Intracellular Sites of Prothyrotropin-releasing Hormone Processing*

Ignacio Perez de la Cruz and Eduardo A. Nillni‡

From the Division of Endocrinology, Department of Medicine, Brown University School of Medicine, Rhode Island Hospital, Providence, Rhode Island 02903

Post-translational processing of proteins plays a key role in regulating their subcellular localization, enzymatic activity, and protein-protein interactions by such diverse mechanisms as phosphorylation, glycosylation, and proteolytic cleavage. The prothyrotropin-releasing hormone (pro-TRH) precursor (26 kDa) undergoes proteolytic cleavage at either of two sites, generating a 15/10-kDa or a 9.5/16.5-kDa N/C-terminal pair of intermediates. Using transfected AtT20 cells encoding a prepro-TRH cDNA, we have previously reported that this initial set of cleavages occurs prior to entry into the secretory granules (Nillni, E. A., Sevarino, K. A., and Jackson, I. M. D. (1993) Endocrinology 132, 1271–1277). In this study, we set out to identify the subcellular compartment where this initial cleavage takes place as well as to determine the sites of processing of the intermediates produced. Our strategy was to block the transport of pro-TRH or its intermediates from one subcellular compartment to the next and to assay for the accumulation of intermediates, presumably because their processing occurs in a post-blockade compartment.

Radiolabeling experiments in AtT20 cells in the presence of the drug brefeldin A, which blocks transport from the endoplasmic reticulum to the Golgi complex, led to an accumulation of the 26-kDa precursor, suggesting a post-endoplasmic reticulum site of processing. When Golgi complex-to-secretory granule transport was blocked at 20 °C, the processing of the 26-kDa precursor was not affected, suggesting a Golgi complex site of processing. At this temperature, the 15-kDa N-terminal intermediate accumulated, suggesting a post-Golgi complex processing site, while the 16.5-kDa C-terminal intermediate was processed in the Golgi complex to produce a 5.4-kDa peptide.

Through post-translational processing, preprothyrotropin-releasing hormone (prepro-TRH)* generates TRH (TRH, pGlu-His-ProNH₂) and seven cryptic peptides with potential biological activity (1–4). The primary function of TRH is to stimulate the synthesis and release of thyrotropin-stimulating hormone, which in turn regulates thyroid function (5). TRH also stimulates the synthesis and release of prolactin and growth hormone from the anterior pituitary (6–8). TRH is widely distributed within the central nervous system (9), where it acts as both a neurotransmitter and a neuromodulator (10, 11). Among the cryptic peptides, prepro-TRH-(160–169) (pST₁₀) has been reported to potentiate thyrotropin-stimulating hormone release from the anterior pituitary and to stimulate thyrotropin-stimulating hormone-β gene promoter activity (2). Furthermore, prepro-TRH-(178–199) is believed to be a corticotropin-releasing inhibitory factor (3), inhibiting both basal and corticotropin-releasing hormone-stimulated ACTH synthesis and secretion. However, a new publication has indicated that this cryptic peptide has no significant inhibitory effect on basal or stimulated ACTH secretion by normal pituitary corticotrophs in vitro (12). More recently, we found that opiate withdrawal in rat periaqueductual gray produced an increase in the level of the N-terminal prepro-TRH-(53–74) (pYT₂₂) peptide, whereas the level of TRH remained unaltered (4), suggesting that this cryptic peptide may have a biological function in periaqueductal gray.

Rat prepro-TRH is a 29-kDa polypeptide composed of 255 amino acids. This precursor contains an N-terminal 25-amino acid leader sequence that targets the protein to the secretory pathway; five copies of the TRH progenitor sequence (Gln-His-Pro-Gly), each flanked by two pairs of basic amino acids (Lys or Arg); and seven cryptic peptides inserted between the TRH sequences (13). Much of the knowledge about the post-translational processing of pro-TRH has come from studies using the AtT20 corticotropin cell line transfected with cDNA encoding prepro-TRH (1). By Western blot analysis, immunoprecipitation followed by SDS-PAGE, and radioimmunooassay, we demonstrated that pre-TRH is present in AtT20 cells as a 26-kDa protein. We further showed by pulse-chase analysis that the 26-kDa precursor is cleaved at two mutually exclusive sites. The first cleavage generates a 15-kDa N-terminal peptide (prepro-TRH-(25–151) or -(25–157)) and a 10-kDa C-terminal peptide (prepro-TRH-(154–255) or -(160–255)). A second (alternative) cleavage generates a 9.5-kDa N-terminal peptide (prepro-TRH-(25–106) or -(25–112)) and a 16.5-kDa C-terminal peptide (prepro-TRH-(109–255) or -(115–225)). In subsequent steps using specific antibodies developed in this laboratory to multiple sequences of the prohormone, we showed that the 15-kDa N-terminal intermediate moiety of pro-TRH was further processed to a 6-kDa peptide corresponding to prepro-TRH-(25–80) and a 3.8-kDa peptide corresponding to prepro-TRH-(83–112). Processing of the remaining 10-kDa C-terminal fragment produced a 5.4-kDa peptide corresponding to the C-terminal flanking peptide prepro-TRH-(208–255). Furthermore, processing of the 9.5-kDa N-terminal fragment arising from the alternative cleavage of the 26-kDa prohormone at residues 107–108 was postulated to account for the production of...
of the N-terminal peptides prepro-TRH-(25–50), prepro-TRH-(53–74), and prepro-TRH-(83–106), while the 16.5-kDa fragment was further processed to produce a 5.4-kDa peptide arising from the C terminus of the prohormone, prepro-TRH-(208–255) (1).

