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 Ser"3, amidated at Phe"2, and sulfated at Tyr"7 or Tyr"103. 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 radio-immunoassays and chromatographic systems that resolve phosphorylated, amidated, and sulfated gastrin products, including intermediates generated prior to amidation (i.e. C-terminal glycine-extended variants). Assays for Phe"2-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 Ser"36 was depressed significantly in fasted rats. The proportions of amidated peptides sulfated at Tyr"7 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 for peptides sulfated at Tyr"103. 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 excised 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-Glu-Asp-Gln-Tyr-Ser) was synthesized by the solid phase method, cleaved from the resin by liquid HF in the presence of anisole and dimethyl sulfoxide 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-

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FIG. 1. Schematic representation of rat preprogastrin showing expanded sequences corresponding to antigenic domains for antibodies used in this study. There is a single copy of the major active product, heptadecapeptide gastrin (G17), which is generated by endopeptidase cleavage, carboxypeptidase B-like cleavage and C-terminal amidation. Cleavage in the N terminus of G17 yields a C-terminal fragment known as minigastrin (possibly the pentadecapeptide G15). Antibodies L303 and L304 react with the C-terminal tryptic fragment of preprogastrin including variants phosphorylated on Ser\(^{26}\) and (in the case of L304) sulfated on Tyr\(^{26}\). Antibody 109-2 reacts with glycine-extended forms of G17 and minigastrin, and L2 with amidated biologically active products.

The tissues were subsequently weighed while deeply frozen and the gastrins then extracted in boiling water as described previously (9).

Characterization of Rat Preprogastrin-Derived Peptides—Tissue extracts were fractionated by gel filtration on Sephadex G-50 (1 × 100-cm Superfine) eluted with 0.05 M ammonium bicarbonate and by ion exchange chromatography on DE52 (1 × 15 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) or 0.05 M ammonium bicarbonate, pH 7.3, 4 h, 37 °C or with arylsulfatase (200–400 μg, Sigma type VIII 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 preprogastrin 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 (CPF-1–IV, in order of elution) after ion exchange chromatography of rat antral extracts (Fig. 2); the other, L303, reacted with CPF-I and II but failed to reveal the two most acidic peptides (CPF-III and IV). Digestion of antral extracts with alkaline phosphatase converted CPF-IV to CPF-III and CPF-II to CPF-I, indicating that the second and fourth peaks were phosphorylated. Further digestion with arylsulfatase converted CPF-III to CPF-I, indicating that the former was sulfated (Fig. 2). Since CPF-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...
Modulation of Gastrin Processing

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 Arg7 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-

Fig. 2. Separation by ion exchange chromatography on DE52 of a rat antral extract. In the top panel is shown a control elution profile assayed with L303 (○) and L304 (●). In the center panel is shown the results of digestion of the extract with alkaline phosphatase, and in the bottom panel the results after further arylsulfatase digestion. The arrow indicates the elution position of synthetic unmodified nonapeptide. Peaks are identified as C-terminal flanking peptide (CFP) of progastrin, I-IV, in order of elution. See "Materials and Methods" for details of chromatography.

Fig. 3. Separation by ion exchange chromatography of antral extracts from fasted and fed rats assayed with antibody L2. Unmodified synthetic rat G17 elutes in the position indicated by the arrow. There are four major peaks in both types of extract, labeled AG-I-IV in order of elution. The peaks correspond to unsulfated minigastrin, unsulfated G17, sulfated minigastrin, and sulfated G17.

Fig. 4. Separation by ion exchange chromatography of antral extracts from fasted and fed rats assayed with antibody 109-2. There are four major peaks in both types of extract, labeled G-Gly-I-IV in order of elution. The peaks correspond to unsulfated glycine-extended minigastrin (I), unsulfated glycine-extended G17 (II), sulfated glycine-extended minigastrin (III), and sulfated glycine-extended G17 (IV).
munoreactive material measured in assays using antibodies L2, L109-2, 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 minigastrin (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 Tyr87. In the case of the C-terminal tryptic peptide of progastrin which contained Tyr103, 84-90% of total immunoactive peptide was in the sulfated form. In contrast, only 54-67% of amidated gastrins containing Tyr87 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 immunoactivity 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 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 Tyr87 the sulfation of amidated minigastrin 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

### Table I

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Fed</th>
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<tr>
<td>Tyr103</td>
<td>59.0 ± 1.1</td>
<td>66.0 ± 3.1</td>
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<tr>
<td>Minigastrin</td>
<td>59.2 ± 2.4</td>
<td>64.6 ± 4.3</td>
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<tr>
<td>G17</td>
<td>69.3 ± 3.1*</td>
<td>77.0 ± 4.5</td>
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<tr>
<td>Minigastrin-glycine</td>
<td>80.8 ± 5.2*</td>
<td>89.2 ± 2.2</td>
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<tr>
<td>G17-glycine</td>
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<td></td>
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<tr>
<td>Tyr87</td>
<td>63.4 ± 1.6</td>
<td>92.6 ± 1.2</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>
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* p < 0.05.

### Table II

<table>
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<tr>
<th>Antibodies</th>
<th>Fed</th>
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<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>
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<tr>
<td>L109-2</td>
<td>1.09 ± 0.92</td>
<td>0.81 ± 0.05</td>
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* NSD, not significantly different.
relative proportions of the sulfated-unphosphorylated C-terminal fragment of progastrin, but there was essentially no difference in the relative proportions of the phosphorylated/sulfated peptide or of the unmodified tryptic peptide (Table III). The four forms of the C-terminal tryptic peptide of progastrin were separated on ion exchange chromatography; see “Results” for identification of each form. The results are presented as the percent contribution of each form to total immunoreactivity with L304. Note the decrease in the relative abundance of phosphorylated/sulfated peptide and the corresponding increase in sulfated/unphosphorylated peptide with fasting. Mean ± S.E. (n = 7 for each group).

<table>
<thead>
<tr>
<th>Form</th>
<th>Fed</th>
<th>Fasted</th>
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</thead>
<tbody>
<tr>
<td>Unmodified</td>
<td>7.2 ± 0.9</td>
<td>6.1 ± 1.3</td>
</tr>
<tr>
<td>Phosphorylated</td>
<td>38.5 ± 3.9</td>
<td>39.5 ± 0.6</td>
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<tr>
<td>Sulfated</td>
<td>38.5 ± 1.5</td>
<td>51.0 ± 2.2</td>
</tr>
<tr>
<td>Phosphorylated/sulfated</td>
<td>50.4 ± 1.6</td>
<td>39.5 ± 1.8</td>
</tr>
</tbody>
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*NSD, not significantly different.

relative proportions of the sulfated-unphosphorylated C-terminal fragment of progastrin, but there was essentially no difference in the relative proportions of the phosphorylated unsulfated peptide or of the unmodified tryptic peptide (Table III).

**DISCUSSION**

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). It is of interest, however, that there were significant differences in the relative proportions of sulfated and unsulfated progastrin products containing Tyr<sup>7</sup> and Tyr<sup>10</sup>. 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<sup>7</sup>). The differences in sulfation between Tyr<sup>7</sup> and Tyr<sup>10</sup> 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<sup>7</sup> in amidated and C-terminal glycine-extended peptides are therefore compatible with the idea of preferential amidation of unsulfated peptides. Previous stud-
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, 5, 27). We have shown previously that in human gastrinoma 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 has 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 (9, 10), 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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