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Insertion of Dibasic Residues Directs a Constitutive Protein to the Regulated Secretory Pathway

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The mechanisms for sorting proteins to the regulated secretory pathway (RSP) remains poorly understood. We recently reported that dibasic sequences that are cleaved by pro-protein convertases (PCs) in pro-neurotensin also acted as sorting signal for the precursor. Here we addressed two questions regarding the role of dibasics as sorting signal: (i) Are dibasics sufficient to direct proteins to the RSP? (ii) Do they sort proteins by virtue of their interaction with PCs? The first question was studied by inserting dibasics in β-lactamase, a constitutively secreted protein and comparing the regulated secretion of β-lactamase to that of its mutant in transfected endocrine cells. The second question was investigated by comparing the regulated release of pro-neurotensin in PC12 cells that are devoid of PCs to that in PC1- and PC2-transfected PC12 cells. The data show that the mutant β-lactamase was indeed targeted in part to the RSP and that pro-neurotensin was sorted to the RSP without the assistance of the PCs, thus indicating that dibasics can act as sorting signal by themselves independently of their interaction with PCs.

Key Words: **sorting; regulated secretory pathway; regulated release; dibasic residues; pro-neurotensin; cleavage sites; processing.**

The ability to secrete peptides and/or proteins under stimulation is a feature shared by all endocrine and neuronal cells, and represents one of the major steps in the regulation of neuro-endocrine processes. It is now established that these cells are endowed with a regulatory secretory pathway (RSP) that coexists with the constitutive secretory pathway present in all cell types (1). Newly synthesized constitutive and regulated proteins enter the Golgi apparatus and are then transported to the *trans*-Golgi network (TGN) where their fate diverges. Constitutive proteins are carried from the TGN to the plasma membrane in light vesicles and are continuously secreted in the medium at a rate that is proportional to their rate of synthesis. In contrast, regulated proteins are sorted into early granules termed immature granules. The latter evolve in mature granules or dense core vesicles in which regulated proteins are stored. Under stimulation, the granules fuse with the plasma membrane and their content is released in the extracellular medium (2). The immature granules also possess the ability to release their content upon stimulation (3, 4). Many of the proteins secreted this way are neuropeptides and polypeptide hormones. Extensive studies demonstrated that most of these active products are synthesized as part of larger inactive precursors. A processing step is then required to obtain the mature forms. This operation requires the cleavage of the precursors at specific sites, usually dibasic amino acid sequences, by a subset of proprotein convertases (PC) specific for the RSP compartments such as PC1 and PC2 (for review, see 5).

Sorting between constitutive and regulated proteins is generally thought to occur in the TGN, although in some instances it has been proposed to take place in the immature secretory granules (6, 7). The sorting mechanisms are not fully understood yet. Two models are currently proposed (reviewed by 6). The first one, termed "sorting for entry," proposes that entry of regulated proteins into the immature granule is an active process involving their aggregation and/or their recognition by a sorting receptor in the TGN. The other one, referred to as the "sorting by retention" model, suggests that all proteins reach the immature granule and that under the conditions that prevail within the maturing granule, there is a selective elimination of constitutive proteins in a "constitutive-like" pathway (8).

Both models imply that there is a structural feature shared by all regulated proteins that would act as a sorting signal by interacting with the sorting/retention machinery. However, the nature of the sorting signal is still a matter of debate. Recently, we presented evidence that dibasics are critical for the sorting of pro-neurotensin (pro-NT/NN) into the RSP (9). In this pre-

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cursor, the sequence of the mature peptides, neuromedin N (NN) and neurotensin (NT), are located in the C-terminus of the precursor where they are flanked and separated by basic doublets (Fig. 1). We showed that deletion of the C-terminal domain containing the mature peptides or removal of all the dibasics within it abolished regulated secretion of the pro-NT/NN constructs. We further demonstrated that fusing the dibasics-containing domain of pro-NT/NN to the C-terminus of β-lactamase, a constitutive protein, efficiently rerouted the fusion protein to the RSP when expressed in the insulinoma β TC7 cell line. These data together with other pieces of evidence from studies with prosomatostatin (10) and prorenin (11) strongly suggest that dibasics play an important role in routing proteins to the RSP.