To generate biologically active TRH or any of the other peptides, pro-TRH is cleaved at paired basic residues by the action of carboxypeptidase H activity to remove the basic residue(s) (17, 18). To generate TRH, the TRH extended form is further processed in the GC, while the 15-kDa N-terminal intermediate is further processed in the SGs.

**Pro-TRH Polypeptide Processing and Sorting**

![Diagram of Pro-TRH Polypeptide Processing and Sorting](image)

**Fig. 1.** Antibodies used in this study and the pro-TRH intermediates they recognize. CRIF, corticotropin-releasing inhibitory factor.

**Experimental Procedures**

**Tissue Culture—**AtT20 cells transfected with a cDNA encoding pro-pre-TRH (29) were grown in 75-cm² flasks at 37 °C in an atmosphere of 5% CO² and 95% air with 90% humidity (30). Each flask was plated with 5 million cells, and cultures were maintained for 6 days in Dulbecco’s modified Eagle’s essential medium (Life Technologies, Inc.) containing 10% fetal calf serum as described previously (30). Culture medium was removed every 2 days and replaced with fresh medium. Experiments were performed at a confluency corresponding to 20–30 × 10⁶ cells.

**Radiolabeling/Pulse-Chase—**The medium was removed from 75-cm² flasks, and 6 ml of leucine-free Dulbecco’s modified Eagle’s essential medium (Life Technologies, Inc.) supplemented with 5% dialyzed fetal calf serum (Life Technologies, Inc.) was added to each and incubated for 30 min at 37 °C. Cells were pulsed with 200 μCi of [3,4,5-³H]leucine (156 Ci/mmol; DuPont NEN) for 3 h at 37 °C in the presence or absence of brefeldin A (Sigma) at 10 μg/ml in the cell medium. For short-term pulse-chase, cells were washed three times with 10 ml of Hanks’ solution after 15 min of pulse and chased in Dulbecco’s modified Eagle’s essential medium containing 10% fetal calf serum and 1 mM unlabeled leucine. For the temperature experiments, the chase was carried out in a 20 °C water bath. At various times, cells were harvested over ice in 2 N acetic acid containing 5 mM EDTA and EGTA and protease inhibitors (phenylmethylsulfonyl fluoride, aprotinin, bacitracin, bestatin, and pepstatin, each at 0.01%). Cells were detached with a cell scraper, and extracts were boiled for 10 min, sonicated, and centrifuged at 17,000 × g for 30 min. The supernatant was separated and lyophilized.

**Antibodies—**The following antibodies were utilized for the immunoprecipitation studies: anti-pCC10, which recognizes prepro-TRH-(25–255) (26 kDa), prepro-TRH-(25–255) (26 kDa), prepro-TRH-(208–255) (16.5 kDa), prepro-TRH-(208–255) (16.5 kDa), and anti-pAC12, which recognizes prepro-TRH-(208–255) (5.4 kDa). Fig. 1 depicts the cryptic peptides within the pro-TRH molecule that are recognized by the various polyclonal antibodies.

**Immunoprecipitation—**Immunoprecipitations were carried out essentially as described previously (1). Briefly, lyophilized cell extracts were resuspended in 500 μl of buffer A (10 mM NaPO₄, pH 7.2, 1 mM EDTA, and 0.1% Triton X-100) and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies. Samples were incubated with 8 μl of primary antibody for 1 h at 37 °C, followed by 24 h at 4 °C. Goat anti-rabbit IgG (8 μl) was added to each sample along with 75 μl of buffer B (500 mM KCl, 50 mM NaH₂PO₄, pH 7.4, 5 mM NaEDTA, and 25 μl of 2% bovine serum albumin. Samples were centrifuged, and the supernatant was divided in two so that immunoprecipitations could be carried out with N- and C-terminal antibodies.
were boiled for 4 min prior to SDS-PAGE.

SDS-Polyacrylamide Gel Electrophoresis—Samples were loaded onto a 1.5-mm discontinuous Tricine-polyacrylamide gel, with 3% cross-linking (acylamide/bisacrylamide solution) stacking gel and 6% cross-linking separating gel (31). Following electrophoresis, gels were cut into 2-mm slices in a gel slicer (Hoeffer Scientific Instruments, San Francisco, CA), and proteins were extracted from gel slices by incubation in 0.5 ml of 2 N acetic acid for 3–4 days at 4 °C. Scintillation fluid (Bio Safe II, RPI, Mount Prospect, IL) was added, and the samples were counted in a scintillation counter.

