Prohormone convertases 1/3 and 2 together orchestrate the site-specific cleavages of progastrin to release gastrin-34 and gastrin-17

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INTRODUCTION

Gastrin is a gastrointestinal hormone that regulates gastric acid secretion and mucosal cell growth (for a review, see [1]). Most gastrin is synthesized in antral G-cells where progastrin, through multiple steps, is processed to α-amidated gastrins of different lengths (Figure 1). Of these, gastrin-34 and gastrin-17 predominate in normal tissue and circulation. Their synthesis requires multiple steps, is processed to α-amidated gastrins of different lengths (Figure 1). Of these, gastrin-34 and gastrin-17 predominate in normal tissue and circulation. Their synthesis requires multiple steps, is processed to α-amidated gastrins of different lengths (Figure 1). Of these, gastrin-34 and gastrin-17 predominate in normal tissue and circulation. Their synthesis requires multiple steps, is processed to α-amidated gastrins of different lengths (Figure 1). Of these, gastrin-34 and gastrin-17 predominate in normal tissue and circulation.
Figure 1  Progastrin and its post-translational maturation in antral G-cells

(A) The mouse progastrin sequence is given in the single letter amino acid code with the three dibasic cleavage sites printed in bold and * indicating the single tyrosine-sulfation site. The bioactive tetrapeptide sequence in the gastrins is boxed. (B) Diagram of the co- and post-translational modification of progastrin in antral G-cells. Activation of the gastrin amidation site (Phe71-Gly72-Arg73-Arg74) occurs via a series of C-terminal cleavages and modifications. Endoproteolytic cleavage by PCs produces the carboxypeptidase E substrate (Phe-Gly-Arg). Carboxypeptidase E then acts in secretory granules to remove the C-terminal arginine residue yielding glycine-extended gastrin (Phe-Gly). Carboxyamidation by PAM subsequently results in the production of bioactive gastrin (-Phe-NH2). Concomitant N-terminal cleavage by PCs produces bioactive gastrins of varying size (e.g. gastrin-34 and gastrin-17). The indications of PC1/3 and PC2 in the diagram is based on the results of the present study and a previous study [27]. A library of sequence-specific antibodies was used in combination with in vitro protease treatment to measure bioactive gastrin and various precursor peptides, as described in the text.

12-week-old PC1/3-null mice and wild-type (+/+) littermates of the same age. The care and treatment of all animals was in accordance with NIH (National Institute of Health) and institutional guidelines.

**Tissue isolation and extraction**

Mice in groups of four were killed by CO2 inhalation, and stomach tissue was rapidly dissected and frozen on dry ice. The
tissue was gently rinsed in PBS on ice before freezing. Tissue extracts for RIA analysis were prepared as previously described [29]. Briefly, frozen tissues were boiled in water (1 ml/mg of tissue) for 20 min, homogenized (polytron) and centrifuged for 30 min at 14000 g. The supernatants were withdrawn and the pellets were re-extracted in 0.5 M acetic acid (1 ml/mg of tissue), rehomogenized, incubated at room temperature (20°C) for 30 min and centrifuged (14000 g for 30 min at 4°C). The neutral water and acid supernatants were stored at −20°C until RIA analysis. The acid extracts, however, turned out to contain only negligible amounts of progastrin and its products. Consequently they were not included in further examinations.

**Chromatography**

Extracts (1 or 2 ml) were applied to Sephadex G-50 superfine columns (10 mm × 1000 mm), which were eluted at 4°C with 0.02 M barbital buffer (pH 8.4), containing 0.1 % BSA. Fractions of 1 ml were collected at a rate of 4.0 ml/h. The columns were calibrated with 125I-albumin (void volume), gastrin-34 and gastrin-17, and with 25NaCl (total volume). The eluted fractions were assayed with a library of sequence-specific RIAs against different progastrin epitopes and fragments using antibody numbers 2604, 2605, 8017, 2145, 5284 and 3208, as detailed below. The percentages of carboxyamidated gastrin were calculated by planimetry. Four different extracts from each of the two groups of mice (PC1/3 knockout and corresponding wild-type controls) were subjected to gel chromatography.

