Localization of Metallocarboxypeptidase D in AtT-20 Cells

POTENTIAL ROLE IN PROHORMONE PROCESSING*

(Received for publication, October 19, 1998, and in revised form, February 10, 1999)

Oleg Varlamov, Francis J. Eng, Elena G. Novikova, and Lloyd D. Fricker‡

From the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

Carboxypeptidase D (CPD) is a recently discovered metallocarboxypeptidase that is predominantly located in the trans-Golgi network (TGN), and also cycles between the cell surface and the TGN. In the present study, the intracellular distribution of CPD was examined in AtT-20 cells, a mouse anterior pituitary-derived corticotroph. CPD-containing compartments were isolated using antibodies to the CPD cytosolic tail. The immunopurified vesicles contained TGN proteins (TGN38, furin, syntaxin 6) but not lysosomal or plasma membrane proteins. The CPD-containing vesicles also contained neuropeptide-processing enzymes and adrenocorticotropic hormone, a product of proopiomelanocortin proteolysis. Electron microscopic analysis revealed that CPD is present within the TGN and immature secretory granules but is virtually absent from mature granules, suggesting that CPD is actively removed from the regulated pathway during the process of granule maturation. A second major finding of the present study is that a soluble truncated form of CPD is secreted mainly via the constitutive pathway in AtT-20 cells, indicating that the luminal domain does not contain signals for the sorting of CPD to mature secretory granules. Taken together, these data are consistent with the proposal that CPD participates in the processing of proteins within the TGN and immature secretory vesicles.

Most peptide hormones and neurotransmitters are produced from larger precursors by limited proteolysis. Initially, the prohormone precursors are processed at multiple basic amino acid cleavage sites by a family of endoproteases collectively known as prohormone convertases (PC)* (1, 2). The subsequent processing step is mediated by carboxypeptidases, which remove the basic amino acids from the C terminus of the peptide to generate either the bioactive product, or a precursor for the formation of the C-terminal amide group (3, 4).

Carboxypeptidase E (CPE, also known as carboxypeptidase H and enkephalin convertase) is the major carboxypeptidase involved with the processing of many peptides (3, 4). Within several tissues, CPE has been localized to the peptide-contain-
distribution of CPD using immunosolubilization and electron microscopic approaches. We have also examined the intracellular distribution of a soluble truncated form of CPD. Although neither the full-length or the truncated form of CPD are efficiently routed to mature secretory vesicles, both forms are present in immature secretory vesicles. These findings strongly support the proposal that CPD functions in the processing of prorhomonones and other proteins that transit either the regulated or constitutive secretory pathways.

MATERIALS AND METHODS

Generation of Constructs and Expression of Proteins in AtT-20 Cells—gp180 and gp170 constructs previously described for baculovirus expression (23) were subcloned into the pCDNA3 expression vector (Invitrogen) and transfected into AtT-20 cells using the standard calcium phosphate precipitation (33). Stable cell lines were selected using 0.6 mg/ml Geneticin (G418). Cells expressing both constructs were identified by Western blot analysis. For each of these constructs, several positive clones were selected and analyzed as described below; the data shown are representative of two or three separate clones.

Antibodies—The antiserum to duck CD recognizes both the soluble and membrane-associated forms of the protein (i.e. gp170 and gp180) but does not cross-react with mouse CPD (24). The antiserum to the C-terminal cytosolic tail of gp17 (17) recognizes both rat and duck antibodies. The antiserum raised against a C-terminal peptide of bovine CPE recognizes the mouse protein (9). Antisera to ACTH and β-endorphin that recognize both precursor and processed peptide (31) were a gift of Dr. Richard Mains (Johns Hopkins University, Baltimore, MD). The antiserum to TGN98 was a gift of Dr. Sharon Milgram (University of North Carolina, Chapel Hill, NC); the antiserum to the furin C-terminal tail was a gift of Dr. Ruth Angeletti (Albert Einstein College of Medicine, Bronx, NY), the monoclonal antibody against syntaxin 6 was a gift of Jason Bock and Dr. Richard Scheller (Stanford University, Stanford, CA), the antiserum to PC1 was a gift of Dr. Iris Lindberg (Louisiana State University, New Orleans, LA), the antiserum to cathepsin D was a gift of Dr. Regina Rau (Albert Einstein College of Medicine, Bronx, NY), the monoclonal antibody to SNAP-25 was a gift of Dr. Peter Davies (Albert Einstein College of Medicine, New York, NY), and the antiserum to calnexin was a gift of Dr. Ari Helenius (Yale University, New Haven, CT).