Western Blotting—Lyophilized samples were resuspended in 300 μl of sample buffer, centrifuged for 10 min in a microcentrifuge, and boiled for 4 min, followed by SDS-PAGE. Gel proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) overnight. The membrane was blocked in 50 ml of 2% nonfat dry milk overnight and washed three times with TTBS (20 ml Tris, 500 ml NaCl, and 0.05% Tween 20, pH 7.5), and 8 μl of primary IgG was added in antibody buffer (TTBS and 1% nonfat dry milk) and incubated overnight at 4 °C. The membrane was washed again three times with TTBS, and secondary goat anti-rabbit antibody was added in a 1:3000 dilution in antibody buffer. Enhanced chemiluminescence analysis was carried out using the Immuno-Lite chemiluminescent kit according to the manufacturer’s specifications (Bio-Rad).

Trichloroacetic Acid Precipitations—Cell content lysates and release media samples (1 ml each) from 6-well plates were centrifuged briefly to remove cell debris, and proteins were precipitated in an equal volume of 10% trichloroacetic acid. The supernatant was removed, and proteins were precipitated in a equal volume of media samples (1 ml each) from 6-well plates were centrifuged briefly to remove cell debris, and proteins were precipitated in an equal volume of media samples (1 ml each) from 6-well plates were centrifuged briefly to

RESULTS

BFA and 20 °C Incubation Block the Vectorial Transport of Proteins to the Secretory Granules—Before studying the effects of the cellular blockades on the processing of pro-TRH, we wanted to determine the effectiveness of our blockades. The strategy was to pulse-chase AtT20 cells and see if the labeled proteins were prevented from reaching the last compartment of the secretory pathway, the SGs, when the chase was carried out in the presence of BFA or temperature blockades. We briefly transfected AtT20 cells with [3H]leucine for 15 min, followed by a chase with unlabeled leucine for 4 h under three different conditions: 37 °C, 37 °C with BFA, and 20 °C to allow the labeled protein to travel to the SGs. We then induced the fusion of the SGs with the plasma membrane to release the stored proteins into the medium by incubating cells with the secretagogue PMA (phorbol 12-myristate ester) for 1 h and without PMA to measure the unstimulated or basal release. Radioactive protein released into the medium was measured after trichloroacetic acid precipitation. Protein released from the SGs was calculated by subtracting basal release protein levels (without PMA) from stimulated release protein levels (with PMA). Fig. 2 shows an inhibition of the amount of protein released into the medium from the SGs in BFA-treated cells and in temperature-blocked cells when compared with control cells. For BFA-treated cells, the protein levels appear as a negative number because basal release in these cells was higher than stimulated release. We conclude that the BFA and 20 °C temperature conditions used in this study result in a blockade of protein transport through the secretory pathway of AtT20 cells.

Processing of the 26-kDa Precursor Occurs in a Post-ER Compartment—Based on our previously described pathway of pro-TRH processing and the proposed sites where PC1 and PC2 produced their endoproteolytic cleavages (14–16), the first cleavage of the 26-kDa precursor can occur at either of two sites to generate either a 15/10-kDa or a 9.5/16.5-kDa pair of N/C-terminal intermediates. This first set of alternative cleavage sites has been shown to occur prior to packaging into the SGs based on subcellular fractionation studies (20). Limitations in this approach prevent the assignment of this first cleavage to either the ER or the GC. To determine how much processing of the 26-kDa precursor if any occurs in the ER, we pulsed cells with [3H]leucine for 3 h to label pro-TRH and its intermediates, in the presence or absence of BFA to block the exit of proteins from the ER to the GC. Whole cell extracts were immunoprecipitated with anti-pC160 antibodies specific for the intact 26-kDa precursor and N-terminal intermediates (see Fig. 1) (1), and immunoprecipitates were resolved by SDS-PAGE. We reasoned that if the pro-TRH precursor (26 kDa) was normally processed upon leaving the ER, blocking its exit from this compartment with BFA would prevent its processing and lead to an accumulation over control levels. Indeed, Fig. 3A shows a BFA-dependent accumulation of the 26-kDa precursor, suggesting that it is normally processed upon leaving the ER. In addition, the 15- and 9.5-kDa N-terminal intermediates produced in BFA-blocked cells accumulate ~4-fold over the levels seen in control cells, suggesting that further processing of these intermediates into smaller peptides occurs in a post-ER compartment (Fig. 3C). The increase in 26-kDa precursor levels was not due to an increase in protein synthesis in response to BFA because we did not observe an increase in the levels of trichloroacetic acid-precipitated material in the presence of BFA (data not shown).

Fig. 3B shows the profile of pro-TRH intermediates from an identical experiment immunoprecipitated with anti-pYER1α antibodies, which detect the 26-kDa precursor along with other C-terminal intermediates (see Fig. 1). As shown above in Fig. 3A, the 26-kDa precursor accumulates in BFA-treated cells over control levels. The 16.5-kDa C-terminal intermediate is produced in BFA-blocked cells and accumulates 2-fold over control levels (Fig. 3C), suggesting that its processing to yield smaller intermediates also occurs in a post-ER compartment. The 10-kDa intermediate accumulates only slightly in BFA-blocked cells, suggesting that it might be further processed in the ER itself or is rapidly degraded as suggested in earlier studies (1).