Since dibasics represent also the cleavage sites recognized by PCs, the question arise as to whether processing by PCs is part of the mechanism by which precursors and their maturation products are targeted to or retained in secretory granules. In this context, it was recently shown that processing of proinsulin at dibasics by PC1 and/or PC2 may contribute to the retention of mature insulin within the secretory granules (12, 13). In another study, it was proposed that processing of prorenin at dibasics by PCs might be linked to its sorting to the RSP (11).

In the present study we addressed two questions about the involvement of the dibasics as a sorting signal. First, do they represent a sufficient signal to direct a protein to the RSP? For this purpose, we mutated β-lactamase to introduce several dibasic residues in its C-terminus, expressed the construct in the β TC7 endocrine cell line and studied its secretion. The results demonstrated that the mutation is sufficient to confer regulated release to the β-lactamase construct. Second, can PCs assist in the storage of any dibasic-containing precursor in the regulated compartments? To address this question, we analyzed the regulated secretion of pro-NT/NN in PC12 cells that endogenously express the precursor but lack processing enzymes, and compared it to that in PC12 cells that have been stably transfected with PC1 or PC2 (14). The results showed that the regulated secretion of pro-NT/NN or pro-NT/NN-derived products was as efficient in wild type PC12 cells as in PC1- or PC2-expressing PC12 cells, thus establishing that neither PC1 nor PC2 influenced the retention of pro-NT/NN in the RSP.

EXPERIMENTAL PROCEDURE

Obtaining of the constructs. The β-lactamase sequence was PCR amplified from the vector pcDNA3 (Invitrogen) with oligonucleotides specifics for sequences in its 3′ (oligo 1) and 5′ (oligo 2) extremities with the addition of restriction sites (*Hin*dIII and *Not*I, respectively). The amplification product was purified, digested and ligated in the polylinker of pcDNA3 under the control of an eukaryotic promoter. To obtain the β-lac/basics mutant, two additional oligonucleotides (Eurogentec) were used. One is very homologous with the 825–858 sequence of the β-lactamase gene (oligo 3), the other is similar to the inverse complementary with the 793–824 nucleotidic sequence (oligo 4). Both contain point mutation that replace codons by lysine- or arginine-encoding codons. They were used in a PCR step with *Pfu* from Promega to amplify the 825-3′ (oligo 1 + oligo 3) and the 5′-1-824 of β-lactamase (oligo 2+ oligo 4). The two PCR fragments were purified and ligated in a 1:1 molecular ratio. The ligation product was amplified with oligos 1 and 2, digested by *Hin*dIII and *Not*I and subcloned in pcDNA3.

Cell culture. The obtaining and characterization of the stably PC1-transfected L1.2 and PC2-transfected E2.16 PC12 cell lines were described elsewhere (14). Wild type and transfected PC12 cells were cultured in DMEM (Life Technologies, Inc.) containing 10% horse serum (Biomedia) and 5% fetal calf serum (Dutscher) with 50 μg/ml gentamycin (Life). The cells were plated in 100-mm dishes the day before the beginning of the experiments. Induction of the pro-NT/NN gene was performed by adding 200 ng/ml NGF, 1 μM Forskolin, 1 μM dexamethasone, and 20 mM $LiCl_2$ from Sigma for 36 h. Prior to the secretion experiments, cells were incubated for 5 h in OptiMEM (Life Technologies, Inc.).

The insulinoma β TC7 cell line has previously been shown to express both PC1 and PC2 and to represent a good model to study the fate of transfected regulated proteins (9). The cells were cultured in DMEM with 10% fetal calf serum and 50 μg/ml gentamycin from Life. Transfection was performed with polyethyleneimine (Fluka) as previously described (9). 48 h after transfection, G418 (Life Technologies, Inc.) was added in the medium to a final concentration of 0.5 mg/ml. The G418 concentration was diminished to 0.25 mg/ml 3 weeks later, when clones become visible. Colonies were picked and cultured to obtain cell lines that were assayed in Western blot for their contents in β-lactamase. Cells were cultured in 0.25 mg/ml G418 throughout until the beginning of the experiments.

To determine constitutive and regulated secretion, cells were incubated for 5 h in OptiMEM (5 h basal condition). The medium was collected and the cells were incubated for another 30 min in normal OptiMEM (basal). The medium was collected and replaced by depolarizing medium (OptiMEM containing 46 mM KCl and 4.4 mM $CaCl_2$). After a 30-min incubation in depolarizing conditions, the medium was collected and the cells were harvested in 0.1 N cold HCl, heated at 95°C for 5 min and centrifuged (13,000 rpm/15 min). The supernatants and extracellular media were kept frozen until use.

