Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 204-211

# MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF THE HUMAN GALLBLADDER CHOLECYSTOKININ A RECEPTOR#

Charles D. Ulrich, Irene Ferber, Eileen Holicky,
Elizabeth Hadac, Gary Buell+, and Laurence J. Miller*

Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, MN 55905
+Glaxo Institute for Molecular Biology S.A., Geneva, Switzerland

Received April 2, 1993

**SUMMARY:** Through binding to cholecystokinin (CCK) A receptors, CCK is an important physiologic regulator of both gallbladder contraction and pancreatic enzyme secretion. In this work, we have used a combination of hybridization screening of a cDNA library and polymerase chain reaction to clone a 2.1 kb cDNA which encodes the human gallbladder $CCK_A$ receptor. Nucleotide sequence analysis revealed an open reading frame encoding a 428 amino acid protein, with seven putative transmembrane domains and a high degree of homology with the rat $CCK_A$ receptor. COS cells transfected with this cDNA clone bound CCK-8 and L-364,718 with high affinities appropriate for the $CCK_A$ receptor, and exhibited a transient increase in intracellular calcium in response to CCK. This should provide an important resource for the analysis of the role of this receptor in human physiology and pathophysiology. 

The cholecystokinin family of peptide hormones have been implicated in numerous important physiologic events. These appear to be mediated through two general classes of receptors classified as $CCK_A$ or $CCK_B$ (gastrin) based on their binding affinities for CCK/gastrin family peptides (1). The $CCK_A$ receptor has a high affinity for CCK-8, with the desulfated peptide and peptides shorter than the C-terminal heptapeptide possessing much lower affinities. In contrast, the $CCK_B$ receptor requires only the C-terminal tetrapeptide for high affinity binding, and recognizes CCK and gastrin almost equally well.

Through binding to $CCK_A$ receptors, CCK is a major physiologic mediator of gallbladder contraction and pancreatic enzyme secretion; appears to play a role in slowing gastric emptying, relaxation of the sphincter of Oddi, and potentiation of insulin secretion;

# Sequence data have been deposited in the GenBank Data Library under Accession No. L13605.

* To whom correspondence and reprint requests should be addressed.

0006-291X/93 $4.00

and has been implicated as a mediator of pancreatic growth and tumorigenesis (2,3). $CCK_A$ receptors have also been described in the anterior pituitary, myenteric plexus, and regions of the central nervous system where they have been implicated in the pathogenesis of feeding disorders, Parkinson's disease, schizophrenia, and drug addiction (2,4).

A number of studies have demonstrated that intraluminal fat and protein stimulates the release of CCK peptides from neuroendocrine cells in the proximal small intestinal mucosa, resulting in a rise in serum CCK levels, and gallbladder contraction. Competition binding studies have confirmed the pharmacologic similarity of pancreatic and gallbladder $CCK_A$ receptors (5-7). Affinity labeling of pancreatic and gallbladder CCK receptors has revealed similar apparent sizes both before ($M_r$=85,000-95,000) and after deglycosylation with endoglycosidase F ($M_r \approx 42,000$), and similar protease peptide maps (6,8,9). Based on these findings, the probable identity of the pancreatic and gallbladder $CCK_A$ receptors is generally accepted.

Until recently, studies attempting to characterize the molecular architecture of the $CCK_A$ receptor had been limited by the sparse number of receptors on native receptor-bearing cells. In 1992, Wank et al. (10) reported the cloning of the rat pancreatic $CCK_A$ receptor cDNA. Analysis of that sequence predicted a 444 amino acid protein with seven hydrophobic segments consistent with being a G protein-coupled receptor. Notable features include a high degree of homology with the $CCK_B$ receptor (11), the presence of four potential N-linked glycosylation sites, and four consensus sites for protein kinase C phosphorylation. While this provides an important tool to study the tissue distribution and physiology of this receptor in the rat, our understanding of human physiology and pathophysiology would be considerably enhanced by molecular characterization of the human $CCK_A$ receptor.

In this work, we report the cloning of a cDNA encoding the human $CCK_A$ receptor from a human gallbladder library. The recombinant protein was expressed in COS cells, characterized pharmacologically through competition binding studies, and demonstrated to be functional based on an intracellular calcium response to CCK stimulation.