For immunosolubilization, polyclonal antibodies against the C-terminal cytosolic tail of CPD were subjected to affinity purification using glutathione-Sepharose-4B by the standard cyanogen bromide method (Sigma). Affinity-purified antibodies were concentrated to 1 mg/ml in PBS, and used for the immunosolubilization procedure as described below.

Immunofluorescence Isolation of CPD/gp180-containing Vesicles—Typically, six confluent 10-cm plates of AtT-20 cells expressing gp180 were used for the immunosolubilization. Cells were washed three times with cold PBS, scraped from the plates, and gently pelleted at 700 × g. The cell pellet was resuspended in 4 ml of cold 0.25 M sucrose, 10 mM HEPES, pH 7.4, containing 1 mM EDTA, 3 mM EGTA, and a mixture of protease inhibitors (1 μM aprotonin, 1 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 15 μM pepstatin A, 22 μM leupeptin, 14 μM E-64, and 40 μM bestatin). The suspension was passed five times through a 22-gauge needle, and then homogenized in a tight-fitting Dounce homogenizer (25 strokes). The resulting homogenate was centrifuged for 7 min at 1000 × g twice, and the supernatant was layered onto 4 ml of 1.6 M sucrose and centrifuged in a SW41 rotor (Sorvall) for 1 h at 200,000 × g. Typically, 1 ml of a microsomal fraction was collected from the interface of the 0.25 and 1.6 M sucrose, and then subjected to immunosolubilization. The affinity resin for the immunosolubilization was prepared by incubation of 100 μl of Pansorbin (Calbiochem) with 100 μg of affinity-purified antibodies to the CPD C-terminal cytosolic tail. An aliquot (300 μl) of the microsomal fraction was incubated for 3 h at 4°C with gentle agitation with 100 μl of the affinity resin in a final volume of 500 μl containing 140 mM NaCl and the protease inhibitors described above. The Pansorbin resin was recovered by low speed centrifugation and was washed three or four times with the same buffer, and then vesicle-associated proteins were subjected to isolation using 1% Triton X-100. IgG-associated gp180 was eluted from the beads by boiling in 1% SDS gel loading buffer for 5 min. Aliquots of each fraction were subjected to Western blot analysis using antisera to various secretory pathway proteins at 1:1000 dilution.

Labeling of AtT-20 Cells with [35S]Met—AtT-20 cells expressing gp170 were labeled with [35S]Met (100 μCi/ml) for 15 min, washed twice with PBS, and then incubated in Dulbecco’s modified Eagle’s medium for different periods of time. Media were removed, cells were washed with PBS, and then frozen in 10 mM NaAc, pH 5.5, with 1 mM phenylmethylsulfonyl fluoride. The cells and the media were then subjected to immunoprecipitation using the antisera to either duck CD or CPE (see above). To test whether the CD-containing compartments contain the neutrophilic precursor POMC and its proteolytic fragments, AtT-20 cells expressing gp180 were either continuously incubated with [35S]Met (100 μCi/ml) for 8 h, or pulsed for only 20 min and then chased for 0 or 90 min in the presence of unlabeled Met. Following the labeling, the cells were washed three times with cold PBS and then subjected to the immunoprecipitation procedure described above. The resulting fractions were subjected to immunoprecipitation using antisera to either ACTH or β-endorphin, and analyzed on 10% polyacrylamide Tricine gels.

Regulated Secretion—To examine whether gp170 is secreted via the regulated pathway, the cells expressing gp170 were grown on 35-mm cell culture dishes to 90% confluence. The cells were washed twice with PBS, then treated with the secretagogue 5 mM 8-Br-cAMP or control media for 30 min and the secreted proteins analyzed by Western blotting. To examine the regulated secretion of [35S]Met-labeled proteins, cells were labeled for 15 min with [35S]Met, chased for 2 h, washed three times with PBS, and then incubated for 30 min in either control media or with 5 mM 8-Br-cAMP. The media were subjected to immunoprecipitation using the antisera to either CPE or duck CD.

