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A TRUNCATED ISOFORM OF HUMAN CCK-B/GASTRIN RECEPTOR GENERATED BY ALTERNATIVE USAGE OF A NOVEL EXON

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An isoform cDNA of CCK-B/gastrin receptor was isolated from human stomach. This cDNA differed from initially cloned cDNA only in the 5'-end region and encoded a truncated isoform (ΔCCK-B) in which the putative N-terminal extracellular domain of the CCK-B/gastrin receptor was completely lost. Isolation of genomic CCK-B/gastrin receptor DNA revealed that this transcript is generated by alternative usage of a novel exon, termed exon 1b. Human stomach expressed both transcripts of ΔCCK-B and entire CCK-B/gastrin receptor (CCK-BR), whereas human stomach cancer cell line AGS exclusively expressed ΔCCK-B transcripts. Transfection of COS-7 with ΔCCK-B cDNA led to the appearance of binding sites for ^{125}I-CCK-8. Its ligand selectivity was different from that of CCK-BR. These results suggest the molecular diversity in CCK-B/gastrin receptor subtypes.

The gastrin and CCK family of peptides share a common C-terminal amino acid sequence and have affinities for common receptors. These receptors are pharmacologically classified into two subtypes, termed CCK-A and CCK-B/gastrin receptor. On the basis of studies using CCK-B/gastrin receptor antagonists, putative physiological activities of these peptides mediated by CCK-B/gastrin receptor subtype include anxiety, panic attacks and opiate-induced analgesia in the central nervous system (1, 2), gastric acid secretion and histamine release in the stomach (3, 4), and trophic actions on several cancer cell lines and gastrointestinal tumors (5-8). Molecular cloning and genomic Southern blot analysis of this receptor subtype suggested that this receptor gene is not heterogeneous in genomic DNA (9). The diversity of physiological effects may reflect the existence of multiple CCK-B/gastrin receptor isoforms resulting from an identical gene. We here report the cDNA cloning and functional expression of a novel isoform of CCK-B/gastrin receptor generated by the alternative usage of the novel exon 1b.

Materials and methods

Oligonucleotides. The oligonucleotide primers used for rapid amplification of 5'-cDNA ends (5'-RACE) and reverse transcriptase-polymerase chain reaction (RT-PCR) were synthesized according to the sequences illustrated in Fig. 1A; H11, 5'-TCG CTG ACT GCC AGT GAG AG-3'; H31, 5'-CTC AAC AGC AGC AGT GTG G-3'; H32, 5'-GAA GGC ATT GGT GAC AGT CC-3'; H33, 5'-CAG GAG AAG AAC TGA ACT GTC C-3'; H34, 5'-TGG GGT ACA TCC ACA AGT GC-3'; H35, 5'-CAC GAT GAT GAG CAT ATT TCC TCC-3'; H37, 5'-TGA TGAGAT GAT GCT CAC GG-3'; H38, 5'-ATC TAT GTG TGT GAG AGG CAG G-3'; and H40, 5'-TATCAG AGG CAT GCC TCC TT-3'

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Isolation of cDNA by 5'-RACE and RT-PCR. 5'-RACE was performed using human stomach poly (A)+ RNA (Clontech) according to the protocol of a 5'-AmpliFinder RACE kit (Clontech). First strand cDNA was synthesized from the RNA using the primer H11. The single-stranded anchor oligonucleotide was ligated to the 3'-end of the cDNA. The anchor-ligated cDNA was then used as a template for PCR amplification, using a primer complementary to the anchor and the nested primers H32 and H35. PCR was performed in 100-μl standard mixture with 10 mM Tris-HCl, pH 9.0/50 mM KCl/2.5 mM $MgCl_2$/0.1% Triton X-100/2.5 units of *Taq* DNA polymerase (Toyobo, Japan) as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 3 min; and a final hold at 72 °C for 10 min.