**RIAs**

A library of six sequence-specific antisera against progastrin and its fragments was used to measure the different forms of gastrin- and progastrin-processing intermediates (Figure 1). The sum of carboxyamidated gastrins was measured using the gastrin-specific antisera number 2604, 125I-gastrin-17 was used as a tracer and gastrin-17 was used as a standard. Antibody number 2604 binds the carboxyamidated gastrin-34 and gastrin-17 with equimolar affinity, irrespective of size and degree of tyrosine O-sulfation [30]. The cross-reactivity with homologous cholecystokinin peptides is negligible [30,31]. Non-sulfated carboxyamidated gastrin-34 and -17 were measured in parallel using antibody number 2605 [32]. Glycine-extended processing intermediates of progastrin were measured using antisera numbers 5284 and 3208 with 125I-glycine-extended gastrin-17 as a tracer and glycine-extended gastrin-17 as a standard [33]. Glycine-arginine-extended intermediate precursors were measured using antisera numbers 5284 and 3208 following enzymatic pretreatment with only carboxypeptidase B [33]. To measure all precursor forms of gastrin, samples were pretreated with both trypsin and carboxypeptidase B followed by RIA measurement using antisera number 5284, as previously described [33]. Carboxypeptidase B mimics the effect of carboxypeptidase E, whereas trypsin mimics the effects of PCs. Antiserum number 2145 was used to measure fragment 38–52 of human progastrin, corresponding to the pyroglutamated N-terminus of gastrin-34 using 125I-gastrin-34 as a tracer and gastrin-34 as a standard [34]. Correspondingly, antiserum number 8017 was used to measure the pyroglutamated N-terminus of human gastrin-17 using 125I-gastrin-17 as a tracer and gastrin-17 as a standard [35]. Owing to species differences in the N-terminal sequences of mammalian gastrin-34 and -17, it was necessary to use antisera numbers 2145 and 8017 for characterization of possible progastrin cleavage products after incubation of human progastrin with recombinant PCs/6. To monitor the purification of recombinant human progastrin, we also used a RIA based on antisera number 94023 directed against the extreme N-terminus of human progastrin together with the corresponding N-terminal decapeptide fragment tyrosylated at its C-terminus as the tracer and standard [36].

**In vitro cleavage of progastrin and progastrin fragments with recombinant PCs/6A**

To produce recombinant human progastrin, an expression vector of human preprogastrin, as previously described [37], was transiently transfected into CHO (Chinese-hamster ovary) cells using a standard FuGENETM6 transfection protocol (Roche Applied Science). After 48 h, culture medium was recovered, separated by gel filtration on a 1000 mm × 50 mm Superdex G50 Superfine column and eluted, as previously described [37]. Recovered fractions were analysed for progastrin using the RIA specific to the N-terminus of human progastrin [36]. Fractions eluting at the position of intact progastrin were pooled and subjected to an *in vitro* PCs/6A cleavage assay. To half of the peptide pool was added 10 units of human PCs/6 (Phenoswitch Bioscience) and reaction buffer [20 mM Bis-Tris (pH 6.5) containing 1 mM CaCl2 and 2 g/l BSA] to a concentration as suggested by Phenoswitch Bioscience and incubated for 4 h at 37°C. To a control pool the reaction buffer only was added and incubated in a similar manner to the reaction pool. Both pools were subjected to gel filtration and analysed using specific RIAs towards the N-terminals of progastrin, gastrin-34 and gastrin-17, as well as a C-terminal assay against glycine-extended gastrin [30,33,34,36].

