Processing of the Gastrin Precursor

MODULATION OF PHOSPHORYLATED, SULFATED, AND AMIDATED PRODUCTS*

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Post-translational processing of the precursor for rat gastrin yields products that include peptides phosphorylated at SerE9, amidated at PheE9, and sulfated at TyrE7 or TyrE103. The phosphorylation site is immediately adjacent to the processing point that gives rise to the biologically active amidated gastrins. We have examined changes in post-translational processing which occur in gastrin cells from rats that are physiologically stimulated (by feeding) or unstimulated (by fasting). Peptides were identified using site-directed radioimmunoassays and chromatographic systems that resolve phosphorylated, amidated, and sulfated progastrin products, including intermediates generated prior to amidation (i.e. C-terminal glycine-extended variants). Assays for PheE9-amidated peptides and for the C-terminal tryptic fragment of progastrin indicated decreases in the total tissue concentrations of immunoreactive peptide with fasting; in contrast, the tissue concentrations of glycine-extended biosynthetic intermediates were similar in fasted and fed rats. Taken together, the data suggest a relative failure in amidation mechanisms in unstimulated cells. The endopeptidase cleavage of progastrin was not influenced significantly by fasting. However, the phosphorylation of peptide products containing SerE99 was depressed significantly in fasted rats. The proportions of amidated peptides sulfated at TyrE7 were generally lower than their corresponding glycine-extended biosynthetic precursors, but in both cases the proportion of peptide in the sulfated form was lower than phosphorylated peptides sulfated at TyrE103. Feeding did not change the sulfation of amidated heptadecapeptide gastrin or its glycine-extended variant. The results suggest that the mechanisms determining phosphorylation and amidation of progastrin-related peptides depend on the patterns of stimulation of gastrin cells. The observation that decreased phosphorylation is associated with a failure to produce active amidated products is consistent with a regulatory function for phosphorylation in gastrin production.

The small biologically active peptides that function as extracellular signaling molecules in neuronal, paracrine, and endocrine systems are synthesized initially as large precursors. In addition to the cleavage steps that liberate the active peptides, the final products are frequently modified by sulfation, amidation, phosphorylation, or glycosylation. The pyloric antral hormone gastrin, which regulates acid secretion, provides a convenient model for studies of the relevant mechanisms since the precursor is relatively small (101-104 residues depending on the species) and contains only a single copy of the active sequence (Fig. 1); moreover progastrin-derived peptides are known to be amidated, sulfated, and phosphorylated (1-4). These modifications subserve several important biological functions. Thus, the presence of a C-terminal amide is obligatory for the biological activity of physiologically relevant products (5). Sulfation modifies the biological activity of gastrin on some tissues and also renders it resistant to the action of some proteases (6, 7). The phosphorylation site is of special interest because it is immediately adjacent to the cleavage site that subsequently yields the important biologically active C-terminally amidated forms of gastrin (8, 9).

It is established that both gastrin release and gastrin gene expression are normally regulated by the gastric luminal contents (4). Whether or not the post-translational processing of progastrin is under physiological control is less clear although it has been suggested that progastrin phosphorylation might be of regulatory significance in these processes (8, 9).

Evidence in favor of this idea has come from studies of the relative proportions of phosphorylated and unphosphorylated progastrin, and its cleavage products, in gastrin-producing tumors and in gastrin cells that have been surgically excluded from contact with the normal contents of the stomach (9, 10). In the present series of experiments we have examined the cleavage, amidated, phosphorylated, and sulfated products of progastrin in pyloric antral gastrin cells in rats that are normally stimulated, i.e. by feeding, or unstimulated, i.e. by fasting. We report here that withdrawal of food is associated with a decrease in tissue gastrin concentrations and with depressed amidation and phosphorylation of progastrin-derived peptides. These are not nonspecific changes because other processing steps, e.g. sulfation of G17, were not different in fasted and fed rats.