For RT-PCR cloning of ΔCCK-B, random-primed first strand cDNA of human stomach was used as a template. First-stage PCR was performed in a 100-μl standard mixture using the primers H33 and H38. Second-stage PCR further amplified the first-stage PCR products using the nested primers H37 and H40. The cycles described above were used, except annealing was at 62 °C for the first-stage.

The 5'-RACE and RT-PCR products were cloned into pCRII vector (Invitrogen), and sequenced by the dideoxynucleotide chain-terminator method. To eliminate PCR misincorporation errors, consensus sequences are reported.

Long PCR of Genomic DNA. Genomic DNA upstream of exon 2 was amplified using the primer H31 or H37 and H11 in 50-μl reaction mixtures with human genomic DNA (0.25 μg; Clontech) in 20 mM Tris-HCl, pH 8.8/10 mM KCl/10 mM $(NH_4)_2SO_4$/2 mM $MgSO_4$/0.1% Triton X-100/ 0.01% BSA/5% gycerol/5.0 units of recombinant *Taq* DNA polymerase (Takara Shuzo, Japan)/5.0 units of *Taq* extender additive (Stratagene). Cycles consisted of a Hot Start using AmpliWax (Perkin Elmer); initial denaturation at 94 °C for 1 min; 35 cycles of denaturation at 94 °C for 10 sec, annealing and extension at 68 °C for 10 min; and a final hold at 72 °C for 10 min.

Human Genomic Southern Blot Analysis. Ten μg of human genomic DNA were digested with either *Bam*HI or *Bam*HI and *Bgl*II, separated by electrophoresis on a 0.7% agarose gel, and transferred to Biodyne A (Pall). The membrane was hybridized with ^{32}P-labeled probe S (Fig. 2D), derived from DNA fragments (nt 29-156) generated by PCR using the primers H33 and H34 overnight at 65 °C, 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml sheared, denatured salmon sperm DNA, and then finally washed in 0.5 × SSC, 0.1% SDS at 65 °C. The blot was reprobed with probe C (Fig. 2D), derived from *Tfi*I/*Bam*HI fragments of CCK-BR cDNA (nt 179 to 952) under the same conditions and washed in 0.2 × SSC, 0.1% SDS at 65 °C. Autoradiographs were prepared with a Bio Imaging Analyzer BAS2000 (Fuji Film, Japan) using phosphor imaging plates.

RT-PCR Analysis and Northern Blot Analysis of mRNA. Poly (A)+ RNAs of cell lines were isolated using oligo(dT)-cellulose from AGS, LoVo, WiDr and Jurkat (American Tissue Culture Collection). Human tissue poly (A)+ RNAs were purchased from Clontech.

RT-PCR analysis of mRNAs were performed using the random-primed cDNA synthesized from each RNA as template. The efficiency of poly (A)+ RNA isolation and cDNA synthesis were estimated by RT-PCR using specific primer sets of glyceraldehyde 3-phosphate dehydrogenase (Clontech; data not shown). To detect each receptor transcript, the downstream primer H32 corresponded to the antisense sequence in exon 2. Two upstream primers, H37 and H31 were used to detect the presence of CCK-BR and ΔCCK-B, respectively. The cycles described above were used, with annealing at 61 °C.

For Northern blot analysis of mRNAs, 6 μg of poly (A)+ RNA was separated by electrophoresis on 1.2% agarose/formaldehyde gel, and transferred to Hybond-N (Amersham). The membrane was hybridized with ^{32}P-labeled probe corresponding to the conserved region downstream of *Tfi*I site (in Fig. 2D) overnight at 42 °C, 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS, and 200 μg/ml sheared, denatured salmon sperm DNA, and then finally washed in 0.1 × SSPE, 0.1% SDS at 55 °C. An autoradiograph was prepared as described above.

