Progastrin processing differs in 7B2 and PC2 knockout animals: a role for 7B2 independent of action on PC2

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Abstract Cellular synthesis of neuroendocrine peptides requires prohormone convertases (PCs). In order to determine the role of PC2 for gastrin synthesis, we examined antral extracts from mice lacking PC2 or its chaperone, 7B2. The overall concentrations of precursors and α-amidated gastrins were similar in all mice. Chromatography, however, revealed that while the K_{53}–K_{54} site was almost fully cleaved in controls and half cleaved in PC2 null mice, only 23% was cleaved in 7B2 null mice. The results show that PC2 and 7B2 both are required for synthesis of the main form of gastrin (gastrin-17), and that 7B2 exhibits effects beyond PC2-mediated cleavages. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

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1. Introduction

The gastrointestinal hormone, gastrin, regulates gastric acid secretion and mucosal cell growth (for reviews, see refs. [1,2]). Most gastrin is synthesized in antral G-cells, where progastrin through multiple modifications matures to bioactive carboxyamidated gastrins of different chain lengths (Fig. 1). Mammalian progastrins harbor three dibasic cleavage sites (R_{36}–R_{37}, K_{53}–K_{54}, and R_{73}–R_{74}) of which cleavage of the K–K site determines the amount of gastrin-17 produced relative to that of gastrin-34. In most mammals, more than 90% of the G-cell gastrin is the heptadecapeptide amide, gastrin-17, a few percent is gastrin-34, and the rest are traces of either the long gastrin-71 or the shorter gastrin-14 and gastrin-6 peptides [3–8]. Outside the antrum, the gastrin gene is expressed at low levels in specific intestinal and pancreatic endocrine cells, in pituitary cortico- and melanotrophs, in hypothalamic neurons, in spermatocytes and in various adenocarcinomas [9–18]. The extra-antral level of expression is, however, low compared to that of the antrum, and in addition the processing is cell-type specific. For instance, there is no cleavage of the K–K site in either ileal TG-cells or pituitary corticotrophs, which accordingly synthesize only gastrin-34 and no gastrin-17 [19,20].

The elaborate posttranslational maturation of progastrin requires several processing enzymes (Fig. 1). While the precise involvement of tyrosyl sulfotransferases, carboxypeptidase E and the amidation enzyme complex (peptidyl-glycine α-amidating monooxygenase (PAM)) by now is well established [21–23], the roles of prohormone convertases (PCs) 1, 2 and other PCs in progastrin processing remains to be defined. Mammals express seven PCs of which PC1 and PC2 are assumed to be responsible for most of the endoproteolytic processing of neuroendocrine proproteins (for reviews, see refs. [24–26]). Accordingly, PC1 and PC2 have been demonstrated immunochemically in human and rat G-cells [27,28]. The PC cleavages typically occur at dibasic sites. Of the three such sites in progastrin, cleavage of the C-terminal R_{73}–R_{74} determines the availability of the glycine-extended precursor for α-amidation (Fig. 1), and hence for gastrin peptides to become bioactive. On the other hand, processing of the two N-terminal sites (R_{36}–R_{37} and K_{53}–K_{54}) determines the length of the N-terminal extensions, and hence whether the carboxyamidated fragments become either gastrin-34 or gastrin-17, the two major gastrins in circulation.

In order to determine the role of PC2, we have now examined the antral G-cell processing of progastrin in PC2 and 7B2 null mice. 7B2 is a neuroendocrine protein, that acts as a cellular binding protein necessary for PC2 activity [26]. An attractive aspect of the study is that progastrin, with its well-defined double-R and double-K sites, may be an instructive substrate for examination of the relative roles of PC1 and PC2 in hormone synthesis.

2. Materials and methods

2.1. Mice

Both PC2 and 7B2 knockout (−/−) and corresponding wild-type (+/+) mice were generated by intercrossing heterozygotes and genotyping the offspring [29,30]. The PC2 knockouts (−/−) [29] and corresponding controls (both C57BL/6J:Sv129 (50:50 mix) mice) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), whereas the 7B2 knockouts (−/−) [30] and controls (both Sv129 mice) were generated in LSUMC (New Orleans, LA, USA). The mice were housed on a 12 h light–dark cycle and freely fed. All experiments were approved by the Animal Ethics committees of the local institutions.

