Disturbed progastrin processing in carboxypeptidase E-deficient *fat* mice

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Abstract The *fat* mouse strain exhibits a late-onset obesity syndrome associated with a mutation in the gene encoding carboxypeptidase E (CPE). Since CPE plays a central role in the biosynthesis of a number of regulatory peptides, including gastrin, we examined the biogenesis and processing of progastrin in *fat/fat* mice by measuring gastrin mRNA, carboxyamidated gastrin and its processing intermediates in the stomach. The tissue concentration of carboxyamidated (i.e. bioactive) gastrin was only slightly reduced (601 ± 28 pmol/g in *fat/fat* mice vs. 715 ± 43 pmol/g in wild-type controls). However, progastrin processing intermediates accumulated excessively with an 86-fold increase in the concentration of the CPE substrate, glycyl-arginine extended gastrin, and a seven-fold increase in the concentration of glycine-extended gastrin. Accordingly, the total progastrin product was doubled, as was the concentration of gastrin mRNA. Plasma concentrations of carboxyamidated gastrin were, however slightly reduced both in fasted *fat/fat* mice and postprandially. The results show that the CPE mutation diminishes the efficiency of progastrin processing, but gastrin synthesis is nevertheless increased to maintain an almost normal production of bioactive gastrins. By comparison with other neuroendocrine prohormones, progastrin processing in CPE-deficient mice is unique. Hence, the increase of glycine-extended gastrin in combination with normal levels of carboxyamidated gastrin suggests that G-cells may have another biosynthetic pathway for gastrin.

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Key words: Progastrin processing; Carboxypeptidase E; *fat* mice

1. Introduction

Peptide hormones and neurotransmitters are synthesized as propeptides that undergo a series of modifications in the trans-Golgi network and the secretory vesicles before they are released as active peptides. The modifications include endo- and exoproteolytic cleavages, sulfation, phosphorylation, amidation and others. Prohormone convertases catalyze cleavages at di- and monobasic sites [1–3] with subsequent trimming by a carboxypeptidase to remove C-terminal basic residues [4,5].

Recently the *fat* mouse mutant was identified as one of the first in vivo models containing a mutation in a prohormone processing enzyme. The *fat* mutation is a missense mutation (Ser[202]Pro) in the carboxypeptidase E (CPE) gene, which appears to abolish enzyme activity [6]. The resulting phenotype is a slowly developing adult-onset obesity with mild diabetes

[6,7]. CPE is involved in the posttranslational maturation of many regulatory peptides. Thus, peptides requiring C-terminal trimming of basic residues may be deficient in this mouse mutant. Accordingly, *fat/fat* mice exhibit changes in the processing of proinsulin, prodynorphin, proneurotensin, promelanin-concentrating hormone and pro-opiomelanocortin showing accumulation of hormone precursors and a marked decline in the production of bioactive peptides [6,8–10]. However, it is difficult to predict the complete range of peptides affected in the *fat/fat* mouse since carboxypeptidase D may compensate for CPE in some tissues [9,11].

Cellular synthesis of the gastrointestinal hormone, gastrin, also requires carboxypeptidase cleavage (Fig. 1). Progastrin processing involves endoproteolytic cleavage by prohormone convertases yielding a C-terminally glycyl-arginine extended gastrin, which by carboxypeptidase removal of the arginyl residues exposes glycine-extended gastrin for amidation [12]. Using the *fat* mouse as an in vivo model, we have now tested the hypothesis that CPE is required for the maturation of gastrin. Moreover, we examined how alterations in progastrin processing might change the biosynthesis to ameliorate the effects of reduced maturation. Finally, we measured plasma concentrations of amidated (i.e. bioactive) gastrin to determine if *fat/fat* mice responded adequately to food.

2. Materials and methods

2.1. Mice

Heterozygous *fat*/+ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous *fat/fat* and wild-type (+/+) mice were generated by intercrossing heterozygotes and genotyping the offspring. Mice were housed on a 12 h light-dark cycle and fed Purine 5008 chow ad lib.

