Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11825–11830, October 1996
Medical Sciences

G protein-coupled cholecystokinin-B/gastrin receptors are responsible for physiological cell growth of the stomach mucosa *in vivo*

(gene targeting/cell proliferation/brain-gut peptide hormone)

AKI NAGATA*†‡, MITSUHIRO ITO*‡, NOBUKO IWATA*, JUNKO KUNO†, HIROSHI TAKANO†, OSAMU MINOWA†, KAZUO CHIHARA*, TOSHIMITSU MATSUI*§, AND TETSUO NODA†

*Third Division, Department of Medicine, Kobe University School of Medicine, Kobe 650, Japan; and †Department of Cell Biology, Cancer Institute, Tokyo 170, Japan

Communicated by J. E. Rall, National Institutes of Health, Bethesda, MD, July 31, 1996 (received for review May 20, 1996)

ABSTRACT **Many peptide hormone and neurotransmitter receptors belonging to the seven membrane-spanning G protein-coupled receptor family have been shown to transmit ligand-dependent mitogenic signals *in vitro*. However, the physiological roles of the mitogenic activity through G protein-coupled receptors *in vivo* remain to be elucidated. Here we have generated G protein-coupled cholecystokinin (CCK)-B/gastrin receptor deficient-mice by gene targeting. The homozygous mice showed a remarkable atrophy of the gastric mucosa macroscopically, even in the presence of severe hypergastrinemia. The atrophy was due to a decrease in parietal cells and chromogranin A-positive enterochromaffin-like cells expressing the H^+,K^+-ATPase and histidine decarboxylase genes, respectively. Oral administration of a proton pump inhibitor, omeprazole, which induced hypertrophy of the gastric mucosa with hypergastrinemia in wild-type littermates, did not eliminate the gastric atrophy of the homozygotes. These results clearly demonstrated that the G protein-coupled CCK-B/gastrin receptor is essential for the physiological as well as pathological proliferation of gastric mucosal cells *in vivo*.**

Cell proliferation and differentiation are regulated by a wide array of factors such as growth factors, cytokines, and hormones (1). Several peptide hormones such as bombesin/gastrin-releasing peptide, angiotensin, and endothelin, and neurotransmitters such as serotonin and adrenaline have been shown to stimulate cell proliferation through their own seven-transmembrane, heterotrimeric guanine nucleotide-binding protein (G protein)-coupled receptors *in vitro* (2–5). Certain serotonin, acetylcholine, or adrenergic receptor subtypes were reported not only to stimulate cell proliferation but also to transform 3T3 fibroblasts in a ligand-dependent manner as do growth factor receptors (3–5). Very recently, G protein-coupled receptors have also been shown to involve tyrosine kinases and the Ras-mitogen-activating protein kinase pathway in their intracellular signaling as do growth factor and cytokine receptors (6–9). Although some peptides could promote the proliferation of a variety of human tumor cell lines *in vivo* as well as *in vitro* (10, 11), the physiological significance of the mitogenic activity through the G protein-coupled receptor superfamily remains to be clarified.

The peptide hormone, gastrin, is well characterized as a stimulant of gastric acid secretion. In addition, there is circumstantial evidence that gastrin presumably functions as a trophic factor for the gastrointestinal tissues (11, 12). Another peptide hormone, cholecystokinin (CCK), is also isolated as a stimulant of enzyme secretion by the pancreas (13). Because of the abundant expression of CCK in the central nervous system as well as in digestive organs, this hormone is also thought to act as a neurotransmitter or modulator in the brain (14). Moreover, the specific receptors for CCK and/or gastrin have been pharmacologically shown to be expressed in various human tumor cells and to stimulate their growth in a ligand-dependent manner (10, 11).

Of the two types of cloned CCK receptors, CCK-A and CCK-B, the latter shows a high affinity for gastrin, as well as CCK. Recent cDNA cloning has revealed that the gastrin receptor in the stomach mucosa is identical to the brain CCK-B receptor belonging to a seven-transmembrane G protein-coupled receptor family (15–17). Moreover, CCK and gastrin have been shown to stimulate the growth of Chinese hamster ovary cells and NIH 3T3 fibroblasts transfected with the cDNA expression vector for the human CCK-B/gastrin receptor in serum-free medium (9, 17).

However, post-translational processing intermediates of gastrin, specifically glycine-extended gastrin, as well as the mature amidated gastrin itself, have very recently been reported to stimulate cell proliferation through a receptor other than the CCK-B/gastrin receptor (18, 19). The concentrations of glycine-extended gastrin in plasma, tissues, and neoplastic cells are reported to be higher than those of amidated gastrin (18, 20). In addition, a low-affinity gastrin-binding protein has been suggested to be involved in hypergastrinemia-induced tumor cell growth and to be a likely target for the growth inhibitory effect of some CCK-B/gastrin receptor antagonists (11, 21). CCK-B/gastrin receptor-selective antagonists did not inhibit the proliferation induced by glycine-extended gastrin. This implies that there could be at least two independent mechanisms that mediate the proliferative action of peptides derived from the gastrin gene. Thus, it is important to determine whether the trophic effect of hypergastrinemia on the gastrointestinal cells *in vivo* is mediated through the G protein-coupled CCK-B/gastrin receptors.

