Distinct Linkage between Post-translational Processing and Differential Secretion of Progastrin Derivatives in Endocrine Cells*

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Prohormones often undergo extensive cellular processing prior to secretion. These post-translational processing events occur in organelles of the constitutive or regulated secretory pathway. The aim of this study was to examine the relationship between post-translational modifications and the secretory pathways taken by peptides derived from progastrin, the prohormone of gastrin, which in vivo is secreted by cells of the pyloric glands and stimulates the release of gastric acid. Targeting progastrin to compartments of the early secretory pathway shows that endoproteolytic processing is initiated in a pre-trans-Golgi network compartment of endocrine but not non-endocrine cells. The resulting N-terminal fragments of progastrin are secreted via the constitutive pathway, whereas endoproteolytically processed C-terminal fragments are secreted via the regulated or constitutive-like pathways. C-terminal fragments derived from progastrin differ in characteristic manners in levels and patterns of carboxyamidation and tyrosine sulfation in accordance with the secretory pathway taken. Point mutations introduced into a sorting motif disrupt these patterns, suggesting that differences in post-translational modifications are attributable to differential intracellular sorting of precursors. The results suggest a two-step sorting mechanism for progastrin leading to differential secretion of processed fragments via different secretory pathways.

Specialized secretory cells, such as neurons, endocrine, and exocrine cells, contain two classes of secretory vesicles that follow different intracellular pathways (1). Both classes of vesicles are formed in the trans-Golgi network (2) and differ in their mode of secretion. Constitutive vesicles release their cargo in an unregulated fashion, whereas mature secretory granules respond to a specific extracellular signaling. The vesicles carry different cargo thus allowing differentiated secretion of cellular products.

The secretory granules are initially formed as immature granules (3) that undergo maturation by removal of redundant material (for review, see Refs. 4, 5). Accordingly, selection occurs both in the TGN2 and in the immature secretory granules (4, 6). The “sorting for entry” model implicates that the TGN is the segregation point for secretory proteins, whereas the “sorting by retention” model ascribes this function to immature secretory granules. In endocrine cells, prohormones are targets for selection, because the processing to active hormones requires modifications by enzymes mainly active within the mature granules (7). These modifications include endoproteolytic cleavage by prohormone convertases, side-chain modifications, and carboxyamidation.

The mechanism behind sorting of secretory proteins has been debated in recent years (for reviews, see Refs. 4, 6, 8–12). It is generally accepted that cargo proteins, irrespective of sorting method, have to interact with a “receptor” that facilitates segregation. Such receptor function has been ascribed to specific membrane-associated proteins, e.g. the processing enzyme carboxypeptidase E (13), but could also be mediated by lipid rafts (14). Alternatively, aggregation and co-aggregation with membrane-attached granins may function in the process (for review, see Ref. 9). Irrespective of the mechanisms, sorting motifs are present in protein cargo to facilitate correct segregation.

Gastrin is a gastrointestinal hormone that regulates gastric acid secretion and growth of the gastric mucosa (for reviews, see Refs. 15, 16). Human progastrin is an 80-amino acid protein that undergoes multiple post-translational modifications to release bioactive peptides (Fig. 1). In the trans-Golgi network, progastrin is partially tyrosine sulfated and in the secretory granules, progastrin is cleaved by the prohormone convertases PC1/3 and PC2 (17). Processing at the dibasic site Arg73-Arg74 followed by removal of residual basic residues by carboxypeptidase E generate the immediate gastrin precursor, often referred to as glycine-extended gastrin, a progastrin fragment suggested to have independent growth-stimulating effects (18). Subsequent carboxyamidation by the peptidylglycine α-amidating monoxygenase (PAM) complex completes the activation of gastrin in terms of ability to stimulate gastric acid secretion. However, even in the gastrin-producing G cells progastrin processing is incomplete, resulting in the occurrence of various processing intermediates. In a recent study we showed that two

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The abbreviations used are: TGN, trans-Golgi network; PC1/3, prohormone convertase 1/3; PC2, prohormone convertase 2; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; ERGIC, endoplasmic reticulum-Golgi intermediate compartment; PAM, peptidylglycine α-amidating monoxygenase complex.
synergistically acting motifs in progastrin mediate sorting to the secretory granules. The most important motif consists of the dibasic sequences, which are also endoproteolytic cleavage sites. Another motif is a stretch of acidic residues close to the active site of gastrin (Fig. 1) (19).