Processing of the 26-kDa Precursor Occurs in the GC—Since the BFA data suggested that cleavage of the 26-kDa precursor occurred in a post-ER compartment, we wanted to determine if this compartment was the GC. We pulsed transfected AtT20
Fig. 3. BFA profiles of generated N- and C-terminal intermediates from [3H]pro-TRH during pulse-labeling experiments. Transfected AtT20 cells were radiolabeled for 3 h with [3H]leucine in the presence or absence of BFA; cell content immunoprecipitates using anti-pCC10 (A) or anti-pYE17 (B) antibodies were electrophoresed on SDS-polyacrylamide gel; and counts were plotted against gel slice. A, the 26-kDa pro-TRH precursor and the 15- and 9.5-kDa N-terminal intermediates accumulated in the ER when their exit from this compartment was blocked by BFA, suggesting that their processing normally occurs in a post-ER compartment. B, the 16.5- and 9.5-kDa C-terminal intermediates also accumulated in the ER when their exit from this compartment was blocked by BFA, suggesting that their processing also occurs in a post-ER compartment. C, shown is a schematic representation of the proposed intracellular processing pro-TRH (26 kDa) at the post-ER level.
cells continuously with $[^3H]$leucine at 20°C to prevent exit of proteins from the GC and checked the levels of the 26-kDa precursor after 1 and 3 h of labeling with anti-pYE17 antibodies. Fig. 4A shows the profile of pro-TRH immunoprecipitates from such an experiment. The level of the 26-kDa precursor did not change from 1 to 3 h, suggesting it had achieved a steady-state level where the rate of formation of the 26-kDa precursor equaled its rate of breakdown. Because the 26-kDa protein cannot exit the GC at 20°C, the amount produced between 1 and 3 h had to be broken down into smaller intermediates in the GC or in a previous compartment. When we repeated the experiment in the presence of BFA as shown in Fig. 4B, the 26-kDa precursor did not achieve steady-state levels and instead accumulated over time in the ER as it did at 37°C, suggesting that when its exit from the ER is blocked, the 26-kDa precursor is not processed. Thus, if the 26-kDa precu-

![Graph showing temperature profiles of generated C-terminal intermediates from $[^3H]$pro-TRH during 1- and 3-h pulse-labeling experiments.](image)

**FIG. 4.** 20°C temperature profiles of generated C-terminal intermediates from $[^3H]$pro-TRH during 1- and 3-h pulse-labeling experiments. Transfected AtT20 cells were radiolabeled for 1 or 3 h with $[^3H]$leucine in the presence (B) or absence (A) of BFA; cell content immunoprecipitates using anti-pYE17 antibodies were electrophoresed on SDS-polyacrylamide gel; and counts were plotted against gel slice. Steady-state conditions were achieved by the 26-kDa precursor at 20°C in control cells (A), but not in the presence of BFA (B). Therefore, the 26-kDa precursor is processed in the GC. Also shown is a schematic representation of the proposed intracellular processing of pro-TRH (26 kDa) at the GC level (C).
Pro-TRH Polypeptide Processing and Sorting

The 15-kDa N-terminal Intermediate Is Processed in Immature Secretory Granules, While the 16.5-kDa C-terminal Intermediate Is Further Processed in the Golgi Complex—Having determined that the 26-kDa precursor is cleaved in the GC, we next wanted to determine the fate of its cleavage products, the 15/10-kDa and the 9.5/16.5-kDa intermediates. We were unable to detect the 10- and 9.5-kDa intermediates in sufficiently high amounts, probably due to their instability or rapid turnover, so our analysis was limited to the 15-kDa N-terminal and 16.5-kDa C-terminal peptides. To determine if these two intermediates were further processed in the GC, we used a 20 °C incubation to block their exit from this compartment. Cells were pulsed with [3H]leucine for 4 h to label pro-TRH intermediates and chased for various times with unlabeled leucine before harvesting. Pro-TRH intermediates were immunoprecipitated with N- and C-terminal antibodies and separated by SDS-PAGE. Fig. 5A shows a 2-fold accumulation of the 15-kDa peptide at 20 °C over control levels when its exit from the GC was blocked (Fig. 5C), suggesting that processing of the 15-kDa intermediate normally occurs in a post-Golgi compartment, i.e. the SGs. In contrast, Fig. 5B shows a time-dependent decrease of the 16.5-kDa intermediate at 20 °C over control levels. This rapid processing suggests that this intermediate is normally processed in the GC prior to packaging into the SGs (Fig. 5C).

The Proconverting Enzymes That Process the 15-kDa Intermediate Are Not Inhibited by Reduced Temperatures—It is
possible that the 15-kDa intermediate is normally processed in the GC and that the accumulation observed at 20 °C was not due to a blockade of the Golgi complex, but to an inhibition of the proconverting enzymes by the reduced temperature. To test this possibility, we wanted to see if the 15-kDa intermediate would also accumulate at 25 °C, when the temperature is also low, but proteins can exit the GC into immature SGs. We reasoned that if the low temperature and not the blockade was responsible for the accumulation of the 15-kDa intermediate, then we might expect to see this accumulation at 25 °C. Fig. 6 shows the N-terminal immunoprecipitates after a short-term pulse with [3H]leucine, followed by a 4-hour chase at different temperatures. The levels of the 15-kDa intermediate for the 25 °C chase were identical to those at 37 °C, while the levels at 20 °C showed an accumulation of the 15-kDa intermediate. These results suggest that the accumulation of the 15-kDa N-terminal intermediate is not due to an inhibition of proconverting enzymes by low temperature, but is instead due to the inability of the 15-kDa intermediate to reach the SGs at 20 °C, where it would normally be processed. It is interesting that the 15-kDa intermediate is processed faster at 20 °C than at 37 °C. This might be due to a parallel accumulation of processing enzymes in the TGN.