2.2. Tissue isolation and extraction

Mice between 2 and 6 months of age in groups of five or eight were sacrificed by CO_2 inhalation, and stomach tissue was rapidly dissected and frozen on dry ice. The tissue was gently cleaned in phosphate-buffered saline on ice before freezing. Tissue extracts for radioimmunoassay (RIA) were prepared as previously described [31]. Briefly,

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Abbreviations: CPB, carboxypeptidase B; PAM, peptidyl-glycine α-amidating monooxygenase; PC, prohormone convertase; POMC, proopiomelanocortin; RIA, radioimmunoassay

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frozen tissues were boiled in water (1 ml/mg) for 20 min, homogenized (polytron) and centrifuged for 30 min at 10 000 rpm. The supernatants were withdrawn and the pellets re-extracted in 0.5 M CH_3COOH (1 ml/mg), rehomogenized, incubated at room temperature for 30 min and centrifuged. The neutral water and acid supernatants were stored at −20°C until RIA analysis.

2.3. Chromatography

1 or 2 ml of extracts were applied to Sephadex G-50 superfine columns (10×1000 mm), which were eluted at 4°C with 0.02 M barbital buffer, pH 8.4, containing 0.1% bovine serum albumin. Fractions of 1.0 ml were collected at a rate of 4.0 ml/h. The columns were calibrated with [^{125}I]albumin (void volume), gastrin-34 and gastrin-17, and with ^{22}NaCl (total volume). The eluted fractions were assayed with a sequence-specific RIA using antibody no. 2604 as described below. The percentages of α-amidated gastrin were calculated by planimetry. Four different extracts from each of the four groups of mice (PC2 knockout, 7B2 knockout and corresponding controls) were subjected to gel chromatography.

2.4. RIAs

A library of sequence-specific antibodies against progastrin was used to measure the different forms of gastrin and its processing intermediates (Fig. 1). The sum of carboxyamidated, bioactive gastrin was measured using the gastrin-specific antiserum no. 2604, with [^{125}I]gastrin-17 as tracer and gastrin-17 as standard [32]. Antibody no. 2604 binds all the carboxyamidated forms of gastrin (except gastrin-6) with equimolar affinity irrespective of size and sulfation. The crossreactivity with homologous cholecystokinin peptides is negligible [32]. Non-sulfated carboxyamidated gastrins were measured in parallel using antibody no. 2605 [33]. Glycine-extended processing intermediates of progastrin were measured using antiserum no. 3208 with [^{125}I]glycine-extended gastrin-17 as tracer and glycine-extended gastrin-17 as standard. Glycine–arginine-extended intermediate precursors were measured using antiserum no. 3208 following enzymatic pretreatment with only carboxypeptidase B (CPB). To measure all precursor forms of gastrin, samples were pretreated with both trypsin and CPB followed by RIA with antiserum no. 3208, as described previously [34]. CPB mimics the effect of carboxypeptidase E, while trypsin mimics the effects of PCs.

3. Results

As shown in Tables 1 and 2, the concentrations of progastrin, its main categories of processing intermediates, and its functional end product, the carboxy- or α-amidated gastrins, were similar in the antral extracts of knockout mice and their corresponding wild-type controls. The degree of tyrosyl O-sulfation of the gastrins was also similar in knockout and wild-type mice. These measurements would seem to indicate, that PC1, tyrosyl-sulfotransferases, carboxypeptidase E and PAM act independently of PC2 and 7B2 in antral G-cells.

Gel chromatography, however, displayed major processing abnormalities in the 7B2 and PC2 knockout mice. As shown in Fig. 2 and Table 3, gastrin-34 constituted by far most of the carboxyamidated gastrin in the antrum of 7B2 knockout mice, PC2 knockout antra contained equal amounts of gastrin-34 and gastrin-17 and the antral gastrin in the wild-type mice was mainly gastrin-17, the predominant antral gastrin of all mammals. Hence, the chromatography shows that the K_{53}–K_{54} cleavage site of progastrin that normally is almost completely processed (i.e. >90%) is only partly cleaved (~50%) in the absence of PC2. But in the absence of 7B2, only 23% of the double-K sites are processed.