MATERIALS AND METHODS

Peptides—The C-terminal nonapeptide of rat progastrin (Ser-Ala-Glu-Glu-Asp-Gln-Tyr-Asn) was synthesized by the solid phase method, cleaved from the resin by liquid HF in the presence of anisole and dimethyl sulfide as scavengers, and extracted with aqueous acetic acid (11). Rat unsulfated heptadecapeptide gastrin (G17) was synthesized by UCB Ltd. and made available by Dr. J. Smith (SKF, Ltd.). The C-terminal hexapeptide of rat G17 extended at the C terminus by glycine was provided by Dr. J. Walsh (UCLA). Other standard peptides used for calibration of chromatographic columns were syn-
thet human 34-residue gastrin (G34; UCB Ltd.) and natural human progastrin (progastrin 22-101) (9).

Radioimmunoassays—Peptides with the C-terminal sequence of G17, which includes the sulfated and unsulfated forms of G17 itself, their C-terminal fragments of 8-9 residues or more, and N-terminally extended forms, e.g. G34, were detected by radioimmunoassay using an antibody (L2) with high specificity for C-terminal phenylalanine amide. This antibody shows low affinity for the related peptide cholecystokinin but reacts similarly with all chemically characterized biologically active forms of gastrin (12). The immediate biosynthetic precursor of amidated peptides, i.e. C-terminally glycine-extended forms, were detected with monoclonal antibody 109-2 generously donated by Dr J. Walsh. This was used in radioimmunoassays at a dilution of 1:4.5 million with gastrin-glycine hexapeptide as described previously (13).

Peptides with structures that included the extended C-terminal portion of rat progastrin were identified by radioimmunoassay using either of two new antibodies. These antibodies were raised to Ser-Ala-Glu-Glu-Glu-Asp-Gln-Tyr-Asn conjugated to thyroglobulin as follows. Synthetic nonapeptide (750 nmol) was conjugated to bovine thyroglobulin (2.5 mg) by the addition of glutaraldehyde (50 ml, 5% solution) in 1.3 ml of phosphate buffer (0.1 M, pH 7.4). The mixture was incubated at 22 °C for 90 min and then dialyzed against distilled water (4.5 liters) for 24 h at 4 °C. The incorporation of peptide into conjugate was 83% based on the recovery of a small amount of radiolabeled nonapeptide added to the reaction. The conjugate was emulsified in Freund’s complete adjuvant, and doses equivalent to 40-nmol peptide were given at multiple intradermal sites to five rabbits. Further immunizations with the equivalent of 20 nmol were given at 6-week intervals; the rabbits were bled from an ear vein 7–10 days after the second and successive immunizations.

Radioimmunoassays for C-terminal progastrin fragments were made using antibodies L303 and L304 and Ser-Ala-Glu-Glu-Glu-Asp-Gln-Tyr-Asn labeled with 125I by the IOD0-GEN method as follows. Peptide (1 nmol in 2.5 ml of ammonium bicarbonate) was added to tubes coated with IOD0-GEN by drying 100 ml of a solution of 40 μg.ml−1 in dichloromethane. Phosphate buffer (50 ml, 0.1 M, pH 7.4) was added followed by 250 μCi of Na125I; after 10 min at 22 °C the radiolabeled peptide products were purified by reverse phase high pressure liquid chromatography on a Hypersil 5-μm column (250 x 4 mm) eluted with a gradient of acetonitrile from 0.1% trifluoroacetic acid. This system resolved unlabeled peptide, monoiodolabeled peptide (specific activity, 2,500 cpm-nmol−1), and diiodolabeled peptide (specific activity, 5,400 cpm-nmol−1). For routine radioimmunoassays monoiododiolabeled peptide (2,000 cpm) was used with dilutions of antibody binding approximately 50% of label (L304, 1:5,000; L303, 1:10,000); assays were incubated in 0.09 M phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.75% (v/v) Baumوجn (Ortho Diagnostics Ltd). Antibody bound and free peptide were separated by the addition of Amberlite CG400 type II (200 μl of a suspension of 2 g in 10 ml) and centrifuging (2,000 x g, 5 min). The synthetic nonapeptide was used as radioimmunoassay standard; the concentration giving 50% inhibition of binding of label was 35.3 ± 1.4 pmol-liter−1 (mean ± S.E., n = 10) with L304 and 41.0 ± 3.3 pmol-liter−1 with L303 (n = 8). The results of arylsulfatase digestion indicated that the relative molar potency of the sulfated progastrin-flanking peptide with L304 was 0.65 compared with unsulfated peptide, and appropriate corrections were made in determining the tissue concentrations of C-terminal fragments of progastrin.