Analysis of the fractions. Radioimmunoassay procedures have been described previously (15). Briefly, the NN-Ah antibody is specific for the free N-terminus of NN. To detect cryptic immunoreactive NN (iNN), i.e., NN sequences present within large precursor forms, an additional step is required that entails Arg-directed tryptic digestion of the samples to be assayed. As the NN sequence contains a N-terminal Lys residue that is critical for the recognition by the antibody, this procedure implicates a reversible protection of the lys residues by citraconylation prior to the digestion. The whole procedure is referred to as CTD (for citraconylation, trypsin digestion) procedure. Immunoreactive NN detected after CTD is termed CTiNN and accounts for both free iNN and iNN within the precursor forms. Processing at the level of the dibasic before NN is determined by the ratio of iNN (processed form) to CTiNN (processed + unprocessed forms). As NN immunoreactivity corresponds to processed precursor, the amount of unprocessed large forms is determined by subtracting iNN to CTiNN. The stimulated release is expressed as the ratio of released material under stimulation over that in basal conditions.

For Western blot detection, the intracellular lysate was freeze-dried and resuspended in Laemmli buffer with the addition of 50 μl/ml β mercaptoethanol. Extracellular media were precipitated by addition of trichloroacetic acid at a final concentration of 10% (w/v). After 15 min on ice, extracts were centrifuged at 16,000*g* for 15 min. The pellet was washed twice with 1 ml cold acetone and resuspended in Laemmli buffer with the addition of 50 μl/ml β mercaptoethanol.

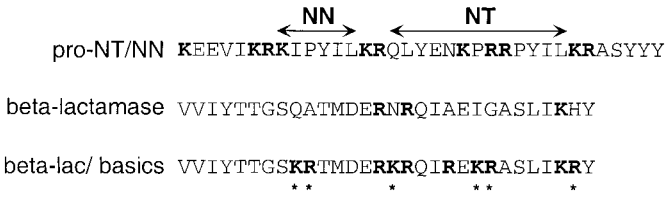

FIG. 1. C-terminal sequences of pro-NT/NN, β-lactamase, and β-lac/basics. The NN and NT sequences in the C-terminal domain of pro-NT/NN are indicated. Basic residues are represented in bold. Inserted basic residues in the β-lac/basic mutant are indicated by an asterisk and their position was chosen so as to mimic the dibasic arrangement seen in pro-NT/NN.

Aliquots of each medium were loaded on 12% acrylamide gel. After migration, the gel was transferred on a nitrocellulose membrane and incubated for 12 h in PBS/milk 5% at 4°C, then for 2 h in PBS/milk 5% containing anti β-lactamase antibody from 5′ (1:2000). Nonspecific binding was eliminated by three washes in PBS/Tween 0.05%. The specific binding was detected by incubating the membrane in PBS/milk 5% containing peroxidase-coupled goat anti-rabbit antibody from Jackson (1:10,000) for 45 min. The peroxidase activity was detected using the ECL kit from Amersham under the manufacturer's procedure.

RESULTS

The β-lactamase mutant with a C-terminal basic cluster transits through the regulated pathway. βTC7 cells stably expressing wild type β-lactamase or β-lactamase with a basic cluster in its C-terminus (β-lac/basics) (Fig. 1) were assayed for their ability to sort the construct to the RSP. For this purpose, cells were incubated first in OptiMEM for 30 min (nonstimulated) and then in depolarizing medium for an additional 30-min period (stimulated). Constitutive secretion was determined by the extracellular amount of products secreted in 5 h. Intracellular and extracellular media were analyzed for the presence of wild type and mutated β-lactamase by Western blotting (Fig. 2). Analysis of cell extracts show that wild type β-lactamase was present intracellularly as a single 29-kDa protein (Fig. 2A). The protein was readily secreted in a constitutive manner as 76% of its intracellular content was released in 5 h (Figs. 2A and 2B). The secretion of β-lactamase amounted to 10% of its intracellular content after a 30 min incubation in basal condition and was not increased after a 30-min incubation in stimulation medium (Figs. 2A and 2C), attesting that β-lactamase did not reached the RSP compartments. These observations are consistent with the constitutive behavior of the bacterial β-lactamase when expressed in eukaryotic cells (16).