Gastrin-34 from Sigma and the peptides GPASHHRRQLG-FQGP (progastrin cleavage site for gastrin-34 N-terminus) and PEELVHRVKKRRADPDPMK (PC substrate [38]) were custom-synthesized by Schafer-N. Incubation of the peptides was performed as suggested by PhenoSwitch in a total volume of 100 μl of 20 mM Bis-Tris buffer (pH 6.5), containing 1 mM CaCl2, BSA (2 g/l), 100 μM peptide (substrate) and 10 units of PCs/6 for 2 h at 37°C. The reaction was terminated by the addition of 10 μl of 10 % trifluoroacetic acid. The reaction mixture was separated by HPLC using a 2.1 mm × 150 mm C18 column (Vydac) eluted with a gradient from 10–40 % acetonitrile over 30 min; the elution buffers contained 0.1 % trifluoroacetic acid. The chromatograms were monitored at 214 nm and peaks were collected manually. The peptides collected were analysed by MALDITOF (matrix-assisted laser-desorption ionization–time-of-flight) MS (AutoFlex II, Bruker) using α-cyano-4-hydroxycinnamic acid as the matrix.

**Immunocytochemistry**

To examine the cellular co-localization between gastrin and PC1/3 or PC2, the stomach was removed from three wild-type mice aged 3–4 months, cut open, washed and immersion-fixed overnight in 4 % (w/v) paraformaldehyde. Transverse paraffin sections (6 μm) were cut, mounted on superfrosted glass slides and baked overnight at 60°C. Antigen retrieval was performed by boiling the slides in a microwave oven for 3 min at 800 W, followed by 3 × 5 min at 400 W in a 10 mM Tris-buffered EGTA solution at pH 9.0. Preblocking was performed in 10 % ovine serum in TBS for 20 min. The sections were incubated with PC1/3 or PC2 antisera diluted 1:10000 overnight at 4°C [39,40]. The primary antisera was detected with Envision®+ (Dako) for 1 h, followed by incubation for 1 h with a Cy3-conjugated anti-horseradish peroxidase antibody (Jackson) diluted 1:400. Gastrin was detected with overnight incubation with antisera number 8007 (dilution 1:1000) at 4°C and subsequently reacted to Alexa Fluor® 488-conjugated donkey-anti rabbit Fab-fragments.
(dilution 1:1000; Molecular Probes). Antiserum number 8007 is specific to the carboxyamidated C-terminus of the gastrins [31]. Cover slips were mounted with Aquamount (Dako). Analysis of the sections was performed with a Zeiss LSM510 confocal microscope.

**In situ hybridization**

Serial sections were probed with the PC5/6 antisense probe, gastrin antiserum number 8007 [31] and the PC5/6 sense probe. *In situ* hybridization was performed as previously described [41]. In brief, mouse stomach was fixed in 4% (w/v) paraformaldehyde, 8 μm sections were placed on SuperFrostRPlus slides (Menzel), deparaffinized and paraformaldehyde-fixed, and treated with proteinase K (P2308; Sigma), post-fixed [4% (w/v) paraformaldehyde], prehybridized for 1 h at 50°C, and incubated overnight with the labelled PC5/6 probe (50°C) specific to the nucleotide. Visualization was achieved with anti-digoxigenin-AP (where AP is alkaline phosphatase; Roche Diagnostics) and the chromogens BCIP (5-bromo-4-chloroindol-3-yl phosphate; Sigma B-8503) and NBT (Nitro Blue Tetrazolium; Sigma N-6876).

**RESULTS AND DISCUSSION**

**Progastrin processing in PC1/3- and PC2-knockout mice**

As shown in Table 1 and Figure 2, the antral concentrations of progastrin and its products are grossly altered in PC1/3-knockout mice. The concentration of progastrin itself is increased nearly 3-fold, whereas concentrations of the processing intermediates are reduced to one-third, and the bioactive, carboxyamidated gastrins are reduced to one-fourth of the concentrations in wild-type mice.