For immunoisolation, stably transfected AtT-20 cells were cultured on growth-supporting glass coverslips (Fisher Scientific). Cells were washed with PBS, fixed in 4% paraformaldehyde for 15 min, and then permeabilized for 15 min in 0.1% Triton X-100 in PBS. After 1 h of blocking in 3% bovine serum albumin, the cells were immunostained for 1 h with the primary antisera (1:1000 dilution). Cells were washed with PBS containing 0.2% Tween 20 and then incubated with fluorescein-labeled anti-mouse or rhodamine-labeled anti-rabbit secondary antibody (Vector Laboratories Inc., 1:200 dilution) for 1 h, followed by extensive PBS washing. Immunofluorescence staining was examined using a Bio-Rad confocal microscope.

Electron Microscopy Analysis—Stably transfected AtT-20 cells expressing either gp180 or gp170 were fixed for 30 min at room temperature in 4% formaldehyde, 0.2% glutaraldehyde in PBS, then dehydrated, embedded in LR-White resin according to standard procedures, sectioned, and mounted on either gold or nickel grids. Each grid was incubated for 30 min in blocking solution containing 10% goat serum, 2.5% bovine serum albumin, 0.1% Tween 20 in PBS at pH 8.2, and then incubated for 1 h in the same solution containing the antisera to either duck CD or CPE (final dilution 1:200). Cells were extensively washed with PBS, then postfixed in 1% OsO4, dehydrated in a series of acetone and embedded in LR-White resin. Ultrathin sections (70 nm) were cut at the intracellular distribution of gp180, gp170, and CPE, electron micrographs that were immunogold-labeled with antibodies to either duck CD or CPE were scanned in random order. In total, about 1000 gold particles/grid were analyzed, and the percentage of total label that was found in specific compartments was determined. The main morphological criterion to distinguish different subclasses of the secretory granules was the size of the vesicles and the size of the dense cores (34), which were measured from negatives taken at magnification ×54,000. An additional morphological criteria was the presence of the lighter core in immature granules surrounded by a broad electron-lucid peripheral zone (this zone was much broader in granules at the low state of condensation). Light aggregates of condensing proteins were observed in the dilated cisterns of the TGN, consistent with previous studies that formation of the dense core aggregates begins at the level of the TGN in AtT-20 cells (35). These electron-dense granules at the low state of condensation, which may represent cross-sections of the TGN or early immature vesicles, had an average diameter of 280 nm and dense cores that were too variable to accurately determine an average. Electron-dense granules at an intermediate state of condensation had an ovoid or spherical shape, and were in the process of detaching or already detached from the TGN. The average diameters of these granules were 170 nm, and their dense cores were 120 nm. These vesicles closely resemble immature secretory granules previously described in AtT-20 cells (35). Another type of vesicle was found in close proximity to the plasma membrane; these vesicles had a regular spherical shape and highly condensed dense cores, which are characteristic features of mature secretory granules in endocrine cells (35). The av-
Average diameters of mature secretory granules were typically 150 nm and their dense cores were 110 nm, which is consistent with previous studies on AtT-20 cells (28, 36, 37).

RESULTS

We have previously demonstrated that endogenous CPD is predominately localized to the perinuclear region in AtT-20 cells (25). To biochemically determine the proteins that co-localize with CPD, we used an immunoisolation technique. For these studies, microsomes from cells stably transfected with full-length duck CPD (gp180) were isolated at the interface between a 1.6 M and a 0.25 M sucrose layer (Fig. 1A, Ms).

Typically, about 30% of the total gp180 in the cell homogenate was recovered in the microsomal fraction (Fig. 1B). Similarly, 20–30% of the calnexin (an ER membrane protein), SNAP-25 (a plasma membrane protein), and syntaxin-6 (a TGN protein) were also recovered in this microsomal fraction (Fig. 1B). None of these proteins were detected in the 0.25 M or the 1.6 M sucrose layers (Fig. 1B), indicating no enrichment or loss of these organelles during the preparation. For immunoisolation, Pansorbin was precoated with either affinity-purified antibodies to the CPD C-terminal cytosolic tail (αCPD) or rabbit IgG (IgG). The aliquots of bound (B) and non-bound (NB) fractions were subjected to Western blot analysis using antisera either to the N-terminal portion of duck CPD or to different secretory pathway markers. The positions of prestained protein standards are indicated. Except for the analysis of cathepsin B, calnexin, and SNAP-25, which was performed twice, the entire experiment was repeated five times with similar results.