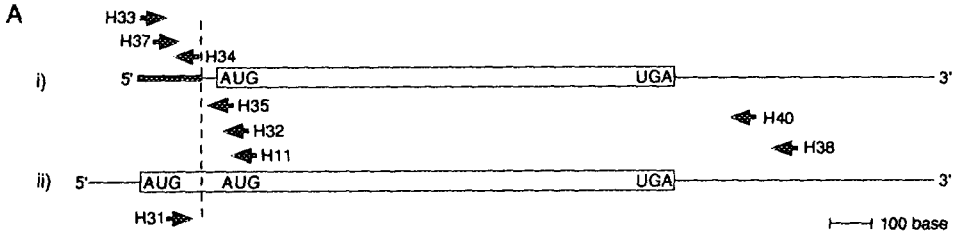
Transfection and Binding. The plasmid for expression of ΔCCK-B was constructed by insertion of ΔCCK-B cDNA into the eukaryotic expression vector pEF-BOS (10). COS-7 were exposed to the plasmid DNA with DEAE-dextran for 14 hr and then chloroquine for 2.5 hr. After 2-day culture, transfected cells were rinsed with ice-cold 25 mM HEPES-buffered Hanks' balance salt solution, pH 7.4, containing 0.1% bovine serum albumin, scraped from the plate, centrifuged, and suspended in the same solution. Suspended cells (1-2 × 10^5 cells) were incubated at 37 °C for 60 min with 0.5 nM of ^{125}I-CCK-8. Cells were washed with ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.01% bovine serum albumin by filtration

on glass fiber filters (Pharmacia). Filters were measured for γ radioactivity. Nonspecific binding was determined in the presence of 10^{-5} M of CCK-8.

Results

We performed 5'-RACE of human stomach poly (A)+ RNA to determine nucleotide sequence of 5'-cDNA ends of human stomach CCK-B/gastrin receptor. The amplified cDNAs using the primer H32 and the anchor primer were analyzed by Southern hybridization with the entire coding region of CCK-BR cDNA (11) as a probe. Two broad bands were detected at about 250 and 350 bp. These PCR products were further amplified using the nested primer H35 and the anchor primer, then isolated and cloned. Sequencing of 30 independent clones showed the existence of two distinct transcripts. Twenty transcripts were identical to CCK-BR cDNA, whereas the other 10 carried a unique region at the 5'-cDNA end (Fig. 1B). We further performed RT-PCR cloning with the upstream primers corresponding to this unique region and the downstream primers corresponding to the 3'-flanking noncoding region of CCK-BR cDNA. Sequencing of isolated clones revealed the existence of a transcript which differs from CCK-BR cDNA only in the 5'-cDNA end. This cDNA has the longest open reading frame coding for a 381-amino acid polypeptide. The deduced amino acid sequence is completely identical to the C-terminal region of CCK-BR. This transcript encodes a truncated receptor which completely loses the putative N-terminal extracellular domain, termed ΔCCK-B.

Compared to genomic DNA sequence of CCK-BR (12, 13), our cloned cDNA differed in the region corresponding to exon 1. To determine whether this cDNA is encoded on genomic DNA of CCK-BR, we performed human genomic Southern blot analysis. As shown in Fig. 2A, an identical genomic *Bam*HI-digested fragment was hybridized with probes representing the unique 5'-end region (probe S, in Fig. 2D) and the common region (probe C, in Fig. 2D).



B

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ACAGAACAAATACAGATCTTCTAAACACCAGGAGAAGAACTGAACTGTCCCAATCTTCTGAGTCGCCTGGTCTGA   75
TGCCAGTTTTCCTCATCCCCAGGAAGATGATGAGATGATGCTCACGGGTAGACTTATCAAGGCACTTGTGGATGT  150
ACCCCAGCAGAATTGGAGCTGGCCATTAGAATCACTCTTTACGCAGTGATCTTCCTGATGAGCGTTGGAGGAAAT  225
                                                    M  S  V  G  G  N
ATGCTCATCATCGTG                                                               240
M  L  I  I  V
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Fig. 1. Structure of ΔCCK-B transcript. A) Schematic illustration of the structure of ΔCCK-B (i) and CCK-BR transcript (ii) and the positions of the primers used in this study. A bold line indicates the region specific to ΔCCK-B. The primers are indicated by arrows with their names. B) Nucleotide and deduced amino acid sequence of 5'-end of ΔCCK-B transcript. Nucleotides different from CCK-BR are enclosed with a solid line. Underlined sequence is complementary to the primer H35 for 5'-RACE.

