Human Colorectal Cancers Express a Constitutively Active Cholecystokinin-B/Gastrin Receptor That Stimulates Cell Growth*

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Although ectopic expression of the cholecystokinin B/gastrin receptor (CCK-BR) is widely reported in human colorectal cancers, its role in mediating the proliferative effects of gastrin1-17 (G-17) on these cancers is unknown. Here we report the isolation of a novel splice variant of CCK-BR that exhibits constitutive (ligand-independent) activation of pathways regulating intracellular free Ca\(^{2+}\) and cell growth. The splice variant (designated CCK-BR\(_{i4sv}\) for intron 4-containing splice variant) is expressed in colorectal cancers but not in normal colonic mucosa adjacent to the cancer. Balb3T3 cells expressing CCK-BR\(_{i4sv}\) exhibited spontaneous, ligand-independent, oscillatory increases in [Ca\(^{2+}\)]\(_i\), whereas cells expressing wild-type CCK-BR did not. Primary cultures of cells isolated from resected colorectal cancers also exhibited a similar pattern of spontaneous [Ca\(^{2+}\)]\(_i\) oscillations. For both Balb3T3 and primary tumor cells, application of G-17 (10 and 200 nM, respectively) caused an increase in [Ca\(^{2+}\)]\(_i\). Selective CCK-BR antagonists blocked the G-17-stimulated Ca\(^{2+}\) responses but not the spontaneous [Ca\(^{2+}\)]\(_i\) oscillations. Cells expressing CCK-BR\(_{i4sv}\) exhibited an increased growth rate (~2.5-fold), in the absence of G-17, compared with cells expressing wild-type CCK-BR. The selective pattern of expression, constitutive activity, and trophic action associated with CCK-BR\(_{i4sv}\) suggest that this variant may regulate colorectal cancer cell proliferation though a gastrin-independent mechanism.

Colorectal cancers are the third leading cause of cancer deaths in the United States (1). It is estimated that approximately 130,000 new cases of colorectal cancer will be diagnosed in the United States this year. Despite major advances in uncovering the basic biochemical and genetic alterations involved in the development and progression of colorectal cancers (2), currently, treatment of this disease still relies predominantly upon surgical resection. Prognosis for survival is determined primarily by stage of disease at the time of diagnosis. Since the majority of patients with colorectal cancers have serosal penetration and nodal involvement at the time of operation, more effective adjuvant therapies are required. A better understanding of the molecular mechanisms regulating colorectal cancer cell proliferation would greatly facilitate the development of novel therapeutic agents.

In addition to regulating gastric acid secretion, the peptide hormone, gastrin (G-17), and its non-amidated precursor, glycine-extended gastrin (G-Gly), stimulate the growth of some colorectal cancers (3–6). The growth-promoting effects of these peptides have been reported in vivo using human colon cancer xenografts (4, 7) and in vitro in various cell lines derived from human colorectal cancers (8). The cholecystokinin B/gastrin receptor (CCK-BR), a member of the G protein-coupled receptor superfamily (9, 10), mediates many of the biological actions of G-17, including stimulation of gastric acid secretion by parietal cells in the oxyntic mucosa (11) and enterochromaffin-like cell proliferation (12). Although ectopic expression of CCK-BR has been widely reported in colorectal cancers (13, 14), a role for CCK-BR in mediating the trophic effects of G-17 and related peptides on colorectal cancer cell growth remains controversial. The controversy is due, in part, to variability in the reported prevalence of CCK-BR expression in colorectal cancers (12, 15, 16) and to the observations that the receptor(s) mediating the mitogenic effects of G-17 and G-Gly does not always satisfy the biochemical and pharmacokinetic criteria of CCK-BR (17, 18).