In control experiments, when rabbit IgG was used instead of antibodies to the CPD tail, most of the gp180 immunoreactivity and secretory markers were recovered in non-bound fractions (Fig. 1C, left panels), indicating that nonspecific binding of microsomes to the affinity carrier is very low. When using antibodies to the CPD tail, most of the gp180 (typically >95%) is recovered in the bound fraction (Fig. 1C, top). Furin, TGN38, and syntaxin 6, which have been previously localized to the TGN and immature secretory granules (38–40), are exclusively associated with gp180-containing vesicles (Fig. 1C, right panels). In contrast to the TGN proteins, CPE and PC1 which were previously found within mature secretory granules (5, 41, 42), demonstrate only a partial co-localization with the gp180 compartment (Fig. 1C, right panel). Typically, 20–30% of CPE, and 40–50% of PC1 were recovered in non-bound fractions. To further validate the specificity of our immunoisolation technique, we probed bound and non-bound fractions with antibodies to cathepsin B, calnexin, and SNAP-25 (Fig. 1C). Cathepsin B and SNAP-25 are exclusively recovered in the non-bound fraction (Fig. 1C), indicating that lysosomes and plasma membrane are not present in the immunoisolates. Approximately 30% of the calnexin is associated with the CPD-containing

![Figure 1](image_url)
FIG. 2. Recovery of POMC-derived peptides within the gp180-containing compartment. A, AtT-20 cells expressing gp180 were labeled with [35S]Met for 8 h, and then subjected to the immunoprecipitation procedure. Pansorbin-bound (B) and non-bound (NB) materials were subjected to the immunoisolation procedure with antibodies to ACTH. aCPD, Pansorbin precoated with rabbit IgG. The positions of POMC and ACTH are indicated. The entire experiment was repeated four times with similar results. B, AtT-20 cells expressing gp180 were labeled with [35S]Met for 20 min and then chased for either 0 or 90 min and subjected to vesicle immunoisolation with antibodies to the CPD tail. The aliquots of the cell homogenate (H) and microsome-derived (Ms) immunoisolated fractions were subjected to immunoprecipitation using an antiserum to ACTH. The dried gel was exposed to x-ray film for 2 h (chase 0) or for 20 h (chase 90).
The majority of the CPD-reactive material is concentrated along the antiserum directed against duck CPD (Fig. 4, C–E, open arrowheads), and even in a small number of dense core vesicles located in close proximity to the plasma membrane that resemble mature secretory granules (Fig. 4F, filled arrowheads). The small number of gold particles detected associated with dense core vesicles is likely to reflect the real presence of CPD in these compartments rather than background labeling, as the gold particles were often present in clusters of 3 or more. Additionally, virtually no labeling was detected in mitochondria or nucleus, which are not expected to contain CPD. In control experiments in which the primary antiserum was omitted, only background staining was observed in the TGN and vesicles (data not shown). Quantitation of approximately 1000 gold particles demonstrates that 84% of gp180 is located in the TGN, 10% is found within moderately-condensed granules, 4% is located within granules at an intermediate state of condensation, and only 2–3% is recovered within mature granules (Table I). Taken together, the electron microscopy, immunocytochemical, and immunolocalization results further support the idea that gp180 predominately functions at the level of the TGN, and to a lesser extent in the immature secretory vesicles.

Immunogold electron microscopic analysis was also performed on the AtT-20 cell line expressing gp170 (Fig. 4, G–I). In addition to the TGN localization, gp170 is also found within secretory vesicles containing electron-dense material (Fig. 4F, open arrowheads, and Table I) that are morphologically similar to the gp180-containing vesicles. As with gp180, immunoreactive gp170 was not generally detected in mature granules (Fig. 4H, Table I). A large fraction of gp170 was also found in small clusters (40–50 nm in diameter) distributed throughout the cytoplasm (Fig. 4G, crossed arrows). These structures may represent small constitutive vesicles that have been previously described (43, 44), or cross-sections of the ER. In contrast to the pattern of CPD distribution, the majority of the immunoreactive CPE was localized in the dense core granules that are morphologically similar to immature and mature secretory vesicles (Fig. 4J, Table I).

