Progastrin Is Directed to the Regulated Secretory Pathway by Synergistically Acting Basic and Acidic Motifs

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Bioactivation of prohormones occurs in the granules of the regulated secretory pathway of endocrine cells, which release hormones in response to external stimulation. How secretory granules are formed and how the cargo is selected is still unclear, but it has been shown for several prohormones and processing enzymes that domains within the prohormone structure can act as “sorting signals” for this pathway. The domains mediate interactions with other proteins or with the membrane or facilitate aggregation of the (pro)peptides. We have now searched for domains in progastrin that are active in sorting the prohormone into secretory granules. Truncation studies showed that the N-terminal 30 residues of progastrin are dispensable, whereas the last 49 residues are sufficient for correct biosynthesis of bioactive gastrin. Thus, further N-terminal truncation abolished gastrin expression. C-terminal truncation of 8 residues resulted in an increase in basal secretion as did point mutations in the dibasic processing sites of progastrin. These mutants, however, still responded to secretagogues, suggesting a residual sorting capacity to the regulated pathway. Amino acid substitutions in an acidic, polyglutamate motif within gastrin-17, the main bioactive, cellular gastrin form, did not alter secretion per se, but when these residues were substituted in C-terminally truncated mutants, double mutants increased in basal secretion and did not respond to secretagogue stimulation. This implies that the mutants are constitutively secreted. Our data suggest that the dibasic processing sites constitute the most important sorting domain of progastrin, and these sites act in synergy with the acidic domain.

Peptide hormones are potent signaling molecules that regulate biological key functions. Accordingly, active peptides are primarily synthesized as inactive precursors, and later, after processing to the bioactive peptides, stored in specialized granules to be secreted only after appropriate external stimuli. In the granules belonging to the regulated secretory pathway (1), prohormones are activated by sequential enzymatic reactions including specific endoproteolysis by specific prohormone convertases (2, 3). Thus, the correct sorting of prohormones into the granules is a prerequisite for both activation and timing of hormonal signaling.

The mechanism behind the sorting of prohormones to secretory granules has been debated extensively in recent years (for reviews, see Refs. 4–8). Basically, two models exist for the sorting of cargo to the secretory granules, the “sorting for entry” and the “sorting by retention” models (4). With the former model, entry to the immature secretory granules is the main point of sorting, whereas the latter model proposes that peptides for regulated secretion are retained in immature secretory granules from which other proteins and peptides are removed and constitutively secreted. Several different sorting mechanisms exist that can utilize differently depending on the cell type and protein to be sorted (7). Calcium-mediated aggregation of proteins is important for sorting proinsulin, proatrial natriuretic factor, and chromogranin A to the regulated pathway (9–12). However, aggregation is not the only factor in cargo selection and regulation of protein sorting (6, 7). A “sorting receptor” that recognizes specific domains of cargo proteins and selects these for secretory granules has also been proposed. One candidate for such a receptor, carboxypeptidase E, has been proposed to be involved in the sorting of proopiomelanocortin, proenkephalin, and proinsulin to the regulated pathway (13, 14). Proopiomelanocortin contains a hydrophobic, disulfide loop that is thought to interact with specific residues of carboxypeptidase E (15), and sorting is then mediated by membrane attachment of carboxypeptidase E (16, 17). In chromogranin A and B, similar loops mediate sorting via membrane binding and not by binding carboxypeptidase E (18, 19). This is also the case in the sorting of two enzymes localized in regulatory granules, the prohormone convertases 1/3 and 2 (20, 21). Recent evidence, however, suggests that at least one additional sorting determinant is present in chromogranin A and that protein folding may be important for sorting (22). Also, sorting determinants of other proteins have been described without the identification of binding receptors, e.g. proneuropeptide Y and proneurotensin that both require basic residues for sorting (23, 24).

