The Role of Dibasic Residues in Prohormone Sorting to the Regulated Secretory Pathway

A STUDY WITH PRONEUROTENSIN*

Sylvain Feliciangeli, Patrick Kitabgi‡, and Jean-Noël Bidard§

From the Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, UPR 411, 660 Route des Litoles, 06560 Valbonne, France

The mechanisms by which prohormone precursors are sorted to the regulated secretory pathway in neuroendocrine cells remain poorly understood. Here, we investigated the presence of sorting signal(s) in proneurotensin/neuromedin N. The precursor sequence starts with a long N-terminal domain followed by a Lys-Arg (neuromedin N)-Lys-Arg-(neurotensin)-Lys-Arg sequence and a short C-terminal tail. An additional Arg-Arg dibasic is contained within the neurotensin sequence. Mutated precursors were expressed in endocrine insulinoma cells and analyzed for their regulated secretion. Deletion mutants revealed that the N-terminal domain and the Lys-Arg-(C-terminal tail) sequence were not critical for precursor sorting to secretory granules. In contrast, the Lys-Arg-(neuromedin N)-Lys-Arg-(neurotensin) sequence contained essential sorting information. Point mutation of all three dibasic sites within this sequence abolished regulated secretion. However, keeping intact any one of the three dibasic sequences was sufficient to maintain regulated secretion. Finally, fusing the dibasic-containing C-terminal domain of the precursor to the C terminus of β-lactamase, a bacterial enzyme that is constitutively secreted when expressed in neuroendocrine cells, resulted in efficient sorting of the fusion protein to secretory granules in insulinoma cells. We conclude that dibasic motifs within the neuropeptide domain of proneurotensin/neuromedin N constitute a necessary and sufficient signal for sorting proteins to the regulated secretory pathway.

Whereas all cells secrete proteins constitutively, neuroendocrine and exocrine cells are also able to release a number of proteins and peptides, including hormones and neuropeptides, prohormone convertases (PCs)1 and digestive enzymes, through a regulated secretory pathway (RSP) (1–3). Furthermore, in neuroendocrine cells, neuropeptides and peptide hormones are generally synthesized as part of large precursors in which they are flanked by processing sites, usually pairs of basic residues. The precursors must be cleaved at processing sites by PCs (for review, see Ref. 4) to yield the biologically active peptides that will be secreted under stimulation. Sorting between the constitutive and the regulated secretory pathway is thought to occur in the trans-Golgi network (TGN) and/or in immature secretory granules (IG) that originate from the TGN (for review, see Ref. 5), ultimately leading to the packaging and storage of regulated proteins and peptides into mature secretory granules.

Despite considerable advancement in our understanding of the regulated secretory pathway machinery in specialized cells, the sorting mechanisms into the RSP remain unclear. Moore and Kelly (6) demonstrated that a constitutive protein can be re-routed to the RSP when fused with a regulated protein. This result led to the idea that the targeting of proteins into the RSP is an active process, whereas constitutive secretion occurs by default. Furthermore, it was recognized early that aggregation of secreted proteins occurs in a late Golgi compartment (1), and this led Kelly to suggest that specific aggregation of regulated proteins could direct their sorting to the RSP (3). At least two nonexclusive mechanisms have been proposed to account for the sorting of proteins or protein aggregates to the RSP. The first mechanism, designated sorting-for-entry (5), postulates that upon reaching the TGN, regulated proteins or aggregates are actively directed to IGs by a process that would involve their specific binding to components of the TGN membrane. Thus, because of the homologies between exocytosis and receptor-mediated endocytosis, Orci (7) suggested the existence of TGN receptor(s) that would bind regulated proteins and lead to their RSP sorting. The second mechanism, termed sorting-by-reten-

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‡ To whom correspondence should be addressed. Tel.: 33-4-93-95-77-64; Fax: 33-4-93-95-77-08; E-mail: kitabgi@ipmc.cnrs.fr.
§ Present address: Centre de Biochimie, INSERM, U470, 06108 Nice Cedex 2, France.
1 The abbreviations used in this paper are: PC, prohormone convertase; CPE, carboxypeptidase E; CT, citraconylation/trypsin/unblocking procedure; HPLC, high pressure liquid chromatography; iNN, iNT, iK6L, iE6I, and iH10P, immunoreactive NN, NT, K6L, E6I, and H10P, respectively; NN, neuromedin N; NT, neurotensin; POMC, proopiomelanocortin; BIA, radioimmunoassay; BPP, regulated secretory pathway; TGN, trans-Golgi network; WT, wild type; PCR, polymerase chain reaction; CgB, chromogranin B; IG, immature secretory granule; PBS, phosphate-buffered saline.
the granule membrane. In this case, constitutive proteins could be passively excluded from the granules (12).

Whatever the sorting mechanism, interactions of regulated proteins with other proteins or membrane components in the TGN or in the IGs are thought to play a crucial role in sorting between the constitutive and the regulated pathways. This has led to the search for sequences or structural elements (sorting signal) in regulated proteins that would direct or facilitate their storage in mature secretory granules. However, the nature of these elements is still a matter of debate (recently reviewed in Ref. 12). No consensus amino acid sequence was revealed by sequence alignments of hormone precursors (13). Comparison between hydropathic profiles of regulated proteins suggested that a N-terminal hydrophobic domain might represent a possible sorting signal for some regulated proteins (14). However, experimental evidence to support this hypothesis are lacking. In vitro studies demonstrated the ability of granins to aggregate in the TGN (15). Further studies indicated that a disulfide bond-stabilized loop in the N-terminal region of chromogranin B (CgB) was necessary for its secretion through the RSP (16–18). A disulfide bond-delimited sequence in the N-terminal region of POMC was also found to be essential for addressing this precursor to the RSP (19). In this case, the disulfide-bonded sequence was shown to bind to carboxypeptidase E (CPE), an enzyme involved in late steps of precursor processing, and it was proposed that CPE might be a sorting receptor in the TGN for several hormone precursors (20). This view, however, is in contradiction with earlier studies showing that the N-terminal domain of POMC was not required for its sorting to the RSP but, rather, that cooperation between different internal POMC domains might be involved (21). Furthermore, the proposal of CPE as a sorting receptor for hormone precursors was recently challenged (22, 23). Attention was also paid to the dibasic sequences that are usually numerous in prohormone precursors as possible sorting signals. Thus, although initial studies suggested that the N-terminal domain of prosomatostatin may play a role in precursor targeting to the RSP (24), further work indicated that the somatostatin 28-containing C-terminal region might also be involved in sorting (25) and that a single mutation of the Arg-Lys dibasic that is normally processed to yield somatostatin 14 prevented prosomatostatin from entering the RSP (26). Similarly, following initial studies suggesting that in prorenin no signal was present in the prodomain that may be involved in its routing to the RSP (27–30), a more recent work showed that mutation of the dibasic that is normally processed in prorenin to produce renin prevented sorting of the protein to the RSP (31).