The third cytoplasmic loop domain of G protein-coupled receptors (GPCRs) interacts with heterotrimERIC G proteins and for many receptors, including CCK-BR, plays a critical role in the activation of intracellular signal transduction cascades (19), the regulation of ligand binding affinity, and agonist-induced receptor desensitization (20, 21). Because of the importance of the third cytoplasmic loop in regulating the biochemical and pharmacokinetic properties of GPCRs, we examined this region of CCK-BR, expressed in human colorectal cancers, to determine whether mutations existed that could account for some of the reported inconsistencies in the G-17-induced responses. Here we report the identification and isolation of a novel splice variant of the human CCK-BR that is generated by intron 4 retention during RNA processing. The resulting receptor protein contains 69 additional amino acid residues in its third intracellular loop domain. Expression of the novel splice variant (designated CCK-BR\(_{i4sv}\) for intron 4-containing splice variant) was detected in human colorectal...
cancers and adenomatous polyps but not in the normal colonic mucosa adjacent to the cancer. In addition to having ligand binding properties distinct from the previously characterized wild-type CCK-B (CCK-BRwt), CCK-BRi4sv exhibits constitutive (agonist-independent) activation of pathways regulating the levels of intracellular free Ca\(^{2+}\) (\([\text{Ca}^{2+}]_i\)) and cell proliferation.

**EXPERIMENTAL PROCEDURES**

cDNA Library Construction and Screening—CCK-BRi4sv was isolated from a cDNA library constructed with double-selected (polyA\(^\pm\)) mRNA from a freshly resected human colon cancer. The cDNA library was constructed using the ZAP Express XL Library Construction Kit (Stratagene, La Jolla, CA). Double-stranded cDNA, ranging in size from 2 to 4 kb, was size-selected on a low melting point agarose gel and ligated into the ZAP Express XL Vector. One million plaques were screened by plaque hybridization using a random-primed \([^{32}\text{P}]\)dATP-labeled CCK-BR cDNA probe as described previously (22). After hybridization overnight at 37 °C, the nitrocellulose filter was washed three times (20 min each) in 0.1× SSC and 0.1% SDS at 65 °C and exposed to x-ray film. Plasmid DNA was recovered from the positive phagemids by an in vivo excision reaction. Two cDNA clones of approximately 2.2 kb in length were identified. DNA sequence was determined using an automated sequencer (Applied Biosystems Inc., FIESM). Both clones contained the full-length CCK-BR in which intron 4 was retained. The total nucleotide sequence (2154 bp) of CCK-BRi4sv was submitted to the GenBank\(^\text{TM}\) data base (accession number AF239668).

Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)—Total RNA was isolated using Ultraspec RNA Isolation System (Biotec Laboratories, Inc., Houston, TX) and treated with 1 unit of RNase-free DNase I at 37 °C for 30 min (Promega, Madison, WI). Poly(A\(^\pm\)) mRNA was isolated using Poly A\(^\pm\) Quick mRNA Isolation Kit (Stratagene, La Jolla, CA). 100 ng of mRNA was converted to cDNA using SuperScript II reverse transcriptase (Life Technologies, Inc.) according to previously described methods (23). PCR was performed with 2.5 \(\mu\)l of the RT reaction and the following primers: sense primer, 5'-GGTCTTGGCTTCTGGCTCTCACTCATC-3', and antisense primer, 5'-AACGATCCACGACACGACATTCCGC-3' in a total volume of 100 \(\mu\)l. The PCR conditions were 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 45 °C for 30 s, and 72 °C for 1 min. A 1-kb DNA ladder (10 \(\mu\)l) (Life Technologies, Inc.) was used to determine the relative size of the PCR products.

RNase Protection Assay—Plasmid DNA, containing a 185-bp fragment of intron 4, was linearized with HindIII and used as template for labeling of an antisense RNA probe with MAXIscript in vitro transcription kit (Ambion, Austin, TX). Total RNA (50 \(\mu\)g) isolated from colorectal tumors and, patient-matched, normal mucosa adjacent to the tumor were hybridized with a \([^{32}\text{P}]\)UTP-labeled antisense riboprobe (\(4 \times 10^5\) cpm). Following digestion with RNases, the protected RNA fragments were separated by electrophoresis (6% polyacrylamide gel) and visualized by autoradiography.

**Transfection Technique**—Balb3T3 cells were plated at a density of 8 \times 10\(^5\) cells/100-mm dish in DMEM supplemented with 5% FBS. After 24 h at 37 °C, the cells were transfected with 1–6 \(\mu\)g of either CCK-BRwt, CCK-BRi4sv, or CCK-BRi4sv* plasmid DNA using a 1:2.3 ratio of DNA to FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals). Total RNA was extracted 24 h after transfection using the Ultraspec RNA Isolation System. To eliminate chromosomal DNA contamination, total RNA was treated with RNase-free DNase I at 37 °C for 30 min.