Tables 1 and 2 also reveal differences between the progastrin concentrations, for instance, of the two groups of wild-type controls. Presumably, the differences reflect that different strains of mice were examined. As mentioned above, the PC2 study used C57BL/6J:Sv129 (50:50 mix) mice, and the 7B2 study Sv129 mice. Moreover, the mice in the 7B2 study and its controls were three weeks younger than those in the PC2 study due to the high mortality of 7B2 null mice [30].

4. Discussion

The relative contributions of the various convertases to biologically relevant precursor cleavages is only now beginning to be elucidated. One of the important findings of the present study is that PC2-mediated cleavage occurs at the K_{53}–K_{54} site of progastrin. Cleavage at this site is necessary for synthesis of the major functional product of progastrin, the carboxyamidated gastrin-17 peptide, which is known to be released from antroduodenal G-cells that produce most of the gastrin in mammals. The present in vivo data support the earlier observation from our laboratory that pituitary corticotrophs, which contain only PC1, cannot process the K_{53}–K_{54} site [11,19]. The later study of Sawada et al. [35] also supports this notion. They found that PC2 but not PC1 can accomplish cleavage of this site in transfected AtT-20 cells [35]. Similarly to progastrin, proopiomelanocortin (POMC) also contains a K–K site within β-endorphin which remains unprocessed in AtT-20 cells, but can be processed upon cotransfection with PC2 [36,37]. However, cleavage of the K–K site within proenkephalin is apparently largely mediated by PC1, since it is efficiently cleaved within AtT-20 cells [38]. Examination of the primary sequences of the three precursors (progastrin, POMC, and proenkephalin) reveals extensive differences in the vicinity of the K–K site, which could account for the differential ability of PC1 to recognize these types of processing sites within different contexts.

Since half of the K–K sites within progastrin can be cleaved in the absence of PC2, other convertases are also likely to participate in this cleavage in vivo. Potential candidates for this activity include PC1 and PC5, both expressed in G-cells ([27,28] and Friis-Hansen, unpublished data). The cleavage specificity of PC5 is not known at present. As mentioned above, PC1 is apparently unable to perform this particular cleavage since the site remains uncleaved within AtT-20 cells [19,35].

It is interesting to note that the cleavage profiles of progastrin-derived peptides differ between the PC2 and 7B2 null mice. Since 7B2 is thought to represent a binding partner for PC2, which is absolutely required for the production of

Table 1
Antral progastrin products in PC2 null and corresponding wild-type mice (pmol/g tissue (mean ± S.E.M.) $n = 8$)

	PC2 null	Wild-type
Progastrin	4.2 ± 1.3	2.8 ± 0.7
Gastrin–Gly-Arg-(Arg)	0.0 ± 0.0	0.1 ± 0.0
Gastrin–Gly	1.3 ± 0.3	1.1 ± 0.3
Gastrin (α-amidated)	150.0 ± 28.0	130.1 ± 27.0
Gastrin (α-amidated and non-sulfated)	43.2 ± 8.6	34.0 ± 8.5