With β-lac/basics, two protein bands were presents in the cell. The major band was observed at an apparent molecular mass of 29 kDa, corresponding to what is expected for the whole mutant. Another band that represented 15% of the total intracellular content was also detected at an apparent molecular mass of 26 kDa (Fig. 2A). This form is likely to represent a processed form of β-lac/basics. Indeed, the mutations in the C-terminus of the protein introduce several dibasics that represent potential processing sites. A cleavage after the most N-terminal dibasic would remove a 22-amino-acid fragment, leading to the apparition of a 26.3-kDa protein. The difference observed in Western blot between the apparent molecular mass of the two bands is therefore consistent with the 26 kDa form being a maturation product derived from the 29-kDa full-length construct.

Analysis of extracellular media showed that both forms were constitutively secreted albeit at different rates. Thus, over a 5-h interval, the amounts of constitutively secreted 29 and 26 kDa forms represented 25% and 150% of the total intracellular protein content, respectively (Figs. 2A and 2B). Interestingly, whereas depolarization of the cells did not increase secretion of the 29 kDa protein, it readily stimulated that of the

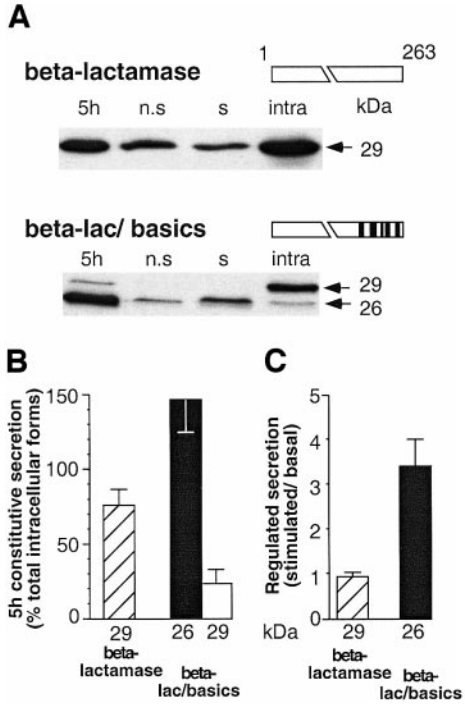

FIG. 2. Analysis of β-lactamase and β-lac/basics secretion. (A) Stable transfectants expressing either the β-lactamase or the β-lac/basics construct were incubated for 5 h and then for 30 min in normal medium (n.s.). After another 30 min incubation in depolarizing medium (s), the cells were harvested and cell extracts and media were analyzed by Western blotting using a β-lactamase antibody. (B) Quantification of constitutive secretion over a 5-h period of time for 26 and 29 kDa β-lac/basics-derived products and 29 kDa β-lactamase expressed as the ratio of the amount of each protein in the 5 h medium to the corresponding total intracellular amounts. (C) Quantification of stimulated release expressed as the ratio of the quantities released under a 30-min stimulation divided by the quantities secreted for the same period of time in basal condition. Values were determined by quantification of Western blots such as that shown in A, and represent the mean ± SEM from 4 to 10 experiments.

TABLE 1

Processing and Sorting in PC12 Cells

	CTiNN (pmol/mg)	iNN		Large forms	
		% CTiNN	stim/basal	stim/basal	% stim/intra
PC12	26.0 ± 2.9	2.5 ± 0.6	2.8 ± 1.1	3.5 ± 1.0	5.6 ± 2.1
PC12/PC1	30.5 ± 6.6	16.8 ± 5.1	2.3 ± 0.2	2.4 ± 0.7	5.4 ± 1.4
PC12/PC2	3.1 ± 0.5	30.9 ± 8.6	4.7 ± 1.2	4.0 ± 1.5	5.4 ± 1.3

Note. Wild type, PC1- and PC2-expressing PC12 cells were incubated for 36 h under conditions that induce the expression of the pro-NT/NN gene. After 5 h in basal medium, they were assayed for their ability to secrete the different processed forms under basal and stimulated conditions. The amounts of iNN (mature form) and CTiNN (processed + unprocessed NN-containing forms) in the different media were assayed. The percentage of mature iNN divided by the intracellular CTiNN value indicates the processing at the level of the dibasic before the NN sequence. Regulated release for the iNN is obtained by dividing the iNN value in the stimulation medium by the value in the basal medium. For the large forms, the amount present in each medium was deduced by subtracting the iNN from the CTiNN values, and their stimulation ratio was determined as above. The percentage of secretion is obtained by dividing the large form amounts released in the stimulation medium by the intracellular content. Values are the mean ± SEM from 4 to 6 experiments.