The hormone gastrin is a major regulator of gastric acid secretion and gastric mucosal growth (25). Human progastrin is an 80-residue polypeptide that is cleaved by the prohormone convertases 1/3 and 2 and then C-terminally trimmed by carboxypeptidase E. Subsequent ε-amidation by the amidation enzyme complex, peptidylglycine ε-amidating monoxygenase, generates the acid stimulatory gastrins, gastrin-17 and gastrin-34 (see Fig. 1). We have previously established a model system to study human gastrin biosynthesis using mutagenesis (26, 27). In the present study, we have analyzed the features of progastrin that are involved in sorting to the regulated secretory pathway. Using N- and C-terminal truncations and amino acid substitutions, we have found that two domains are implicated in progastrin sorting. Either one is by itself sufficient to sort progastrin to secretory granules responding to the secretagogue, 3-isobutyl-1-methylxanthine (IBMX),1 whereas double mutations abolished responsiveness to IBMX.

1 The abbreviation used is: IBMX, 3-isobutyl-1-methylxanthine.
EXPERIMENTAL PROCEDURES

Materials—Culture media and supplements for cell culture were obtained from Invitrogen. Enzymes and materials for PCR and molecular cloning were obtained from Roche Applied Science, New England Biolabs, or Promega. HIT-T15 cells were obtained from ATCC (Manassas, VA).

Expression Vector Constructions and Mutagenesis—The expression vectors in this study were based on two different gastrin expression vectors with similar expression levels. The first utilizes the human UbB promoter to express the human gastrin gene (26), and the second expresses gastrin cDNA cloned as a PCR fragment in pcDNA3.1/Zeo (Invitrogen) from the cytomegalovirus promoter. Mutations in the human gastrin gene or cDNA were made by PCR, either by using nucleotide substitutions in primers followed by direct cloning or by using overlap PCR followed by cloning. All molecular biology methods and enzymatic treatment were conducted according to the supplier’s suggestions and general molecular biology protocols.

Cell Culture and Transfection—The Syrian hamster β-cell line HIT-T15 was cultured and transfected as described previously (28). Basically, 2 × 10⁶ cells were seeded in 10-cm Petri dishes and transfected the following day. Media were changed the following day, and the cells were allowed 2 days for expression before harvesting. Stimulation experiments were initiated 1 day after transfection by the addition of 0.5 mM IBMX (Sigma) and incubated for peptide expression for 24 h, when experiments were initiated 1 day after transfection by the addition of 0.5 mM IBMX (Sigma) and incubated for peptide expression for 24 h, when cells and culture media were harvested. At harvest, culture media were recovered and were removed by the addition of phosphate-buffered saline added to 2 g/liter EDTA, pelleted, and kept at −20 °C until peptide extraction. At the time of harvest, each dish contained about 10⁷ cells. All transfection series were repeated, and data presented are from parallel transfections of four dishes.

Radioimmunoassay—Cells were resuspended in 1 ml of boiling water, and culture media were extracted by boiling at neutral pH for 20 min. Cell debris was removed by centrifugation. Extracts were analyzed by radioimmunoassays using antisera 2604, which is specific for carboxyamidated gastrin and cholecystokinin and is less sensitive toward amino acid substitutions in the gastrin-17 sequence (29). Thus, radioimmunoassay using antibody 2609 is highly specific and has been used in previous mutational analysis of gastrin biosynthesis, including mutations in the polyglutamate region of the gastrin-17 fragment (30). Radioimmunoassays were performed as described in Ref. 30.

Nomenclature—The nomenclature used in this study is as follows (see Fig. 1 for residue numbering and progastrin fragment references). Any carboxyamidated fragment is denoted as gastrin and numbered after the length of the peptide fragment, e.g. gastrin-34. Other fragments are numbered after the numbering in residues. A construct is named after the structure, e.g. progastrin-(1–74)/SAQSQ and denotes a mutant having a wild type progastrin fragment of the first 74 residues followed by the sequence AAQSQ. Mutations in dibasic cleavage motifs are denoted with the exact amino acid substitutions. Lastly, mutations in the polyglutamate region of the gastrin-17 fragment are named after the structure of the polyglutamate stretch. Hence, gastrin-(1–72)-SESEES denotes a mutant having the five glutamates (EEE-EEE, see Fig. 2) substituted with SSEES and having a deletion of the C-terminal flanking peptide.