Tissue Gastrins—Two groups of rats were studied. One was fed ad libitum on normal rat chow, and the other was deprived of food for 48 h. Both groups had free access to water. Animals were killed by decapitation and trunk blood collected for assay of plasma gastrin. The pyloric antrum was then removed rapidly and frozen on dry ice. The tissues were subsequently weighed while deeply frozen and the gastrins then extracted in boiling water as described previously (9).

Characterization of Rat Progastrin-Derived Peptides—Tissue extracts were fractionated by gel filtration on Sephadex G-50 (1 x 100-cm Superfine) eluted with 0.05 M ammonium bicarbonate and by ion exchange chromatography on DE52 (1 x 16 cm) equilibrated with 0.05 M ammonium acetate, pH 5.6, and eluted with a gradient to 1.0 M ammonium acetate, pH 7.2. Phosphorylated and sulfated immunoreactive peptides were identified by digestion of samples with either alkaline phosphatase (1–2 units, Sigma type III from Escherichia coli) in 0.1 M ammonium bicarbonate, pH 7.3, 4 h, 37 °C or with arylsulfatase (200–400 μg, Sigma type VII from Abalone gut) in 0.2 M sodium acetate, pH 5.0, 2 h, 37 °C (9, 14). Control and digested samples were then fractionated by ion exchange chromatography.

RESULTS

Progastrin Antibodies—Two new antibodies raised to the C-terminal nonapeptide fragment of rat progastrin were generated for this study. These antibodies differed markedly in their pattern of cross-reactivity with native peptides. One, L304, revealed four peaks of immunoreactive material (CFP-1–IV, in order of elution) after ion exchange chromatography of rat antral extracts (Fig. 2); the other, L303, reacted with CFP-I and II but failed to reveal the two most acidic peptides (CFP-III and IV). Digestion of antral extracts with alkaline phosphatase converted CFP-IV to CFP-III and CFP-II to CFP-I, indicating that the second and fourth peaks were phosphorylated. Further digestion with arylsulfatase converted CFP-III to CFP-I, indicating that the former was sulfated (Fig. 2). Since CFP-I co-eluted with the unmodified synthetic nonapeptide, the four peaks were identified as unmodified, phosphorylated, sulfated, and both sulfated and phosphorylated, in order of elution. Thus, antibody L304 reacted with phosphorylated and sulfated peptides whereas L303 did not react with sulfated variants.

Gastrin Variants in Rat Antrum—As already noted above, separation of antral extracts by ion exchange chromatography
resolved sulfated and phosphorylated variants. Four major peaks of immunoreactivity were identified in the same column eluates in assays using an antibody specific for C-terminally amidated gastrins (AG-I-IV in order of elution) (Fig. 3). One of these (AG-II) co-eluted with synthetic rat unsulfated heptadecapeptide gastrin (G17); the most acidic peptide (AG-IV) was identified as sulfated G17 because arylsulfatase digestion converted it to AG-II. The other two peaks have been identified previously as rat minigastrin and are thought to correspond to unsulfated (AG I) and sulfated (AG III) variants of the C-terminal pentadecapeptide of G17 generated by cleavage at Arg2 of G17 although this has yet to be confirmed by sequencing (15, 16). Assays using antibodies specific for G17 extended at its C-terminus by a glycine residue (G-Gly) also revealed four major peaks after ion exchange chromatography (Fig. 4). In order of elution, these peaks are attributable to glycine-extended unsulfated minigastrin (G-Gly-I), glycine-extended unsulfated G17 (G-Gly-II), glycine-extended sulfated minigastrin (G-Gly-III), and glycine-extended sulfated G17 (G-Gly-IV). These assignments were confirmed by arylsulfatase digestion, which also indicated that antibodies L2 and 109-2 reacted similarly with sulfated and unsulfated peptides.