26-kDa protein by 3.4-fold (Figs. 2A and 2C). We tested if the 26 kDa form present in the medium could be a degradation product of the secreted 29 kDa protein. For this purpose, a lysate from β-lac/basics-expressing cells (in which the 29 kDa protein represents the main product) was incubated for 5 h on a confluent β TC cell layer. Western blot analysis of the 29 and 26 kDa forms present in the lysate before and after cell exposure revealed no change in their relative proportion (data not shown), indicating that the 29 kDa protein was not converted to the 26 kDa form in the extracellular medium and therefore originated within the cells. Thus, although the 26 kDa protein is present in low intracellular amounts, the high extracellular levels of this form reveal that the intracellular processing leading to the appearance of the 26-kDa protein is quite efficient.

Prohormone convertases do not mediate pro-NT/NN sorting. To see if dibasic cleavage by PCs might assist storage of precursors in the RSP, we compared the processing and regulated secretion of pro-NT/NN in PC12 cells—that endogenously synthesize the precursor but are devoid of PC1 and PC2—to that in PC12 cells stably transfected with either PC1 or PC2 (14). The secretion was determined by incubating the cells for 30 min first in normal medium and then in depolarizing medium. At the end of the latter incubation, the cells were collected and the immunoreactive products in cell extracts and media were determined by RIA (Table 1). Pro-NT/NN expression level as evaluated by total intracellular CTiNN content and cleavage at the dibasic that precedes the NN sequence as measured by the ratio of iNN to CTiNN (Table 1) are in agreement with our previous findings (14). Thus, dibasic cleavage was barely observable in wild type PC12 cells whereas it was markedly enhanced in both PC1- and PC2-expressing PC12 cells. N-terminal iNN was secreted in a regulated manner from all PC12 cell lines. Interestingly, large NN-containing precursor forms, as determined from the difference between CTiNN and free iNN, were also released in a regulated manner with similar stimulation factors compared to the small iNN products. Furthermore, the percentage of secreted large forms relative to the intracellular amounts of those forms was almost identical in the three cell lines.

DISCUSSION

Despite its critical involvement in neuro-endocrine processes, the mechanism responsible for the sorting of hormones and neuropeptides in the RSP that leads to their storage in secretory granules is not elucidated to date. Regardless of the underlying mechanism, it is generally thought that regulated proteins possess a sorting signal that allows them to enter and/or remain in secretory granules. Recently, we provided evidence that the dibasic sequences in pro-NT/NN that serve as cleavage sites for the PCs were crucial for the sorting of the precursor to the RSP (9). This together with the work of others on prosomatostatin (10) and prorenin (11) strongly suggest that dibasics in regulated proteins represent a major sorting signal for the RSP.

In this work we addressed the question as to whether dibasic amino-acid sequences are able by themselves to induce the regulated secretion of a constitutive protein in which they are inserted. For this purpose, we introduced by mutagenesis several dibasic sites in the C-terminus of β-lactamase, a prokaryotic protein that is constitutively secreted when expressed in neuroendocrine cells (16). The sites of mutation were chosen so as to mimic the C-terminus of pro-NT/NN with regard to the positions of basic residues. The resulting mutant proved to be quite efficiently processed yielding a protein that was truncated at the most N-terminal of the introduced dibasic sites. Furthermore, the maturation product exhibited regulated secretion, its release being increased more than threefold by depolarization.

As the only difference between the mutant and wild type β-lactamase (that is not processed and shows only constitutive secretion) is the presence of basic residues, our results indicate that dibasic motifs are able to address a protein to the RSP compartments and strongly support the contention that dibasics represent a sorting signal by themselves.