In the present study, we have analyzed the relationship between intracellular sorting and post-translational processing. To do so we examined the processing of a membrane-bound chimera progastrin molecule in an early secretory compartment. We also examined differences in secretion of products at various stages of the post-translational activation and found that the products are differentially secreted. Moreover, combining these findings with the effect of mutations in a progastrin sorting motif on tyrosine sulfation allows us to propose a two-step sorting model of progastrin to the mature secretory granules.

**EXPERIMENTAL PROCEDURES**

**Progastrin-processing Nomenclature**—The nomenclature of progastrin-derived peptide fragments in this study follows a recent proposal (20). Human and mouse progastrin is an 80-amino acid residue precursor that is endoproteolytically cleaved at dibasic sites. Of these, cleavage at Arg73-Arg74 is necessary for bioactivation (Fig. 1). After cleavage here, residual C-terminal basic residues are removed by carboxypeptidase E leading to N-terminally truncated derivatives of progastrin. The progastrin fragments with a C-terminal glycyl residue (Gly73) are the immediate precursors of bioactive, carboxyamidated gastrins. The sequence of the main product of antral G-cells, gastrin-17, is shown. The residues discussed are shown in **bold italics**, and the three acidic residues that constitute a sorting signal for the mature granules are **underlined**.

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**Cell Culture and Transfections**—The Syrian hamster β-cell line, HIT-T15 (ATCC), was cultured in Dulbecco’s modified Eagle’s medium (with Glutamax and 5 mM glucose) supplemented, with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen), as previously described (21). The cultures were incubated at 10% CO₂ and 37 °C. Cells were split 1 day prior to transfections using phosphate-buffered saline-added EDTA.

**Gene Constructions**—Human gastrin expression vectors were as previously described (21). The ERGIC-progastrin constructs (Fig. 2A) were constructed by ligation of a double-stranded oligonucleotide to the 3′-end of the gastrin gene. This consists of an EcoRII-linker followed by a sequence encoding the last 35 amino acids of the rat ERGIC-53 (22). The sequence includes a 5-amino acid residue spacer, the transmembrane domain, and the entire cytosolic domain, including the KKFF retention motif. Cyan fluorescent protein (CFP) was cloned in the translation frame into the construct using the EcoRI site. The ER and Golgi markers were YFP-expressing vectors obtained from Clontech.

**Expression Studies in Cell Culture**—Construction of gastrin and gastrin mutant vectors and expression of these in HIT-T15 cells have previously been described (21, 23). Expression was transient, using a modified calcium-phosphate transfection procedure. Cells were harvested 3 days after transfection for peptide analysis.

**Microscopy**—For microscopy, cells were seeded and transfected on glass plates at a density of 30,000 cells/cm². Cells were co-transfected using FuGENE (Roche Applied Science) with YFP- and CFP-labeled ERGIC-progastrin construct and organelle marker, respectively. Forty-eight hours after transfection, living cells were analyzed using a Zeiss LSM 510 confocal laser scanning microscope.

**Preparations of Cell Extracts and Chromatography**—At harvest, cells were pelleted and stored at −20 °C until extraction. Peptides were extracted by boiling cell pellets in 1 ml of water for 30 min. Cell debris was removed by centrifugation and extracts stored at −20 °C until chromatography.

Anion-exchange chromatography was performed using a fast protein liquid chromatography system with a Mono Q column (Amersham Biosciences), as previously described (23). Gel filtration was as previously described (21).

