The orexigenic hormone ghrelin is a 28-amino-acid peptide derived from a 99-amino-acid precursor and acylated at Ser-3, which was initially isolated from rat stomach. In addition to stimulating appetite and growth, it also plays various important roles in energy homeostasis and in the cardiovascular and immune systems. Although analysis of its physiological effects has yielded many significant results, much less information is available on its biosynthesis and the mechanism of its acylation.

In this report, we have studied the endoproteolytic processing of this molecule from its precursor (proghrelin) into mature ghrelin in various prohormone convertase null mouse strains generated in our laboratory and have identified the convertase responsible for this event. Using Western blotting, mass spectrometry, and immunocytochemical methods, we have demonstrated that (a) in mouse stomach, prohormone convertase 1/3 (PC1/3) is the endoprotease responsible for the conversion of proghrelin to ghrelin, (b) the acylation of this peptide is processing-independent, and (c) the expression of proghrelin mRNA is increased in the processing-deficient (PC1/3 null) mouse.

Ghrelin, a 28-amino-acid peptide, was initially isolated from rat stomach (1) as a ligand of the growth hormone secretagogue receptor (2). Subsequent studies have revealed that it is a potent orexigenic peptide (3). It is also involved in various functions of the heart (4), pancreatic islets, and insulin secretion (5–10) and also in insulin signaling (11), adipose tissue (12), and the immune system (13). The expression of ghrelin is not limited to the stomach. The duodenum also produces large amounts of ghrelin, and it is also expressed at lower levels in other tissues, such as pancreas, brain, liver, and lung, (14).

Similar to many other peptide hormones, ghrelin is processed from a 94-amino-acid precursor. Octanoylation at a serine residue (Ser-3) is necessary for its action via the growth hormone secretagogue receptor, although the desoctanoyl peptide may have alternative functions (15). Interestingly, recent research has revealed another bioactive peptide named obestatin within the ghrelin precursor (16). Obestatin is a 23-amino-acid peptide and is a cognate ligand for receptor GPR39, which is a member of a ghrelin receptor subfamily (17, 18). Although derived from the same precursor, in contrast to the effects of ghrelin, obestatin suppresses food intake, inhibits intestinal activity, and decreases weight gain (16). It is well established that many bioactive peptide hormones are generated by limited proteolytic processing by members of a secretory pathway-specialized serine protease family related to yeast Kex2 (the subtilisin-like prohormone convertase family SPCs or PCs), which includes PC1/3, PC2, and PC5/6A (19, 20). Much data has accumulated over the past few years on functional aspects of ghrelin. However, biosynthetic studies have been lacking. In this report, we investigate the processing of proghrelin in various prohormone convertase-deficient mouse models available in our laboratory.

**EXPERIMENTAL PROCEDURES**

**Animals**—Mice used in this study were all on the C57BL/6J genetic background. They were maintained under controlled temperature (21–23 °C) and lighting conditions (lights on 7:00 a.m.–7:00 p.m.) with free access to food and water. All experiments were conducted in accordance with the National Institutes of Health guidelines. PC1/3 and PC2 null mice were created as described previously (21, 22). PC5/6A null mice were created by deletion of exon 23A.2 Exon23A encodes a 38-amino-acid C-terminal tail that targets PC5A into secretory granules (23).

**Peptide Extraction**—Peptides were extracted following the procedure described by Kojima et al. (1). Briefly, four-month-old mouse stomachs (~200 mg) were dissected free and opened to remove their contents. They were rinsed with double-distilled water and then boiled for 10 min in 1 ml of double-distilled water to inactivate proteases. The stomachs were then minced, the resulting suspensions adjusted to a final concentration of 1 m acetic acid and 20 mm HCl, and homogenized with a polytron. The mixtures were centrifuged at 200,000 × g for 30 min. The supernatants were collected and the volume reduced to 0.3 ml using a Speedvac before being precipitated by the addition of 0.6 ml of acetone. The precipitates were removed by centrifugation at 13,000 revolutions/min for 10 min and the supernatants dried in vacuo for Western blot analysis.

**Western Blot**—The dried stomach extracts (~10 μg of protein) were resuspended in 30 μl of water and 60 μl of tricine sample buffer. The mixture was boiled for 5 min before 30 μl of each was loaded onto a 20% tris-glycine gel. The gel was then transferred to a polyvinylidene difluoride membrane overnight at 24 volts using glycine buffer in the presence of 20% methanol. The purified antighrelin IgG was a gift from

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2 X. Zhu, unpublished results.
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MHSLGCICSLLLSMWMDMMAMAGSFLSPEHOKAQCOKESKKPKPAKLQTPRLEGWLH

Signal Peptide

PEDRGGAEETEELEIFNAPFDVGIKLSQGQYQQHGRALGKFQDILWEEVKEAPADK

Obestatin

FIGURE 1. Amino acid sequence of mouse proghrelin. Arrows are putative convertase cleavage sites.