We have generated CCK-B/gastrin receptor-deficient mice by gene targeting to investigate the physiological significance of the receptor *in vivo*. In this report, the G protein-coupled CCK-B/gastrin receptor was clearly shown to be essential for the physiological as well as pathological proliferation of gastric mucosal cells *in vivo*.

MATERIALS AND METHODS

Construction of the CCK-B/Gastrin Receptor Gene (*CCKBR*) Targeting Vector. To isolate the mouse *CCKBR* gene, a mouse 129sv genomic library was screened with a human *CCKBR* cDNA probe (17). Five overlapping clones

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Abbreviations: CCK, cholecystokinin; PPI, a proton pump inhibitor; HDC, histidine decarboxylase; ECL, enterochromaffin-like; *CCKBR*, cholecystokinin-B/gastrin receptor gene; ES, embryonic stem.

‡A.N. and M.I. contributed equally to this work.
§To whom reprint requests should be addressed.

contained an 18-kb genomic region including the entire murine *CCKBR* locus. The *Sph*I–*Kpn*I 2.0-kb genomic fragment containing a part of exon 2 and exons 3, 4, and 5 was replaced by LacZ in-frame and a PGK-*neo* cassette (Fig. 1*A*) (22). This replacement deleted most of the seven membrane-spanning CCK-B/gastrin receptor except for the first 108 amino acids containing the first membrane-spanning region. This deletion mutant was expected to impair the entire function of the receptor. The targeting vector included a 1.2-kb upstream homologous region of exon 2 and a 7-kb downstream region of exon 5. It also included a diphtheria toxin A (DTA) fragment cassette at the 5′ end of the short homologous sequence (23).

Generation of the *CCKBR* Knockout Mice. J1 embryonic stem (ES) cells were electroporated with the linearized targeting vector and selected with geneticin, G418, on embryonal fibroblast feeder cells. In total, 1033 of the G418-resistant clones were screened by Southern blot analysis using 5′ external and 3′ internal probes (Fig. 1*B*). Six clones displayed evidence for homologous recombination of the disrupted *CCKBR* gene. Four

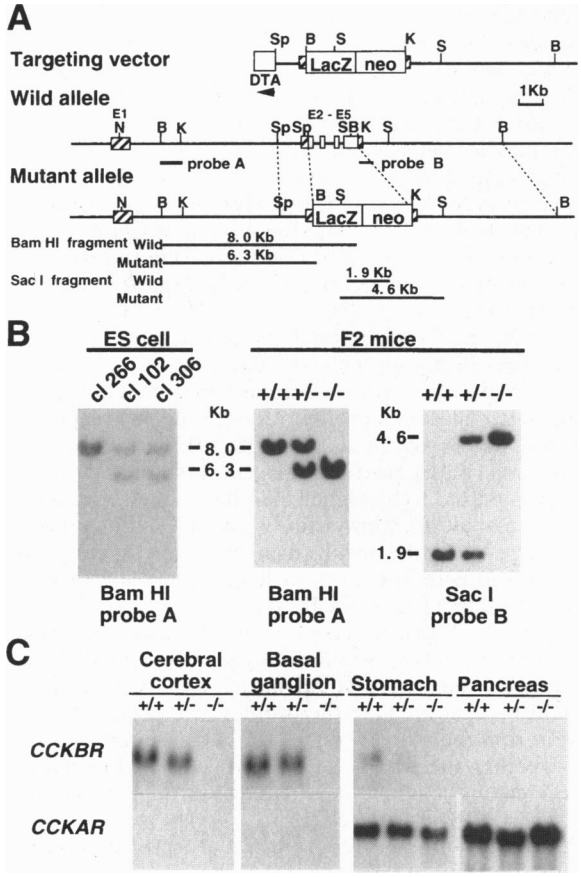

FIG. 1. Disruption of the *CCKBR* gene. (*A*) The targeting vector (*top*) was designed to replace the *Sph*I–*Kpn*I fragment of the mouse *CCKBR* gene (Wild allele) with LacZ and pGK-*neo* cassettes. The predicted mutant allele by homologous recombination is shown as Mutant allele. Cross-hatched boxes indicate exons without replacement. Restriction sites indicated are *Bam*HI (B), *Kpn*I (K), *Nco*I (N), *Sac*I (S), and *Sph*I (Sp). (*B*) Southern blot analysis of homologous recombinant clones and offspring obtained by a heterozygous cross. High molecular weight DNAs of ES cells (wild clone, cl 266; recombinant clones, cl 102 and 306), and F_2 wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice were digested with *Bam*HI and *Sac*I. Each digest was hybridized with 5′ external probe A or 3′ internal probe B. The positions of the bands corresponding to the wild allele (8.0 kb) and the mutant allele (6.3 kb) are indicated. The probe B confirmed a single integration of the targeting vector in the genomic DNA. (*C*) The expression of CCK-B/gastrin receptor (*CCKBR*) and CCK-A receptor (*CCKAR*) mRNAs in the cerebral cortex, basal ganglion, stomach, and pancreas of each indicated genotype.