Relative Concentrations of Different Forms and Fragments—On Sephadex gel filtration the major peaks of im-
munoreactive material measured in assays using antibodies L2, L309-2, and L304 showed similar chromatographic properties to G17, G17-Glycine extended, and the C-terminal tryptic fragment of progastrin, respectively (Fig. 5). The gel filtration elution patterns were similar for tissue extracts from fed and fasted rats. This system does not adequately resolve G17 and mini-gastrin (or their glycine-extended forms); it does, however, separate intact progastrin, G34 and G17. The gel filtration data therefore suggest that the major tissue forms detected in these three assays have the properties of peptides generated by cleavage of progastrin at pairs of basic residues (allowing for carboxypeptidase B-like cleavage, or amidation, where relevant). The total tissue concentrations measured with L304 were closely similar to the sum of the concentrations of C-terminally amidated peptides (antibody L2) and their glycine-extended immediate precursors (antibody 109-2).

Marked differences were found in the relative proportions of unsulfated and sulfated peptides containing Tyr₁₀³ and Tyrₛ₇. In the case of the C-terminal tryptic peptide of progastrin which contained Tyr₁₀³, 84-90% of total immunoreactive peptide was in the sulfated form. In contrast, only 54-67% of amidated gastrins containing Tyrₛ₇ were in the sulfated form although 69-89% of their glycine-extended precursor peptides were sulfated. These differences were significant (t test; p < 0.05) (Table I). The estimate of Tyr₁₀³ sulfation found on ion exchange chromatography (assays with L304) agreed well with estimates of concentration of C-terminal progastrin immunoreactivity made using L303, which reacts only with peptides unsulfated at this residue (Table II).

Changes in Concentrations and Forms with Fasting—Plasma concentrations measured with L2 decreased from 50.1 ± 3.9 pmol liter⁻¹ in freely fed rats to 10.2 ± 1.7 in rats fasted for 48 h (mean ± S.E., n = 6). The tissue concentrations of progastrin-derived peptides measured with antibodies L2, L304, and L303 also decreased significantly with fasting. However, the concentrations of glycine-extended variants did not change (Table I). Thus, in rats fed ad libitum, 63.8 ± 7.4% of peptides containing the Phe₉² occurred in the amidated form (the remainder being C-terminal Phe-Gly) whereas in fasted rats only 43.4 ± 5.3% of peptides containing Phe₉² were amidated at this residue (p < 0.05). The pattern of molecular forms detected after ion exchange chromatography also revealed changes in sulfation and phosphorylation between fasted and fed rats (Figs. 3, 4, and 6). There was no change in sulfation at Tyr₁₀³, but at Tyrₛ₇ the sulfation of amidated mini-gastrin increased from 54 to 67% (p < 0.05) (Table I). The sulfation of G17 and of glycine-extended forms was unchanged. The phosphorylation of progastrin C-terminal tryptic peptides decreased with fasting; in fed rats, approximately 50% of the tryptic peptide was both phosphorylated and sulfated, but in fasted rats this decreased significantly (p < 0.05). There was a corresponding increase in the

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**Table I**

Percent sulfation of different progastrin-derived peptides

Results are presented as the percentage of each peptide occurring in the tyrosine-sulfated form. Note that with the exception of mini-gastrin, the proportions of sulfation variants did not change significantly with fasting. However, higher proportions of glycine-extended G17 and mini-gastrin are sulfated compared with the corresponding amidated form (p < 0.05, t test). In addition, a higher proportion of phosphorylated C-terminal progastrin tryptic peptide is sulfated compared with the unphosphorylated product (footnote *). Values are mean ± S.E. (n = 4 for each group).