**Competition Binding Studies**—Balb3T3 cells were plated in 12-well plates, initial density of 3 \times 10\(^4\) cells per well, in 2 ml of DMEM supplemented with 5% FBS. After 24 h, the cells were transfected with plasmid DNA containing either CCK-BRi4sv* or CCK-BRwt cDNA as described above. Binding experiments were performed 24 and 48 h after transfection. For competition analyses, cells were washed twice with 1 ml of binding buffer (1× Hank’s balanced salt solution, 10 mM HEPES, pH 7.4, 0.1% BSA) and then incubated (1 h at 30 °C) in binding buffer containing 0.05 nm \([^{125}\text{I}]\)-labeled G-17 (specific activity = 2200 Ci/mmol, Amersham Pharmacia Biotech) and various concentrations of unlabeled competitors (1 pm to 10 \(\mu\)M). The binding reaction was terminated by the addition of cold washing buffer. The cells were centrifuged at 1000 \(g\) for 4 min (4 °C). The resulting cell pellets were counted in a Cobra II gamma counter (Packard Instrument Co.). Total binding averaged approximately 6% of the total counts added to the assays for each receptor.

Non-specific binding was defined as the amount of radioactivity detected in the presence of 10 \(\mu\)M unlabeled G-17. Each data point was determined in triplicate, and the graphs represent the mean ± S.E. of four independent experiments.

**Preparation of Primary Tumor Cells and Calcium Imaging**—Tumor tissue was collected immediately following resection and placed into a sterile tube containing DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Tissues were homogenized in 1 ml of DMEM plus FBS and antibiotic. After mincing, the tissue was placed into a 15-ml Falcon tube containing 2 ml of media supplemented with 1000 units/ml collagenase II (Sigma), incubated for 10 min (37 °C), and mixed on a vortex for 10 s. 2 ml of media/cells was removed and added to an equal volume of 100% FBS. This procedure was repeated 3 times. The dissociated cells were collected by centrifugation (250 \(\times\) g) for 10 min, resuspended in DME supplemented with 20% FBS and 1% penicillin/streptomycin, and plated on glass coverslips (25 mm). Single cell calcium imaging was performed using the calcium-sensitive dye fura-2. The cells were washed with a physiological medium (KRH) containing NaCl (125 mM), KCl (5 mM), KH\(_2\)PO\(_4\) (1.2 mM), MgSO\(_4\) (1.2 mM), CaCl\(_2\) (2 mM), glucose (6 mM), HEPES (25 mM), pH 7.4, and loaded with 2 \(\mu\)M fura-2 AM (Molecular Probes, Eugene, OR) for 50 min at 25 °C. Single cell recordings were collected using a Nikon Diaphot inverted microscope (Garden City, NY) coupled to a dual monochrometer system via a fiberoptic cable (Photon Technology International, South Brunswick, NJ). Fluorescence was detected using an intensified CCD camera (Dage-MTI, Inc., Michigan City, IN). Image frames were acquired every 1–8 s and analyzed using ImageMaster software (Photon Technology International).

**RESULTS**

**Cloning of CCK-BRi4sv**—The novel CCK-BR splice variant was isolated from a cDNA library constructed with double-selected polyA\(^\pm\) mRNA from a freshly resected human colon cancer. The screening of 1 million plaques, using a random primed \([^{32}\text{P}]\)dATP-labeled CCK-BR cDNA probe, produced two cDNA clones of approximately 2.2 kb in length. The DNA sequences of the sense and antisense strands for each clone were determined and found to contain a single large open reading frame encoding a CCK-BR in which intron 4 was retained (CCK-BRi4sv). Retention of intron 4 did not cause a frameshift in the open reading frame or the introduction of a stop codon. Consequently, the amino acid sequence of CCK-BRi4sv, predicted from the cDNA, encodes a receptor protein with a 69-amino acid insertion in its putative tertiary cytoplasmatic domain (Fig. 1A). The amino acid sequence upstream and downstream of the intron 4 insertion site is identical to wild-type CCK-BR (CCK-BRwt) with the exception of one residue at position 64. A point mutation in the nucleotide sequence (T to C) resulted in a change in the amino acid residue at this position from an isoleucine in CCK-BRwt to a threonine residue in CCK-BRi4sv (Fig. 1A). Additionally, DNA sequence analysis revealed that retention of intron 4 was not due to mutations in either the 5′ or 3′ splice site consensus sequences (Fig. 1B).