The aim of the present study was to look for structural elements that could be responsible for the routing of pronoertensin/neuromedin N (pro-NT/NN) into the RSP. This molecule is the common precursor of two bioactive neuropeptides, neuromedin N (NT) and neuromedin N (NN) (32). Pro-NT/NN is 169 amino acids long and starts with a 22-residue signal peptide not shown here. Amino acid residues in pro-NT/NN are numbered from 1, starting with the first residue of the prosequence. The Cys residues in positions 39 and 88 in pro-NT/NN are linked by a disulfide bridge. The positions of the dibasic sites are indicated by vertical thick lines. The main processing products generated by PC1 (NT and large NN), PC2 (NN, NT, and large E61) and PC5-A (NT, large NT, and large NN) are represented (33, 34).

**Fig. 1.** Diagrammatic representation of rat pro-NT/NN. Rat prepro-NT/NN is 169 amino acids long and starts with a 22-residue signal peptide not shown here. Amino acid residues in pro-NT/NN are numbered from 1, starting with the first residue of the prosequence. The Cys residues in positions 39 and 88 in pro-NT/NN are linked by a disulfide bridge. The positions of the dibasic sites are indicated by vertical thick lines. The main processing products generated by PC1 (NT and large NN), PC2 (NN, NT, and large E61) and PC5-A (NT, large NT, and large NN) are represented (33, 34).
terminal region of pro-NT/NN, we first demonstrated the existence of an intramolecular disulfide bridge in the precursor and investigated its function by mutating the cysteine residues or deleting the sequence in between (Fig. 2). Then, the role of the long N-terminal region upstream of NN and of the short C-terminal tail downstream of NT was studied with deletion. Finally, deletions and mutations were also made in the region that contains the dibasic sites and the active peptide sequences. All the constructs were transiently transfected in beta TC7 cells, an insulinoma cell line able to process endogenous insulin and to sort it to the RSP (35). We then studied the intracellular processing pattern of wild type and mutated pro-NT/NN and determined the secretion pathway of the maturation products. Our results demonstrate that neither the disulfide bridge nor the N- and C-terminal flanking domains are necessary for the correct routing of the precursor to the RSP. In contrast, the neuropeptide-encoding region of pro-NT/NN was shown to contain structural elements that are critical for sorting to the RSP and those elements were identified as the dibasic sequences within this region. Thus, the C-terminal domain of pro-NT/NN contains sorting motifs that is further demonstrated by fusing this domain to the C term of a protein that is constitutively secreted when expressed in eukaryotic cells (36), and showing that the fusion protein was redirected to the RSP in beta TC7 cells.

EXPERIMENTAL PROCEDURES

Disulfide Bond Analysis—Pro-NT/NN contains two cysteine residues in positions 39 and 88. To see if they form a disulfide bond, large E6I,3 the 1–117 pro-NT/NN fragment (Fig. 1), was partially purified from rMTC 6–23 cells as reported previously (37) and 70 pmol of this material were submitted to Arg-directed cleavage using the citraconylation, trypsin digestion, and unblocking methods (CT procedure) described previously (37). The CT-treated material was divided in two portions, one of which was reduced with 3% (v/v) b-mercaptoethanol. This generated several peptides, among them the 9–56 and the 65–89 pro-NT/NN fragments, each of which contains one of the Cys residues. If a disulfide bond links the Cys, both fragments should migrate as a single entity on reverse phase HPLC prior to reduction, whereas they should elute as two peaks after reduction.

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Site-directed Mutagenesis of Pro-NT/NN—Wild-type (WT) prepro-NT/NN cDNA was kindly provided by Dr. P. R. Dobner (University of Massachusetts, Worcester, MA). Deletions within the N-terminal domain of pro-NT/NN (D39–88, D14–88, D14–117, D171–117) (Fig. 2) were performed using the Ex-site method (Stratagene, La Jolla, CA). Point mutations, apart from C39S and C88S performed with the USE mutagenesis kit (Amersham Pharmacia Biotech), were done with the Quickchange kit from Stratagene (C39S/C88S, K118Q, R127Q, R136Q).

3 Precursor peptide fragments are designated as K6L, E6I, and H10P, in which the first letter corresponds to the single-letter code of the first amino acid, the middle number corresponds to the number of amino acids residues in the peptide, and the final letter corresponds to the single-letter code of the last amino acid.

\( \text{EC}_{50} \text{ and } \text{EC}_{100} \text{ of the assay were (in fmol/tube) 24, 122, and 625, respectively.} \)

**Fig. 2. Schematic representation of the pro-NT/NN mutants.** The upper part represents the mutations performed in the wild type precursor, and the lower part represents the mutations in large NT. Internal deletions are indicated by dashed lines. Punctual mutations are represented by open circles.
the ampicillin resistance gene (TEM-1) from pcDNA3. We used a 36-mer oligonucleotide (oligo 1), the first 24 5’ nucleotides identical with the 3’ end sequence of β-lactamase, and the other 12 identical with the sequence encoding for the 109–112 domain of pro-NT/NN. Another 36-mer oligonucleotide was used (oligo 2), the first 24 5’ nucleotides of which were identical to the sequence encoding for the 109–116 domain of pro-NT/NN and the other 12 reverse complementary to the 3’ end of the β-lactamase nucleotide sequence. The β-lactamase sequence was PCR-amplified with oligo 1 and an oligonucleotide identical with the 5’ end of the β-lactamase sequence (apart from a punctual mutation giving rise to a HindIII restriction site). The 109–147 segment of pro-NT/NN was amplified with Sp6 oligo (3’ end of the polylinker) and oligo 2 from the WT pro-NT/NN-encoding plasmid. The 109–125-encoding sequence was obtained with the same oligos on the previously obtained Δ126–147-encoding plasmid as matrix. The β-lactamase fragment and the C-term pro-NT/NN segment were purified, mixed at a 1:1 molecular ratio, and a PCR amplification (with the Sp6 oligo and the oligonucleotide specific for the 5’ of the β-lactamase sequence) allowed the obtention of the β-lactamase/pro-NT/NN sequence. This product was purified and subcloned in HindIII/Not-digested pcDNA3.