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Fed</th>
<th>Fasting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyr₁₀³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minigastrin</td>
<td>59.0 ± 1.1</td>
<td>66.0 ± 3.1</td>
</tr>
<tr>
<td>G17</td>
<td>59.2 ± 2.4</td>
<td>64.6 ± 4.3</td>
</tr>
<tr>
<td>Minigastrin-glycine</td>
<td>69.3 ± 3.1*</td>
<td>77.0 ± 4.5</td>
</tr>
<tr>
<td>G17-glycine</td>
<td>80.8 ± 5.2*</td>
<td>89.2 ± 2.2</td>
</tr>
<tr>
<td>Tyrₛ₇</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFP unphosphorylated</td>
<td>84.4 ± 1.7</td>
<td>89.2 ± 2.2</td>
</tr>
<tr>
<td>CFP phosphorylated</td>
<td>92.9 ± 1.8*</td>
<td>92.6 ± 1.2</td>
</tr>
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</table>

* p < 0.05.

**Table II**

Tissue concentrations of progastrin-derived peptides measured with three different antibodies

Antibodies L303 and L304 react with C-terminal tryptic peptide of progastrin; L2, with C-terminally amidated biologically active gastrins; and 109-2, with their glycine-extended biosynthetic precursors. Mean ± S.E. (n = 8 for each group).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fed</th>
<th>Fasted</th>
</tr>
</thead>
<tbody>
<tr>
<td>L304</td>
<td>2.92 ± 0.24</td>
<td>1.12 ± 0.13</td>
</tr>
<tr>
<td>L303</td>
<td>0.34 ± 0.06</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>L2</td>
<td>1.79 ± 0.25</td>
<td>0.69 ± 0.13</td>
</tr>
<tr>
<td>109-2</td>
<td>1.02 ± 0.02</td>
<td>0.81 ± 0.05</td>
</tr>
</tbody>
</table>

* NSD, not significantly different.
The present study has shown changes in the phosphorylation and amidation of peptides derived from progastrin when the normal gastrin cell stimulus (feeding) is withdrawn. In particular, in fasted rats there is decreased phosphorylation of the C-terminal portion of progastrin and decreased conversion of glycine-extended biosynthetic intermediates into amidated peptides. These changes are associated with diminished plasma and tissue concentrations of amidated gastrins, indicating that they occur at a time when there is an overall decrease in gastrin production. Sulfation patterns depend on the particular site and molecular species concerned, but for the most part there were no changes with fasting. The data indicate that there is physiological control of selected post-translational modifications of progastrin-derived peptides.

We have reported previously that in several species there are approximately equimolar tissue concentrations of the C-terminal tryptic fragment of progastrin and of the major biologically active product of progastrin, namely G17 (8, 17-19). The two peptides are derived from the same precursor, and so the stoichiometry is not surprising. There is a departure from this pattern in the rat, since antral extracts from this species contain appreciable quantities of glycine-extended G17, which is the biosynthetic intermediate generated immediately prior to amidation of the biologically active forms (13, 20-22). The rat is also relatively unusual in possessing substantial concentrations of minigastrin. This is thought to be generated by cleavage at a unique arginine in the N terminus of G17 (15, 16). The sum of the concentrations of amidated gastrins and their glycine extended precursors is roughly equal to that of the progastrin C-terminal fragment, which is consistent with the stoichiometry mentioned above.

In fasted rats, there was a decrease in tissue concentrations of total amidated peptide and C-terminal progastrin fragments. However, there was no change in tissue concentrations of glycine-extended intermediates. The data indicate, therefore, that conversion of glycine-extended peptides into biologically active amidated products was depressed. Since these changes occur when the G-cell is deprived of its normal stimulus the observations suggest that the conversion of glycine-extended peptides into the amidated products is under physiological control. These findings extend previous studies in canine antral mucosa that was surgically excluded from contact with the gastric contents (10). This operation, like fasting in rats, leads to a decrease in gastrin release associated with a failure of conversion of glycine-extended intermediates. However, in the excluded antrum, there is G-cell hyperplasia, and it was not possible to determine whether the changes in these cells reflected normal gastrin cell functions. The present studies indicate that modulation of amidation by changes in the gastric luminal environment can be evoked in normal G-cells.