**Radioimmunoassay**—Sequence-specific radioimmunoassays for human progastrin and its processing products were used, as previously detailed. Carboxyamidated gastrins were measured in a radioimmunoassay using ab.2609, which recognizes tyrosine sulfated as well as non-sulfated gastrins (24). The glycine-extended gastrins were measured using a radioimmunoassay based on ab.7270 (25). To verify the identity of peaks after anion-exchange chromatography, fractions were also measured with radioimmunoassays using ab.2145 and ab.8017, which are specific for the N-terminal sequences of gastrin-34 and gastrin-17, respectively (26, 27). N-terminal progastrin fragments were measured in a radioimmunoassay using ab.94023 raised against the N terminus of human progastrin (31).

**Sulfation Ratios**—Sulfation ratios, defined as percent sulfated of total amount of peptide, were determined on the basis of chromatograms, as previously described (23, 28). The two radioimmunoassays used for determination of sulfation were ab.2609 and ab.7270. During calculation, differences in cross-reactivity of the antisera were taken into account. Thus, ab.2609 cross-reacts 131% with sulfated gastrin-17, but only 63% with sulfated gastrin-34. Likewise, ab.7270 cross-reacts 133% with

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FIGURE 1. A schematic diagram of the human progastrin structure. Progastrin-(1–80) is shown, including the major proteolytic processing sites. CTPF denotes the C-terminal flanking peptide. The black bars show the major circulating carboxyamidated, gastric acid-releasing gastrins. The sequence of the most important motif consists of antral G-cells, gastrin-17, is shown. The residues discussed are shown in **bold italics**, and the three acidic residues that constitute a sorting signal for the mature granules are **underlined**.
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### Results

#### Early Processing Fragments Are Constitutively Secreted

To investigate the initiation site of proteolytic processing, we constructed a chimera of progastrin and the C-terminal domain of the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) marker, ERGIC-53. This gene construct, denoted ERGIC-progastrin, contains the entire progastrin sequence followed by an ERGIC-53-derived transmembrane domain, including a KKFF retention motif that prevents further progression within the secretory pathway (Fig. 2A). Any secretion of fragments from this membrane-anchored protein requires proteolytic processing, and secreted fragments are thus indicators of proteolytic processing in an early secretory compartment. The β-cell line HIT-T15 was transiently transfected with ERGIC-progastrin or with wild-type progastrin, and cells and culture media were analyzed for progastrin-derived peptides. Radioimmunoassays using antisera specific for the N terminus of progastrin and for carboxyamidated gastrins showed that both ERGIC-progastrin and wild-type progastrin were well expressed, and that N-terminal fragments from ERGIC-progastrin were secreted into the media as a result of proteolytic processing (Table 1). However, only small amounts of carboxyamidated gastrins were measured after expression of ERGIC-progastrin. To determine the pathway that secreted N-terminal fragments, we stimulated secretion from the cells using 3-isobutyl-1-methylxantine as secretagogue (Fig. 2B). In the case of ERGIC-progastrin, secretion of N-terminal fragments did not respond to the secretagogue, suggesting that all N-terminal fragments were secreted constitutively. In contrast, when transfected with wild-type gastrin, the secretion of both N-terminal fragments and fully processed carboxyamidated gastrins increased in response to 3-isobutyl-1-methylxantine, although basal secretion of N-terminal fragments was higher. The results show that more N-terminal fragments are secreted constitutively-like than the carboxyamidated gastrins. In addition, N-terminal fragments are released constitutively when originating from processing in a pre-sorting compartment. Chromatography of the media using the N-terminal-specific antisera showed that secreted fragments arise from cleavage at Arg19 and/or Arg36-Arg37 in progastrin (Fig. 2C).

#### Early Processing Fragments Localize to an Early Secretory Compartment

Finally, we examined whether early processing was specific for endocrine cells. In Chinese hamster ovary cells transfected with the ERGIC-progastrin construct we were unable to detect secreted products, although high level expression could be detected within the cells (data not shown). This finding suggests that early processing was specific for endocrine cells.