FIGURE 2. Proghrelin is not processed in the PC1/3 null stomach. A, Western blot of mouse stomach extract. B, mass spectrometric trace of the 11-kDa band recovered from the gel.

Roy Smith, which was raised against octanoyl-ghrelin (24). The second antibody was NA934V, ECL anti-rabbit IgG, horseradish peroxidase-linked.

Immunocytochemistry—Stomachs were harvested and embedded in OCT (catalog number 4583, Sakura Finetek USA, Inc., Torrance, CA) and frozen under −20 °C 2-methylbutane for 20 min. Thin sections with a thickness of 5 μm were produced with a cryomicrotome. They were then fixed with 4% paraformaldehyde for 10 min at room temperature followed by three washes of phosphate-buffered saline. The sections were treated with 10% normal donkey serum in phosphate-buffered saline containing 0.01% Triton X-100. For ghrelin staining, 1 g of anti-ghrelin IgG was conjugated to Alexa Fluor 488 (Molecular Probes, Inc., Eugene, OR). The conjugated IgG was then diluted into 500 μl of phosphate-buffered saline and used for staining. For PC1/3 staining, the sections were treated with PC1/3 antibody (1:600 dilution, a gift from Iris Lindberg) followed by treatment with donkey anti-rabbit IgG-conjugated Alexa594 (1:100 dilution, Molecular Probes). The sections were examined with a fluorescence microscope (Olympus BX51).

Northern Blot—Fresh mouse stomach was rinsed and minced before it was homogenized in 5 volumes (~1 ml) of cold TRIzol (Invitrogen) with a polytron and then centrifuged at 13,000 revolutions/min using a table top microcentrifuge for 15 min. Two hundred microliters of chloroform were added to the supernatant and mixed by vortexing and then centrifuged at 13,000 revolutions/min, again to collect the supernatant. RNA was precipitated by adding an equal volume of isopropyl alcohol. The pellet was washed once with 80% ethanol. RNA was resuspended in diethylpyrocarbonate water, and the concentration was estimated by A260. Samples (100 μg each) were loaded onto a 1.3% agarose gel containing 10% formaldehyde. After electrophoresis (5 V/cm, 3 h), the RNA was transferred to a nylon membrane (Ambion, catalog number 1940). The RNA probe was labeled with P-32 by transcribing ghrelin cDNA with a T7 promoter. The membrane was washed and exposed to a phosphorimaging device, and the intensity of each band was quantified and expressed as arbitrary units.

RESULTS

Ghrelin Is Not Processed in PC1/3 Null Mouse Stomach—Ghrelin was derived from a 94-amino-acid proprotein (Fig. 1). To test whether PC1/3 is involved in this process, we analyzed ghrelin processing in PC1/3 null mouse stomachs. Stomachs from two PC1/3 null and two wild-type four-month-old mice were extracted, resolved, and Western blotted as described under “Experimental Procedures.” The results (Fig. 2A) show that a band of 3.2 kDa corresponding in size to mature ghrelin is present in wild-type samples. However, in the PC1/3 null stomach samples, there is no detectable 3.2 kDa band, even after extended exposure. However, a band of 11 kDa corresponding to the predicted size of the precursor is present in the PC1/3 null samples. To verify the identity of this 11-kDa band, PC1/3 null mouse stomach extract was immunoprecipitated with ghrelin antibody before it was resolved by SDS-PAGE. The 11-kDa band was then excised and the protein extracted by electroelution. The sample was then subjected to mass spectrometric analysis using the linear positive ion mode with a proteomics analyzer (Applied Biosystems, 4700). A peak with a mass of 10,836 Da (Fig. 2B) corresponding to the predicted mass of octanoylated proghrelin (10,832 Da) was observed, confirming the identity of the 11-kDa band. The results thus strongly indicate that PC1/3 is the convertase responsible for the processing of ghrelin from proghrelin in vivo.

Ghrelin Is Co-localized with PC1/3 in Mouse Stomach—To examine the ghrelin expression pattern in relation to that of PC1/3 in stomach, we cryosectioned wild-type stomachs and immunostained sections with both ghrelin antibody and PC1/3 antibody. The ghrelin antibody was labeled with Alexa Fluor 488, and PC1/3 antibody was labeled with Alexa 594. These labels are fluorogenic, and when excited appropriately during microscopy, the ghrelin will appear as a green signal, and PC1/3 will be red. Fig. 3A shows the distribution of ghrelin immunoreactivity, and Fig. 3B illustrates the pat-
tern of PC1/3 expression in the stomach. Merging of these images demonstrates that the expression of ghrelin parallels the expression of PC1/3 (Fig. 3C). These results indicate that ghrelin is co-localized with PC1/3 in the ghrelin-producing cells of the stomach.