However, despite its regulated secretion, the processed form of the mutant β-lactamase was readily secreted in a constitutive manner, at a higher rate even than the wild type protein. This behavior is different from that of regulated proteins that are usually efficiently stored in secretory granules and exhibit little if any constitutive secretion. Therefore, the dibasics inserted in β-lactamase do not appear to fully reproduce the behavior of regulated proteins. Two hypotheses could account for this observation. First, it could be that soon after reaching the Golgi apparatus, a large fraction of the 29-kDa mutant β-lactamase would undergo rapid cleavage at the most N-terminally inserted dibasic by PCs such as furin or PC1 which unlike PC2 are active in this compartment (for review, see 5). This would generate the 26-kDa protein before it can enter the RSP and would lead to its rapid constitutive secretion. Only a small fraction of the 29-kDa protein would reach the RSP where it would be processed and exhibit regulated secretion. Second, the 29-kDa mutant protein would enter the early compartments of the RSP, i.e., the immature secretory granules, where it would be processed but would not be efficiently retained in the maturing granules from which a large fraction of the 26 kDa would escape through the constitutive-like secretory pathway. Further work is needed to test these hypotheses. Whatever the explanation, our data suggest that, although we took care to mimic in β-lactamase the arrangement of dibasics seen in pro-NT/NN, either their positioning is not optimal or other structural elements may be necessary for the full targeting of the protein to the RSP. This is in keeping with previous observations (9, 11) that dibasics must be in a proper structural environment to function as a sorting signal for the RSP.

As recalled in the introduction, PCs have been proposed to assist in the retention of insulin in maturing secretory granules (13) and sorting of prorenin to the RSP has been hypothesized to be linked to its processing by PCs (11), suggesting a role for these enzymes in the sorting/retention of regulated proteins in the RSP possibly through an interaction with dibasic residues. Therefore, we investigated whether this could be generalized to other precursors using pro-NT/NN as a model. Secretion of the precursor and its maturation products was assayed both in wild type PC12 cells devoid of the enzymes able to process it, and in stably transfected PC12 cells expressing either PC1 or PC2. We observed that the precursor and, when processed, its maturation products, were all secreted under stimulation in a similar quantitative fashion. This study demonstrates that sorting and storage of pro-NT/NN in secretory granules is not enhanced by processing and excludes that either PC1 or PC2 might play a role in the sorting of this precursor.

Altogether, our data show that dibasics are essential for the targeting of proteins to the RSP. We have so far demonstrated this to be true for pro-NT/NN (9), for a β-lactamase fusion protein that was extended at its C-terminus with the dibasic-containing domain of pro-NT/NN (9) and in the present work for a β-lactamase mutant in which dibasic sequences were inserted. Others have provided evidence that dibasics could operate as a sorting signal for prosomatostatin and prorenin (10, 11). We further show in the case of pro-NT/NN that sorting and processing are two independent steps even though they require the same structural motifs to operate. This raises the question as to how dibasics function as a sorting signal. Two non mutually exclusive types of protein-protein interactions have been suggested to play a role in targeting proteins to immature secretory granules: homo- or hetero-aggregation of regulated proteins in the TGN and/or binding of the regulated proteins or aggregates to a sorting receptor in the TGN membrane (7). Thus, dibasic motifs might be involved in pro-NT/NN aggregation although preliminary studies in our laboratory suggest that the protein is soluble and unable to form homo-aggregates in the pH and ionic environment of the TGN. It remains possible that the precursor could aggregate with other proteins destined to the RSP. Alternatively, dibasics might interact with a sorting receptor. Carboxypeptidase E, a RSP enzyme involved in trimming C-terminal basic residues of hormones and neuropeptides after they have been released from their precursors by PCs in secretory granules, was proposed to act as a sorting receptor (17). There is however much controversy surrounding this proposal (18). PCs do not appear to fulfill the role of sorting receptor as shown here. Therefore, the search for such receptor(s) is still open. Clearly, more work is needed in order to resolve these issues. The various pro-NT/NN and β-lactamase constructs and the stably transfected β TC7 and PC12 cell lines we have developed in the past (9, 14, 19) and in the present study should prove very useful in this regard.

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REFERENCES

1. Palade, G. (1975) Intracellular aspects of the process of protein synthesis. *Science* **189,** 347–358.
2. Halban, P. A., and Irminger, J. C. (1994) Sorting and processing of secretory proteins. *Biochem. J.* **299,** 1–18.