**TABLE 1**

| Carboxyamidated Gastrins | N-terminal fragments |
|--------------------------|----------------------|
| Cells | Media | Cells | Media |
| Wild-type progastrin | 1489 ± 179 | 1718 ± 112 | 1592 ± 224 | 4150 ± 477 |
| ERGIC-progastrin | 35 ± 6 | 89 ± 5 | 937 ± 175 | 258 ± 31 |

Sulfated, glycine-extended gastrin-17-Gly and 78% with gastrin-34-Gly. Statistical analysis was performed using an unpaired t test, with or without Welch’s correction using Prism 4.0 (GraphPad, Inc.).
TGN-specific markers were analyzed by confocal microscopy (Fig. 3). Overlays demonstrated that ERGIC-progastrin is co-localized with the ER marker, whereas no overlap was found with the TGN marker. This suggests that the processing occurs in an early pre-TGN compartment of the secretory pathway. To rule out that the lack of processing at Arg73-Arg74 was due to steric hindrance due to the close proximity to the transmembrane domain, peptide analysis was performed on the CFP-ERGIC-progastrin fusion, which showed an identical pattern to that of ERGIC-progastrin (data not shown).

Carboxyamidated Gastrins Are Secreted Differently from their Immediate Precursors—Because secretion of N-terminal wild-type progastrin fragments responded to secretagogue but displayed a higher basal secretion than carboxyamidated gastrins, we examined whether other fragments of the C-terminal part of progastrin were also differently secreted. Fig. 4 shows that the immediate precursors of the bioactive carboxyamidated gastrins, the glycine-extended gastrins, were secreted at a higher basal level than the carboxyamidated forms. Like the N-terminal fragments from wild-type progastrin, secretion of the glycine-extended gastrins responded to the secretagogue, but the N-terminal fragments aresecreted at a higher basal level than that of glycine-extended gastrins. Thus, the glycine-extended gastrins are secreted in a more constitutive-like manner than the carboxyamidated gastrins.

Sulfation Stoichiometry Varies between Progastrin Derivatives—As the progastrin fragments appear to be differentially secreted, we examined whether differences in intracellular trafficking would influence other post-translational processing events. Tyrosine sulfation is an early modification that occurs in the TGN. Therefore, we compared the sulfation of the completely processed, carboxyamidated forms with the sulfation of glycine-extended gastrins.

To analyze the sulfation stoichiometry of progastrin derivatives, human progastrin was transiently expressed in HIT-T15 cells. This cell line processes progastrin into gastrin-17 and gastrin-34 as well as into glycine-extended gastrin-17 and -34 at high levels. Extracts of transfected cells were subjected to anion-exchange chromatography (Fig. 5). From the chromatography, different progastrin-derived fragments were quantified, and the sulfation stoichiometry was determined (Fig. 6A).

The quantification showed that glycine-extended gastrin-17 was sulfated to a significantly higher degree than both glycine-extended gastrin-34 (p < 0.002) and carboxyamidated gastrin-17 (p = 0.014). In contrast, the sulfation ratio of gastrin-17 was similar to that of gastrin-34 (p = 0.2160).

Sulfation Pattern in Alanine Scan of the Progastrin Polyygalutamate Motif—In previous studies we have examined the sulfation stoichiometry of carboxyamidated fragments of progastrin mutants substituted in the primary structure surrounding the sulfation site (23). We now analyzed these mutants for the individual sulfation stoichiometry of gastrin-17 and -34, as well as their precursor forms, glycine-extended gastrin-17 and -34.
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A 

Carboxyamidated gastrins

| Fraction number | pmol/l | Carboxyamidated gastrins |
|-----------------|--------|--------------------------|
| 0               | 0      | gastrin-17 (ns)           |
| 5               | 0      | gastrin-34 (ns)           |
| 10              | 0      | gastrin-17 (s)            |
| 15              | 0      | gastrin-34 (s)            |

B 

Immediate gastrin precursors

| Fraction number | pmol/l | Immediate gastrin precursors |
|-----------------|--------|-----------------------------|
| 0               | 0      | gastrin-17-gly (s)           |
| 5               | 0      | gastrin-34-gly (ns)          |
| 10              | 0      | gastrin-17-gly (s)           |
| 15              | 0      | gastrin-34-gly (ns)          |

FIGURE 5. A representative anion exchange chromatogram of cell extracts from HIT-T15 cells transfected with a vector expressing the human gastrin gene analyzed with specific antisera. Panel A shows the elution profile of carboxyamidated gastrins, whereas panel B shows the glycine-extended gastrins. The different progastrin products were quantified taking into account the varying cross-reactivities of the antisera. "(s)" denotes sulfated peptide, and "(ns)" the non-sulfated forms.