RESULTS

The N-terminal Part of Progastrin Is Not Necessary for Sorting to the Regulated Secretory Pathway—In several prohormones, sorting domains necessary for intracellular localization to the regulated secretory pathway are localized in the N-terminal region of the prohormones. As part of the N-terminal end of progastrin is well conserved during evolution, we did N-terminal truncations to analyze whether this region is necessary for sorting to the regulated secretory pathway. We performed sequential N-terminal truncations of 10 residues of progastrin (Fig. 1), with the exception of the very N-terminal seryl residue of progastrin, which was kept intact to ensure correct removal of the signal peptide. The constructs were then transiently transfected into the Syrian hamster β-cell line, HIT-T15, and cells and media were analyzed for gastrin expression using a sequence specific gastrin radioimmunoassay.

Expression of human progastrin mutants having sequential N-terminal truncations of 10 residues (see Fig. 1)

| Cellular concentration of amidated gastrins | Media concentration of amidated gastrins | Secretion ratio |
|--------------------------------------------|------------------------------------------|-----------------|
| pmol/liter                                 | pmol/liter                                |                 |
| Wild type gastrin                         |                                          |                 |
| 3987 ± 975                                | 4988 ± 972                                | 1.04 ± 0.03     |
| Wild type gastrin + IBMX                   |                                          |                 |
| 1499 ± 65                                 | 7469 ± 384                                | 5.00 ± 0.27     |
| Δ10-progastrin                            |                                          |                 |
| 2088 ± 191                                | 2148 ± 208                                | 1.03 ± 0.01     |
| Δ10-progastrin + IBMX                      |                                          |                 |
| 1040 ± 29                                 | 3273 ± 200                                | 3.15 ± 0.19     |
| Δ20-progastrin                            |                                          |                 |
| 2379 ± 87                                 | 2131 ± 74                                 | 0.90 ± 0.03     |
| Δ20-progastrin + IBMX                      |                                          |                 |
| 938 ± 126                                 | 3657 ± 164                                | 4.14 ± 0.64     |
| Δ30-progastrin                            |                                          |                 |
| 1039 ± 37                                 | 1359 ± 157                                | 1.32 ± 0.19     |
| Δ30-progastrin + IBMX                      |                                          |                 |
| 635 ± 22                                  | 2887 ± 117                                | 4.52 ± 0.16     |
| Δ40-progastrin                            |                                          |                 |
| 46 ± 6                                    | 0                                        | 0.00            |
| Δ40-progastrin + IBMX                      |                                          |                 |

Figure 1. Structure of human progastrin, main gastrin forms, and schematic diagram of N-terminal truncations. The structure of human progastrin is shown with indications of the main proteolytic processing sites (open bar). Amino acids are numbered from the 1st residue of progastrin. The main bioactive, carboxyamidated gastrins, gastrin-17 and gastrin-34, are shown above progastrin (black bars). The C-terminal end of progastrin, CTFP, denotes the C-terminal flanking peptide. The gray bars show the truncated progastrins examined.
We analyzed the formation of the bioactive carboxyamidated gastrins and the ability to secrete these peptides in response to external stimuli, two hallmarks of the regulated secretory granules. To analyze regulated secretion, we used IBMX because this had proved to be the most potent secretagogue in previous experiments with HIT-T15 cells (28). Table I shows that truncation of the 30 most N-terminal residues of progastrin had little effect on the formation of carboxyamidated gastrin and the ability to increase secretion of amidated gastrin in response to IBMX stimulation. In contrast, removal of an additional 10 N-terminal residues severely reduced expression of carboxyamidated gastrins. These data show, on the one hand, that the N-terminal region is not necessary for formation of gastrin, and on the other hand, that the 49 most C-terminal residues are sufficient to express the active peptide and to confer responsiveness to external stimuli. We therefore concluded that the 49 C-terminal residues contain the motifs for sorting to the regulated pathway. Fig. 2 shows the structure of this 49-amino-acid human progastrin fragment aligned to the corresponding sequences of other mammalian gastrins. The fragment consists of the gastrin-34 sequence flanked by N- and C-terminal sequences, and the alignment shows that this progastrin fragment is evolutionarily well conserved. Conservation of the bioactive gastrin-17 is expected, but because the C-terminal flanking peptide is also well conserved, we performed C-terminal truncation studies to examine contributions to the intracellular sorting of this region.