Cell Culture and Transfection—Beta TC7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 50 µg/ml gentamycin (Biomedia). Cells were maintained at 37 °C in 5% CO2 atmosphere. Culture media were changed every other day, and cells were passed once a week. The day before transfection, cells were plated in 35-mm dishes (1 × 105 cells/dish). Beta TC7 cells were transfected with polyethylenimine (800 µl/dish; Fluka) with few modifications to the protocol described (39). For each plate, DNA (2 µg) was diluted in 100 µl of OptiMEM (Life Technologies, Inc.) and incubated for 30 min at room temperature with 100 µl of OptiMEM containing 15 µl of polyethylenimine (0.9 mg/ml). Mix was then incubated with 800 µl of OptiMEM and added to the cells. After 5 h, 1 ml of Dulbecco’s modified Eagle’s medium containing 20% serum was added. 18–20 h later, the supernatant was replaced with 2 ml of fresh complete culture medium. Transiently transfected cells were analyzed 48 h after transfection.

Betatransfection was used for regulated secretion studies, the cells were incubated first with 500 µl of OptiMEM (30-min basal), then with 500 µl of OptiMEM containing 46 mM KCl and 4.4 mM CaCl2 (30-min stimulation) and the incubation media were kept frozen until further use. Finally, the cells were washed with phosphate-buffered saline (PBS from Eurobio) and extracted in 500 µl of cold 0.1 N HCl. The extractions were centrifuged at 10,000 × g for 10 min at 95 °C, centrifuged, and the supernatants were kept frozen. Protein amounts were determined using the Bio-Rad protein assay reagent under the manufacturer’s conditions.

Analysis of Pro-Neurotensin Processing Products—HPLC and RIA procedures have been described previously (37, 40). Briefly, the NT 29G and NT 28H antisera (kind gifts of Jean-Claude Cuber, INSERM U 45, University, New Orleans, LA) Western blots with the PC2 antisera generously provided by Iris Lindberg (Louisiana State University, New Orleans, LA). Western blots with the β-lactamase were

The graph shows intracellular amounts of immunoreactive NT (filled bar), N-terminal NT (shaded bar), and C-terminal NT (open bar) expressed as the ratio of immunoreactive material over intracellular CTiNN. The right part represents the stimulated release of immunoreactive NT (filled bar) and C-terminal INT (open bar) expressed as the ratio of released material under stimulated conditions over that in basal conditions. Values are the mean ± S.E. from 14 experiments. C, reverse phase HPLC analysis of intracellular immunoreactive NT (open circles), N-terminal NT (filled squares), and C-terminal NT (open squares). Arrows indicate the elution position of synthetic NT and NT.
performed similarly, except that proteins (50 μg) were separated by electrophoresis on 15% polyacrylamide minigels. The β-lactamase antibody was purchased from 5 Prime, Inc. (Eppendorf-5Prime; Boulder, CO) and used at a 1:1000 dilution.

**Table I**

Expression of pro-NT/NN mutants in transiently transfected beta TC7 cells

| CTiNN     | pmol/mg | n  |
|-----------|---------|----|
| Wild type | 1.0 ± 0.2| 14 |
| Pro-NT/NN mutants | | |
| C39S,C88S | 0.6 ± 0.3 | 5  |
| Δ39–88   | 0.9 ± 0.4 | 3  |
| Δ14–88   | 1.0 ± 0.1 | 8  |
| Δ141–147 | 3.0 ± 0.6 | 12 |
| Δ126–147 | 0.6 ± 0.1 | 7  |
| K118Q,R127Q,R136Q | 0.4 ± 0.1 | 8  |
| Large NT (Δ141–147) mutants | | |
| K118Q    | 1.4 ± 0.3 | 4  |
| R127Q    | 14.1 ± 7.0 | 4  |
| R136Q    | 14.0 ± 5.7 | 3  |
| K118Q,R127Q | 7.0 ± 2.1 | 3  |
| K118Q,R136Q | 1.5 ± 0.8 | 4  |
| R127Q,R136Q | 3.2 ± 0.5 | 3  |
| K118Q,R127Q,R136Q | 7.9 ± 0.6 | 3  |

**RESULTS**

Characterization of Pro-NT/NN Processing in the Mouse Insulinoma Beta TC7 Cell Line—The beta TC7 cell line was derived from mouse pancreatic beta cells and shown to retain the properties of insulin-secreting cells (35, 41). Proinsulin processing into mature insulin has been shown to result from the action of two prohormone convertases, PC1 and PC2, that are normally expressed in pancreatic beta cells (42, 43). Western blot analysis of beta TC7 cell extracts revealed that the cell line does express both PC1 and PC2 (Fig. 3A). Pro-NT/NN was transiently transfected in beta TC7 cells and the transfected cells were analyzed for their ability to process the precursor and to secrete the maturation products in a regulated manner. Intracellular levels of precursor (processed + unprocessed) as determined by CTiNN levels amounted to 1 pmol/mg of protein (Table I). Pro-NT/NN was efficiently processed to yield similar intracellular amounts of immunoreactive NT and NN (iNT and iNN) that represented 70–80% of synthesized precursor (Fig. 3B).