**CCK-BRi4sv Expression in Human Colorectal Cancers and Adenomatous Polyps**—To begin to assess the prevalence of CCK-BRi4sv expression in human colorectal cancers, we have screened DNase I-treated mRNA isolated from the resected cancers of eight patients (Table I) by reverse transcription-polymerase chain reaction (RT-PCR) assays. The oligonucleotide primers, used in the PCR reaction, were complementary to sequences found in the receptor’s fifth and sixth transmembrane domains, flanking the third cytoplasmic loop. By using these primers, CCK-BRi4sv expression would be indicated by the presence of a 554-bp band (bp) PCR product (Fig. 2A), whereas the expression of CCK-BRwt would yield a 547-bp product (Fig. 2A and B). A single PCR product of 554 bp in length, was detected in all eight cancers, indicating expression of CCK-BRi4sv (Fig. 2C, T1-T8). DNA sequence analysis and Southern blotting confirmed the presence of intron 4 sequence within the 554-bp PCR product (data not shown). After 40 cycles of amplification, we did not detect expression of either CCK-BRwt (347 bp) or CCK-BRi4sv (554 bp) in paired, normal
colonic mucosa adjacent to the cancers (Fig. 2C, N1–N8). Because the CCK-BR gene contains three other introns (Fig. 2A), we also analyzed the RNA from tumor and normal tissue samples for the presence of these other introns. We did not detect any of the other three introns in the RNA samples from either cancers or normal mucosa (data not shown), indicating selective retention of intron 4.

Confirmation of the selective expression of CCK-BRi4sv in human colon cancers was further demonstrated by RNase protection assays. After treatment with RNase, a 185-bp fragment, corresponding to a portion of intron 4, was protected from digestion in tumor RNA samples isolated from patients 1, 5, 6, and 8 (Fig. 2D, T1, T5, T6, and T8). A protected band was not detected in matched RNA samples isolated from normal colonic mucosa (Fig. 2D, N1, N5, N6, and N8). CCK-BRi4sv expression was not detected by RNase protection assays in cancers from patients 2, 3, 4, and 7. These samples also showed a relatively faint 554-bp band by RT-PCR (Fig. 2C).

Adenomatous polyps are an early manifestation of dysregulated growth control of the colonic epithelium and a predispos-
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Radiolabeled Ligand Binding Studies—To assess the effects of intron 4 retention and the subsequent 69 amino acid insertion on the binding and signaling properties of CCK-BRi4sv*, we expressed either the splice variant or CCK-BRwt in mouse Balb3T3 fibroblasts. Although CCK-BRi4sv was the only form of CCK-BR detected in human colorectal cancers, examination of the cDNA revealed normal intron 4 splice site consensus sequences (Fig. 1B). Therefore, we reasoned that expression of CCK-BRi4sv in a non-tumorigenic cell line, such as Balb3T3 fibroblasts, would result in intron splicing and the generation of CCK-BRwt. To ensure that we were characterizing the properties of CCK-BRi4sv*, and not CCK-BRwt, we introduced point mutations into the previously identified splice sites of intron 4 (24). The single nucleotide changes modified the splice consensus sequences without changing the amino acid composition of the protein (Fig. 3A). To confirm that the splice site mutant receptor (designated CCK-BRi4sv*) was expressed in transfected Balb3T3 cells, we performed RT-PCR analysis. As expected, before the splice sites were modified, a fraction of the CCK-BRi4sv transcript was converted to CCK-BRwt as indicated, before the splice sites were modified, a fraction of the CCK-BRi4sv transcript was converted to CCK-BRwt as indicated by direct competition of 125I-labeled G-Gly binding on CCK-BRi4sv* (Fig. 3B). Therefore, we reasoned that expression of CCK-BRi4sv in a non-tumorigenic cell line, such as Balb3T3 fibroblasts, would result in intron splicing and the generation of CCK-BRwt. To ensure that we were characterizing the properties of CCK-BRi4sv*, and not CCK-BRwt, we introduced point mutations into the previously identified splice sites of intron 4 (24). The single nucleotide changes modified the splice consensus sequences without changing the amino acid composition of the protein (Fig. 3A). To confirm that the splice site mutant receptor (designated CCK-BRi4sv*) was expressed in transfected Balb3T3 cells, we performed RT-PCR analysis. As expected, before the splice sites were modified, a fraction of the CCK-BRi4sv transcript was converted to CCK-BRwt as indicated by the appearance of both the 554- and 347-bp PCR products (Fig. 3B, CCK-BRi4sv*). After introduction of point mutations at the splice boundaries, only a 554-bp PCR product corresponding to the CCK-BRi4sv* transcript was detected (Fig. 3B, CCK-BRi4sv*). The effects of intron 4 retention on radiolabeled ligand binding and intracellular signaling were assessed using CCK-BRi4sv*.