The C-terminal Flanking Peptide of Progastrin Is Important for Regulated Secretion of Gastrin—Truncation from the C-terminal part of progastrin was performed by removal of the last 8 C-terminal residues (Fig. 2, denoted as C-terminal flanking peptide). The construct was then expressed in HIT-T15 cells, and cellular and culture media concentrations of gastrin were analyzed. The secretion ratio of wild type gastrin and the truncated progastrin-(1–72) was calculated as the media-to-cell-content ratio. Truncation of the 8 most C-terminal residues (progastrin-(1–72)) resulted in an increase in the basal secretion ratio of gastrin, suggesting constitutive-like secretion (Fig. 3). This again suggests that the C-terminal flanking peptide contains an active sorting domain. Inspection of the 8 residues disclosed, apart from the dibasic proteolytic cleavage site, an evolutionarily conserved motif of acidic residues (Fig. 2). These were point-mutated in progastrin to give progastrin-(1–74)-SAQNQN, which substitutes the acidic residues with neutral residues but still has 8 C-terminal residues in the C-terminal flanking peptide. The secretion ratios of these mutants were similar to that of the wild type, suggesting that these residues are not involved in prohormone sorting. If just the last 6 residues were truncated, leaving the dibasic motif at the C terminus, this construct, progastrin-(1–74), showed a secretion ratio similar to that of wild type gastrin (Fig. 3). This suggests that the basic residues of the C-terminal proteolytic processing site serve as a sorting domain in progastrin.

Dibasic Processing Sites Are Mediating Progastrin Sorting—Finding effects of the sorting of the dibasic motif of the C-terminal flanking peptide prompted us to examine the effects of all three dibasic cleavage sites of progastrin (Fig. 1). First, we did point mutations of the dibasic cleavage sites at Arg-36–Arg-37 and Lys-53–Lys-54 to Asn-36–Asn-37 and Gln-53–Ser-54, respectively, and expressed this construct in HIT-T15 cells. The secretion ratio of amidated gastrins from this construct is shown in Fig. 4A. Basal secretion ratios of gastrins are affected by the double mutations by 2-fold, but mutant gastrins are still responsive to external stimuli, although the response is weaker. To examine the effect of all three dibasic cleavage sites, we simultaneously mutated all 3 basic residues by mutating Arg-73–Arg-74 to Asn-73–Asn-74 in addition to the two N-terminal dibasic cleavage sites. This construct was expressed and analyzed using a different assay than the gastrin assay because the mutant is incapable of processing progastrin to carboxyamidated gastrins. This assay utilizes an antisem that recognizes the N terminus of gastrin-52, a fragment released by cleavage at Arg-19 of progastrin, but which cross-reacts with progastrins unprocessed at this site. Thus, this assay would monitor the smallest mutant derivative of the progastrin mutant, progastrin (Asn-36–Asn-37, Gln-53–Ser-54, Asn-73–Asn-74). The secretion ratio of this construct is shown in Fig. 4B. Basal secretion ratios of the triple mutant showed a further increase when compared with the mutant lacking the two N-terminal processing sites and a 5-fold in-

**Fig. 2. Alignment of C-terminal parts of mammalian progastrins.** Primary amino acid sequences of mammalian progastrins were aligned using Clustal W. Identical residues are marked by asterisks, and one or two dots denote a lower or higher degree of conservation in amino acid substitutions. The residues of gastrin-17 and the C-terminal flanking peptide are boxed, and the motifs examined in this study are marked in gray boxes.
creases when compared with wild type gastrins. However, the fragments still responded to IBMX stimulation, suggesting that mutant peptide is still at least partially sorted to the regulated secretory pathway.