ES clones were microinjected into blastocysts of C57BL/6J females. Finally, two independent ES clones generated germ-line chimeras. The chimeras were bred to C57BL/6J and 129sv mice to generate heterozygous mutant F1 mice.

^{125}I-CCK-8 Binding Assay. To prepare cell membrane fractions, the whole brain was homogenized in 10 vol of 50 mM Tris·HCl (pH 7.4) at 4°C. The homogenates were centrifuged at 42,000 × *g* for 15 min at 4°C. The pellets were washed with 50 mM Tris·HCl (pH 7.4), centrifuged, and then resuspended in 10 vol of incubation medium (118 mM NaCl/4.7 mM KCl/5 mM $MgCl_2$/1 mM EGTA/10 mM Hepes/5 mg/ml bovine serum albumin/0.25 mg/ml bacitracin). The pancreas was homogenized similarly, and the pellet was finally resuspended in 400 vol of incubation medium containing 5 mM dithiothreitol.

Freshly prepared membrane fractions (300 μl per sample) were incubated with 70 pM of ^{125}I-CCK-8 (2000 Ci/mmol; 1 Ci = 37 GBq; Amersham) in the presence or absence of sulfated CCK-8 (Peninsula Laboratories). Non-specific binding was defined by the addition of 1×10^{-6} M CCK-8 to the incubation medium. After 2 hr of incubation at 24°C, the samples were centrifuged at 10,000 × *g* for 2 min at 4°C. The pellets were washed with ice-cold incubation medium. The radioactivities bound to the membrane fraction were measured with a gamma counter. Non-specific binding was subtracted from total binding to yield specific binding. Specific binding to the brain and pancreas membranes was confirmed to be saturable in saturation studies described previously (24).

RNA Blot Analysis. Total RNAs isolated from various tissues by the guanidine thiocyanate method were subjected to an RNA blot analysis as described (17). The probes used were a 2.3-kb fragment of mouse CCK-B/gastrin receptor cDNA, a 1.1-kb fragment of mouse CCK-A receptor cDNA, a 2.3-kb fragment of mouse histidine decarboxylase (HDC) cDNA, a 0.7-kb fragment of mouse H^+,K^+-ATPase cDNA, a 1.8-kb fragment of mouse chromogranin A cDNA, and a 1.1-kb fragment of G3PDH cDNA (CLONTECH).

Preparation of Tissues for Histological Analysis. Stomachs were opened along the greater curvature, gently rinsed three times with ice-cold saline, and weighed. For hematoxylin–eosin staining, the stomach was fixed with 10% formaldehyde overnight, and embedded in paraffin.

To measure protein content, the whole glandular stomach was homogenized with 1 ml of buffer containing 10 mM sodium phosphate (pH 7.5), 1 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 0.1% aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were clarified by centrifugation at 10,000 × *g* for 30 min at 4°C. The protein concentration of each supernatant was measured with a protein assay kit (Bio-Rad).

To quantify the DNA content, high molecular weight DNAs were isolated by digestion of the stomach with proteinase K, followed by phenol-chloroform extraction.

For immunohistochemistry, mice anesthetized with sodium pentobarbital were perfused intracardially with ice-cold Zamboni's solution (4% paraformaldehyde/0.5% picric acid in 0.1 M PBS). The removed stomach was post-fixed for 2 hr in the perfusion medium, and transferred to 30% sucrose in 0.1 M PBS. After 12 hr at 4°C, the stomach was mounted in Tissue-Tek O.C.T. Compound (Miles), frozen at −80°C, and then cut into 8-μm sections with a cryostat. Immunostaining was performed by using an anti-mouse chromogranin A antibody kindly provided by T. Watanabe (Osaka University, Suita, Japan) and a Vectastain ABC-AP kit (Vector Laboratories).

Measurement of Gastric Acid Secretion. Shay's method was employed to examine gastric acid secretion (25). Eight-week-old male mice fasted for 3 hr were anesthetized with ether for the epigastric laparotomy. After ligating the pylorus, the abdominal incision was sutured. The stomach was removed 4 hr later to collect the gastric juice. The acid output was determined by titrating the gastric juice with 0.1 M NaOH to pH 7.0.

Proton Pump Inhibitor (PPI) Treatment. Eight-week-old female mice, 18–24 g body weight, were daily administrated orally with a PPI, omeprazole (400 nM/g body weight in 0.1% methylcellulose solution) for 10 weeks. The control group was given only 0.1% methylcellulose solution.

RESULTS

Expression of CCK-A and CCK-B/Gastrin Receptors in the *CCKBR* Targeted Mice. Of 118 neonates obtained by crosses between mice heterozygous for the *CCKBR*, 24 (20.3%) were wild type ($CCKBR^{+/+}$), 62 (52.5%) were heterozygous ($CCKBR^{+/-}$), and 32 (27.1%) were homozygous mutants ($CCKBR^{-/-}$). This distribution is thought to follow the Mendelian rule. Thus, the targeted disruption of the *CCKBR* gene did not impair embryogenesis. $CCKBR^{-/-}$ mice are fertile, and their growth evaluated by body weight was the same as that of wild-type littermates (data not shown). Now the oldest $CCKBR^{-/-}$ mice have reached the age of 24 months without obvious abnormality in their general appearance compared with their $CCKBR^{+/+}$ littermates.