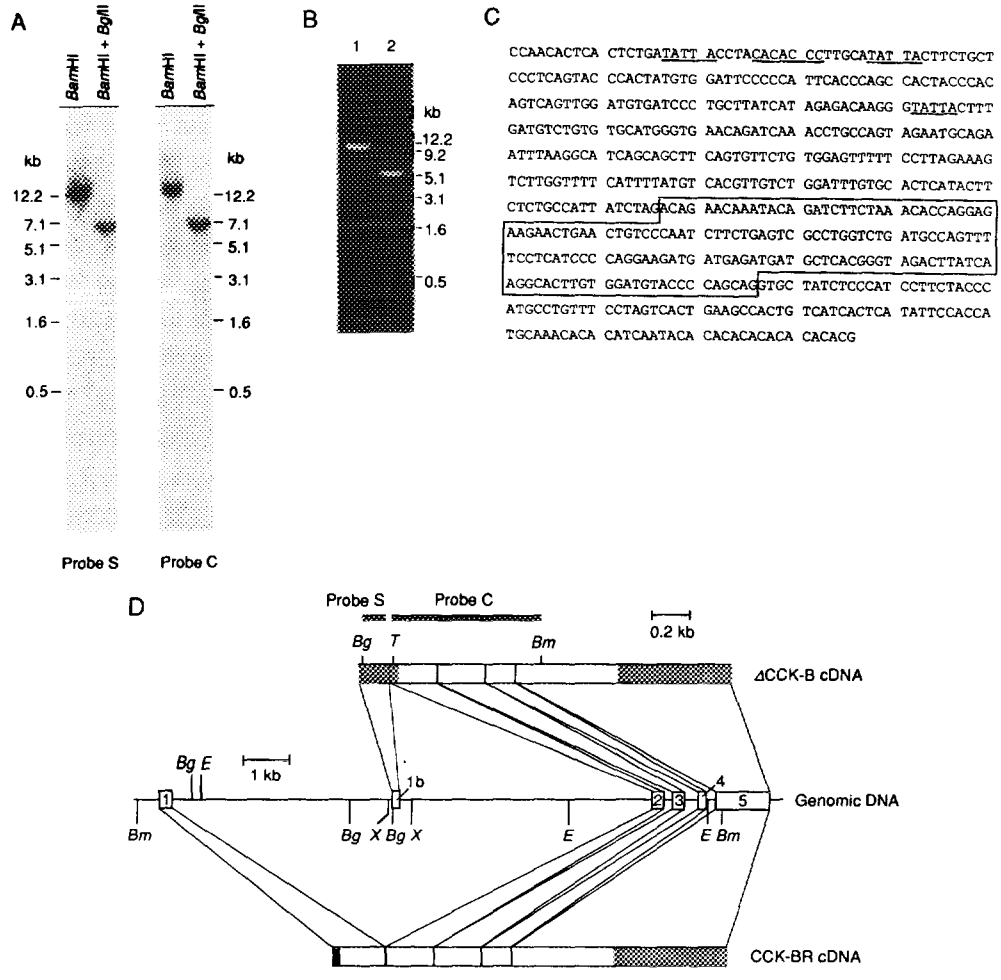

Fig. 2. Genomic structure of ΔCCK-B. A) Human genomic Southern blot analysis. Ten μg of human genomic DNA was digested with either *Bam*HI or *Bam*HI and *Bgl*II, separated on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized under conditions described in "Materials and methods" with the probe S or C indicated in Fig. 2D. B) Long PCR of genomic DNA upstream of exon 2. Upstream primers were H31 (lane 1) and H37 (lane 2), specific to exon 1 and exon 1b, respectively. The common downstream primer H11 is specific to exon 2. C) Nucleotide sequence of the region surrounding exon 1b. TATA-like and CACCC sequences are underlined. Sequence corresponding to exon 1b is enclosed with a solid line. D) Exon arrangement of human CCK-B/gastrin receptor gene. Genomic organization upstream of exon 1 and downstream of exon 2 are cited from *ref.* 13 (GenBank accession no. D21218-9). Exons are represented by numbered boxes. In both cDNAs, stippled boxes indicate the 5'- and 3'-noncoding regions. Bold lines indicate the probes used in genomic Southern blot analysis. Restriction sites are marked: *Bg*, *Bgl*II; *Bm*, *Bam*HI; *E*, *Eco*RI; *T*, *Tfi*I; *X*, *Xba*I.