![Figure 2](https://example.com/figure2.png)

**Figure 2**  Average percentages of the processing parameters measured in antral extracts of PC1/3 knockout mice

The parameters (progastrin, gastrin-Gly-Arg, gastrin-Gly and bioactive gastrin-amide) are compared with those measured in PC2-knockout mice and in carboxypeptidase E-deficient (CPE) mice [18,27], as well as with their respective wild-type controls (broken line at 100%).

### Table 1 Progastrin and its products in antral mucosa of PC1/3 and PC2 (data from [27]) null mice, carboxypeptidase E-deficient mice (data from [18]) and corresponding wild-type mice (pmol/g of tissue; means ± S.E.M.)

<table>
<thead>
<tr>
<th>Hormone/by-product</th>
<th>PC1/3 knockout (n = 4)</th>
<th>Wild-type (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progastrin</td>
<td>98.0 ± 49.6</td>
<td>35.8 ± 7.3</td>
</tr>
<tr>
<td>Gastrin-Gly-Arg</td>
<td>0.5 ± 0.3</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>Gastrin-Gly</td>
<td>3.5 ± 1.8</td>
<td>10.3 ± 1.8</td>
</tr>
<tr>
<td>Gastrin-amide</td>
<td>145.5 ± 65.0</td>
<td>568.3 ± 135.6</td>
</tr>
<tr>
<td>(b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormone/by-product</td>
<td>PC2 knockout (n = 8)</td>
<td>Wild-type (n = 8)</td>
</tr>
<tr>
<td>Progastrin</td>
<td>21.0 ± 6.5</td>
<td>14.0 ± 3.5</td>
</tr>
<tr>
<td>Gastrin-Gly-Arg</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Gastrin-Gly</td>
<td>6.5 ± 1.3</td>
<td>5.5 ± 1.5</td>
</tr>
<tr>
<td>Gastrin-amide</td>
<td>750.0 ± 140.5</td>
<td>650.5 ± 131.0</td>
</tr>
<tr>
<td>(c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormone/by-product</td>
<td>CPE-deficiency (n = 8)</td>
<td>Wild-type (n = 8)</td>
</tr>
<tr>
<td>Progastrin</td>
<td>0.0 ± 0.0</td>
<td>11.6 ± 5.2</td>
</tr>
<tr>
<td>Gastrin-Gly-Arg</td>
<td>1054.0 ± 122.0</td>
<td>12.2 ± 6.3</td>
</tr>
<tr>
<td>Gastrin-Gly</td>
<td>34.7 ± 7.1</td>
<td>4.8 ± 0.5</td>
</tr>
<tr>
<td>Gastrin-amide</td>
<td>601.0 ± 28.0</td>
<td>715.0 ± 43.0</td>
</tr>
</tbody>
</table>
controls. The reductions contrast with previous findings in PC2-knockout mice and the carboxypeptidase E-deficient (CPE<sup>−/−</sup>) mice, in which the antral concentrations of the bioactive, carboxyamidated gastrins are maintained at a normal level in spite of major disturbances at earlier steps in the processing pathway and in the ratio between gastrin-34 and gastrin-17 (Table 1, Figure 2 and [18,27]).

Gel chromatography of neutral antral extracts revealed an unchanged molecular pattern of carboxyamidated gastrins in the PC1/3-knockout mice, despite the marked difference in tissue concentrations (Figure 3). Hence, the ratios between gastrin-34 and gastrin-17, and between tyrosine-sulfated and non-sulfated gastrins were as in the wild-type controls (Figure 3). The low concentrations excluded chromatographic examination of the processing intermediates, but it was possible to study the progastrin immunoreactivity, which showed a significant difference between the null mice and their wild-type littermates (Figure 4).