2.2. Genotyping

Genomic DNA was prepared from tail biopsies [13]. The *fat* and wild-type CPE alleles were detected using an allele-specific polymerase chain reaction (PCR) assay. Primer CPE-R (5' CGC TCC GTG TCT CAT CAT ATG GGT AAT) was paired with primer WT (5' GGA CAT TCC ATT TGT GCT T̲T̲) or FAT (5' GGA CAT TCC ATT TGT GCT T̲C̲) to amplify an 80 bp fragment from the wild-type or *fat* alleles, respectively. (Underlined primer sequences highlight the single nucleotide difference between the two alleles.) PCR reactions contained 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 3.5 mM (WT) or 3 mM (*FAT*) $MgCl_2$; 0.01% (w/v) bovine serum albumin; 0.2 mM dATP, dCTP, dGTP and dTTP; *Taq* polymerase; and 1.0 μM (CPE-R, *FAT*) or 2.0 μM (WT) primers. The cycle parameters were: (i) 94°C 5 min, (ii) 40 cycles of 94°C 45 s, 62°C 45 s, 72°C 1 min, (iii) 72°C 3 min. The amplified products were detected by electrophoresis in 5% agarose [3:1 NuSieve GTG (FMC BioProducts):ultra PURE agarose (Gibco-BRL)] and ethidium bromide staining.

2.3. Tissue isolation and extraction

Eight mice 2–6 months old were anesthetized, and stomach tissue was rapidly dissected and frozen in liquid nitrogen. The tissue was gently cleaned in phosphate buffered saline, on ice, before freezing. Tissue extracts for radioimmunoassay (RIA) were prepared as previ-

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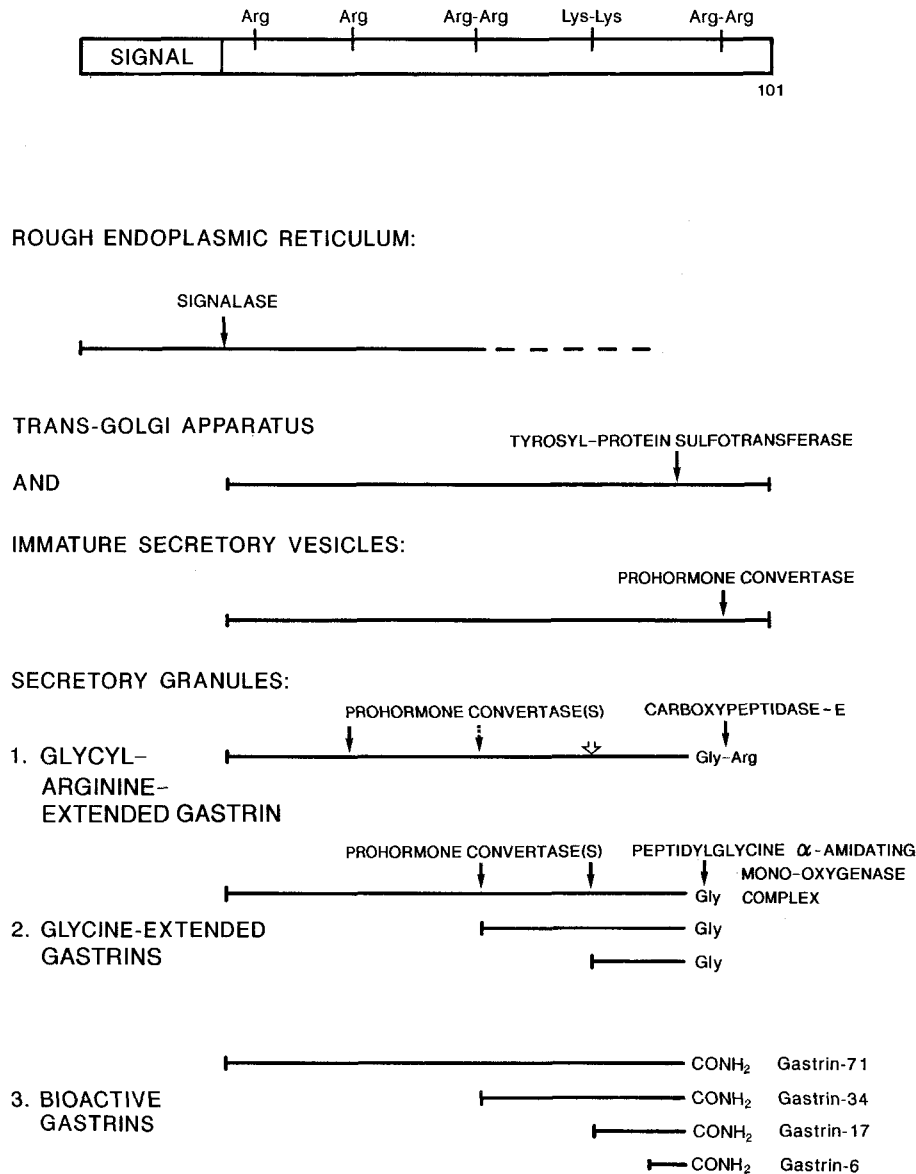E-mail: rehfeld@rh.dk