**Fig. 4.** Evidence of an intracellular disulfide bridge in the prorregion of pro-NT/NN. A, schematic representation of the products obtained after successive Arg-directed (CT procedure) and Met-directed (CNBr) cleavages of large E6I, leading to the exposure of the H10P and K6L sequences. B, HPLC analysis of immunoreactive H10P (circles) and K6L (squares) prior to (open symbols) and after (closed symbols) reduction of cleaved large E6I. K6L immunoreactivity was directly assayed in aliquot portions of each HPLC fraction, whereas H10P immunoreactivity was assayed after CNBr treatment of portions of the fractions.

**B**

The disulfide bridge is not necessary for pro-NT/NN processing and sorting—As the primary sequence of pro-NT/NN contains two cysteine residues (Fig. 1), we checked whether they formed a disulfide bridge. For this purpose, partially purified large E6I was cleaved by Arg-directed tryptic digestion between the two cysteines, thus generating several peptides, among them the 9–56 and the 65–89 fragments each containing one of the cysteines (Fig. 4A). The digested material was then treated or not with β-mercaptoethanol and the reduced and nonreduced products were submitted to HPLC. The fractions were analyzed by RIA using two antibodies, each specific for one of the tryptic fragments (Fig. 4A). Fig. 4B shows that immunoreactivities coeluted under nonreducing conditions, whereas they separated under reducing conditions. These results are consistent with the existence of a disulfide bridge between the fragments. To demonstrate that it is an intramolecular bond, pro-NT/NN was transiently expressed in COS M6 cells and cell extracts were electrophoresed under reducing and nonreducing conditions and immunoblotted with a pro-NT/NN antiserum. In both cases, a 17-kDa band was observed (data not shown), which corresponds to the expected molecular mass of pro-NT/NN deduced from its amino acid sequence. Hence, products were stored within secretory granules. Thus, beta TC7 cells represent a good endocrine cell line model for studying the processing and targeting to the RSP of pro-NT/NN and pro-NT/NN mutants following transient cell transfection.
The various constructs depicted in the figure were transiently transfected in βTC7 cells. 48 h later, cells were incubated for 30 min first in normal medium and then for another 30 min in depolarizing medium. Cell extracts were also collected. Processing at the level of the indicated dibasic sequences is expressed as the ratio of immunoreactive peptide generated after cleavage at the level of this doublet over intracellular CTiNN. The right column represents the stimulated release of immunoreactive CTiNN expressed as the ratio of released material under stimulated conditions over that in basal conditions. The values are the mean ± S.E. from the number of experiments given in Table I. ns, not significantly different from wild type pro-NT/NN; *, p < 0.05 when compared with wild type pro-NT/NN; °, not significantly different from 1.

pro-NT/NN was synthesized as a monomer. Taken together, the data demonstrate the existence of an intramolecular disulfide bridge in the precursor.

To assess the importance of the disulfide bridge and the disulfide-bonded sequence for pro-NT/NN processing and sorting to the RSP, two mutant precursors were constructed in which either both cysteine residues were mutated to serine (C39S/C88S) or the 39–88 precursor sequence was deleted (Δ39–88) (Fig. 2). The mutants were transiently transfected in βTC7 cells. Intracellular CTiNN levels of both mutants were comparable to those of wild type pro-NT/NN (Table I). The C39S/C88S mutant behaved quite similarly to wild type pro-NT/NN with regard to both the extent of processing and the regulated secretion (Fig. 5). The Δ39–88 mutant exhibited a 10–30% reduction in processing efficiency, as compared with wild type pro-NT/NN (Fig. 5). However, the regulated secretion pattern of processing products was similar to that of the wild type precursor (Fig. 5). These data show that the disulfide bond and the sequence it delimits in pro-NT/NN are not necessary for precursor sorting and processing into the RSP.

The N-terminal Region of Pro-NT/NN Is Not Involved in Sorting and Processing—We then investigated the role in sorting and processing of the N-terminal region of pro-NT/NN that extends from residue 14 after the signal peptide to residue 117 just prior to the dibasic that flanks the N terminus of NN. A first Δ14–117 deletion mutant was constructed and transfected in βTC7 cells. However, this mutant was not expressed to any detectable level in the cell line (data not shown), likely because its short size did not allow signal peptide removal (44), which possibly led to the degradation of the abnormal protein. Two other deletion mutants, Δ14–88 and Δ71–117, were then constructed (Fig. 2) and transiently transfected in βTC7 cells. Both mutant precursors were expressed (Table I) and efficiently processed to give iNN and iNT, and the maturation products were secreted under stimulation, relevant to their sorting to the RSP (Fig. 5). Hence, no sorting signal was present in the N-terminal region of pro-NT/NN.

Unexpected results were obtained with the Δ71–117 mutant. First, this mutant exhibited regulated secretion levels higher than the first mutants (Fig. 5). Second, it generated substantial amounts of immunoreactive K6L material (80 ± 3% of CTiNN, n = 4), indicating that the first Lys63-Arg64 dibasic site was cleaved as efficiently as the other dibasic sequences. This cleavage was not observed with wild type pro-NT/NN nor with any of the mutants that contained the KR-K6L sequence (data not shown).

The KR-NT Sequence Contains Essential Information for Sorting—As deletions of the N-terminal region did not prevent pro-NT/NN processing and sorting to the RSP, we surmised that the C-terminal precursor region that contains the neuropeptide sequences and the dibasic sequences that flank them might play a role in sorting. To test this hypothesis, C-terminal deletions were performed to yield large NT (Δ141–147 mutant) and large NN (Δ126–147 mutant) (Fig. 2). Both deletion mutants were transiently transfected in βTC7 cells (Table I), and their processing and regulated release were analyzed (Fig. 5). Large NT was processed to yield iNT and iNN in proportions similar to those obtained with wild type pro-NT/NN and secretion was enhanced upon stimulation (Fig. 5). Therefore, the KR-tail sequence is not by itself necessary for pro-NT/NN processing and sorting into the RSP. In contrast, large NN was not processed, i.e. cleavage of the Lys118–Arg119 dibasic sequence that normally proceeds to greater than 80% in wild-type pro-NT/NN was markedly reduced to less than 10% in the deletion mutant (Fig. 5). HPLC analysis confirmed that large NN was the main intracellular product (data not shown). Furthermore, large NN was not secreted in a regulated manner by βTC7 cells (Fig. 5). Thus, large NN was not stored in the RSP compartments where processing takes place. Altogether, the data obtained with large NT and large NN show that the C-terminal KR-NT sequence of large NT contains elements that are essential for storage in the RSP.