3. Tooze, S. A., Flatmark, T., Tooze, J., and Huttner, W. B. (1991) Characterization of the immature secretory granule, an intermediate in granule biogenesis. *J. Cell Biol.* **115,** 1491–1503.
4. Tooze, S. A. (1991) Biogenesis of secretory granules. Implications arising from the immature secretory granule in the regulated pathway of secretion. *FEBS Lett.* **285,** 220–224.
5. Seidah, N. G., and Chretien, M. (1997) Eukaryotic protein processing: Endoproteolysis of precursor proteins. *Curr. Opin. Biotechnol.* **8,** 602–607.
6. Arvan, P., and Castle, D. (1998) Sorting and storage during secretory granule biogenesis: Looking backward and looking forward. *Biochem. J.* **332,** 593–610.
7. Glombik, M. M., and Gerdes, H. H. (2000) Signal-mediated sorting of neuropeptides and prohormones: Secretory granule biogenesis revisited. *Biochimie* **82,** 315–326.
8. Kuliawat, R., and Arvan, P. (1992) Protein targeting via the "constitutive-like" secretory pathway in isolated pancreatic islets: Passive sorting in the immature granule compartment. *J. Cell Biol.* **118,** 521–529.
9. Feliciangeli, S., Kitabgi, P., and Bidard, J. N. (2001) The role of dibasic residues in prohormone sorting to the regulated secretory pathway. A study with proneurotensin. *J. Biol. Chem.* **276,** 6140–6150.
10. Brakch, N., Cohen, P., and Boileau, G. (1994) Processing of human prosomatostatin in AtT-20 cells: S-28 and S-14 are generated in different secretory pathways. *Biochem. Biophys. Res. Commun.* **205,** 221–229.
11. Brechler, V., Chu, W. N., Baxter, J. D., Thibault, G., and Reudelhuber, T. L. (1996) A protease processing site is essential for prorenin sorting to the regulated secretory pathway. *J. Biol. Chem.* **271,** 20636–20640.
12. Arvan, P., Kuliawat, R., Prabakaran, D., Zavacki, A. M., Elahi, D., Wang, S., and Pilkey, D. (1991) Protein discharge from immature secretory granules displays both regulated and constitutive characteristics. *J. Biol. Chem.* **266,** 14171–14174.
13. Kuliawat, R., Prabakaran, D., and Arvan, P. (2000) Proinsulin endoproteolysis confers enhanced targeting of processed insulin to the regulated secretory pathway. *Mol. Biol. Cell.* **11,** 1959–1972.
14. Rovere, C., Barbero, P., and Kitabgi, P. (1996) Evidence that PC2 is the endogenous pro-neurotensin convertase in rMTC 6-23 cells and that PC1- and PC2-transfected PC12 cells differentially process pro-neurotensin. *J. Biol. Chem.* **271,** 11368–11375.
15. Bidard, J. N., de Nadai, F., Rovere, C., Moinier, D., Laur, J., Martinez, J., Cuber, J. C., and Kitabgi, P. (1993) Immunological and biochemical characterization of processing products from the neurotensin/neuromedin N precursor in the rat medullary thyroid carcinoma 6-23 cell line. *Biochem. J.* **291,** 225–233.
16. Harrison, T. M., Chidgey, M. A., and Uff, S. (1996) Novel markers for constitutive secretion used to show that tissue plasminogen activator is sorted to the regulated pathway in transfected PC12 cells. *Cell.-Biol. Interact.* **20,** 293–299.
17. Cool, D. R., Normant, E., Shen, F., Chen, H. C., Pannell, L., Zhang, Y., and Loh, Y. P. (1997) Carboxypeptidase E is a regulated secretory pathway sorting receptor: Genetic obliteration leads to endocrine disorders in Cpe(fat) mice. *Cell* **88,** 73–83.
18. Thiele, C., Gerdes, H. H., and Huttner, W. B. (1997) Protein secretion: Puzzling receptors. *Curr. Biol.* **7,** R496-R500.
19. Barbero, P., Rovere, C., De Bie, I., Seidah, N., Beaudet, A., and Kitabgi, P. (1998) PC5-A-mediated processing of Pro-neurotensin in early compartments of the regulated secretory pathway of PC5-transfected PC12 cells. *J. Biol. Chem.* **273,** 25339–25346.