Competition binding studies with Balb3T3 cells transiently transfected with CCK-BRi4sv* revealed two binding sites for G-17 as follows: one site that was competed by relatively low concentrations of G-17 (IC50 of 0.12 nM) and a second site that required higher concentrations (IC50 of 315 nM) (Fig. 3C). The selective CCK-BRwt antagonist L365,260 (L-60) exhibited IC50 95% confidence interval R2

| Binding Conditions | IC50 (nM) | 95% Confidence Interval | R2 |
|--------------------|----------|-------------------------|----|
| CCK-BRi4sv*        | G-17     | 0.12 ± 0.05             | 0.99 |
|                    | G-Gly    | 1.3 ± 0.2               | 0.99 |
|                    | L-60     | 3.6 ± 0.4               | 0.99 |

Summary of competition binding studies

Effects of CCK-BRi4sv* Expression on Intracellular Ca2+

Expression of CCK-BRi4sv (554 bp), but not CCK-BRwt, was detected in all four polyps (Fig. 2E). Additional analyses of polyps from both FAP and non-FAP patients are required; however, these data do suggest a possible role for CCK-BRi4sv in early events leading to the development of colorectal cancer.

Comparison of G-17 and G-Gly binding to CCK-BRi4sv* and CCK-BRwt reveals that CCK-BRi4sv* exhibits a constitutive activation profile similar to that of CCK-BRwt. The single base changes introduced at the splice boundaries, only a 554-bp PCR product corresponding to the CCK-BRi4sv* transcript was detected (Fig. 3B, CCK-BRi4sv*). The effects of intron 4 retention on radiolabeled ligand binding and intracellular signaling were assessed using CCK-BRi4sv*.

Competition binding studies with Balb3T3 cells transiently transfected with CCK-BRi4sv* revealed two binding sites for G-17 as follows: one site that was competed by relatively low concentrations of G-17 (IC50 of 0.12 nM) and a second site that required higher concentrations (IC50 of 315 nM) (Fig. 3C). The selective CCK-BRwt antagonist L365,260 (L-60) exhibited a similar potency for both receptors (IC50 = 2.3 nM for CCK-BRi4sv* and 3.6 nM for CCK-BRwt). However, L-60 competed only the labeled G-Gly bound to the high affinity G-17 site on CCK-BRi4sv* (Fig. 3C). G-Gly binding to CCK-BRi4sv* was confirmed by direct competition of 125I-labeled G-Gly binding by G-Gly (IC50 = 312 nM) (Fig. 3E). Radiolabeled G-Gly binding to CCK-BRi4sv* was also insensitive to inhibition by the selective antagonists L-60 (1 μM) and CAM1028 (1 μM) (Fig. 3F), suggesting that G-Gly binds to the low affinity G-17-binding site on CCK-BRi4sv*.
Signaling—Binding of G-17 to CCK-Bwt is coupled, in part, to the mobilization of intracellular Ca\(^{2+}\) through the activation of heterotrimeric G proteins of the G\(_q\) subfamily and the phospholipase C-dependent generation of inositol 1,4,5-trisphosphate (IP\(_3\)) (10). By using single-cell [Ca\(^{2+}\)]\(_{\text{i}}\) imaging, we found that collagenase-dissociated colorectal cancer cells exhibited both spontaneous, non-synchronous, and oscillatory changes in [Ca\(^{2+}\)]\(_{\text{i}}\) as well as G-17-stimulated increases in [Ca\(^{2+}\)]\(_{\text{i}}\) (Fig. 4A). Balb3T3 cells transfected with CCK-BRi4sv* exhibited a similar pattern of ligand-independent and G-17-dependent increases in [Ca\(^{2+}\)]\(_{\text{i}}\) (Fig. 4B). In contrast, cells expressing either CCK-BRwt or the empty expression vector, pcDNA3.1, did not exhibit spontaneous [Ca\(^{2+}\)]\(_{\text{i}}\) oscillation (Fig. 4C and D); however, a G-17-stimulated response was evoked in cells expressing CCK-BRwt (Fig. 4C). Because increases in GPCR signaling, in the absence of agonist, can result from receptor overexpression (25), we assessed the level of receptor expression using radiolabeled ligand binding. These studies showed similar levels of G-17 binding to both CCK-BRi4sv* and CCK-BRwt cells expressed similar levels of receptors (TB, total binding; NS, nonspecific binding). F, treatment of colon cancer cells with the CCK-BR-selective antagonist, CAM1028, blocked a G-17-induced [Ca\(^{2+}\)]\(_{\text{i}}\); however, CAM1028 had no effect on the spontaneous, ligand-independent [Ca\(^{2+}\)]\(_{\text{i}}\) activity. G and H, Balb3T3 cells expressing CCK-BRi4sv* were treated with G-17 in the presence and absence of CAM1028.