Cleavage of the 16.5-kDa Intermediate Produces a 5.4-kDa C-terminal Product—We have shown that the 16.5-kDa C-terminal intermediate is further processed in the GC. Processing of this intermediate results in a 5.4-kDa C-terminal intermediate and a putative 9.5-kDa N-terminal intermediate (see Fig. 1). Thus, we expected that at 20 °C, these peptides might accumulate. Because our antibodies were not raised against epitopes from the 9.5-kDa region of pro-TRH, our analysis was limited to the 5.4-kDa fragment. To check for accumulation of this intermediate at 20 °C, we pulsed cells for 15 min in [3H]leucine and chased them with unlabeled leucine for 2 h. We immunoprecipitated cell content proteins with the C-terminal antibodies anti-pYE17, which recognizes the 26-, 16.5-, and 5.4-kDa intermediates, and anti-pAC12, which recognizes the 5.4-kDa cryptic peptide. Fig. 7 shows the peptide profile after a 2-h chase. The levels of the 16.5-kDa intermediate dropped at 20 °C versus 37 °C as shown earlier. In addition, we observed a marked accumulation of the 5.4-kDa peptide, produced from cleavage of the 16.5-kDa peptide. These results suggest that the 16.5-kDa intermediate is cleaved at the base of the residues 206 and 207 to yield a 5.4-kDa C-terminal product in the GC. We did not analyze what happens with the cryptic peptides.

The 41-kDa Protein—In the course of our experiments with BFA, we observed the appearance of a new peak never previously observed, corresponding to a protein of ~41 kDa. As shown in Fig. 3 (A and B), the levels of this protein are very low in control cells, but accumulate markedly in BFA-treated cells. We were very much intrigued by the identity of this new peak. If this peak were a cytoplasmic protein, one would not expect that a BFA-dependent blockade of ER-to-GC transport would lead to its accumulation. In fact, one would expect its levels to be lower in BFA-treated cells since protein synthesis is reduced in these cells. Thus, we believe the 41-kDa protein to be a secretory protein that accumulates in the rough ER when its exit from this compartment is blocked.

Interestingly, this protein was immunoprecipitated by two completely different sets of polyclonal antibodies, the N-terminal directed anti-pCC10 (Fig. 3A) and the C-terminal directed anti-pYE17 (Fig. 3B), suggesting that its immunoprecipitation is not the result of nonspecific binding by the antibodies. However, our antibodies were not affinity-purified, so we cannot exclude this possibility. Immunoprecipitation of this 41-kDa protein could result from a stable interaction with the 26-kDa pro-TRH precursor, which both sets of antibodies recognize. To check this possibility, we performed Western blotting on control and BFA-treated cell lysates. We reasoned that if the 41-kDa protein was being immunoprecipitated with the 26-kDa precursor, it should not be detected on a Western blot since only proteins that are bound directly by the antibodies are identified by this technique. Fig. 8 demonstrates that the 41-kDa protein is recognized directly by anti-pCC10 antibodies. These results also confirm the increase of this protein under BFA treatment as well as the accumulation of the 26- and 15-kDa intermediates observed in the pulse experiments (Fig. 3, A and B).

DISCUSSION

In the last decade, it has become clear that peptides play an important role in the fine control of the central nervous system, and monoamine and amino acid function in the central nervous system is mediated by peptides. Even more astounding is the discovery that many sets of these neuropeptide hormones with distinct physiological functions arise from the processing of a single polypeptide precursor (16, 32–34). This is achieved throughout a differential processing mechanism by the action of specific processing enzymes acting in specific compartments. This concept of differential processing associated with differential subcellular localization and specific cell type is further reinforced by the observation that certain regions in the brain can give rise to several different pro-TRH-derived peptides in addition to, or instead of, TRH. For example, the reticular nucleus of the thalamus contains abundant pro-TRH mRNA and several of the pro-TRH-derived peptides, but does not contain TRH (35, 36). Moreover, the N-terminal extended forms of TRH, prepro-TRH-(172–199) and prepro-TRH-(154–169), have been found to be the major end products of pro-TRH processing in the olfactory lobe (37–39), but not in the hypothalamus, where pro-TRH is completely processed to cryptic peptides and TRH (37).

Similar observations have been reported for pro-opiomelanocortin (POMC). This prohormone is processed primarily to ACTH, β-endorphin, and N-POMC-(1–77) in the anterior pituitary (corticotrophs). In turn, these products are further processed to α-melanocyte-stimulating hormone, β-endorphin-(1–31), N-POMC-(1–49), and γ-melanocyte-stimulating hormone.