Fig. 1. Diagrammatic presentation of the co- and posttranslational modification of preprogastrin in the stomach. Activation of the gastrin amidation site (R_1-Phe-Gly-Arg-Arg-R_2) occurs via a series of carboxy-terminal cleavages and modifications. Endoproteolytic cleavage by prohormone convertases produces the carboxypeptidase E substrate (R_1-Phe-Gly-Arg). Carboxypeptidase E then acts in secretory granules to remove the C-terminal arginine residue yielding glycine-extended gastrin (R_1-Phe-Gly). Carboxyamidation of glycine-extended gastrin by α-amidating mono-oxygenase results in the production of bioactive gastrin (R_1-Phe-NH_2). Concomitant N-terminal cleavage by prohormone convertases produces pro- and bioactive gastrins of varying sizes (e.g. gastrin-71, gastrin-34, etc.). A library of sequence-specific antibodies was used in combination with in vitro protease treatments to measure bioactive gastrin and various precusor peptides, as described in Section 2.

ously described [14]. Briefly, frozen tissues were boiled in water (1 ml/ mg) for 20 min, homogenized (polytron) and centrifuged for 30 min at 10000 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 water and acid supernatants were stored at −20°C until RIA analysis.

2.4. Chromatography

One or two ml of extracts were applied to Sephadex G-50 superfine column (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 [125I]albumin (void volume), human gastrin-71, -34 and -17 (both sulfated (s) and non-sulfated (ns) gastrin-17), and

with $^{22}NaCl$ (total volume). The elutions were monitored with sequence-specific radioimmunoassays as described below.

2.5. Radioimmunoassay

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 were measured using the gastrin-specific antiserum no. 2604, with [125I]gastrin-17 as tracer and gastrin-17 as standard [15]. Antibody no. 2604 binds all the carboxyamidated forms of gastrin with equimolar affinity irrespective of size and sulfation. Crossreactivity with homologous cholecystokinin (CCK) peptides is negligible [15]. Nonsulfated carboxyamidated gastrins were measured in parallel using antibody no. 2605 [16]. Glycine-extended intermediates of gastrin were measured using antiserum no. 3208 with [125I]glycine-extended
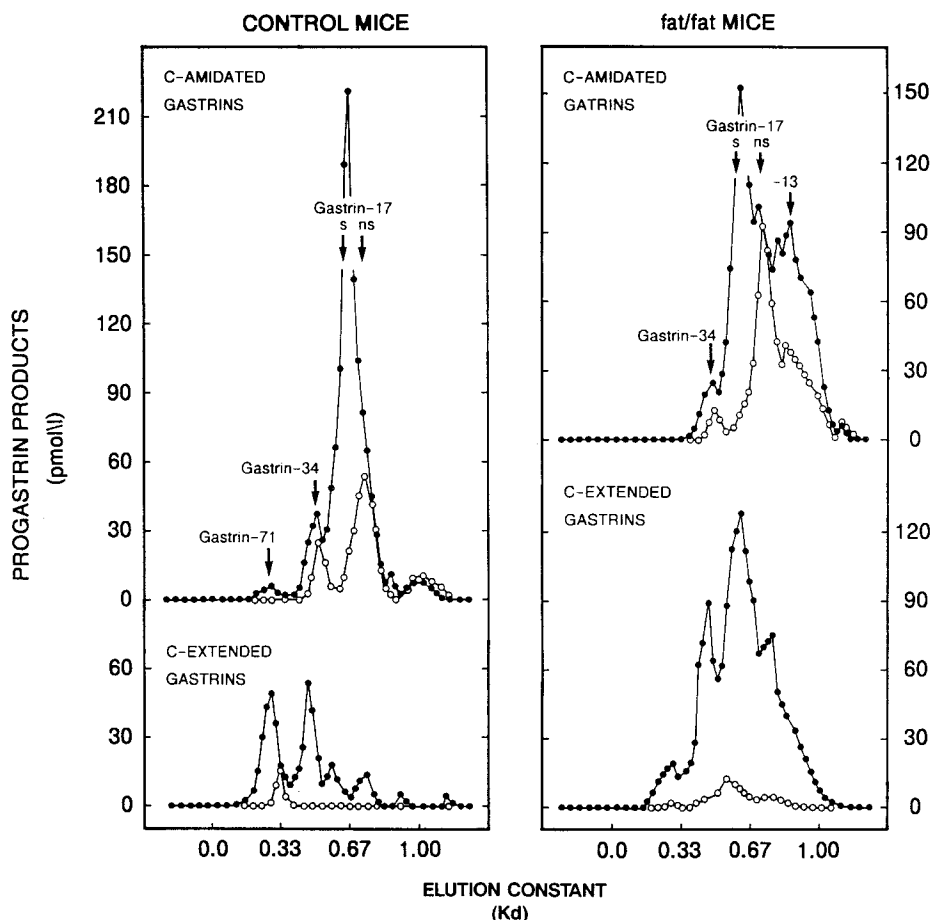
Fig. 2. Gel chromatography on Sephadex G-50 superfine columns of stomach extracts from wild-type control mice (left) and the CPE-mutant *fat/fat* mice (right). The upper panels show the elution of carboxyamidated gastrins as measured using Ab. no. 2604 (●), which binds both sulfated and non-sulfated gastrins and Ab. no. 2605 (○), which binds only non-sulfated gastrins. The lower panels show the elution of glycine-extended gastrins (Ab. no. 3208, ○) and further C-terminally extended gastrins (Ab. no. 3208 after trypsin and carboxypeptidase B treatment, ●). The arrows indicate the elution positions of corresponding human gastrins used for calibration.