To further explore the routing pathway of gp170, we determined the secretion kinetics of the transfected gp170 and the endogenous CPE in AtT-20 cells. Cells were labeled for 15 min with [35S]Met, chased for different periods of time, and then both cells and media were subjected to immunoprecipitation using antibodies to either duck CPD or mammalian CPE. The secretion rate of gp170 is fairly rapid; after a 2-h chase approximately 90% of the labeled gp170 is present in the media (Fig. 5, A and B). In contrast, a large portion of CPE (50%) is retained within the cells after 2 h of the chase period, and the CPE secretion approaches a plateau (Fig. 5, A and B). These results are consistent with electron microscopy data, demonstrating that the bulk of gp170 does not usually enter dense core secretory granules, whereas CPE is targeted to this compartment (Fig. 4).

To further characterize the gp170 pathway, we tested whether the secretion of gp170 could be induced by secretagogues. Two different approaches were used to study the secretion of gp170. First, cells were incubated for 30 min in the presence or absence of Br-cAMP and then media were analyzed by Western blot (Fig. 6, left). Although the secretion of CPE was stimulated 2.5–3-fold by the secretagogue treatment, the secretion of gp170 was not significantly affected by the secretagogue treatment (Fig. 6, left). Two other secretagogues (forskolin and a phorbol ester) also did not significantly stimulate the secretion of gp170 from the AtT-20 cells (data not shown). These
results indicate that the bulk of gp170 undergoes constitutive secretion. As a small amount of radiolabeled gp170 was detected within the cells after several hours of chase (Fig. 5A), we tested whether this pool of gp170 could undergo regulated release in response to a secretagogue. Cells were labeled with [35S]Met for 15 min, chased for 2 h, washed, and then incubated for an additional 30 min in either control media or media containing Br-cAMP. Both gp170 and CPE are released to the media in the regulated fashion (Fig. 6, right). Quantitation of three independent experiments demonstrated a statistically significant stimulated release of both gp170 (Fig. 6, right) and CPE (data not shown) in response to the secretagogue, indicating that a small fraction of the gp170 undergoes regulated exocytosis. These findings are consistent with the electron microscopy and immunocytochemistry data, and taken together suggest that the bulk of gp170 does not enter mature secretory vesicles in AtT-20 cells.

DISCUSSION

CPD was previously localized by light microscopy to a perinuclear compartment that overlaps with the distribution of the TGN endopeptidase furin in AtT-20 cells (25). In the present study, biochemical and electron microscopic analysis supports the localization of CPD to the TGN, and in addition has provided evidence that a fraction of the CPD is present in immature secretory granules. The processing of many neuroen-

**FIG. 4.** Electron microscopy of AtT-20 cells. Stably transfected cells expressing either gp180 (A–F) or gp170 (G–J) were processed for the electron microscopy as described under "Materials and Methods," and then stained with either antibodies to duck CPD (B–I) or CPE (J). A, cells subjected to the conventional electron microscopy. B–J, immunogold analysis. The criteria for the following assignments are described under "Materials and Methods": filled arrows, TGN; open arrows, TGN dilations containing protein aggregates; open arrowheads, immature secretory granules; filled arrowheads, mature granules; crossed arrows, small vesicles containing gp170. The entire experiment was repeated using two different clones. Bar, 200 nm.

### TABLE I

Quantitation of immunogold labeling: intracellular distribution of carboxypeptidases in AtT-20 cells (percentage of total)

| Compartment                   | Diameter          | Total gold particles |
|-------------------------------|-------------------|----------------------|
|                               | Vesicle Dense core | gp180 gp170 CPE      |
| trans-Golgi network           |                   | 83.5 ± 1.5 16 ± 4    |
| EDG<sub>low</sub>             | 280               | 9.5 ± 1.5 34 ± 1.5   |
| EDG<sub>int</sub>             | 170               | 4.0 ± 2.0 4.0 ± 1.0  |
| Mature secretory granules     | 150 110           | 2.5 ± 0.5 1.0 ± 0.3  |
| Small vesicles                | 40–50 None        | <0.5 45 ± 8 <0.5     |
| Nuclei                        | <0.5 <0.5         | <0.5 <0.5 <0.5      |
| Mitochondria                  | <0.5 <0.5         | <0.5 <0.5 <0.5      |
Localization of Carboxypeptidase D in AtT-20 Cells