The absence of CCK-B/gastrin receptor mRNA in $CCKBR^{-/-}$ mice was first confirmed by using total RNAs extracted from the cerebral cortex, brain basal ganglion, and stomach, which abundantly express the transcripts in $CCKBR^{+/+}$ mice (Fig. 1*C*). The disruption of the *CCKBR* gene neither affected the expression level of CCK-A receptor mRNA in these tissues or in the pancreas.

We employed a ^{125}I-CCK-8 binding assay to confirm the lack of a functional receptor protein (Fig. 2). The cell membrane fractions prepared from the whole brain of wild-type mice showed high affinities for CCK-8, as well as for gastrin I. The IC_{50}s were 0.9×10^{-9} M and 9.9×10^{-9} M, respectively. These results are consistent with previous reports that the brain dominantly expresses CCK-B/gastrin receptors (14). In contrast, cell membrane fractions prepared from the brains of $CCKBR^{-/-}$ mice had no specific ^{125}I-CCK-8 binding site displaced by excess CCK-8 or gastrin I. This means that the CCK-B/gastrin receptors in $CCKBR^{-/-}$ mice were functionally completely disrupted. Although the maximum ^{125}I-CCK-8 binding per microgram of protein in the brain of $CCKBR^{+/-}$ mice was diminished, the affinities for CCK-8 and gastrin I were not significantly changed (IC_{50}s were 0.5×10^{-9} M and 9.2×10^{-9} M, respectively).

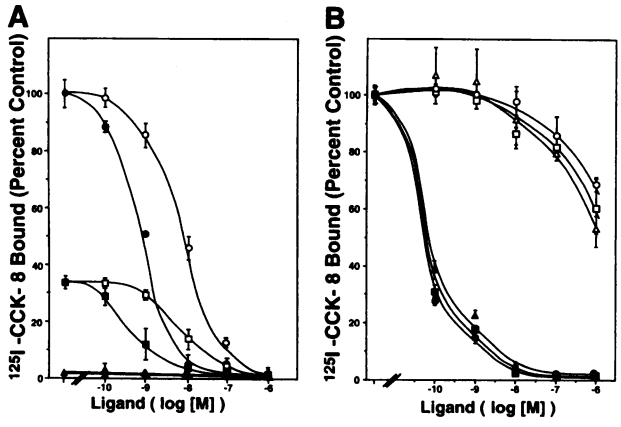

FIG. 2. Disruption of CCK-B/gastrin receptor binding sites in $CCKBR^{-/-}$ mice. Competitive inhibition of ^{125}I-CCK-8 binding to cell membrane fractions of the brain (*A*) and pancreas (*B*) by CCK-8 (closed symbols) and gastrin I (open symbols) was determined in $CCKBR^{+/+}$ (circles), $CCKBR^{+/-}$ (squares), and $CCKBR^{-/-}$ (triangles) mice. Data are presented as percent saturable binding (total binding in the presence of labeled CCK-8 alone minus binding in the presence of 1×10^{-6} M CCK-8) to cell membrane fractions of $CCKBR^{+/+}$ mice. Values are the means ± SEM of triplicate samples. These results were reproducible in three independent experiments.

^{125}I-CCK-8 binding sites of the pancreas showed high affinities for CCK-8 and low affinities for gastrin I in both the $CCKBR^{-/-}$ and $CCKBR^{+/+}$ littermates. These results indicate that the deficiency in CCK-B/gastrin receptors had no effect on the CCK-A receptor expression at the protein level in the pancreas.

Hypochlorhydria and Hypergastrinemia in *CCKBR* Targeted Mice. CCK-B/gastrin receptors on the gastric parietal cells are thought to play an important role in the regulation of gastric acid secretion, in cooperation with the histamine H_2 and muscarine M_3 receptors (26–28). The basal acid output in $CCKBR^{-/-}$ mice (3.8 ± 2.1 mEq/hr) was remarkably inhibited, compared with that of $CCKBR^{+/+}$ mice (13.1 ± 3.3 mEq/hr) ($P < 0.05$, $n = 6$). Gastric luminal acidity is known to inhibit gastrin release from G cells in the antrum (29). Thus, we also determined serum gastrin levels by using Gastrin RIA kit II (Dainabot, Tokyo). As expected, serum gastrin levels in 12–20-week-old $CCKBR^{-/-}$ mice fasted for 6 hr (1582 ± 257 pg/ml) were about 5 times higher than those of wild-type mice (293 ± 60.6 pg/ml) ($P < 0.01$, $n = 25$).