Dibasic Sites Are Essential for Pro-NT/NN Sorting—The C-terminal neuropeptide-encoding region of pro-NT/NN contains four dibasic sequences (Fig. 1): the three Lys-Arg dibasic sequences that flank and separate NT and NN, and an Arg-Arg dibasic in position 8–9 of the NT sequence (position 135–136 in pro-NT/NN) that is not normally processed in tissues. As three out of the four dibasic sequences are removed in large NN, we hypothesized that these motifs might alone or in combination play a role in pro-NT/NN sorting to the RSP. Since large NT, which contains three of the four dibasic sequences was efficiently processed and targeted to the RSP as shown above, it appears that the dibasic site that flanks the C terminus of NT is not alone necessary for pro-NT/NN sorting to the RSP. To assess the role of the remaining three dibasic sequences in large NT, we constructed all possible combinations of large NT in which either one, two, or all three dibasic sequences were mutated on one of the basic residues (Fig. 2), transiently transfected the mutants in βTC7 cells, and analyzed precursor

| Processing (%)CTiNN | Release stimulated/basal |
|---------------------|--------------------------|
| WT                 | 85.4 ± 4.9               | 62.4 ± 11.3               | 67.3 ± 11.3               | 5.9 ± 1.3       |
| C39,88S            | 81.1 ± 10.5              | 52.4 ± 9.0                | 51.7 ± 12.5              | 6.6 ± 2.4NS     |
| delta 39-88        | 52.9 ± 6.7               | 48.1 ± 11.4              | 38.3 ± 6.9               | 6.2 ± 1.8NS     |
| delta 14-88        | 91.3 ± 12.2              | 73.6 ± 10.0              | 75.7 ± 10.5              | 14.5 ± 1.8*     |
| delta 71-117       | 92.2 ± 4.6               | 60.1 ± 11.7              | 51.3 ± 10.9              | 21.2 ± 4.5°     |
| delta 141-147      | 79.0 ± 14.5              | 51.7 ± 6.4               | 55.4 ± 10.9              | 3.6 ± 0.3NS     |
| delta 126-147      | 10.5 ± 3.6               | n.d                       | n.d                       | 1.3 ± 0.1°      |

Fig. 5. Processing and regulated release of disulfide bond, N-terminally deleted, and C-terminally deleted pro-NT/NN mutants. The various constructs depicted in the figure were transiently transfected in βTC7 cells. 48 h later, cells were incubated for 30 min first in normal medium and then for another 30 min in depolarizing medium. Cell extracts were also collected. Processing at the level of the indicated dibasic sequences is expressed as the ratio of immunoreactive peptide generated after cleavage at the level of this doublet over intracellular CTiNN. The right column represents the stimulated release of immunoreactive CTiNN expressed as the ratio of released material under stimulated conditions over that in basal conditions. The values are the mean ± S.E. from the number of experiments given in Table I. ns, not significantly different from wild type pro-NT/NN; *, p < 0.05 when compared with wild type pro-NT/NN; °, not significantly different from 1.
processing and regulated secretion (Fig. 6).

All the large NT mutants were well expressed in beta TC7 cells, as evaluated by CTiNN measurements (Table I). Extracts from all of the large NT mutant-expressing cells (Fig. 6) reacted positively with the C-terminal NT antiserum, consistent with the fact that the mutants possess a free C-terminal NT sequence. In all cases, the dibasic sequences that flank NN were processed to yield N-terminal iNN and N-terminal INT when they were left intact, whereas their mutation totally prevented cleavage. N-terminal iNN levels ranged from 75% to 90% of intracellular CTiNN levels, indicating that cleavage at the dibasic that precedes NN proceeded near completion. N-terminal iNT concentrations amounted only to 30% of CTiNN, suggesting that processing at the dibasic that separates NN and NT was somewhat hindered by mutations of either of the adjacent dibasic. All the single and double mutants yielded immunoreactive products that could be released in a regulated manner (Fig. 6). In contrast, the large NT construct with the triple mutation was not secreted upon cell stimulation. These results indicate that the integrity of at least one of the three dibasic sequences in large NT is necessary and sufficient for its storage into secretory granules.

To see if adding the C-terminal KR-tail sequence to large NT bearing the triple mutation would restore regulated secretion, a triple pro-NT/NN mutant was constructed and transiently transfected in beta TC7 cells. The mutant was well expressed as indicated by intracellular CTiNN levels (Table I). Interestingly, although the dibasic that follows NT was efficiently processed to yield C-terminal INT (69.7 ± 11%, n = 4), the immunoreactive product generated by the cleavage was not released upon cell stimulation (Fig. 6). This suggests that cleavage of the Lys141-Arg142 dibasic sequence occurred before sorting could take place, possibly through the action of PC1 since this enzyme has been shown to be activated in the endoplasmic reticulum of neuroendocrine cells, in contrast to PC2, which is activated much later in secretory granules (45–47).