**Fig. 4**. Colon cancer cells and Balb3T3 cells expressing CCK-BRi4sv* exhibit both constitutive (ligand-independent) and G-17-stimulated increases in [Ca\(^{2+}\)]. A, primary cultures of cells isolated from human colorectal tumors exhibit both spontaneous and G-17-stimulated increases in [Ca\(^{2+}\)]. The change in [Ca\(^{2+}\)], is expressed as the ratio of fura-2 fluorescence at 340/380 nm. The tracings from 5 to 10 individual cells are shown. The black bar indicates the period of time the cells were exposed to indicated ligands. B, Balb3T3 cells expressing CCK-BRi4sv* exhibited both spontaneous and G-17-stimulated increases in [Ca\(^{2+}\)]. C, Balb3T3 cells expressing CCK-BRwt showed a G-17-stimulated increase in [Ca\(^{2+}\)] but did not exhibit spontaneous fluctuations in [Ca\(^{2+}\)]. D, cells transfected with the empty expression vector, pcDNA3.1 (Invitrogen, Carlsbad, CA), did not displace spontaneous or G-17-stimulated [Ca\(^{2+}\)] activity. E, radiolabeled G-17 binding assays demonstrated that both CCK-BRi4sv* and CCK-BRwt cells expressed similar levels of receptors (TB, total binding; NS, nonspecific binding). F, treatment of colon cancer cells with the CCK-BR-selective antagonist, CAM1028, blocked a G-17-induced [Ca\(^{2+}\)]; however, CAM1028 had no effect on the spontaneous, ligand-independent [Ca\(^{2+}\)] activity. G and H, Balb3T3 cells expressing CCK-BRi4sv* were treated with G-17 in the presence and absence of CAM1028.
CCK-BRi4sv expression was confirmed using rat NRK-49F cells transiently transfected with either CCK-BRi4sv, CCK-BRwt, or pcDNA3.1. One day after transfection, the NRK-49F were replated, and single cells were identified. Three days later, the number of cells arising from the previously identified single cell was determined. We observed an approximately 2-fold increase in the number of cells per cell cluster in CCK-BRi4sv-expressing cells compared with cells transfected with either CCK-BRwt or the empty vector, pcDNA3.1 (Fig. 5, B and C) (CCK-BRi4sv, 28 ± 2.4; CCK-BRwt, 17 ± 2.0; pcDNA3.1, 14 ± 1.9). Together these data demonstrate that expression of CCK-BRi4sv alone, in the absence of agonist stimulation, is sufficient to stimulate cell proliferation.