gastrin-17 as tracer and glycine-extended gastrin-17 as standard [17]. Glycine-arginine-extended intermediate precursors were measured using antiserum no. 3208 following enzymatic pretreatment with carboxypeptidase B (CPB). To measure all three precursor forms of gastrin, samples were pretreated with trypsin and CPB followed by RIA with antiserum no. 3208, as described previously [17]. CPB mimics the effect of CPE, while trypsin mimics the effects of prohormone convertase. Control measurements of homologous CCK peptides were performed with a CCK-specific RIA using the new antibody no. 92128 (Rehfeld, unpublished).

2.6. RNA analysis

Tissue for RNA analysis was isolated from animals fasted overnight. Total RNA was extracted from frozen tissue by a guanidine

thiocyanate homogenization-CsCl centrifugation method [18]. For Northern blot analysis, total stomach RNA samples (10 μg) were electrophoresed in agarose gels containing 2.2 M formaldehyde and transferred to Zeta-Probe nylon membrane (BioRad). Probes were ^{32}P-labeled and hybridized to filters as previously described [19]. Final wash conditions were 0.5×SSC (0.075 M NaCl, 7.5 mM trisodium citrate, pH 7.0) and 0.1% SDS at 60°C. Imaging and quantitation were performed on a GS-250 Molecular Imager (BioRad). The gastrin probe was a 422 bp cDNA [20]. After hybridization, filters were stripped and rehybridized with a mouse ribosomal protein L32 probe [21] to control for RNA loading.

2.7. Plasma hormone measurements

To examine changes in circulating gastrin concentrations in re-

Table 1
Progastrin peptide products in the stomach from *fat/fat* and wild-type mice (pmol per g tissue (wet weight); mean ± S.E.M., $n = 8$)

Peptide	Wild-type	*fat/fat*
Carboxyamidated gastrins		
sulfated	506 ± 37	413 ± 31
non-sulfated	209 ± 11	188 ± 17
total amidated	715 ± 43	601 ± 28
C-terminally extended gastrins		
glycine-extended	4.8 ± 0.5	34.7 ± 7.1
glycine-arginine-extended	12.2 ± 6.3	1054 ± 122
further extended	11.6 ± 5.2	0
Total progastrin product	744	1690

sponse to feeding, mice were fasted for 24 h before blood collection by retro-orbital bleeding. A second group of mice was fasted and then refed for 40 min before bleeding. Blood was collected into tubes containing 10 mM EDTA. Plasma concentrations of carboxyamidated gastrins were measured radioimmunochemically using antibody no. 2604 as previously described [15,22].

2.8. Statistics

Statistical analysis was carried out using an unpaired t-test with the SYSTAT software. All values are expressed as means ± S.E.M.