On the N-terminal site of the sulfation target, at Tyr\(^{66}\), human progastrin has a characteristic sequence of five glutamates (Fig. 1). Except for the substitution of the most proximal residue to the tyrosine, Glu\(^{64}\), an alanine scan of these acidic residues revealed only small changes in the overall sulfation ratio of carboxyamidated mutant forms (23). Peptides from the Glu\(^{64}\) mutant are highly sulfated, and therefore the major forms display only little difference in sulfation (Fig. 6B). Substitution of Glu\(^{63}\) with alanine reduces the overall sulfation slightly, but the individual forms all appeared equally sulfated, and the characteristic pattern of sulfation differences of wild-type gastrin processing products was absent (Fig. 6C). Similar patterns were found in substitutions of Glu\(^{62}\) and Glu\(^{64}\) (Fig. 6, D and E). However, when Glu\(^{66}\) was substituted with alanine, the sulfation levels were similar to that of wild-type gastrins, and the characteristic sulfation pattern was re-established (Fig. 6F).

Thus, the AEEEEAY glycine-extended gastrin-17 was sulfated to a significantly higher degree than both AAEEEAY glycine-extended gastrin-34 and the carboxyamidated AEEEEAY-gastrin-17 (\(p = 0.002\) and \(p = 0.008\), respectively). In addition, AAEEEAY-gastrin-17 was more sulfated than AEEEEAY-gastrin-34 (\(p = 0.001\)). Therefore, the alanine scan showed that substitution of Glu residues 61–63 within the polypeptide stretch affects the characteristic sulfation pattern of the major gastrin forms. This again suggests that there are important differences in the processing of the mutants.

DISCUSSION

The aim of this study was to examine the relationship between post-translational processing steps and intracellular transport of the different products of a prohormone, progastrin. The results show that N-terminal cleavage of progastrin is initiated early in the secretory pathway. The early cleavages lead to constitutive secretion of progastrin-(1–19) and progastrin-(1–35). The results also show that glycine-extended intermediate products are constitutively secreted to a higher degree than the completely processed carboxyamidated gastrins. Finally, the tyrosine sulfation pattern of progastrin derivatives mutated in one of two sorting motifs showed differences in processing that result from differences in the intracellular transport.

N-terminal Processing Begins in the Early Secretory Pathway—Peptide hormones require processing for activation. One of the most common processing events is endoproteolytic cleavage by prohormone convertases (29). Of these, PC1/3 and PC2 are the most important in endocrine cells and neurons. PC2 is mainly active in mature secretory granules at an acidic pH, whereas PC1/3 is active earlier in the secretory pathway at a neutral pH (7). To examine possible processing of progastrin in the early secretory pathway, we constructed a chimera of progastrin and the ER-Golgi-intermediate compartment marker, ERGIC-53. The C-terminal part of the chimera contains the transmembrane domain and the C-terminal KKFF retention signal of ERGIC-53 that secures ER exit and retains ERGIC-53 in the early secretory pathway (30). Because the ERGIC-progastrin chimera is membrane-attached, secretion of progastrin fragments after expression requires proteolytic cleavage. Expression analysis of ERGIC-progastrin in endocrine cells showed that N-terminal fragments were secreted and hence, that significant endoproteolytic cleavage did occur. Using a radioimmunoassay against the N terminus of progastrin on chromatographic fractions showed that endoproteolytic processing had occurred at both mono (Arg\(^{17}\)) and dibasic (Arg\(^{26}\)-Arg\(^{27}\)) sites and that processing is specific for endocrine cells.