Precursor Mutants That Lack Regulated Secretion Are Efficiently Secreted in a Constitutive Manner—To further investigate the cellular fate of those pro-NT/NN mutants that lacked regulated secretion, i.e. the large NN deletion mutant and the triple large NT mutant, the constructs were stably transfected in beta TC7 cells and metabolic studies were performed. The K118Q large NT mutant was also stably transfected in beta TC7 cells. The latter was chosen as a control in this series of experiments because, as shown above, it is processed at the Lys126-Arg127 dibasic to yield a large precursor form that presumably is identical to large NN except for the K118Q substitution and, unlike large NN, undergoes regulated secretion. Three clones, each expressing high levels of one of the mutants, were pulse-labeled for 30 min and chased for 4 h. The medium was collected at varying time intervals and replaced with fresh normal medium for the first 210 min of chase and with depolarizing medium for the last 30-min chase interval. Cell lysates and media were immunoprecipitated with the pro-NT/NN antiserum and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 7). For the K118Q large NT mutant (Fig. 7A), a 30-min pulse without chase led to the intracellular labeling of a 17-kDa protein corresponding in size to the whole pro-NT/NN mutant sequence minus the signal peptide (lane i). After a 4-h chase, the 17-kDa band was no longer observed in the cell lysate but a 15-kDa protein corresponding in size to the whole pro-NT/NN mutant sequence minus the signal peptide (lane h). A fraction of the 17-kDa protein was secreted during the first 60 min of chase (lanes c–f). The protein was barely detectable at longer chase times (lanes c–f) and could not be released upon depolarization (lane h). Similarly, a fraction of the 15-kDa protein was secreted during the chase period with a peak at 60 min and a progressive decline thereafter (lanes a–f). However, unlike the 17-kDa protein, it was readily released upon cell depolarization with a 4-fold stimulation (compare lane g with lane f) in amounts that represented 15% of the material labeled during the pulse. Altogether, these data are consistent with the above data derived from RIA measurements. They show that the K118Q large NT mutant is processed intracellularly to yield a K118Q large NN product that is largely stored in secretory granules and released upon cell stimulation.

| Precursor Mutants | processing | release |
|-------------------|------------|---------|
|                  | (%) C|      |        |stimulated | basal |
| delta 141-147     | 118-119   | 79.0 ± 14.5 | 51.7 ± 6.4 | 3.6 ± 0.3 |
|                   | 126-127   |         |        |            |        |
| delta 141-147     | 118-119   | 79.0 ± 14.5 | 51.7 ± 6.4 | 3.6 ± 0.3 |
|                   | 126-127   |         |        |            |        |
| K118Q             | 118-119   | 3.6 ± 1.0  | 30.7 ± 5.2 | 2.6 ± 0.4 |
|                   | 126-127   |         |        |            |        |
| R127Q             | 118-119   | 88.0 ± 7.5 | 0.4 ± 0.1 | 7.3 ± 1.5*|
|                   | 126-127   |         |        |            |        |
| R136Q             | 118-119   | 92.3 ± 6.0 | 42.0 ± 4.6 | 5.3 ± 1.0 |
|                   | 126-127   |         |        |            |        |
| K118Q, R127Q      | 118-119   | 1.0 ± 0.3  | 0.7 ± 0.5  | 2.3 ± 0.4 ns |
|                   | 126-127   |         |        |            |        |
| K118Q, R136Q      | 118-119   | 1.9 ± 1.1  | 33.0 ± 7.8 | 5.1 ± 1.9 ns |
|                   | 126-127   |         |        |            |        |
| R127,136Q         | 118-119   | 76.3 ± 12.9 | 1.0 ± 0.6  | 5.2 ± 1.3*|
|                   | 126-127   |         |        |            |        |
| K118Q, R127,136Q  | 118-119   | 1.8 ± 0.2  | 0.9 ± 0.6  | 1.3 ± 0.1° |
|                   | 126-127   |         |        |            |        |

Fig. 6. Processing and regulated release of dibasic mutants. Each construct is schematically depicted with the indication of its mutation(s). All possible mutations of the 118–119, 126–127, and 135–136 dibasic sequences were performed in large NT (Δ141–147) as depicted in the top part of the figure. In the bottom line is depicted the triple pro-NT/NN mutant in which the 118–119, 126–127, and 135–136 dibasic sequences were mutated. Processing and release were analyzed as described in Fig. 5. The values are the mean ± S.E. the number of experiments given in Table I. ns, not significantly different from large NT (Δ141–147); *, p < 0.05 when compared with large NT (Δ141–147); °, not significantly different from 1.
With the Δ126–147 pro-NT/NN mutant (large NN) (Fig. 7B), a unique intracellular 15-kDa protein, corresponding in size to unprocessed large NN, was labeled during the 30-min pulse (lane i) and was still present in cell lysates after 4 h of chase (lane h), although at much reduced levels (15%). Most of the 15-kDa protein (>75%) was secreted from the cells during the first 210 min of chase (lanes a–f) and no stimulated release could be observed (lane g). Similar results were obtained with the triple large NT mutant (Fig. 7C). A single 17-kDa intracellular protein with the expected size of the unprocessed mutant was labeled during the 30-min pulse (lane i) and was still present at low levels (10%) after 4 h of chase (lane h). The protein was progressively secreted from the cell for the first 210 min of chase lane (lanes a–f), and no stimulated release was observed during the last 30-min chase interval (lane g). These results are in full agreement with the above RIA data showing that both mutants were not processed and lacked regulated secretion. They further show that the mutants were not stored intracellularly but were readily secreted in a constitutive manner, thus indicating that they reached the TGN and were not blocked in the endoplasmic reticulum as misfolded proteins.

The C-terminal Pro-NT/NN Domain Is a Transferable Sorting Signal—The above results demonstrate the critical importance of the C-terminal encoding-peptide domain of pro-NT/NN for its addressing to secretory granules. We then sought to investigate whether this domain could by itself reroute a constitutive protein to the RSP. The chosen constitutive protein was β-lactamase (also called ampicillinase), a bacterial protein that possesses a signal peptide and has been shown to be constitutively secreted when expressed in neuroendocrine cells (36). Two chimeric proteins depicted in Fig. 8A were constructed by fusing to the C terminus of β-lactamase either the C-terminal 109–147 pro-NT/NN domain (β-Lac-109–147) or the 109–125 pro-NT/NN fragment with a C-terminal NN moiety (β-Lac-109–125). These constructs were stably transfected in beta TC7 cells. Clones that express high levels of the constructs were selected and tested for their ability to process and release the chimeric proteins. The results clearly show that processing and regulated secretion readily occurred for β-Lac-109–147 and, in contrast, were markedly impaired for β-Lac-109–125. Thus, iNN measurements in cell extracts indicated that cleavage of the Lys\(^{118}\)-Arg\(^{119}\) dibasic sequence that precedes NN proceeded to greater than 50% with β-Lac-109–147, whereas it was less than 10% for β-Lac-109–125 (Fig. 8A). Western blot analysis using a β-lactamase antiserum (Fig. 8B) revealed that in β-Lac-109–147-expressing cells, both the intact 34-kDa fusion protein and a 29-kDa processing product corresponding in size to β-lactamase minus its pro-NT/NN-derived C-terminal extension were present. Furthermore, the 29-kDa product was released upon cell depolarization, whereas the 34-kDa precursor was not found in the medium of either unstimulated or stimulated cells. In contrast, the β-Lac-109–125 chimera was expressed as a single 31-kDa band corresponding in size to the full sequence of the fusion protein (Fig. 8B). The protein was constitutively released in the medium but lacked regulated secretion. Immunofluorescence studies coupled to confocal microscopy were performed using the β-lactamase antiserum and an antiserum directed against secretogranin II, a secretory granule marker (Fig. 9). Secretogranin II labeling in beta TC7 cells exhibited a highly punctate distribution and was heavily concentrated in a cytoplasmic region extending from the nucleus to the plasma membrane (Fig. 9B). β-Lactamase staining in β-Lac-109–147-expressing cells exhibited the same punctiform and subcellular distribution as secretogranin II (Fig. 9B). In marked contrast, β-lactamase labeling in β-Lac-109–125-expressing cells was diffuse throughout the cytoplasm of the cells (Fig. 9C).