Because the achlorhydria induced by PPI-treatment has been shown to cause hypergastrinemia in rodents (30), we compared the hypergastrinemia induced by PPI treatment with that of $CCKBR^{-/-}$ mice. Serum gastrin levels of $CCKBR^{+/+}$ mice were significantly elevated by PPI treatment (562.7 ± 73 pg/ml), in comparison with those treated by vehicle alone (201.3 ± 40.4 pg/ml, $n = 10$, $P < 0.02$). In contrast, the hypergastrinemia observed in $CCKBR^{-/-}$ mice was not increased by PPI treatment (1565 ± 139 pg/ml, $n = 10$).

Atrophy of the Gastric Mucosa in *CCKBR* Targeted Mice. In our previous reports, the CCK-B/gastrin receptors were shown to be capable of transmitting mitogenic signals in a ligand-dependent manner *in vitro* (9, 17). To clarify the physiological significance of the mitogenic activity *in vivo*, we investigated the growth of fundic mucosal cells that are thought to express the receptor. As shown in Fig. 3, marked atrophy of the corpus mucosa was obvious upon macroscopic examination of the stomachs from $CCKBR^{-/-}$ mice. Because the corpus of the $CCKBR^{-/-}$ stomach is as thin as the antrum, it is sufficiently translucent to see the color of the cardboard on which the stomach was pinned. Especially in the minor curvature side, the folds of the stomach from $CCKBR^{-/-}$ mice were remarkably flattened and decreased in number compared with those of the $CCKBR^{+/+}$ littermates.

To quantify the atrophy, we measured the wet weight and the protein and DNA contents of the glandular stomach (Fig. 4). The mean wet weight in $CCKBR^{-/-}$ mice was significantly lower than that of $CCKBR^{+/+}$ mice. The protein content in $CCKBR^{-/-}$ mice was about 70% that of $CCKBR^{+/+}$ mice. The DNA content in $CCKBR^{-/-}$ mice was also significantly lower than that of $CCKBR^{+/+}$ mice. These results strongly suggest a decrease in the number of cells in the $CCKBR^{-/-}$ stomach.

PPI treatment induced hypertrophy of the gastric mucosa in wild-type mice, as well as in the rat (31) (Fig. 4). All of the

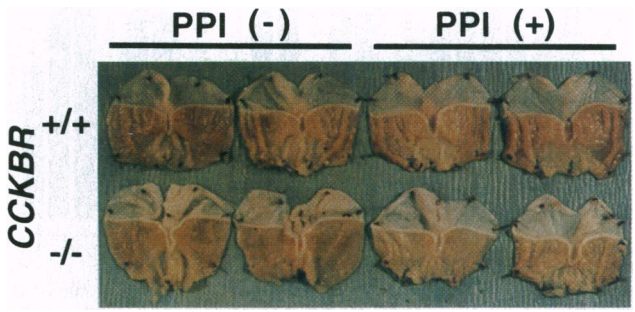

FIG. 3. Gastric atrophy in $CCKBR^{-/-}$ mice. The stomachs of 18-week-old female $CCKBR^{+/+}$ (*Upper*) and $CCKBR^{-/-}$ (*Lower*) mice were pinned on a piece of cardboard to be the same size. The stomachs of the mice treated with a PPI for 10 weeks are shown on the right.

parameters (the mean wet weight and the protein and DNA contents) of the glandular stomach in the *CCKBR*$^{+/+}$ mice treated with PPI were significantly greater than those of the *CCKBR*$^{+/+}$ mice treated with vehicle alone. However, PPI treatment did not eliminate the atrophic change of the *CCKBR*$^{-/-}$ stomach (Fig. 3). The wet weight and protein and DNA contents of the *CCKBR*$^{-/-}$ stomach were not affected by PPI-treatment (Fig. 4).

Decrease in Parietal and Chromogranin A-Positive Enterochromaffin-Like (ECL) Cells in the *CCKBR* Targeted Mice. The atrophy of the fundic mucosa in *CCKBR*$^{-/-}$ mice was further confirmed microscopically (Fig. 5). The fundic mucosa from *CCKBR*$^{-/-}$ mice was remarkably thinner than that of wild-type mice. The thicknesses of the isthmus and neck regions were remarkably decreased in *CCKBR*$^{-/-}$ mice, whereas the thicknesses of the gastric pit region composed of surface mucous cells and the base region were not decreased as much. Especially, the number of parietal cells, easily identified by their large size and clear or acidophilic cytoplasm, decreased at the fundic glands. On the other hand, the mucous neck cells characterized by a rectangular nucleus and mucin granules seemed to be relatively abundant. Moreover, the trophic effect of PPI treatment was confirmed in *CCKBR*$^{+/+}$ mice microscopically, but not in *CCKBR*$^{-/-}$ mice (Fig. 5).