3. Results

3.1. Progastrin processing

The concentrations of carboxyamidated (i.e. bioactive) gastrins in the stomach was insignificantly lower in *fat/fat* mice compared to wild-type controls (Table 1). A large percentage of carboxyamidated gastrins are sulfated in normal mice (71%, Table 1). The similar degree of sulfation in *fat* mice (69%) indicates that the early posttranslational modification by tyrosyl-protein sulfotransferase is unaffected by CPE. To determine the effect of the CPE mutation on further progastrin processing, we measured the concentrations of a number of processing intermediates (Fig. 1) in gastric extracts. In contrast to the almost normal concentration of carboxyamidated gastrin, those of some progastrin processing intermediates were grossly increased in the mutant. The concentration of the progastrin-derived substrate for CPE, glycyl-arginine-extended gastrin, was 86-fold elevated in *fat/fat* mice (12.2 vs. 1054.0 pmol/g, Table 1). Surprisingly the concentration of glycine-extended gastrin was also markedly increased (from 4.8 to 34.7 pmol/g, Table 1). In contrast *fat/fat* mice were apparently devoid of further C-terminal extended gastrin (Table 1). Summing up the total progastrin product, *fat/fat* mouse stomachs contain twice the amount of wild-type controls.

Gel chromatography showed marked differences in the N-terminal proteolytic processing of progastrin in *fat/fat* and wild-type mice (Fig. 2). Carboxyamidated, glycine-extended

Fig. 4. Gastrin mRNA abundance in *fat/fat* mice. Displayed are the results from Northern blot analysis of stomach RNA hybridized with a gastrin probe from two wild-type (+/+) and two *fat/fat* (*f/f*) mice. The top half shows hybridization with the gastrin probe, while the bottom half shows the same filters after stripping and rehybridizing with the rpl32 probe to control for loading.

and glycyl-arginine-extended gastrins were cleaved at N-terminal processing sites to a greater extent in *fat/fat* mice, so that shorter molecular forms constituted a larger fraction of the progastrin products. The molecular pattern suggests that CPE influences endoproteolytic prohormone convertase cleavage, but not the early tyrosyl protein sulfotransferase modification (Table 1, Figs. 1 and 2).

3.2. Plasma gastrin

The basal plasma concentration of carboxyamidated gastrin in *fat* mice was not insignificantly lower than in wild-type controls (Fig. 3). To examine whether *fat/fat* mice exhibit the normal postprandial rise, we also measured gastrin concentrations in plasma after refeeding. In wild-type mice gastrin concentrations significantly increased 40 min after refeeding. However, this response was attenuated in *fat/fat* mice. Although circulating gastrin concentrations in *fat/fat* mice rose after refeeding, postprandial concentrations were 2.4-fold lower than in the control mice.

3.3. Gastrin mRNA levels

Gastrin transcript levels measured by Northern blot analysis were two-fold higher in *fat/fat* mice ($P < 0.05$; $n = 4$ per group) (Fig. 4). This is in accordance with the two-fold increase in progastrin processing intermediates (Table 1).

4. Discussion

The results of this study demonstrate that CPE is heavily involved in the maturation of progastrin. However, the processing differs from that of other prohormones examined so far [6,8–10], as CPE-deficient mice are able to maintain essentially normal tissue concentrations of bioactive α-carboxyamidated gastrin. Analysis of gastrin processing revealed a marked increase in some precursor peptides, including the excessive 86-fold accumulation of glycyl-arginine-extended gastrin. We expected the defect in carboxypeptidase activity to result in an accumulation of glycyl-arginine-extended gastrin, since this is the substrate for CPE. Previous studies in *fat/fat* mice demonstrated reduced levels of mature peptides, such as insulin, neurotensin, melanin-concentrating hormone, dynorphin and ACTH, with a more modest accumulation of arginine-extended peptides [6,8–10], and with no significant changes in other processing intermediates. We have also found such processing abnormalities for cholecystokinin, a hormone ho-
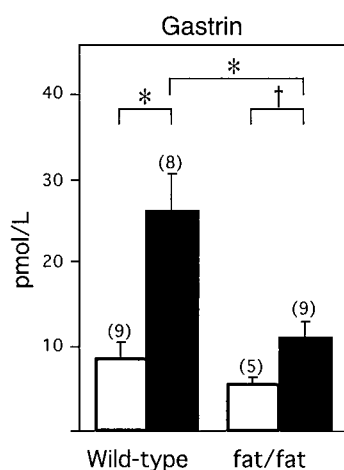