DISCUSSION

In this work, we provide strong evidence that basic residues within the neuropeptide-containing region of pro-NT/NN are essential for precursor entry and/or retention into the RSP, whereas the large N-terminal domain and the disulfide bond it contains are not required for products from pro-NT/NN to reach the RSP.

As recalled in the Introduction, a disulfide bond-stabilized loop in the N-terminal region of CgB was shown to be necessary for its sorting to the RSP (16, 17). A disulfide bond-delimited sequence in the N-terminal region of POMC was also proposed to be essential for the sorting of this precursor to the RSP (19) through binding to a receptor identified as CPE (20). Because pro-NT/NN contains two cysteine residues, we investigated the presence of a disulfide bond in the precursor and its role as a sorting signal. Our data clearly established the existence of a

![Fig. 7. Release of newly synthesized pro-NT/NN mutants. Beta TC7 cells were stably transfected with the K118Q large NT (A), Δ126–147 (large NN) (B), or triple large NT mutants (C). Cells were pulse-labeled with [\(^{35}\)S]methionine/cysteine for 30 min. After the pulse, one dish for each mutant was harvested for determining the intracellular content of labeled proteins after immunoprecipitation with the pro-NT/NN antisera, SDS-polyacrylamide gel electrophoresis, and autoradiography (lane i). Each autoradiogram is from one representative experiment with each pro-NT/NN mutant. Below, the autoradiograms were quantitated by densitometry. The data in lanes a–h are expressed as the percentage of material present in the cells after the pulse (lane i) and are the means from four separate experiments in A and B, and two separate experiments in C.](http://www.jbc.org/)

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disulfide bridge within the N-terminal domain of pro-NT/NN and demonstrated that the disulfide bond and the loop it defines were not involved in precursor sorting and processing into the RSP. In fact, virtually all of the large N-terminal region upstream of the neuropeptide-containing sequence in pro-NT/NN could be removed without affecting the regulated secretion of processing products. Thus, the N-terminal region of pro-NT/NN comprising more than three-quarters of the precursor sequence is clearly not involved in sorting. Furthermore, since deleting large portions of the disulfide bridge-containing N-terminal domain of pro-NT/NN is likely to affect the secondary and tertiary structures of the precursor, it would appear that pro-NT/NN sorting to the RSP is independent of its global conformation and does not involve N-terminal conformational elements, contrary to what was reported for CgB (16) and POMC (19).

This led us to investigate the possibility that the neuropeptide-encoding C-terminal region of pro-NT/NN contains discrete motifs that would be involved in its sorting to the RSP. Sequential C-terminal deletions of the precursor revealed that the large NT construct was processed and addressed to the RSP as efficiently as wild type pro-NT/NN, indicating that the C-terminal tail played no essential role in sorting. In contrast, the large NN fragment was neither processed nor targeted to the RSP. The latter finding supports the conclusion that the N-terminal domain of pro-NT/NN is not involved in targeting the precursor to the RSP and shows that essential sorting elements are contained within the KR-NT sequence of pro-NT/NN. Further mutagenesis studies showed that the three dibasic sequences in the C-terminal region of large NT played an important role in sorting as mutating all of them prevented large NT from reaching mature secretory granules. Interestingly, mutating any combinations of two of the three dibasics yielded large NT constructs that were sorted to the RSP, indicating that only one intact dibasic site in the C-terminal domain of large NT is required for sorting. As the large NN deletion mutant that contains the Lys118-Arg119 dibasic site but lacks the 126–140 region of large NT was not sorted to the RSP, we conclude that in pro-NT/NN the minimal sorting signal consists of a dibasic motif located in the neuropeptide-containing region extending from residue 118 to 140 of the precursor and that, within this sequence, any one of the three 118–119, 126–127, and 135–136 dibasic sites can function as the sorting signal.

It could be argued that the pro-NT/NN mutants that lacked regulated secretion, i.e. large NN and the triple large NT mutant, might, as a consequence of the mutations, be unable to reach the TGN and be trapped as misfolded proteins in the ER where they would either remain or be degraded. Metabolic labeling studies performed with these mutants clearly rule out such a possibility. Thus, following their biosynthesis, both mutants were readily secreted in a constitutive manner from beta TC7 cells and totally lacked regulated secretion, indicating that they reached the TGN but could not enter or remain within secretory granules. In these experiments, the K118Q large NT mutant was used as a control. As shown both by RIA and metabolic labeling experiments, the mutant was processed to yield a K118Q large NN product that is identical to large NN except for the K118Q mutation. Interestingly, unlike large NN, this product was very efficiently stored in secretory granules wherefrom it could be released in a regulated manner. Therefore, the sorting signal defined above, i.e. the C-terminal precursor domain and the dibasic sequences it contains, represent a strong and efficient (all or none) structural requirement for allowing storage of pro-NT/NN in mature secretory granules.