We next examined the expression levels of several marker genes for parietal and ECL cells, because these cells are thought to express the CCK-B/gastrin receptors (15, 32). The expression of H^+,K^+-ATPase mRNA in parietal cells was significantly reduced in coordination with the decreased cell number in the *CCKBR*$^{-/-}$ stomach. The HDC and chromogranin A mRNAs expressed in ECL cells were more remarkably reduced in *CCKBR*$^{-/-}$ mice, in comparison with those in the *CCKBR*$^{+/+}$ littermates (Fig. 6*A*). The expression of G3PDH mRNA in the same blots was not affected in either genotype. A decreased expression of HDC mRNA in *CCKBR*$^{-/-}$ mice was not observed in the cerebral cortex or basal ganglion, which normally express many more CCK-B/gastrin receptors than does the stomach (data not shown). These results indicate that the decreased expression of the HDC gene is specific to the stomach.

Moreover, PPI treatment increased the expression of HDC and chromogranin A mRNAs in the *CCKBR*$^{+/+}$ stomach but not in the *CCKBR*$^{-/-}$ stomach as it did with H^+,K^+-ATPase mRNA (Fig. 6*A*). The specific changes of these marker gene expressions were statistically confirmed by repeated RNA blot analyses using total RNAs extracted from 10 mice in each group and an image analyzer (Fig. 6*B*). Endocrine cells expressing

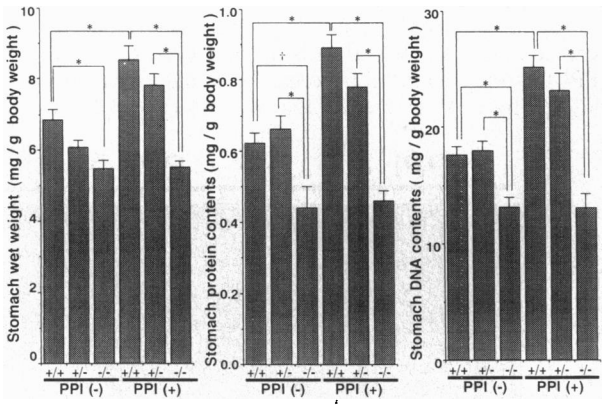

FIG. 4. Decreased wet weight, protein, and DNA contents of the glandular stomach in *CCKBR*$^{-/-}$ mice. Wet weight (mg), protein contents (mg), and DNA contents (mg) of the glandular stomach in the 18-week-old mice without (−) or with (+) PPI treatment were measured. Results are expressed as means ± SEM of 10 mice; * and †, significance at $P < 0.01$ and $P < 0.02$, respectively.

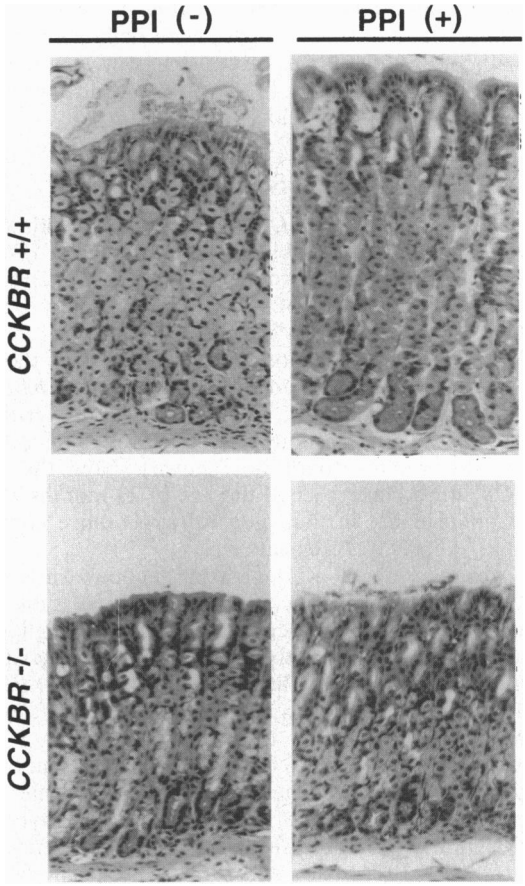

FIG. 5. Histological analysis of the gastric atrophy in *CCKBR*$^{-/-}$ mice. Hematoxylin- and eosin-stained sections of the fundic glands derived from *CCKBR*$^{+/+}$ (*Upper*) and *CCKBR*$^{-/-}$ (*Lower*) mice with (*Right*) or without (*Left*) PPI treatment are shown. (×100.)

chromogranin A mRNA include not only ECL cells but also somatostatin-producing D cells. However, the former is a major population (33), and the expression of somatostatin mRNA was not decreased by the *CCKBR* gene disruption at all (data not shown). Thus, ECL cells expressing HDC and chromogranin A also seemed to decrease in *CCKBR*$^{-/-}$ mice.

To confirm the decrease in ECL cells, we employed an immunohistochemical analysis using an anti-chromogranin A antibody (Fig. 7). The number of chromogranin A-positive cells in the gastric fundic glands was significantly less in *CCKBR*$^{-/-}$ mice (2.8 ± 1.3 cells per field) than in *CCKBR*$^{+/+}$ mice (19.1 ± 1.1 cells per field, $n = 10$, $P < 0.01$). Moreover, PPI treatment increased the number of chromogranin A-positive cells in the stomachs from *CCKBR*$^{+/+}$ mice (23.6 ± 1.5 cells per field, $n = 10$, $P < 0.05$), but not in the stomachs from *CCKBR*$^{-/-}$ mice (3.7 ± 1.5 cells per field, $n = 10$, $P > 1.0$). Thus, the proliferation of chromogranin A-positive ECL cells, as well as that of parietal cells, was significantly reduced in *CCKBR*$^{-/-}$ mice (Fig. 7).