Additional digestion of genomic DNA with *Bgl*II, which can divide at the 5'-end of the unique region (nt 14 in Fig. 1A), still did not distinguish fragments hybridized with each probe. These results suggest that ΔCCK-B cDNA results from alternative usage of a novel exon in CCK-BR gene. To confirm the existence of the novel exon, we further performed isolation of genomic DNA upstream of exon 2 using the long PCR method (14). The upstream primers H31 and H37 were specific to exon 1 and to the unique region, respectively. The common downstream

primer specific to exon 2, H11, was used. The long PCR amplified about 10-kb fragments using primer sets of H31 and H11, and about 5.5-kb fragments using H37 and H11 (Fig. 2B), suggesting that the novel exon exists downstream of exon 1. Sequencing, restriction mapping and nested PCR of the amplified 10-kb fragment revealed that the unique 5'-end region exists as single exon, termed exon 1b, at about 5.5 kb upstream of exon 2 (Fig. 2D). Genomic organization of human CCK-BR has been reported by Song *et al.* (12) and Matsui *et al.* (13). Different lengths of intron 1 were reported, namely about 2 kb in the former report and about 10 kb in the latter. Our results are well consistent with the latter. The sequences of exon 1b and its adjacent region are shown in Fig. 2C. The sequence of the splice donor site in exon 1b conformed to the consensus GT/AG sequence of exon-intron junctions. The 5'-flanking region of exon 1b contained three potential TATA box and one CACCC sequence.

To determine the distribution of ΔCCK-B and CCK-BR, we performed RT-PCR with poly (A)+ RNA from several human tissues and cell lines which appear responsive to gastrin/CCK ligands. As shown in Fig. 3A, RT-PCR with the specific primer H33 for ΔCCK-B cDNA revealed expression of ΔCCK-B transcripts in brain, stomach, pancreas, and the stomach cancer cell AGS and the colon cancer cell LoVo. In contrast, CCK-BR transcripts were detected in brain, stomach, pancreas, LoVo and the T-lymphoblastoma Jurkat, but not AGS. Thus, AGS express ΔCCK-B, and Jurkat express CCK-BR, exclusively. To confirm the size of ΔCCK-B mRNA, we performed Northern blot analysis of mRNA using a probe corresponding to the conserved region. Approx. 2-kb mRNA was detected in AGS (Fig. 3B), suggesting that the length of our unisolated 3'-end is about 0.4 kb, well consistent with that of