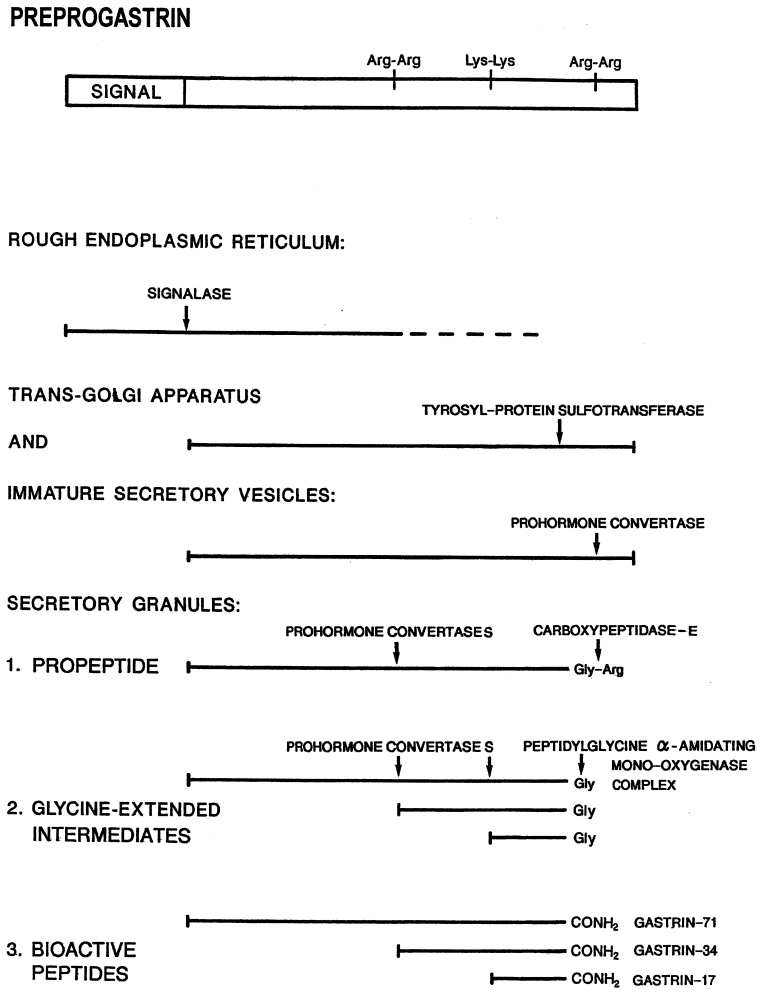

Fig. 1. Diagrammatic presentation of the co- and posttranslational modification of preprogastrin in the stomach. Activation of the gastrin amidation site (–Phe-Gly-Arg-Arg–) occurs via a series of carboxy-terminal cleavages and modifications. Endoproteolytic cleavage by PCs produces the carboxypeptidase 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 of glycine-extended gastrin by α-amidating monooxygenase results in the production of bioactive gastrin (–Phe-NH_2). Concomitant N-terminal cleavage by PCs produces bioactive gastrins of varying size (e.g. gastrin-34 and gastrin-17). A library of sequence-specific antibodies was used in combination with in vitro protease treatments to measure bioactive gastrin and various precursor peptides, as described in the text.

active enzyme [39], one might expect that the absence of either protein would yield a similar PC2-less processing profile. The data of this study indicate that cleavage of the two R–R sites (at positions 36–37 and 73–74) occurs without interruption in the two null animals. Therefore, cleavage at these sites is presumed to be mediated by PC1 [26,40]. However, cleavage of the K–K site mentioned above is significantly reduced in the 7B2 null animals as compared to the PC2 null animals. This unexpected result may indicate a role for 7B2 which extends beyond its function as an activating protein for proPC2. Such a role is hinted at by previous studies showing that 7B2 null animals hypersecrete ACTH from the intermediate lobe of the pituitary, while PC2 null animals do not ([30] and Lindberg, unpublished results). This role is unlikely to represent binding to other convertases, as no convertase other than PC2 was able to interact with 7B2 in co-immunoprecipitation experiments [40]. Proenkephalin is apparently similarly processed in PC2 and 7B2 null animals [30,41]; however, preliminary results indicate that processing of POMC differs somewhat between PC2 and 7B2 nulls (Laurent and Lindberg, unpublished results). Proglucagon processing in both null models has been studied using pulse-chase analysis [30,42]

Table 2
Antral progastrin products in 7B2 null and corresponding wild-type mice (pmol/g tissue (mean ± S.E.M.) $n = 5$)

	7B2 null	Wild-type
Progastrin	7.0 ± 1.4	6.8 ± 2.8
Gastrin–Gly-Arg-(Arg)	0.0 ± 0.0	0.3 ± 0.1
Gastrin–Gly	1.4 ± 0.4	1.8 ± 0.6
Gastrin (α-amidated)	120.0 ± 41.0	160.0 ± 48.2
Gastrin (α-amidated and non-sulfated)	36.1 ± 8.8	57.1 ± 24.0