DISCUSSION

We report here that a targeted disruption of the mouse *CCKBR* gene results in a remarkable, macroscopically observable atrophy of the gastric mucosa, even in the presence of severe hypergastrinemia. The atrophy was due to the reduced proliferation of parietal and chromogranin A-positive ECL cells expressing the H^+,K^+-ATPase and HDC genes, respectively. Administration of PPI, which induced elevation of the serum gastrin levels and hypertrophy of the gastric mucosa in

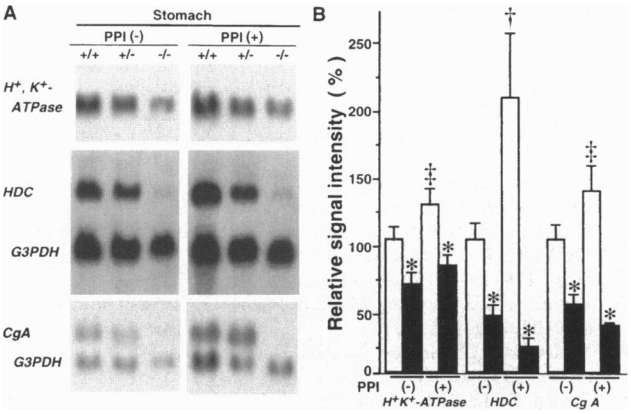

FIG. 6. Decreased expression of H^+,K^+-ATPase, HDC, and chromogranin A mRNAs in the $CCKBR^{-/-}$ stomach. (*A*) RNA blot analyses of total RNAs isolated from the stomachs of each genotype with (+) or without (−) PPI treatment. The cDNA probes are shown at left. (*B*) Relative signal intensities of RNA blot analyses as shown in *A* were quantified with an image analyzer BAS2000 (FUJIX, Tokyo). The results are expressed as a percentage of the mean expression level of each mRNA in 10 wild-type mice without PPI treatment. The expression level of G3PDH mRNA was used for standardization. *, $CCKBR^{-/-}$ mice (solid bars), $P < 0.02$ vs. the $CCKBR^{+/+}$ mice (open bars). † and ‡, $CCKBR^{+/+}$ mice with PPI treatment (+) $P < 0.02$ and $P < 0.05$ vs. $CCKBR^{+/+}$ mice without PPI treatment (−).

wild-type littermates, did not eliminate the gastric atrophy of the mutant mice. These findings were confirmed in all of the mutants derived from the two independent ES-targeted cell lines. Thus, the present studies clearly demonstrate that the G protein-coupled CCK-B/gastrin receptor plays a key role in the regulation of physiological cell growth of the stomach mucosal cells. Moreover, this receptor is responsible for the PPI-induced hypertrophy of the stomach mucosa.

The ligand-dependent activation of several G protein-coupled receptors, such as the angiotensin, bradykinin, serotonin, noradrenaline, and endothelin receptors, has been reported to cause mitogenic stimulation *in vitro* (2, 11). Several mice lacking these mitogenic ligands or their receptors have been established by gene targeting methods, but most of them have shown little evidence that the gene products were essential for physiological cell growth *in vivo* (34–37). In studies of mice deficient in endothelin and its receptor, newborn mutants died with a failure of the normal development of several neural crest-derived cell lineages, indicating that the physiological roles of the mitogenic activity in adults are poorly understood (38, 39). Although the diabetic rat OLEFT congenitally lacks CCK-A receptors, the pathophysiological significance of the mitogenic effect of the CCK-A receptor has not been reported (40).

Mutations causing activation or inactivation of G protein-coupled receptors for thyrotropin and luteinizing hormone have been reported to be responsible for the development of human diseases (41–43). Very recently, a missense mutation of the luteinizing hormone receptor gene was shown to cause Leydig cell hypoplasia leading to pseudohermaphroditism. Moreover, targeted disruption of a common subunit of luteinizing hormone and thyrotropin, the pituitary glycoprotein hormone α-subunit, impaired both gonadal and thyroid development (44). Taken together, our studies confirm that the mitogenic action of a member of the G protein-coupled receptor superfamily is essential for physiological cell growth of certain cell types in adults.