**DISCUSSION**

The possibility that the gastrointestinal peptide hormone, gastrin, and its biosynthetic precursor, G-Gly, play a role in the development of colorectal cancers has aroused considerable interest over the past several years. Most colorectal cancers and derived cell lines produce amidated G-17 and/or G-Gly (26, 27). Also, many colorectal cancers exhibit a proliferative response upon application of exogenous peptides. Together these observations have led to the hypothesis that G-17 and its non-amidated precursors act as autocrine and/or paracrine growth factors to stimulate colorectal cancer cell proliferation. In this model, G-17 and/or G-Gly are released from cancer cells into the interstitium where they bind to specific cell-surface receptors on the same or adjacent cells and stimulate proliferation (18). Here we have reported the isolation and partial characterization of a novel splice variant of CCK-BR, designated CCK-BRi4sv, that stimulates cell growth in a G-17-independent manner. Our data show that colorectal cancers and adenomatous polyps, but not the normal colonic mucosa, express CCK-BRi4sv and that cells expressing this receptor exhibit spontaneous, oscillatory increases in [Ca\(^{2+}\)], in the absence of agonist stimulation.

The constitutive, ligand-independent activation of pathways regulating [Ca\(^{2+}\)], and cell proliferation exhibited by CCK-BRi4sv may contribute to its potential pathophysiological role in colorectal cancers. Recently, other GPCRs exhibiting constitutive activation of intracellular signaling pathways have been identified as causative factors in several human diseases including some cancers (28, 29). The human Kaposi's sarcoma-associated herpesvirus encodes aGPCR, with homology to the human interleukin-8 receptor, which constitutively activates the IP\(_3\)/[Ca\(^{2+}\)] pathway and, when transfected into rat NRK-49F cells, increases their rate of growth in the absence of agonist stimulation (29). We have shown that CCK-BRi4sv may stimulate the rate of cell growth in the absence of agonist stimulation which suggests that, like Kaposi's sarcoma-associated herpesvirus, constitutive action of the IP\(_3\)/[Ca\(^{2+}\)] pathway may be involved in the regulation of cell proliferation. Several other G protein-coupled receptors that signal through Go/IP\(_3\)/[Ca\(^{2+}\)] pathway have been shown to stimulate DNA synthesis and induce a transformed-like phenotype in fibroblasts. These include the serotonin 1c, muscarinic acetylcholine m1, m3, and m5, and the a1b-adrenergic receptors (30–33). However, unlike CCK-BRi4sv and Kaposi's sarcoma-associated herpesvirus, the stimulation of DNA synthesis and transformation, by these other receptors, required the continued presence of agonist.

Various types of reconstitution studies and experiments with natural membrane systems have shown that GPCRs can spontaneously activate G proteins in the absence of agonist (34, 35). A thermodynamic scheme for receptor-G protein interactions has been presented (36, 37) as an extension of the well known ternary complex model of receptor-effector interactions. In this model, a receptor, in the absence of ligand, can exist in so-called...
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inactive (Ri) and active (Ra) states with respect to its predisposition to associate spontaneously with G protein. The activated receptor (Ra) can spontaneously form a complex with G protein (Ra•G) protein. Our data support the hypothesis that the structural alterations associated with the addition of 69 amino acid residues to the third intracellular loop domain of CCK-BRi4sv cause it to favor the Ra conformation and, therefore, increase its predisposition to spontaneous coupling to G protein. The consequences of the increased spontaneous coupling to G protein is reflected by the transient elevations in [Ca\(^{2+}\)]. Another important aspect of this model, which is also supported by our data, is that an agonist has two different binding states of the receptor to choose from, namely Ri and Ra (and Ra•G protein). CCK-BRi4sv* expressed in Balb3T3 cells clearly exhibits two binding states for G-17. Additionally, this model predicts that a ligand that destabilizes Ra•G protein will act as an inverse agonist and inhibit spontaneous signaling by the receptor. Inverse agonists for CCK-BRi4sv could prove to be useful therapeutic agents.

The similarities in both the pattern and pharmacology of the [Ca\(^{2+}\)] responses observed in Balb3T3 cells transfected with CCK-BRi4sv* and primary cultures of collagenase-dissociated colorectal cancer cells strongly suggest that CCK-BRi4sv is the mediator of the response in the latter. Although the identification of CCK-BRi4sv does not preclude the existence of other G-17/G-Gly receptors on human colorectal cancers, its selective pattern of expression, constitutive (ligand-independent) activity, and growth-promoting effects provides compelling evidence for a significant role of CCK-BRi4sv in colorectal carcinogenesis. The unique properties of CCK-BRi4sv suggest that it may stimulate colorectal cancer cell proliferation though a gastrin-independent mechanism and make it an attractive target for the development of novel drugs such as inverse agonists, which, in the future, may provide more effective adjuvant therapies for this devastating disease.

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