Ghrelin Processing Is Not Altered in PC2 and PC5A Mouse Stomach—To further determine whether PC2 or PC5/6A may affect ghrelin processing, we analyzed stomach extracts from PC2 or PC5/6A nulls by Western blot. The results (Fig. 4) show that a band corresponding to mature ghrelin is present in both PC2 and PC5/6A null stomach extracts, and there is no proghrelin detectable, even after extended exposure. These results indicate that PC2 and PC5/6a are not involved in the processing of proghrelin to ghrelin in this tissue.

Ghrelin mRNA Level Is Elevated in PC1 Null Mice—Of the various peptide hormones normally processed by PC1/3 in vivo, such as proopiomelanocortin and growth hormone-releasing hormone, we noticed that, in earlier studies of PC1/3 null animals, their expression was up-regulated (20). To determine whether ghrelin expression is also up-regulated in PC1/3 null mice, total gastric RNA was extracted from two animals at 4 months of age and analyzed by Northern blotting (Fig. 5A). Fig. 5B shows a quantitative analysis of the Northern blot after normalization for loading (against GAPDH) and expressed as arbitrary units. Bars indicate standard deviation.

DISCUSSION

Ghrelin is processed from a 94-amino-acid precursor through limited proteolytic cleavage at a single arginine. In this

FIGURE 3. Ghrelin is co-localized with PC1/3 in the mouse stomach. A, ghrelin staining. B, PC1/3 staining. C, merged image of A and B.

FIGURE 4. PC2 and PC5A are not involved in the processing of proghrelin in stomach. Shown is a Western blot of stomach extract of PC2 and PC5A null mice.

FIGURE 5. Ghrelin expression is increased in the PC1/3 null mice stomach. A, phosphorimage of Northern blot of ghrelin mRNA extracted from wild-type (WT) and PC1/3 null (KO) stomach. B, quantitative analysis of A using Opti-Quant (Packard Instrument, Inc., Meridian, CT). The intensity of each band was calculated and normalized for loading (against GAPDH) and expressed as arbitrary units. Bars indicate standard deviation.
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report, we have demonstrated that prohormone convertase 1/3 is the enzyme responsible for this event in vivo. Western blot results indicated that, in our PC1/3 null mice, only intact full-length precursor (proghrelin) is present in stomach extracts. This finding was confirmed by mass spectrometry. Immunohistochemical data also confirm that PC1/3 is co-localized with ghrelin in gastric ghrelin-positive cells. These three lines of evidence all support the conclusion that PC1/3 is the only convertase responsible for post-translational processing of proghrelin to ghrelin in the stomach. The presence of a proline preceding the C-terminal arginine provides an explanation for the lack of further processing by carboxypeptidase E-like carboxypeptidases (19).

Our results also indicate that post-translational acylation, i.e. the addition of an 8 (or 10) carbon fatty acid to the peptide occurs independently of proteolytic processing; that is, the octanoylation of ghrelin does not require prior processing of the peptide, as indicated by our mass spectrometric results, which show that the proghrelin in the PC1/3 null stomach is acylated. In the mass spectrometric analysis, a second peak with a molecular mass of 10,868 Da was also present. We speculate that this could represent decaenoyl- or decenoyl-proghrelin or a combination of these two forms (25). Our results also suggest that it is highly likely that PC1/3 is involved in the processing of obestatin as well, as we detected only the full-length precursor but not any intermediate products in PC1/3 nulls. Thus, either PC1/3 is capable of cleaving at the single Arg sites Arg-52 and -74 (Fig. 1) to generate obestatin or prior cleavage at Arg-28 is required to expose these processing sites for other convertases when obestatin is produced in the same cells. We cannot rule out the possibility that obestatin is processed from non-acylated proghrelin in other stomach cells.

Of the convertases normally present in neuroendocrine secretory granules, only PC1/3, PC2, and PC5/6A appear to be functionally significant. Our studies showing that PC1/3 is the only enzyme involved in the processing of ghrelin in vivo is consistent with in vitro data showing that PC1/3 is able to cleave bacterially produced recombinant proghrelin, whereas PC2 is not. Although the favored cleavage site for PC1/3 is at paired basic amino acid residues (Lys-Arg or Arg-Arg), it is also capable of cleaving after single basic residues, such as in the processing of proglucagon to GLP-1 (26), pro-CCK to CCK8, CCK58, and at the neurophysin-glycopeptide junction of provasopressin (27). Here we provide another example of PC1/3 processing at a single Arg, in this case a single Arg preceded by a Pro, which may make it a uniquely favored substrate for PC1/3.

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