In several species the C-terminal flanking peptide of progastrin has been shown to occur in phosphorylated and unphosphorylated forms (8, 9, 18). The rat is unique so far in that there is also a sulfation site in the C-terminal flanking peptide. Previously, Wu et al. (23) have reported the isolation and chemical characterization of the unmodified C-terminal nonapeptide of rat progastrin. Our data suggest that this material is only about 10% of total and that most of the rest is sulfated or phosphorylated (or both). However, some antibodies (of which L303 is one) fail to react with the sulfated variant and this might explain why Wu and co-workers failed to find the major molecular species generated by tryptic like cleavage of progastrin. It is of interest that the amino acid sequence adjacent to the sulfation site in this peptide is closely similar to that in G17, i.e. Glu-Glu-Glu-X-Tyr (where X is Ala or Gln). It is known that acidic residues to the N-terminal side of Tyr are required for sulfotransferase activity (24, 25). Moreover, we found that amidated peptides were less likely to be sulfated than their glycine-extended precursors, even though they share the same tyrosine (Tyr*). The differences in sulfation between Tyr* and Tyr** may be explained by differences in the tertiary structure of progastrin around these sites which influence sulfotransferase activity. Sulfation occurs in the Golgi (25, 26) and probably precedes cleavage. The differences in sulfation of Tyr** in amidated and C-terminal glycine-extended peptides are therefore compatible with the idea of preferential amidation of unsulfated peptides. Previous studi-
ies in pig did not find a difference between amidated and glycine-extended gastrins in their sulfation patterns (21). However, the concentrations of glycine-extended gastrins are much higher in rat than pig so that the amidating mechanisms in the latter may be relatively robust and less readily influenced by factors such as sulfation of the substrate.

In previous studies it has been suggested that phosphorylation might play a role in regulating progastrin processing (8, 9). The phosphorylation site is of interest because it is immediately adjacent to an important processing site and is well conserved in gastrin and cholecystokinin (4, 8, 27). We have shown previously that in human gastrin extracts, which often contain appreciable quantities of progastrin, the proportion of precursor in the phosphorylated state was less than that of the corresponding C-terminal flanking peptide (9). This suggests that phosphorylated progastrin might be more readily cleaved than unphosphorylated progastrin to yield the flanking peptide. Immunogold studies in the EM level indicate that cleavage of progastrin occurs in the trans-Golgi and in immature granules (28, 29). The latter are also the first level in the secretory pathway at which amidated gastrins can be found. It is fairly well established that as secretory granules mature in endocrine cells, there are changes in the composition of the granule interior which include, for example, an acidification (30). Some of the endopeptidase and carboxypeptidase D-like enzymes that cleave hormone precursors have pH optima in the acid range and would be able to act in the acid milieu of the secretory granule (31, 32). In contrast, in the case of peptide α-amidating monooxygenase which converts glycine-extended peptides to their amidated forms there are well defined pH optima around neutrality and pH 5, and this enzyme also requires copper and ascorbate (33–35). The relatively stringent requirements for optimal peptide α-amidating monooxygenase activity, coupled with the observation of high concentrations of Gly-extended gastrins stored in rat antrum, suggest that changes in the interior of maturing G-cell granules create an environment that precludes further amidation. Taken together with previous findings (8, 9), the present data support the idea that phosphorylation of progastrin is associated with early and rapid endopeptidase cleavage and consequent presentation of glycine-extended intermediates to peptide α-amidating monooxygenase at a time when the interior of the granule is compatible with optimal enzyme activity. These events are evidently under physiological control although the way in which they are influenced by changes in the gastric lumen remains to be established. Since gastrin cells are stimulated by gastrin-releasing peptide and acetylcholine and inhibited by somatostatin (4), these substances are possible mediators of the effects described here.

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REFERENCES
Processing of the gastrin precursor. Modulation of phosphorylated, sulfated, and amidated products.

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