To examine the localization of ERGIC-progastrin, another chimera, with CFP fused between the progastrin and ERGIC-53 domains, was constructed. Expression showed that ERGIC-progastrin mainly localizes to the ER. This suggests that the processing observed occurred in a pre-TGN compartment of the secretory pathway.

Different Progastrin Products Are Differentially Secreted—In wild-type gastrin, secretion of both carboxyamidated gastrins and N-terminal fragments increased in response to a secretagogue, but the basal secretion of N-terminal fragments was significantly (~3-fold) higher than for the amidated forms. In contrast, ERGIC-progastrin N-terminal processing products did not respond to secretagogue stimulation. We therefore conclude that N-terminal fragments released in the early secretory pathway, as in the case of ERGIC-progastrin, are constitutively secreted. This is in agreement with the finding that the N-ter-
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We also compared the secretion of glycine-extended gastrins with that of the carboxyamidated gastrins. The basal secretion of wild-type glycine-extended gastrin was higher than that of amidated gastrins (~1.5-fold), but they still responded strongly to secretagogue. Thus, the results suggest that the three different classes of processing products of progastrin are secreted in a differentiated manner.

Peptide Segregation in the Regulated Secretory Pathway Affects Gastrin Sulfation—Further examination of the relations between processing and intracellular localization included studies of tyrosine sulfation of the progastrin products that enter the regulated secretory pathway, i.e. the carboxyamidated gastrins and the glycine-extended precursors. The results revealed a sulfation pattern of wild-type gastrin in which the glycine-extended gastrins are more sulfated than the corresponding carboxyamidated forms. Previously, a similar difference was observed in rat antral tissue (32), whereas identical sulfation ratios were reported in the hog (33). In addition, shorter forms, gastrin-17 and glycine-extended gastrin-17, are more sulfated than the longer forms. This confirms previous findings that tyrosine sulfation influences the cleavage at Lys53-Lys54 (34), catalyzed by PC2 (17). That study, however, did not discriminate between carboxyamidated and glycine-extended gastrins (34).

The observation of differences in tyrosine sulfation of the amidated and the glycine-extended gastrins is puzzling. Tyrosine sulfation is one of the earliest processing events in the TGN (35), whereas amidation occurs in the secretory granules. Hence, amidation is unlikely to affect sulfation. However, tyrosine sulfation of progastrin is also unlikely to influence carboxyamidation of glycine-extended gastrins by PAM, because this enzyme complex has half of all peptide hormones as substrates and accordingly a broad specificity (36). We therefore assume that the sulfation differences should be ascribed to segregation of amidated and glycine-extended gastrins in the regulated secretory pathway and that tyrosine sulfation continues along the path predominantly followed by the glycine-extended gastrin forms. The most likely point of segregation is the immature secretory gran-
ules, as is further supported by our finding that glycine-extended gastrins are secreted more constitutive-like than the carboxyamidated gastrins. The difference in sulfation of the gastrin suggests that the sulfotransferases are sorted to immature granules, from where they are routed back to the TGN, for instance through constitutive-like secretory vesicles. More glycine-extended gastrins than carboxyamidated gastrins are present in constitutive-like secretory vesicles, and would thus be target for sulfation for a longer period than peptides sorted to the mature granules. Alternatively, the sulfotransferases are present in both the mature granules and the constitutive-like pathway, but the co-substrate needed for sulfate transfer, adenosine 3’-phosphate 5’-phosphosulfate, is excluded from the mature granules during the condensation process of immature granules.

Following this notion, we analyzed the tyrosine sulfation pattern of progastrin fragments of different mutations in a region of gastrin-17, which harbors a sorting signal for regulated secretion (19). The AEEEAY-gastrin mutant derivatives displayed a differentiated sulfation pattern similar to that of wild-type gastrin. In contrast, progastrin derivatives of EEEEAAY-gastrin displayed identical sulfation ratios of all forms due to an increased sulfation in the TGN. Interestingly, sequential alanine scanning of the acidic residues Glu\textsuperscript{61}-Glu\textsuperscript{63} did not increase sulfation compared with wild-type gastrin but displayed an atypical sulfation pattern with identical sulfation ratios of all peptide products.