Fig. 6. Profile of generated N-terminal intermediates from [3H]pro-TRH in a short-term pulse-chase experiment. Transfected AtT20 cells were pulsed for 15 min at 37 °C with [3H]leucine and chased for 4 h at 37, 25, or 20 °C. Cell content immunoprecipitates using anti-pCC10 antibodies were electrophoresed on SDS-polyacrylamide gel, and counts were plotted against gel slice. Shown is an almost identical profile of the 15-kDa intermediate generated at 25 °C and 37 °C, while at 20 °C, the 15-kDa intermediate accumulated.
in the intermediate lobe and brain (32); this differential processing of a common polypeptide precursor is dependent upon the type of post-translational processing enzymes present in each specific cell type. Proenkephalin, which contains seven identical copies of Met-enkephalin, is processed to large forms in the adrenal medulla, whereas this precursor is cleaved primarily to the pentapeptides of Met-enkephalin in the brain (33). Actions of substance P (SP) on behavior appear to depend on the enzymatic processing of this precursor by the processing enzymes prolyl endopeptidase, SP-(5–11), and endopeptidase (EC 3.4.24.11), SP-(1–7). While SP-(1–7) acts as an analgesic, inhibits aggression, and enhances learning and memory, SP-(5–11) is a pain transmitter, blocks analgesia, stimulates aggression, and blocks learning and memory (34). This differential processing observed with pro-TRH, POMC, SP, and proenkephalin appears to be one of the mechanisms through which cells regulate the maturation of certain specific peptides to fulfill different physiological functions, a mechanism analogous to the alternative splicing of mRNA. In agreement with this paradigm, in this study, we have investigated the intracellular processing of pro-TRH and shown that certain intermediate peptides have a distinct way to be processed to their end products.

In a series of temperature and BFA blockades, we were able to identify the intracellular sites of pro-TRH processing. Fig. 9 shows a schematic representation of the proposed processing of pro-TRH in relation to the compartments of the secretory pathway. When transfected AtT_{20} cells expressing a proprepro-TRH cDNA were treated with BFA to block ER-to-GC transport, the 26-kDa pro-TRH precursor accumulated over control levels, indicating a post-ER site of processing. However, some processing of the 26-kDa precursor did occur under BFA treatment to produce the 15- and 9.5-kDa N-terminal intermediates (Fig. 3A) as well as the 16.5- and 10-kDa C-terminal intermediates (Fig. 3B). These results suggest that some processing of the 26-kDa precursor might begin in the ER, although the majority of the processing would occur in a post-ER compartment. Alternatively, this partial processing might be an artifact caused by the collapse of resident Golgi proteins, including possible endopeptidases, into the ER as observed in BFA-treated cells (40) or simply represents partial degradation of the 26-kDa precursor during protein extraction.

Under an identical BFA blockade (Fig. 3B), the accumulation of the 16.5-kDa intermediate is not as great as that of the 15-kDa intermediate when compared with control levels (4-fold for the 15-kDa intermediate and ~2-fold for the 16.5-kDa intermediate). This differential accumulation might arise from a greater stability of the 15-kDa versus 16.5-kDa intermediate when trapped in the ER, which is consistent with the earlier processing of the 16.5-kDa C-terminal intermediate (in the GC) versus the 15-kDa N-terminal intermediate (in the SGs). Alternatively, the differential levels observed might not be the result of the stabilities of the two intermediates, but due instead to different rates of their formation; the high Ca^{2+}, high pH conditions of the ER (41) might favor initial cleavage of the 26-kDa precursor to produce the 15/10-kDa intermediates over cleavage to produce the 9.5/16.5-kDa pair, leading to higher levels of the 15-kDa versus 16.5-kDa peptide.

In later experiments, with a combination of temperature blockade and BFA treatment, we were able to show that the 26-kDa precursor protein was processed in the GC (possibly in the TGN) to generate the 15/10-kDa and the 9.5/16.5-kDa N/C-terminal pairs of intermediates. In the same compartment, the 18-kDa C-terminal intermediate was further processed to generate a prepro-TRH-(208–255) (5.4 kDa) C-terminal peptide by undergoing a cleavage at the pair of basic residues 206 and 207. In contrast, the 15-kDa N-terminal intermediate seems to be...
processed in a post-GC compartment, i.e. the SGs. In support of these findings, our recent immunocytochemical studies using the same transfected cell line indicated that pro-TRH as well as N-terminal intermediate products were located in the GC and in budding SGs, whereas cryptic peptides and TRH were seen in the SGs along the plasma membrane and cellular processes (42). On the other hand, C-terminal peptides were seen mostly along the plasma membrane and cellular processes. Cryptic peptides derived from the C-terminal portion were predominant in the cell processes (42). Furthermore, we found that in primary cultures of hypothalamic neurons (an endogenous source of pro-TRH), N-terminal intermediate peptides have a different subcompartiment distribution as compared with intermediate peptides derived from the C-terminal side (16). This differential processing phenomenon observed for pro-TRH intermediates might serve as a mechanism to regulate the timing of production of TRH and the potentially active end products so far identified, namely, pST10, corticotropin-releasing inhibitory factor, and pYT22. For example, the 16.5-kDa pro-TRH intermediate that we have shown in this study to be processed in the TGN contains corticotropin-releasing inhibitory factor and pST10. These peptides might be formed prior to their entry into the SGs, perhaps allowing a fraction to exit the cell via the constitutive pathway to maintain a basal level of release independent of TRH secretion.