The Influence of Mutations in the Polyglutamate Motif of Gastrin-17 on Prohormone Sorting—Protein aggregation is a common theme in the sorting of peptide hormones and their precursors to secretory granules. A recent report has shown that a motif within a polyglutamate stretch of gastrin-17 (Glu-61–Glu-65) binds divalent cations (31). We wondered whether this sequence could mediate aggregation and thereby improve sorting to the regulated pathway. We therefore mutated the polyglutamate stretch and analyzed the expression of these constructs in HIT-T15 cells (Fig. 5A). Mutants, named after mutations in the 5 glutamates residues, showed that the basal secretion ratios of the mutants were similar to that of wild type progastrin. To further analyze any effect, we made two double mutants with mutations within the polyglutamates and truncations of the C-terminal flanking peptide. One double mutant, progastrin-(1–72)-SSESS, had 4 polyglutamates substituted with seryl residues, whereas the other, progastrin-(1–72)-SSEES, had 3 gultamates substituted. We also performed parallel transfections of single and double mutants with and without stimulation (Fig. 5B). Secretion ratios of both stimulated and unstimulated progastrin-SSESS and progastrin-SSEES were identical to those of wild type progastrin. However, when both mutants were C-terminally truncated, the basal secretion was highly increased, and the progastin-(1–72)-SSESS mutant did not respond to IBMX. Similarly, progastrin-(1–72)-SSEES only showed a weak response to secretagogue. Thus, neither of the double mutants are correctly sorted to the regulated secretion pathway, but are constitutively secreted.

DISCUSSION

To mature and be secreted in response to appropriate external stimuli, prohormones need to be sorted to the regulated secretory pathway. The mechanism of this sorting event is not completely understood because different prohormones use different sorting mechanisms and because different cell lines may have different machinery for sorting into the regulated secretory pathway (7). In this study, we provide evidence that human progastrin have sorting domains localized in the C-terminal region of the prohormone. Moreover, our study indicates that more than one domain is active in sorting so that progastrin can be sorted by synergistic mechanisms.

N-terminal deletions showed that a signal peptide in combination with the 49 C-terminal residues is sufficient to direct progastrin to the regulated secretory pathway. Additional truncation of progastrin abolishes gastrin synthesis, suggesting that the peptides are degraded rather than sorted to the regulated pathway. A general feature of prohormones is that the precursors are considerably larger than the active hormones. It has been speculated that long polypeptides are necessary to direct the prohormones through the secretory pathway (32), and our studies suggest that the prohormones indeed require a length of 40–50 residues to prevent degradation in the secretory pathway. Generally, prohormones are larger than this size. The smallest prohormone we are aware of is proneuromedin B, which is 52 residues in size (33). Together, these data suggest that the minimal size of a secreted peptide in endocrine cells will be about 50 residues.