This point was further demonstrated by fusing the C-termi-
nal domain of pro-NT/NN to the C terminus of β-lactamase, a prokaryotic protein that has been shown to be constitutively secreted when expressed in neuroendocrine cells (36). The fusion protein expressed in beta TC7 cells clearly exhibited a subcellular localization and a pattern of secretion consistent with it being efficiently sorted to the RSP. In contrast, fusion of the C-terminal motif of large NN (KR-NN) to β-lactamase yielded a protein that displayed a diffuse cellular distribution, was constitutively secreted, and lacked regulated secretion. Therefore, it is clear that the C-terminal neuropeptide-encoding domain of pro-NT/NN contains elements that are necessary and sufficient to direct a protein to the RSP or to prevent it from exiting IGs, independently of the nature of the protein to which it is attached.

Our data show that not all dibasic sites in pro-NT/NN may serve as processing site and/or sorting signal. Thus, in the Δ126–147 deletion mutant (large NN) and the triple large NT and pro-NT/NN mutants, the Lys63-Arg64 dibasic site in the middle region of the proteins did not function as an alternative processing site nor did it target the mutants to the RSP. Interestingly, bringing this site closer to the C-terminal domain of pro-NT/NN as in the Δ71–117 deletion mutant led to its efficient processing, indicating that the C-terminal neuropeptide-containing region of pro-NT/NN is more accessible to processing enzymes than the N-terminal region of the precursor. In any case, it may be suggested that dibasic sequences have to be in a proper structural environment to function as sorting signals. A similar conclusion was reached concerning prorenin (31). This precursor contains, in addition to the Lys-Arg processing site adjacent to renin, two other Lys-Arg sequences in the N-terminal part of the proregion. Mutating the processing site prevented prorenin processing and regulated secretion. However, fusing the N-terminal 16 amino acid sequence of prorenin that contains the silent dibasic sequences to a constitutive protein yielded a fusion protein that was processed and targeted to the RSP. It was concluded that the two N-terminal dibasic sequences could not be used as alternative processing sites and sorting signals in prorenin because they were not in a proper environment.

It was further shown in this study (31) that mutation of the dibasic sequences in the fusion protein abolished both processing and regulated secretion, leading to the interesting suggestion that targeting to the RSP might be linked to processing. In this context, a recent study on insulin and proinsulin suggested that dibasic cleavage may increase storage of the hormone in secretory granules (48). In the present study, virtually all of the pro-NT/NN mutants that were sorted to the RSP were processed at at least one of the dibasic sequences proposed to be involved in sorting pro-NT/NN to the RSP. The only exception is the K118Q,R127Q large NT mutant in which the only intact dibasic site is Arg135-Arg136 within the NT sequence, a site that is not processed in normal tissues that express pro-NT/NN. This mutant was sorted to the RSP. However, we were not able to assess whether the Arg135-Arg136 dibasic site remained intact in beta TC7 cells that express the K118Q,R127Q large NT mutant due to the lack of adequate antibodies. Therefore, we cannot definitively conclude that processing is linked to sorting for pro-NT/NN. We can only suggest that dibasic sequences in the C-terminal domain of pro-NT/NN might interact with other regulated proteins, among which the PCs represent only a possibility, and that this interaction might play a role in the sorting of the precursor. Should this be the case, it would represent a crucial sorting step since our data show that deleting or mutating the dibasic sequences abolishes regulated secretion.

As already mentioned, dibasic sequences have been proposed to act as sorting signals for prosomatostatin and prorenin (26). Sorting motifs have also been investigated in POMC and conflicting results were obtained. One group reported that POMC sorting involved N-terminal conformational elements delimited by a disulfide bridge (19). Others reported that the disulfide bond-containing domain of POMC was not involved in RSP sorting (21) and further showed that deletion of internal precursor sequences abolished POMC targeting to secretory granules (49). As these sequences contain a number of dibasic sequences, it cannot be excluded that such motifs were necessary for POMC sorting. CgB was shown to be addressed to the RSP through a disulfide bond-delimited loop in its N-terminal domain (17, 50). However, CgB appears to play a role by itself in assisting the packaging of certain hormone and neuropeptide precursors into secretory granules (51). It is therefore conceivable that the sorting mechanism of CgB might differ from that of classical prohormones and proneuropeptides. Clearly, more work is needed to answer the question as to whether sorting of hormone and neuropeptide precursors to the RSP through dibasic sequences is a general mechanism. It is, however, an appealing suggestion since sorting to the RSP is a property shared by all neuroendocrine cells and although hormone and neuropeptide precursors exhibit widely different structures they have in common the presence of dibasic motifs in their sequence. Thus, in addition to serving as processing sites for the limited set of prohormone convertases present in neuroendocrine cells, dibasic sequences could also function as recognition sequence for the sorting machinery.

As mentioned in the Introduction, two models termed sorting-for-entry and sorting-by-retention are currently viewed as possible mechanisms for segregating regulated proteins in secretory granules. The latter model can be further subdivided in two schemes: one in which retention of regulated proteins in IGs is an active process and the other in which retention would be passive while exclusion of constitutive proteins from the IGs is the active process (5, 12). Our present data do not allow to conclude as to which mechanism might operate for the sorting of pro-NT/NN. However, because of the all-or-none effect of the C-terminal domain of pro-NT/NN on the sorting of the precursor and the high efficiency of this domain in routing a constitutive protein (β-lactamase) to the RSP, our data are difficult to reconcile with a passive mechanism for retaining regulated proteins in IGs. Rather, we would favor the hypothesis that the C-terminal region of pro-NT/NN and the dibasic sequences it contains play an active role in precursor sorting through protein-protein interactions. Further work will be needed to determine the type of interactions involved: binding to a putative sorting receptor(s), formation of protein aggregates or cleavage-condensation assisted by PCs. The beta TC7 cell lines developed here that stably express various pro-NT/NN constructs or the β-lactamase fusion proteins should provide useful tools in this notice.

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The Role of Dibasic Residues in Prohormone Sorting to the Regulated Secretory Pathway: A STUDY WITH PRONEUROTENSIN
Sylvain Feliciangeli, Patrick Kitabgi and Jean-Noël Bidard

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