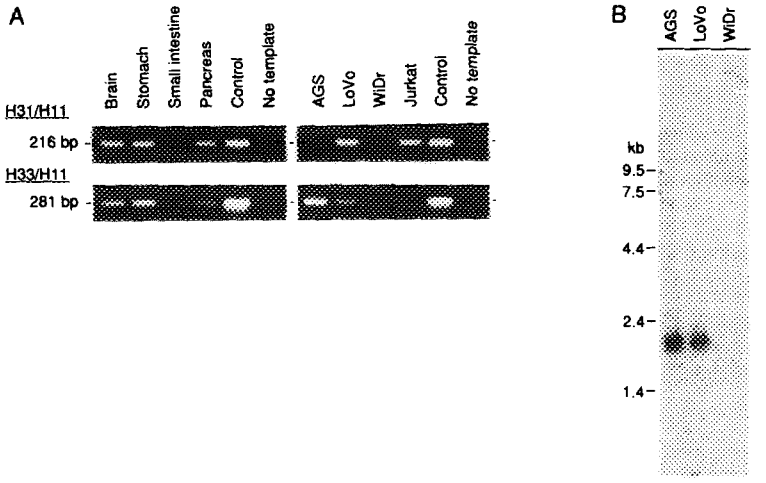

Fig. 3. Distribution of mRNA. A) RT-PCR analysis. RT-PCR were performed using the primer sets H31/H11 (216-bp fragment) and H33/H11(281-bp fragment). The upstream primers H31 and H33 are specific to exon 1 and exon 1b, respectively. The common downstream primer H11 is specific to exon 2. PCR products were separated on a 2% agarose gel. Cloned cDNA was used as control. B) Northern blot analysis. Poly (A)+ RNA (6 μg) was separated by electrophoresis on a 1.2% agarose/formaldehyde gel, transferred to a nylon membrane, and hybridized under conditions described in "Materials and methods" with ^{32}P-labeled probe corresponding to the conserved region, downstream of *Tfi*I site (Fig. 2D).

the 3'-end of CCK-BR cDNA (9). The size of mRNA detected in LoVo was not distinguishable from that detected in AGS, suggesting the mRNA size of ΔCCK-B is close to that of CCK-BR. No signal was detected in the colon cancer cell WiDr, consistent with RT-PCR analysis.

AGS has been found to be positive for the CCK-B/gastrin-like receptor (5, 6, 15). We also confirmed specific binding sites for CCK-8 in AGS: ^{125}I-CCK-8 bound to AGS at $15.5 \pm 1.9 \times 10^3$ and $7.5 \pm 0.2 \times 10^3$ cpm/well in the absence and presence of 10^{-6} M CCK-8, respectively. Since AGS was negative for CCK-A receptor mRNA by RT-PCR analysis (data not shown), the binding sites in AGS may be ΔCCK-B. In addition, we detected specific binding of ^{125}I-CCK-8 to COS-7 transfected with ΔCCK-B cDNA. In competition studies using the antagonists L-365,260 and L-364,718, ΔCCK-B showed the properties of a classical "CCK-B/gastrin" subtype (Fig. 4). However, competition studies using the agonists CCK-8 and gastrin revealed that the binding properties of ΔCCK-B were different from those of CCK-BR: ΔCCK-B and CCK-BR showed approx.100-fold and 10-fold higher affinities for CCK-8 than for gastrin, respectively.

Discussion

CCK-B/gastrin receptor is a member of the superfamily of G protein-coupled receptors with seven transmembrane domains. Among this receptor superfamily, several receptors with

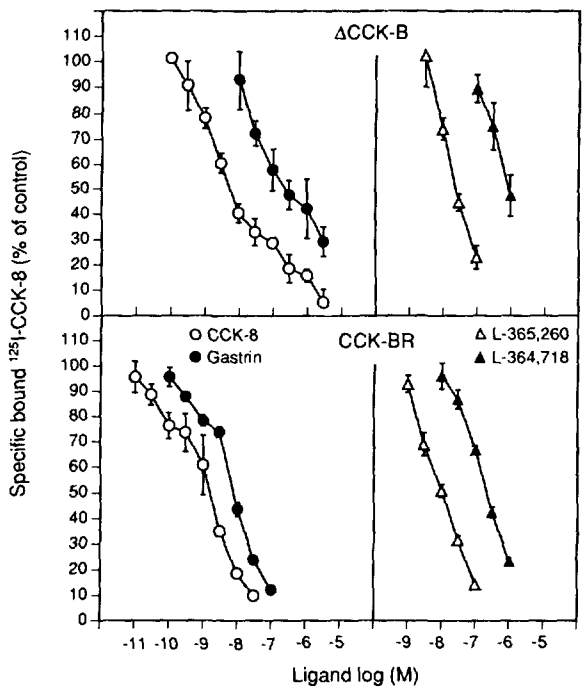

Fig. 4. Competition study of ^{125}I-CCK-8 binding. COS-7 were transiently transfected with ΔCCK-B cDNA (*upper*) and CCK-BR (*lower*; cited from *ref.* 11). ^{125}I-CCK-8 was incubated either alone or with indicated concentrations of CCK-8 (O), gastrin (●), L-365,260 (Δ) or L-364,718 (▲). The results are means ± SE of 4 to 8 determinations.