Another important finding of the present study is that the soluble form of CPD (gp170) also enters immature secretory vesicles, but is not abundant in mature vesicles. As a result, the bulk of this protein is secreted constitutively from AtT-20 cells. A soluble form of CPD has been previously detected in various tissues including liver, kidney, and brain (17). It is likely that this form resembles the truncated gp170 form, as the endogenous soluble form is 170 kDa and does not bind the antiserum to either duck CPD or CPE. Interestingly, potential casein kinase II phosphorylation sites are also found within an acidic residue-rich cluster of the CPD cytosolic tail. Mutation of these sites altered the trafficking of CPD and resulted in detectable staining of the mutant protein in tips of AtT-20 cell processes (24). This suggests that a similar mechanism of retrieval of immature secretory granules is involved in CPD trafficking. Our findings support the idea that CPD is progressively removed from immature granules in the process of their maturation. The retrieval of CPD from immature vesicles may be required for the targeting of CD to the recycling pathway and its efficient return to the TGN (Fig. 7).

Another important finding of the present study is that the soluble form of CPD (gp170) also enters immature secretory vesicles, but is not abundant in mature vesicles. As a result, the bulk of this protein is secreted constitutively from AtT-20 cells. A soluble form of CPD has been previously detected in various tissues including liver, kidney, and brain (17). It is likely that this form resembles the truncated gp170 form, as the endogenous soluble form is 170 kDa and does not bind the antiserum to either duck CPD or CPE. Interestingly, potential casein kinase II phosphorylation sites are also found within an acidic residue-rich cluster of the CPD cytosolic tail. Mutation of these sites altered the trafficking of CPD and resulted in detectable staining of the mutant protein in tips of AtT-20 cell processes (24). This suggests that a similar mechanism of retrieval of immature secretory granules is involved in CPD trafficking. Our findings support the idea that CPD is progressively removed from immature granules in the process of their maturation. The retrieval of CPD from immature vesicles may be required for the targeting of CD to the recycling pathway and its efficient return to the TGN (Fig. 7).

Another important finding of the present study is that the soluble form of CPD (gp170) also enters immature secretory vesicles, but is not abundant in mature vesicles. As a result, the bulk of this protein is secreted constitutively from AtT-20 cells. A soluble form of CPD has been previously detected in various tissues including liver, kidney, and brain (17). It is likely that this form resembles the truncated gp170 form, as the endogenous soluble form is 170 kDa and does not bind the antiserum to either duck CPD or CPE. Interestingly, potential casein kinase II phosphorylation sites are also found within an acidic residue-rich cluster of the CPD cytosolic tail. Mutation of these sites altered the trafficking of CPD and resulted in detectable staining of the mutant protein in tips of AtT-20 cell processes (24). This suggests that a similar mechanism of retrieval of immature secretory granules is involved in CPD trafficking. Our findings support the idea that CPD is progressively removed from immature granules in the process of their maturation. The retrieval of CPD from immature vesicles may be required for the targeting of CD to the recycling pathway and its efficient return to the TGN (Fig. 7).