Further analysis of progastrin sorting was carried out by C-terminal deletions and specific point mutations. To assay the sorting of the peptides, we measured the intracellular concentration and related that to the media concentrations of transfectected cells. This ratio is the sorting index of the mutants. Moreover, as a characteristic of the regulated granules, we examined the ability to respond to external stimuli, in this case the secretagogue IBMX. Because our experiments were carried out as steady state experiments, the sorting ratio is a relative index, and in order for these results to be constitutively secreted, we expected secretion to be independent of IBMX stimulation. To get an idea of the sorting ratio in constitutive secretion, we expressed progastrin in Chinese hamster ovary and COS cells, which lack a regulated secretory pathway, and found the secretion ratio of unprocessed progastrin in these cell lines to be 10–15 (data not shown). This ratio may, however, vary between different cell lines. C-terminal deletions and point mutations showed that the 2 basic residues located in the C-terminal flanking peptide in particular affected the secretion
ratio. Similarly, we found that substitutions in the dibasic cleavage sites, Arg-36–Arg-37 and Lys-53–Lys-54, influence secretion ratios. However, although basal secretion ratios were increased when all three dibasic sites were substituted, we still observed a response to secretagogue stimulation, suggesting residual sorting to regulated granules. We therefore analyzed the influence of a motif of 5 glutamic acid residues within the gastrin-17 sequence and found no effect of these residues per se. However, when substitutions in the acidic motif were combined with C-terminal truncations, the mutant peptide was mis-sorted to the constitutive secretory pathway. The synergistic effects of the two types of domains suggest that they contribute to sorting through different mechanisms.

One mechanism depends on basic residues for sorting. Several prohormones and proteins have recently been shown to use dibasic sites as sorting domains. Examples are prosomatostatin (34), prorenin (35), proneurotensin (24), proneuropeptide Y (23), and prohormone convertase 1/3 (36). The exact mechanism behind sorting mediated by basic residue is unknown, but it is noteworthy that interactions between propeptides and their processing enzymes may by themselves mediate sorting to the regulated pathway. Such a mechanism has already been proposed for the sorting of proopiomelanocortin by carboxypeptidase E (13). In that case, however, contact is apparently independent of both the catalytic site of the enzyme and the target residues of the substrate (15). Alternatively, basic residues could interact with acidic groups of the phospholipids of the membrane (37) or lipid rafts, as recently suggested (36). Considering the growing number of propeptides that get sorted by basic residue motifs, these possibilities will be subject for future studies.

The other mechanism by which progastrin can get sorted depends on a specific domain of acidic residues that is located within the gastrin-17 sequence. This region is highly conserved in mammalian gastrins, but the only known function of the stretch of 5 glutamates is influencing tyrosine sulfation of progastrin (27). Tyrosine sulfation is not necessary for biological activity, but it does increase the degree of proteolytic processing at Lys-53–Lys-54 by PC2 (38, 39). However, previous studies have shown that dramatic substitutions can be made in this region without affecting sulfation, suggesting that conservation of this domain is independent of tyrosine sulfation requirements (27). Instead, it has recently been shown that the acidic residues are implicated in binding divalent cations such as Ca2+ and Mg2+ in a mechanism that may facilitate the folding of the propeptide to its mature conformation before it reaches the regulated secretory pathway. This mechanism provides a potential explanation for the differential sorting properties of progastrin mutants, as illustrated in Figure 5B.
as ferric ions (31). Thus, two ferric ions are bound by Glu-62 and Glu-63–Glu-64, respectively. In relation to our mutants, this implies that progastrin-SSEES can bind a single ferric ion, whereas progastrin-SSESS is incapable of binding ferric ions. When expressed as double mutants lacking the C-terminal flanking peptide, the former mutant is a weak responder to secretagogue and the latter does not respond, suggesting that ion binding indeed may play a role in progastrin sorting. This finding is potentially important for understanding of another well described mechanism of sorting propeptides to the regulated pathway, protein aggregation. It is well known that binding of zinc ions is important for storage of insulin in pancreatic secretory granules (40), and calcium ions are important for aggregation of members of the gramin family (41–43). We therefore suggest that the stretch of 5 glutamates in progastrin may contribute in a similar manner to the aggregation of progastrin. A similar mechanism was described in the sorting of atrial natriuretic peptide to secretory granules. In this case, sorting is mediated by aggregation that requires a diacidic motif in the proregion of the propeptide (44).

Two models exist to describe the mechanism behind sorting to the regulated secretory pathway, the sorting for entry and the regulated secretory pathway. The latter does not respond, suggesting that ion binding indeed may play a role in progastrin sorting. This implication that progastrin-SSESS can bind a single ferric ion, whereas progastrin-SSEES is incapable of binding ferric ions.

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