Thus the progastrin molecule is only partly cleaved at the dibasic Arg<sup>36</sup>Arg<sup>37</sup> site and even less at the Lys<sup>53</sup>Lys<sup>54</sup> site in the PC1/3-null mouse (Figure 4, upper panel), whereas progastrin in the controls is almost completely processed to shorter fragments (Figure 4, lower panel). The modest differences in antral concentrations of progastrin and its processing products between the three groups of wild-type controls (Table 1) supposedly reflect differences in mouse strains and age. Hence, the mice used in the present study are younger (3 months of age) than those used in the studies of carboxypeptidase E-deficiency and PC2 knockout that were up to 6 months of age [18,27]. As shown previously, the
J. F. Rehfeld and others

Figure 4 Gel chromatography on Sephadex G-50 superfine columns of antral extracts from PC1/3-knockout mice (KO, upper panel) and littermate wild-type controls (WT, lower panel)
The chromatographic elutions were monitored using antibody 5284 that binds glycine-extended gastrins after trypsin and carboxypeptidase B treatment of each fraction. Antral extracts from four different mice in each of the two groups (PC1/3 and controls) were subjected to chromatography. Those shown are characteristic of each group.

The antral concentration of progastrin declines with age in rodents [42].

The processing observed in PC1/3-knockout mice indicates that PC1/3 initiates cleavage at the N-terminal di-arginine site (Arg36Arg37) at an early stage in the processing, since more progastrin and a large fragment corresponding to sequence 38–83 of progastrin were found in the antral extract of PC1/3-null mice (Figure 4). Subsequently, PC1/3 continues to cleave the C-terminal di-arginine site Arg73Arg74. PC1/3, however, appears to be without effect on the di-lysine site, Lys53Lys54 (Figure 3). This endoproteolytic cleavage pattern of progastrin is opposite to that of PC2 (Table 2, Figure 2 and [27]). Thus PC2 only cleaves the single di-lysine site of progastrin, Lys53Lys54, the cleavage for half of which PC2 appears to be responsible [27]. The results hence support the contention that PC1/3 generally prefers arginine-containing cleavage sites, whereas PC2 is activated later in the processing pathway to cleave mainly lysine-containing dibasic sites at the lower pH characteristic of mature secretory granules [20].

The results of the present study support our earlier observation that pituitary corticotrophs which contain only PC1/3 and no PC2 cannot process the Lys53Lys54 site of progastrin. Therefore, corticotrophs synthesize gastrin-34, but no gastrin-17 [9,10,13]. This notion is also supported by Sawada et al. [43] who found that PC2 transfected into AtT-20 cells but not PC1/3 can accomplish cleavage of the di-lysine site in progastrin [43]. Also POMC (pro-opiomelanocortin) contains a Lys-Lys site within β-endorphin which remains unprocessed in AtT-20 cells but can be processed upon co-transfection with PC2 [44,45]. However, cleavage of the Lys-Lys site within proenkephalin is apparently largely mediated by PC1/3 since it is efficiently cleaved in AtT-20 cells [46]. The primary structures of the three precursors (progastrin, POMC and proenkephalin) reveals extensive differences in the vicinity of the Lys-Lys sites, which could account for the differential ability of PC1/3 to recognize this type of processing site within different contexts.

A surprising finding in the present study was the increased concentration of progastrin in the antrum of PC1/3-null mice, whereas the concentrations of the processing products were drastically reduced (Table 1 and Figure 2). It is obvious that the lack of PC1/3 reduces the processing of progastrin severely, but it is an unsolved question why the total translational product in PC1/3-null mice is reduced, whereas it is increased in other processing enzyme deficiencies [18]. The C-terminal dibasic cleavage sites in progastrin are, however, part of the motif for sorting to the regulated secretory pathway [47]. Moreover, PC1/3 has been shown to enhance the post-Golgi sorting of some prohormones [48]. It is therefore possible that the lack of PC1/3 itself reduces the storage of progastrin and its products in the secretory granules of G-cells.