Another important finding of the present study is that the soluble form of CPD (gp170) also enters immature secretory vesicles, but is not abundant in mature vesicles. As a result, the bulk of this protein is secreted constitutively from AtT-20 cells. A soluble form of CPD has been previously detected in various tissues including liver, kidney, and brain (17). It is likely that this form resembles the truncated gp170 form, as the endogenous soluble form is 170 kDa and does not bind the antiserum to either duck CPD or CPE. Interestingly, potential casein kinase II phosphorylation sites are also found within an acidic residue-rich cluster of the CPD cytosolic tail. Mutation of these sites altered the trafficking of CPD and resulted in detectable staining of the mutant protein in tips of AtT-20 cell processes (24). This suggests that a similar mechanism of retrieval of immature secretory granules is involved in CPD trafficking. Our findings support the idea that CPD is progressively removed from immature granules in the process of their maturation. The retrieval of CPD from immature vesicles may be required for the targeting of CD to the recycling pathway and its efficient return to the TGN (Fig. 7).
plasma membrane and the TGN (24, 25) raises the possibility that the full-length form of CPD functions within endocytic compartments. Interestingly, it has been demonstrated that furin cleaves precursor proteins in both the exocytic and endocytic pathways (49). Several viral coat proteins including influenza hemagglutinin and human immunodeficiency virus gp160 are processed by furin when co-expressed with the endopeptidase (49). Furin-mediated cleavage of many toxins occurs either at the plasma membrane or in endosomes (49). As furin cleaves to the C-terminal side of basic amino acids, the product of furin cleavage will contain C-terminal basic residues. If these need to be removed for the products to be biologically active, as is the case for most neuroendocrine peptides, CPD is a likely candidate for this activity based on its tissue distribution, intracellular distribution, and pH optimum. The involvement of CPD in important biological functions is supported by the fact that the mutations in the Silver gene, the Drosophila homologue of CPD, are embryonic lethal (21).

The finding that gp170 is primarily secreted in a constitutive manner from AtT-20 cells also suggests that the luminal domain of CPD is not sufficient for the efficient targeting of the protein to mature secretory granules, although it is sufficient for entry into immature secretory vesicles. In contrast, soluble forms of PAM are efficiently packaged into storage granules in AtT-20 cells, indicating that the luminal domain of PAM plays a major role in sorting into the regulated pathway (32). The mechanism of the protein sorting to the regulated secretory pathway remains unclear, and two alternative models have been proposed. The active sorting model postulates that regulated proteins and hormones bind to the sorting receptor in the TGN, and then are delivered to immature secretory vesicles. Proteins lacking specific signals for sorting into the regulated pathway follow the TGN-derived constitutive pathway. In the passive sorting model, protein sorting in the TGN is not selective and both the constitutive and regulated proteins enter immature secretory granules. As sorting proceeds, proteins of the regulated pathway undergo further condensation and packaging into mature secretory granules. The finding that only a small fraction of CPD is present in mature granules indicates that CPD may fail to undergo further condensation during granule biogenesis.

In conclusion, we have previously demonstrated that CPD is predominantly localized to the TGN, and also cycles between the TGN and the plasma membrane in AtT-20 cells (25). In the present study, we demonstrate that CPD enters immature secretory granules containing neuroendocrine peptides (Fig. 7, ISG). We propose that CPD is progressively removed from immature granules in the process of their maturation and enters recycling endosomes (Fig. 7, RE). The soluble form of CPD (gp170) may follow a similar immature granule-mediated sorting pathway, and then is efficiently removed from the regulated pathway via constitutive-like vesicles (CLV) (Fig. 7). Alternatively, only a small fraction of gp170 may enter the regulated pathway, whereas the bulk of it follows a constitutive secretion (Fig. 7, CV).
Localization of Carboxypeptidase D in AtT-20 Cells

41. Guest, P. C., Ravazzola, M., Davidson, H. W., Orci, L., and Hutton, J. C. (1991) Endocrinology 129, 734–740
42. Hornby, P. J., Rosenthal, S. D., Mathis, J. P., Vindrola, O., and Lindberg, I. (1993) Neuroendocrinology 58, 555–563
43. Hohl, I., Robinson, D. G., Chrispeels, M. J., and Hinz, G. (1996) J. Cell Sci. 109, 2539–2550
44. Thorens, B. and Roth, J. (1996) J. Cell Sci. 109, 1311–1323
45. Shennan, K. I. J., Taylor, N. A., Jermany, J. L., Matthews, G., and Docherty, K. (1995) J. Biol. Chem. 270, 1402–1407
46. Greene, D., Das, B., and Fricker, L. D. (1992) Biochem. J. 285, 613–618
47. Dittie, A. S., Thomas, L., Thomas, G., and Tooze, S. A. (1997) EMBO J. 16, 4859–4870
48. Klumperman, J., Kuliawat, R., Griffith, J. M., Geuze, H. J., and Arvan, P. (1998) J. Cell Biol. 141, 359–371
49. Nakayama, K. (1997) Biochem. J. 327, 625–635