Although our temperature experiments concentrated on the sites of processing of the 15- and 16.5-kDa intermediates, our results from the BFA blockade suggest that the 9.5-kDa N-terminal intermediate, which is formed upon cleavage of the 26-kDa precursor to give the 16.5-kDa C-terminal intermediate (Fig. 3A), is processed in the SGs like its 15-kDa N-terminal counterpart since it accumulates slightly at 20 °C (Fig. 7). The 6-kDa extreme N-terminal intermediate, which could be produced from cleavage of the 15- or 9.5-kDa N-terminal intermediate, also accumulates slightly at this temperature (Fig. 7), suggesting that its further processing to give the 4-kDa (pYE27) and 3-kDa (pFT22) cryptic peptides occurs in a post-GC compartment. We believe that the 6-kDa intermediate that accumulated in the GC at 20 °C originated mostly from the 9.5-kDa versus 15-kDa fragment since the 9.5-kDa intermediate appears to be less stable based on previous work (1). However, further studies are needed to determine its origin. Western blot analysis of material extracted from steady-state cultures confirmed the increase in the 26- and 15-kDa intermediates under BFA treatment as well as the accumulation of the 41-kDa protein. This larger protein was also present in primary cultures of hypothalamic neurons (16), which are an endogenous source of pro-TRH. We are currently investigating the origin of this protein by purifying and subjecting it to analysis by two-dimensional gel electrophoresis and microsequencing.

Among the proconverting enzymes involved in the processing of prohormones, PC1 and PC2, which are members of the family of subtilisin/kexin-like proteases (43–45), can correctly process pro-TRH into its predicted products in vitro (14, 15). Maturation of PC1 begins in the ER and continues in the TGN (26, 41, 45). In contrast, pro-PC2 maturation begins in the TGN and continues in the SGs to yield the active form PC2 in the SGs, where it accumulates (28, 41, 46). Thus, the compartments where these enzymes are active are regulated by a differential timing in their respective maturation events. In our studies with hypothalamic neurons, we have found that pro-TRH was coexpressed with PC1 (16). In the same study, we proposed that the 87-kDa form of PC1 may be the one that initiates the processing of pro-TRH at the GC level, followed by the action of PC1 and PC2 at later stages of the secretory pathway. In addition, our current studies using a recombinant vaccinia virus vector to coexpress PC1 and PC2 with pro-TRH indicated that while PC1 and PC2 were equally efficient in producing intermediate forms from the N-terminal side of the prohormone (15- and 3.8 kDa), PC2 had a higher proclivity for processing the intermediate form prepro-TRH-(25–74) to prepro-TRH-(25–50) (pYE27) and prepro-TRH-(50–74) (pYT22) peptides. Moreover, the data suggest that PC2 produced TRH from the N-terminal prepro-TRH-(74–106) (3.8 kDa) peptide, which contains TRH and the sequence prepro-TRH-(83–106) attached. These cleavages are most likely produced in the immature SG or mature SG (Fig. 9). We are also currently conducting cotransfection experiments with PC1, PC2, and pro-TRH cDNAs to test these hypotheses.

Due to the redundancy of TRH and diverse end products, pro-TRH is one of the most complex peptide precursors for

---

3 N. G. Seidah and E. A. Nillni, unpublished data.
processing analysis. Nevertheless, we were able to describe with a certain degree of detail the intracellular processing of this prohormone. We demonstrated that the 26-kDa pro-TRH precursor is cleaved early in the secretory pathway, beginning in the ER, to yield the 15/10-kDa and the 9.5/16.5-kDa pairs of N/C-terminal intermediates. The 16.5-kDa C-terminal and 9.5-kDa N-terminal intermediates are further cleaved in the TGN prior to their packaging into the SGs. These last data strongly support a differential processing mechanism for the C-terminal intermediates, which may have physiological significance. We also propose that the 15- and 6-kDa N-terminal intermediates are cleaved upon their entry into the SGs.

Acknowledgment—We thank Dr. Jackson for providing antibodies against the pro-TRH sequence.