There is now growing evidence that all cells in the gastric mucosa, including ECL cells, are likely to be the product of a common gastric stem cell (45, 46). The vast majority of the mucosal cells are repopulated by active division of immature cells located at the proliferative zone in the isthmus. When the

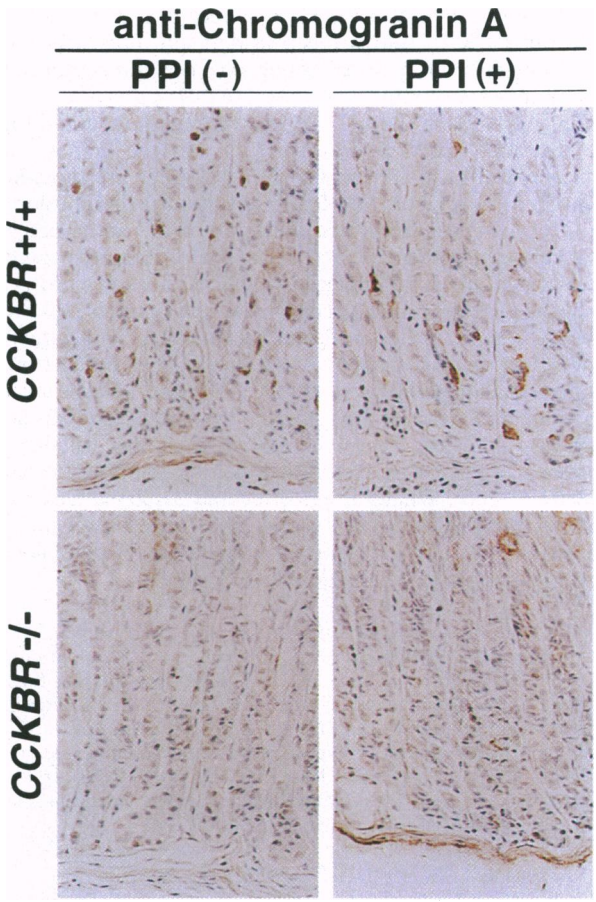

FIG. 7. Decrease in chromogranin A-positive endocrine cells in the $CCKBR^{-/-}$ stomach. Immunohistochemical staining was performed using the stomachs of $CCKBR^{+/+}$ (*Upper*) and $CCKBR^{-/-}$ (*Lower*) mice with (*Right*) or without (*Left*) PPI treatment. Sections were stained with an anti-mouse chromogranin A antibody. (×200). Rabbit polyclonal IgG (Vector Laboratories) was used as a negative control (data not shown).

immature cells migrate out of the proliferative zone, they differentiate and gradually lose their proliferative capacity. The differentiated mature parietal, chief, and surface mucous cells have ceased to divide. Thus, the decrease in mature parietal cells in $CCKBR^{-/-}$ mice appears to be due to the loss of the proliferation and/or differentiation of immature parietal cells, but not pluripotent stem cells. Very recently, the specific ablation of parietal cells has been shown to lead to the loss of other gastric epithelial cells (47, 48). Thus, the decreased mature parietal cells in $CCKBR^{-/-}$ mice might also affect the growth of other cell types in the gastric mucosa. Because the ECL population could be renewed through self-replication of mature ECL cells (49), the mitogenic effect through the CCK-B/gastrin receptor could also act on these mature cells, as well as their precursor cells.

Although CCK-B/gastrin receptors are also abundantly expressed in the cerebral cortex, no obvious abnormalities were detected in either the morphology or the histopathology of the $CCKBR^{-/-}$ brain (data not shown). The receptors in the central nervous system are thought to be concerned with behavioral actions, such as feeding and anxiety (14). There were no differences between the body weights of $CCKBR^{+/+}$ and $CCKBR^{-/-}$ mice for 42 weeks. Antagonists for CCK-A as well as CCK-B receptors were reported to increase the frequency and the amount of food intake in the rat (50). However, the expression of CCK-A receptor mRNA in the $CCKBR^{-/-}$ brain was the same as that of the wild-type littermates. The loss of a functional CCK-B receptor in mice might be compensated

by molecules other than the CCK-A receptor. More detailed studies of CCK-B/gastrin receptor-deficient mice will facilitate clarifying the functional importance of the receptor in the central nervous system.

The signals from the CCK-B/gastrin receptors could regulate the actin stress fiber formation and the activity of a component of focal adhesions, $p125^{FAK}$, which are thought to be involved in the control of cell motility or cell adhesion (9, 51). Thus, the migration and adhesion of the immature mucosal cells expressing the receptor at the proliferative zone of the stomach might be impaired in the mutant mice. Alternatively, the growth of mucosal epithelial cells in the proliferative zone is thought to be regulated by autocrine and paracrine growth factors, as well as by endocrine hormones. The intracellular signaling pathways of the CCK-B/gastrin receptor could cross-talk with those of the tyrosine–kinase type growth factor receptors (9, 51, 52). Thus, it is of interest to know whether the expression of the growth factors and their receptors is affected in *CCKBR*$^{-/-}$ mice. Whatever autocrine and paracrine factors are involved, the present studies clearly show that the G protein-coupled CCK-B/gastrin receptor is essential for the physiological growth of certain gastric mucosal cells *in vivo*.

We are grateful to Drs. H. Takanashi, T. Watanabe, and K. Miyata for measurement of gastric acid secretion, anti-chromogranin A antibody, and omeprazole, respectively. We also thank Dr. K. Sugano for his helpful discussion, and H. Yamanaka and T. Kobayashi for their excellent technical assistance. M.I. is a Research Fellow of the Japan Society for the Promotion of Science. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan and by a research grant from the Uehara Memorial Foundation.

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