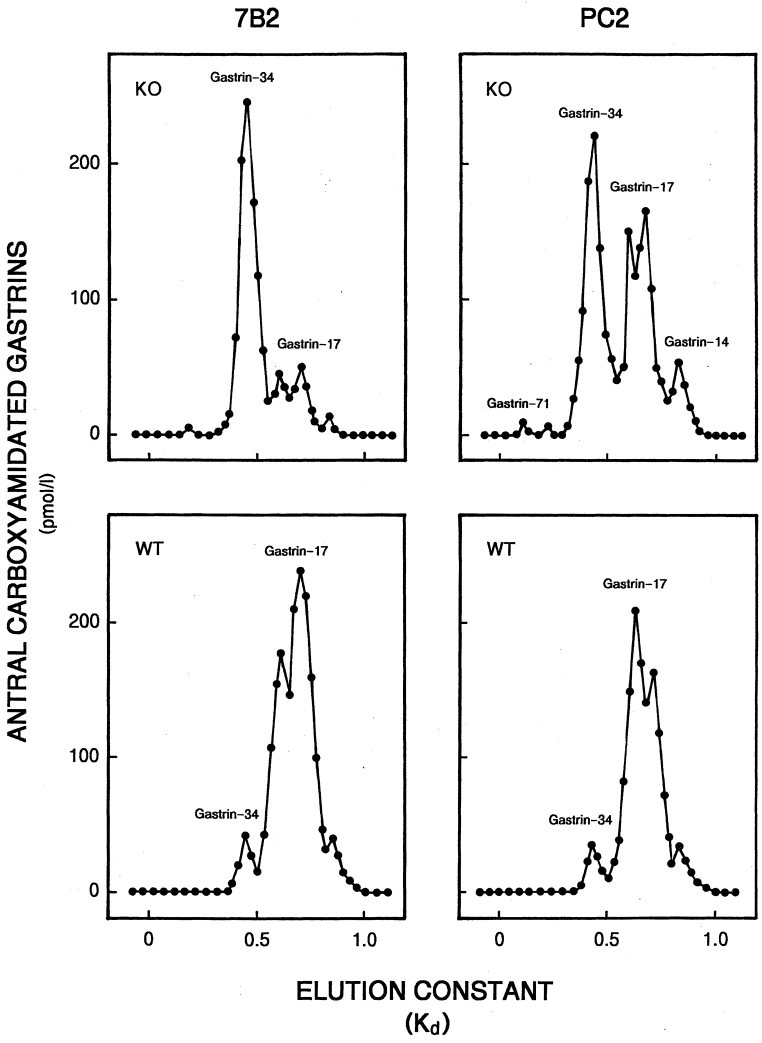

Fig. 2. Gel chromatography on Sephadex G-50 superfine columns of stomach extracts from 7B2 and PC2 knockout mice (upper panels) and from corresponding wild-type control mice (WT; lower panels). The chromatographic elutions were monitored using antibody 2604 (●–●), that binds carboxyamidated gastrins (the bioactive gastrins) irrespective of the degree of tyrosyl-sulfation. Stomach extracts from four different mice in each of the four groups (7B2 and controls, PC2 and controls) were subjected to chromatography. Those shown above are characteristic of each group.

and has been shown to be slightly more extensive in the PC2 null than in the 7B2 null mice, results opposite to those presented here. Thus, the contribution of supplementary roles for 7B2 (i.e. beyond those of assisting PC2) to overall processing appears to vary by the structure of the precursor to be processed, and will require additional investigation. Given the recent identification of a binding partner for PC1, proSAAS [43], the differential contributions of convertase binding proteins to precursor processing is likely to represent a fruitful area for further study.

Table 3
Antral α-amidated gastrins in PC2 null, 7B2 null and wild-type mice (% (mean ± S.E.M.))

	7B2 null	PC2 null	Wild-type
Gastrin-34	75.8 ± 17.1	46.4 ± 8.9	5.0 ± 1.6
Gastrin-17	22.6 ± 6.5	44.3 ± 7.4	91.1 ± 7.6
Gastrin-14 and -71	1.6 ± 0.9	9.3 ± 3.2	3.9 ± 1.1

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