How does the sulfation pattern of the alanine scan of Glu\textsuperscript{61}-Glu\textsuperscript{63} arise? We recently showed that progastrin is sorted to the regulated secretory pathway by two sequentially acting motifs: the dibasic residues constituting the main sites for endoproteolytic processing and the central acidic residues in the five polyglutamates in the gastrins (19). Regulated secretion was monitored by measurement of both basal secretion and response to a secretagogue, and the two motifs acted in synergy, suggesting temporal separation of the action of the sorting motifs. However, mutagenesis in the acidic motif alone showed little effect on the sorting in terms of changes in secretory response, suggesting that this motif acts when the peptide is already in a secretagogue responding compartment, i.e. the immature granules. We therefore propose that three of the glutamates, Glu\textsuperscript{61}-Glu\textsuperscript{63}, function as a sorting signal and that mutations in this domain will affect the sorting to the mature secretory granules, thereby reducing the segregation of glycine-extended and carboxyamidated forms that was observed in wild-type gastrin.

Processing and Secretion of Progastrin Products Relate to Their Intracellular Transport—Based on the present results, on the knowledge of the location and activity of the processing enzymes on progastrin maturation and on the function of the two sorting domains of progastrin, we can predict the localizations of the different progastrin-processing products (Table 2): N-terminal fragments are secreted via constitutive vesicles, whereas uncleaved forms will be sorted to the regulated pathway by C-terminal signals. Carboxyamidated forms require complete C-terminal processing, including the action of PAM, which acts in the regulated secretory pathway. Accordingly, amidated gastrins can be secreted via the mature or immature secretory granules or, to some extent, via the non-responsive constitutive-like secretory pathway. Constitutive-like secretory granules arise from the condensing process of the immature secretory granules to form mature secretory granules (4). Generally, glycine-extended gastrins are found in the same compartments as the amidated form, but the higher basal secretion suggests that more glycine-extended peptides are secreted via the constitutive-like secretory granules or maybe the constitutive vesicles.

Sorting to the regulated secretory pathway of endocrine, exocrine, and neuronal cells has been extensively studied and reviewed (4, 6, 8, 9, 11, 12). Present models of sorting to the regulated pathway involve a single sorting step. The sorting for entry model suggests sorting from the TGN to immature granules, i.e. from a compartment insensitive to stimulation to a responsive compartment, whereas the sorting by retention model predicts sorting within stimulation-responsive granules. We propose that sorting of progastrin occurs at both levels using different sorting signals.

Two-step sorting of progastrin is not simply a matter of incomplete processing. Secretion via the constitutive-like compartment offers the possibility of independent secretion of different progastrin derivatives. Although glycine-extended gastrins also are secreted in response to external stimuli both in cell lines and in vivo (37), constitutive-like secretion may give a higher basal secretion than the carboxyamidated gastrins. Glycine-extended gastrin has aroused considerable interest as a possible growth factor with mitogenic effects on different tissues and cancers (16). Thus, independent secretion of carboxyamidated gastrin and glycine-extended gastrins might be expedient. Recently, the C-terminal flanking peptide has also been proposed to be bioactive (38). It might be another example of a value of differentiated secretion of progastrin-processing products. It was recently shown that the Rho homologous guanine exchange factors, Kalirin and Trio, can modulate secretion response of the constitutive-like vesicles and the mature granules (39). Thus, secretion of glycine-extended gastrins over gastrins might be subjected to specific regulation.
In conclusion, we present a model of sorting progastrin to the mature secretory granules in two steps involving different sorting motifs. This sorting model may explain the abundance of the glycine-extended gastrins in circulation and, in addition, explain the different sulfation patterns of different progastrin derivatives. It also suggests that tyrosylprotein sulfotransferase activity is not restricted to the TGN but continues through later compartments in the regulated secretory pathway.

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