REFERENCES

1. Nilini, E. A., Sevarino, K. A., and Jackson, I. M. D. (1993) Endocrinology 132, 1260–1270
2. Roussel, J.-P., Hollande, F., Bulant, M., and Astier, H. (1991) Neuroendocrinology 54, 559–565
3. Redei, E., Hilderbrand, H., and Aird, F. (1995) Endocrinology 136, 3557–3563
4. Legradi, G., Rand, W. M., Hitz, S., Nilini, E. A., Jackson, I. M. D., and Lechan, R. M. (1996) Brain Res., in press
5. Jackson, I. M. D. (1994) Regulation of Thyrotropin Secretion, Raven Press, Ltd., New York
6. Bowers, C. Y., Friessen, H. G., Hwang, P., Guyda, H. J., and Folkers, K. (1971) Biochem. Biophys. Res. Commun. 45, 1033–1041
7. Wilber, J. F., and Utiger, R. D. (1967) Proc. Soc. Exp. Biol. Med. 127, 488–490
8. Takahara, J., Arimura, A., and Schally, A. V. (1974) Proc. Soc. Exp. Biol. Med. 146, 831–835
9. Lechan, R. M. (1993) Thyroid Today 16, 1–11
10. Metcalf, G. (1974) Nature 252, 310–311
11. Hedner, J., Hedner, T., Jonason, J., and Landberg, D. (1981) Neurosci. Lett. 24, 317–320
12. Nicholson, W. K., and Orth, D. N. (1996) Endocrinology 137, 2171–2174
13. Lechan, R. M., Wu, P., Jackson, I. M. D., Wolfe, H., Cooperman, S., Mandel, G., and Goodman, R. H. (1986) Science 231, 159–161
14. Nilini, E. A., Friedman, T. C., Todd, R. B., Birch, N. P., Loh, Y. P., and Jackson, I. M. D. (1995) J. Neurochem. 65, 2462–2472
15. Friedman, T. C., Loh, Y. P., Huang, S. S., Jackson, I. M. D., and Nilini, E. A. (1995) Endocrinology 136, 4462–4472
16. Nilini, E. A., Luo, L. G., Jackson, I. M. D., and McMillan, P. (1996) Endocrinology, in press
17. Hook, V. Y. H., and Loh, Y. P. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2776–2780
18. Scott, R. E. M., and Pintar, J. E. (1993) Mol. Endocrinol. 7, 585–596
19. Eipper, B. A., Stoffers, D. A., and Mains, R. E. (1992) Annu. Rev. Neurosci. 15, 85–116
20. Nilini, E. A., Sevarino, K. A., and Jackson, I. M. D. (1993) Endocrinology 132, 1271–1277
21. Holms, J. B., and Rhodman, J. E. (1992) Nature 360, 352–354
22. Klausner, R. D., Donalson, J. G., and Lippincott-Schwartz, J. (1992) J. Cell Biol. 116, 1071–1080
23. Saraste, J., and Kuusmanen, E. (1985) Cell 38, 55–54
24. Griffiths, G., Pfeiffer, S., Simans, K., and Matlin, K. (1985) J. Cell Biol. 101, 949–964
25. Kulawat, R., and Arvan, P. (1992) J. Cell Biol. 118, 521–529
26. Milgram, S. L., and Mains, R. E. (1994) J. Cell Sci. 107, 737–745
27. Xu, H., and Shields, D. (1993) J. Cell Biol. 122, 1169–1184
28. Zhou, A., and Mains, R. E. (1994) J. Biol. Chem. 269, 17440–17447
29. Sevarino, K. A., Goodman, R. H., Spiess, J., Jackson, I. M. D., and Wu, P. (1989) J. Biol. Chem. 264, 21529–21535
30. Nilini, E. A., Sevarino, K. A., Wu, P., and Jackson, I. M. D. (1991) in Neurosciences: Peptide Technology (Conn, P. M., ed) pp. 51–69, Academic Press, New York
31. Schagger, H., and Von Jagow, G. (1987) Anal. Biochem. 166, 368–379
32. Eipper, B. A., and Mains, R. E. (1980) Endocr. Rev. 1, 1–27
33. Liston, D., Patey, G., Rossier, J., Verbanck, P., and Vanderhaegen, J. J. (1984) Science 225, 734–736
34. Hall, M. E., and Stewart, J. M. (1983) Peptides 4, 763–766
35. Lechan, R. M., Wu, P., and Jackson, I. M. D. (1987) Endocrinology 121, 1879–1891
36. Segerson, T. P., Hoeffer, H., Childers, H., Wolfe, H. J., Wu, P., Jackson, I. M. D., and Lechan, R. M. (1987) Endocrinology 121, 88–107
37. Bulant, M., Delfour, A., Vaudry, H., and Nicolas, P. (1988) J. Biol. Chem. 263, 17189–17196
38. Cocks, S. M. (1990) FEBS Lett. 264, 253–256
39. Bulant, M., Beauvillain, J. C., Delfour, A., Vaudry, H., and Nicolas, P. (1990) Endocrinology 127, 1978–1985
40. Dom, R. W., Russ, G., and Yewdell, J. W. (1989) J. Cell Biol. 109, 61–72
41. Shennan, K. I. J., Taylor, N. A., Jermyany, J. L., Matthews, G., and Docherty, K. (1995) J. Biol. Chem. 270, 1402–1407
42. Nilini, E. A., Verdier, P. A., Huang, S. H., and Jackson, I. M. D. (1998) Mol. Biol. Cell 9, 368–379
43. Shennan, K. I. J., Taylor, N. A., Jermyany, J. L., Matthews, G., and Docherty, K. (1995) J. Biol. Chem. 270, 1402–1407
44. Thomas, L., Leduc, R., Thorne, B. A., Smeekens, S. P., Steiner, D. F., and Thomas, G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 340–344
45. Seidah, N. G., Chretien, M., and Day, R. (1994) Biochimie (Paris) 76, 197–209
46. Guest, P. C., Arden, S. D., Bennett, D. L., Clark, A., Rutherford, N. G., and Hutton, J. C. (1992) J. Biol. Chem. 267, 22401–22406