### Progastrin cleavage by PC5/6

Since half of the Lys-Lys sites within progastrin are cleaved in the absence of PC2 [27], and since 30% of the two di-arginine sites are cleaved in the absence of PC1/3 (Table 1), other convertases might participate in the in vivo endoproteolytic processing of progastrin in normal G-cells. A candidate for this activity is PC5/6, which has been shown to be expressed in endocrine cells of the gastrointestinal tract [25,49]. As shown in the present study and in the rat, PC5/6 gene expression can be demonstrated in antral G-cells (Figure 7 and [23]). PC5/6 is known to cleave basic processing sites [19,20]. In order to examine possible effects of PC5/6, we first looked at the processing of progastrin in the SK-N-MC cell line, since PC5/6-knockout mice were not available and since SK-N-MC cells have been suggested to express the PC5A isofrom but neither PC1/3 nor PC2 [50]. Recent re-examinations, however, have shown that SK-N-MC cells may indeed express PC1/3 at the protein level (J. R. Bundgaard, unpublished work) and also PC2, although without its chaperone 7B2 [51].

### Table 2 The site-specific cleavages at dibasic sequences in progastrin by PCs expressed in antral G-cells

<table>
<thead>
<tr>
<th>PC</th>
<th>Arg36Arg37</th>
<th>Lys53Lys54</th>
<th>Arg73Arg74</th>
</tr>
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<tbody>
<tr>
<td>PC1/3</td>
<td>++</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>PC2</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>PC5/6</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

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Prohormone convertases in gastrin biosynthesis

Figure 5 Gel chromatography on Sephadex G-50 superfine columns of human progastrin before and after incubation with recombinant PC5/6

The chromatographic elution was monitored by an RIA specific to the intact N-terminus of human progastrin using antiserum number 94023 [36]. The upper panel shows the elution without prior treatment with PC5/6. The lower panel shows the elution after treatment with PC5/6, which has been shown to cleave a relevant control peptide.

Consequently, we decided to exclude the results obtained for progastrin processing in this cell-line.

Instead we examined directly in vitro the substrate activity of PC5/6 against intact progastrin. Human progastrin was expressed in CHO cells, which do not cleave progastrin. Progastrin was subsequently purified by size-exclusion gel chromatography, and recovered peptide was incubated in vitro with recombinant PC5/6A. Products were subjected to repeated chromatography and analysed using RIAs that are specific to the dibasic cleavage sites of human progastrin [34,35]. Enzyme-treated progastrin eluted identically with untreated control fractions (Figure 5), and the site-specific RIAs did not detect processing at any of the cleavage sites. Moreover, digestion with recombinant PC5/6 of gastrin-34 and the synthetic peptide spanning the N-terminus of gastrin-34 within progastrin resulted in no cleavage, whereas the general PC substrate [38] used as control was cleaved as expected. Consequently, we conclude that PC5/6 is without effect on progastrin, and that the processing of progastrin originally observed in SK-N-MC cells is due to endoproteases other than PC5/6.

PC expression in G-cells

As shown in Figure 6, both PC1/3 and PC2 are expressed in normal antral G-cells in mice. The immunocytochemical results are in accordance with earlier results in human and rat antrum [22,23]. In addition, as shown in the present study by in situ hybridization (Figure 7), PC5/6 also is expressed in murine antral G-cells.

The interplay of PC1/3 and PC2 and the role of PC5/6 in G-cells

Since no cleavage was found in the in vitro studies using PC5/6 against intact progastrin and progastrin fragments containing the dibasic cleavage sites, and since the same PC5/6 was fully active in cleaving a control peptide, PC5/6 hardly plays a significant role in the G-cell maturation of progastrin. It is possible that PC5/6...
is instead involved in the processing of other neuroendocrine G-cell products such as chromogranin A and neuropeptide W [52–54]. Thus the endoproteolytic maturation of progastrin as seen in normal G-cells appears to require an interplay, primarily between PC1/3 and PC2. We believe that the processing begins with PC1/3, which is solely responsible for the cleavage of Arg^{57}Arg^{58}. Subsequently, PC1/3 cleaves the crucial Arg^{57}Arg^{58}. Later, in secretory granules, PC2 performs the partial cleavage of Lys^{59}Lys^{64} to ensure the production of gastrin-17.

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Prohormone convertases in gastrin biosynthesis 43

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