introns in their coding region have multiple isoforms generated by alternative usage of exon, such as dopamine D_2 receptor (16), prostaglandin EP_3 receptor (17) and pituitary adenylate cyclase-activating polypeptide receptor (18). Existence of such splice variants could partially explain the multiple function of a G protein-coupled receptor. Human CCK-B/gastrin receptor gene also has 4 introns in its coding region, and is alternatively spliced in exon 4 to generate two isoforms which differ by 5 amino acids in the third cytoplasmic loop (12, 13). Our isolated transcript is generated by alternative usage of the novel exon 1b, and encodes a truncated CCK-B/gastrin receptor, ΔCCK-B, with different binding properties compared to CCK-BR. This structure is unique among splice variants of G protein-coupled receptor reported to date: many splice variants have variations in cytoplasmic C-terminal domain, or in the third cytoplasmic loop. The alternative usage of first exon suggests that expression of ΔCCK-B transcript may be regulated by a distinct promoter. Among G protein-coupled receptors, the existence of an alternative exon 1 and promoter have been reported for platelet-activating factor receptor (19). However, the alternative usage of this exon and promoter causes no change in translated receptor protein, because this exon corresponds to the 5'-noncoding region. N-terminal truncation using alternative exon 1 has been reported for several proteins such as progesteron receptor (20) and IkBγ (21), but not for G protein-coupled receptor superfamily. Recently, the presence of a N-ternimal-truncated CCK-A receptor was demonstrated by photoaffinity labeling studies (22) although the mechanism that generates this isoform remains unknown. Since the N-terminal extracellular domain of CCK-BR includes three putative N-glycosylation sites, N-terminal truncation changes molecular size and perhaps structural conformation. However, ΔCCK-B has affinities for gastrin/CCK ligands. In addition, its binding properties is different from those of CCK-BR. These results suggest that the N-terminal extracellular domain is not essential for ligand binding, but is important for ligand selectivity.

The expression of each transcript of CCK-B/gastrin receptor was heterogenous in several cell lines: AGS and Jurkat exhibited exclusive expression of ΔCCK-B and CCK-BR, respectively, whereas LoVo expressed both transcripts. These results support the possibility of distinct promoters for gene expression of each transcripts. AGS (5, 6, 15), LoVo (7) and Jurkat (23) have been reported to be positive for CCK-B/gastrin-like receptor. In particular, AGS and LoVo show proliferative responses to gastrin/CCK peptides. Thus, ΔCCK-B may mediate the trophic actions of these peptides. Small cell lung carcinoma H510 has been reported to form colonies in response to these peptides, and to be positive for CCK-B/gastrin receptor by Northern blot analysis (24). As shown in "Results", two isoforms of CCK-B/gastrin receptor cannot be distinguished by this analysis. It is of interest to estimate the expression of each transcript in this cell line using RT-PCR analysis. In contrast to the results in cell lines, ΔCCK-B transcripts were detected in all tissues expressing CCK-BR transcripts, suggesting that restricted areas or cells in tissues may express ΔCCK-B transcripts. In rat stomach, gastrin receptors have been divided into two classies: high affinity sites detectable in fundus and low affinity sites detectable only in the antrum of aged rats (15). In addition, affinity crosslink studies using pig stomach membrane have suggested the existence of several gastrin-binding

proteins with different molecular weight (25). Such CCK-B/gastrin-like receptors with different properties may be related with the truncated isoform reported in the present study.

We here report the existence of the N-terminal truncated isoform of CCK-B/gastrin receptor generated by alternative usage of the novel exon 1b. However, the physiological function of this isoform remain unclear. Further investigation is required to determine the cellular localization of each transcript in native tissue and the function of ΔCCK-B in cancer cells.

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