPC3) and bovine intermediate lobe secretory membranes. Arch Biochem Biophys 316:5–13
30. **Hill RM, Ledgerwood EC, Brennan SO, Pu L-P, Loh YP, Christie DL, Birch NP**, Comparison of the molecular forms of the Kex2/subtilisin-like serine proteases SPC2, SPC3 and furin in neuroendocrine secretory vesicles reveals differences in carboxyl terminus truncation and membrane association. J Neurochem, in press
31. **Nillni EA, Sevarino KA, Wu P, Jackson IMD** 1991 Assays for biosynthesis of thyrotropin-releasing hormone. In: Conn PM (eds) Neuropeptide Technology: Synthesis, Assay, Purification and Processing. Academic Press, New York, pp 51–69
32. **Loh YP, Tam WWH, Russell JT** 1984 Measurement of delta-pH and membrane potential in secretory vesicles isolated from bovine pituitary intermediate lobe. J Biol Chem 259:8238–8245
33. **Estivariz FE, Friedman TC, Chikuma T, Loh YP** 1992 Processing of adrenocorticotropin by two proteases in bovine intermediate lobe secretory vesicle membranes. J Biol Chem 267:7456–7463
34. **Bradford M** 1988 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
35. **Wu P, Jackson IMD** 1991 Detection and purification of thyrotropin-releasing hormone precursor peptides using antisera generated against synthetic peptides. In: Conn PM (eds) Neuropeptide Technology: Synthesis, Assay, Purification and Processing. Academic Press, New York, pp 337–353
36. **Nillni EA, Sevarino KA, Jackson IM** 1993 Processing of proTRH to its intermediate products occurs before the packing into secretory granules of transfected AtT20 cells. Endocrinology 132:1271–1277
37. **Rhodes C, Lincoln B, Shoelson S** 1992 Preferential cleavage of des-31,32-proinsulin over intact proinsulin by the insulin secretory granule type II endopeptidase. J Biol Chem 267:22719–22727
38. **Shennan K, Smeekens S, Steiner D, Docherty K** 1991 Characterization of PC2, a mammalian Kex2 homologue, following expression of the cDNA in microinjected *Xenopus* oocytes. FEBS Lett 284:277–280
39. **Zhou Y, Lindberg I** 1993 Purification and characterization of the prohormone convertase PC1(PC3). J Biol Chem 268:5615–5623
40. **Rufaut NW, Brennan SO, Hakes DJ, Dixon JE, Birch NP** 1993 Purification and characterization of the candidate prohormone processing enzyme SPC3 produced in a mouse L cell line. J Biol Chem 268:20291–20298
41. **Benjannet S, Rondeau N, Paquet L, Boudreault A, Lazure C, Chretien M, Seidah NG** 1993 Comparitive biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulphation and identification of the intracellular site of prosegement cleavage of PC1 and PC2. Biochem J 294:735–743
42. **Hatsuzawa K, Murakami K, Nakayama K** 1992 Molecular and enzymatic properties of furin, a Kex2-like endoprotease involved in precursor cleavage at Arg-X-Lys/Arg-Arg sites. J Biochem (Tokyo) 111:296–301
43. **Lindberg I, Lincoln B, Rhodes CJ** 1992 Fluorometric assay of a calcium-dependent, paired-basic processing endopeptidase present in insulinoma granules. Biochem Biophys Res Commun 183:1–7
44. **Martens GJM, Braks JAM, Eib DW, Zhou Y, Lindberg I** 1994 The neuroendocrine polypeptide 7B2 is an endogenous inhibitor of prohormone convertase PC2. Proc Natl Acad Sci USA 91:5784–5787
45. **Alarcon C, Cheatham B, Lincoln B, Kahn CR, Siddle K, Rhodes CJ** 1994 A Kex2-related endopeptidase activity present in rat liver specifically processes the insulin proreceptor. Biochem J 301:257–265
46. **Zhou Y, Lindberg I** 1994 Enzymatic properties of carboxyl-terminally truncated prohormone convertase 1 (PC1/SPC3) and evidence for autocatalytic conversion. J Biol Chem 269:18408–18413
47. **Loh YP, Parish DC, Tuteja R** 1985 Purification and characterization of a paired basic residue-specific pro-opiomelanocortin converting enzyme from bovine pituitary intermediate lobe secretory vesicles. J Biol Chem 260:7194–7205
48. **Nillni EA, McMillan P, Jackson IMD** 1993 Distribution and targeting of pro-TRH in transfected AtT20 cells exposed to glucocorticoids. Mol Biol Cell [Suppl] 4:1840 (Abstract)
49. **Hook VYH, Loh YP** 1984 Carboxypeptidase B-like converting enzyme activity in secretory granules of rat pituitary. Proc Natl Acad Sci USA 81:2776–2780
50. **Nillni EA, Luo LG, Jackson IMD** 1994 Coexpression of proTRH with prohormone convertase 1 (PC1) in primary cultures of neuroendocrine tissues. Mol Biol Cell 5:209a (Abstract)
51. **Pu LP, Ma W, Barker JL, Loh YP**, Anatomical relationship between genes encoding prohormone convertases and pro-TRH in adult rat brain: implication in differential processing of pro-TRH. 24th Annual Meeting of the Society for Neuroscience, Miami Beach, FL, 1994 (Abstract 472.6)