Fig. 3. Plasma concentrations of carboxyamidated bioactive gastrins in fasted and refed mice. Fasted mice were food deprived for 24 h, and refed mice were fasted then refed for 40 min before plasma collection. Open bars represent fasted mice and solid bars represent refed mice. The mean values and S.E.M. are expressed as pmol per liter plasma. The number of mice is shown for each group in parentheses. *$P < 0.01$; †$P < 0.05$.

mologous to gastrin (unpublished). Further examination of gastrin processing revealed an unexpected seven-fold accumulation of glycine-extended gastrin and the absence of other C-terminally extended gastrin precursors. The massive accumulation of gastrin processing intermediates indicates that gastrin biosynthesis is upregulated in the mutant to compensate for the processing deficiency. Thus, the effects of CPE dysfunction on progastrin processing appear to be unique in comparison with the processing of other prohormones.

The seven-fold increase in glycine-extended gastrin is surprising since this intermediate is the product of carboxypeptidase cleavage and thus expected to be readily amidated. The accumulation of glycine-extended gastrin indicates that the peptidylglycine α-amidating mono-oxygenase complex is not readily available for amidation following carboxypeptidase cleavage, suggesting an alternative processing pathway for progastrin in the *fat/fat* mutant. There are recent data to suggest that CPE may function as a sorting receptor to partition peptides into the regulated secretory pathway [10]. Loss of sorting receptor in the *fat* mutant could result in the misdirection of progastrin into a different cellular compartment, removed from other modifying enzymes, producing more widespread disturbances in peptide processing than would be predicted from reduced carboxypeptidase activity alone.

To investigate possible mechanisms for the upregulation of gastrin synthesis, we measured gastrin mRNA in the stomach and found that transcript levels doubled in *fat/fat* mice. This change in gastrin mRNA is similar to increases observed when gastric acid secretion is blocked by the H^+/K^+-ATPase antagonist, omeprazole, or by surgical removal of the acid-secretory portion of the stomach [23,24]. In these models, increased gastrin mRNA is also associated with hypergastrinemia, while in *fat/fat* mice, the plasma concentration of carboxyamidated gastrin was slightly reduced. The observed increase in gastrin mRNA in *fat/fat* mice could result from a proliferation of gastrin producing cells (G-cells), or from increased gene transcription or mRNA stability. A prolonged state of gastric acid hyposecretion can lead to an increase in the number of G-cells in the gastric mucosa. Indeed, G-cell numbers are increased two-fold in a mutant mouse strain which does not express gastrin receptors [25]. Alternatively, an accumulation of transcripts within individual G-cells could also lead to elevated gastrin mRNA levels. Somatostatin, the primary negative regulator of gastrin release, inhibits gastrin gene expression by decreasing transcription and mRNA stability [26–29]. Thus, a reduction in gastrin and gastric acid output in *fat/fat* mice could reduce somatostatin secretion, thereby lessening the inhibitory mechanisms regulating gastrin biosynthesis. However, we measured somatostatin in stomach extracts and found no differences between *fat/fat* mice and wild-type controls (data not shown).

During the preparation of this paper, Udupi et al. reported a similar study on gastrin processing in *fat/fat* mice [29]. Their results generally correspond to those presented here, but they deviate in two important respects. First, they did not measure glycine-extended gastrin, which is necessary for interpreting changes in G-cell processing caused by the CPE mutation. The unexpected increase in the concentration of glycine-extended gastrin found in our study (Table 1) is a key argument for the existence of an alternative processing pathway. In addition, the possible growth effects of glycine-extended gastrin [30,31] have drawn particular attention to its synthesis.

Second, Udupi et al. found a significant reduction in the concentration of carboxyamidated gastrin.

Maintaining physiologic concentrations of gastrin is important for normal stomach function. Measurement of plasma concentrations showed that although the basal concentration of bioactive gastrin is normal in *fat/fat* mice, meal-stimulated gastrin secretion is attenuated. Forty minutes after a meal, gastrin concentrations were 2.4-fold lower in *fat/fat* mice compared to controls. This reduction in postprandial gastrin levels suggests that meal-stimulated acid secretion may also be attenuated. Thus, the attenuation of postprandial hormone secretion in the *fat/fat* mouse suggests that the CPE processing defect may affect gastric function.

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