## METHODS

**Human Gallbladder cDNA Library Preparation.** Human gallbladders were obtained from patients with cholelithiasis by laparoscopic cholecystectomy, the mucosa removed, and the remaining tissue snap frozen in liquid nitrogen. Sections of all organs appeared normal histologically. RNA was prepared by the single-step guanidinium thiocyanate method (12), with high quality of the total RNA assured by analysis on 1% agarose/formaldehyde gel electrophoresis. Following oligo(dT)-cellulose chromatography, eleven micrograms of poly(A)$^+$ RNA was used for library construction in pcDNA1 (Invitrogen, San Diego, CA). The cDNA species greater than 1.5 kb in length were

separated by agarose gel electrophoresis, and unidirectionally ligated into the **BstXI - NotI** sites of the vector.

**Polymerase Chain Reaction (PCR) Probe Amplification.** Oligonucleotide primers analogous to nucleotides 319-335 and the complement to 519-539 of the published cDNA sequence encoding the rat $CCK_A$ receptor (10) were synthesized using the beta-cyanoethylphosphoramidite method and purified using OPC cartridges (Applied Biosystems, Inc.). DNA amplification was performed according to the manufacturer (Perkin-Elmer Cetus) using AmpliTaq recombinant **Taq DNA polymerase** and the custom-synthesized human gallbladder cDNA library as a template. Following 1% agarose gel electrophoresis, a band of the predicted size was excised, purified, and used as template to generate a $^{32}P$-labeled probe (13).

**cDNA Library Screening and PCR Cloning.** Competent **E. coli** (MC1061/P3) were transformed with the human gallbladder cDNA library and screened by hybridization with the $^{32}P$-labeled probe. One million colonies were initially screened, and potential positives were purified by another round of hybridization. Plasmids containing the target sequences were isolated and verified by restriction mapping and double-stranded DNA sequencing. This identified a clone which, when compared to the nucleotide sequence of the rat $CCK_A$ receptor, lacked a 262 bp fragment. Oligonucleotide primers flanking the suspected deletion were used in PCR amplification with the human gallbladder cDNA library as template. This yielded a product of 786 bp, representing 262 bp larger than the 524 bp product expected if the deletion were correct. Both this PCR product and the original clone were cleaved with **BstEII** and **BspEI**, the reaction products gel purified, and ligation performed with **T4 DNA Ligase**. Plasmids containing the expected 2.1 kb cDNA insert were then isolated and sequenced in both directions by a double-stranded DNA method using **Sequenase v.2.**

**Transfection and Membrane Preparation from COS Cells.** The pcDNA1 clone of interest was purified by the alkaline lysis method. Transient transfection into COS-1 cells utilized a modified DEAE-dextran protocol (14). These were harvested 72 hours post-transfection. The cells were washed twice with phosphate buffered saline, scraped into a 50 ml conical tube, and pelleted at 2500 rpm for 2 minutes. The cell pellet was resuspended in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor (STI) and 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed with 5 strokes in a Potter-Elvehjem tissue homogenizer. Enough 2.0 M sucrose was then added to bring the final concentration to 1.3 M sucrose. This homogenate was then overlaid with 0.3 M sucrose and centrifuged for 1 h at 225,000 x g in a fixed angle rotor. Membranes floating to the 0.3-1.3 M interface were collected, washed, and stored at -70°C in a modified Krebs-Ringer-Hepes buffer (KRH) with protease inhibitors (25 mM Hepes, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM $KH_2PO_4$, 0.2% bovine serum albumin, 0.01% STI, and 1 mM PMSF).

**Receptor Binding Studies.** D-Tyr-Gly-[($Nle^{28,31}$)CCK-26-33] was used as radioligand. It was synthesized and oxidatively radioiodinated, as we previously described (15). This CCK analogue has been shown to be fully biologically active and to bind to CCK family receptors with affinities and specificity identical to native CCK-8 (15). In standard binding assays, cell membranes and 3-5 pM radioligand were incubated in KRH, in the presence or absence of competing CCK-8, gastrin, L-364,718, or L-365,260. All binding studies were performed at steady state, attained after 1 hour at room temperature. Bound was separated from free radioligand by rapid filtration with a Skatron cell harvester (Sterling, VA), using receptor-binding filtermats. Nonspecific binding, determined in the presence of excess unlabeled CCK-8 (0.1 $\mu$M), was less than 30% of total binding.

**Measurement of $[Ca^{2+}]_i$.** 72 hours after transfection, COS cells were loaded with fura-2/AM in KRH buffer, and stimulated with hormonal agonists as described (11,16).

Changes in $[Ca^{2+}]_i$ after stimulation with 1 nM CCK-8 were determined from fluorescence emission ratios at 340/380 nm.

## RESULTS AND DISCUSSION

Following construction of a human gallbladder cDNA library, we utilized the combination of colony hybridization screening and PCR cloning to obtain a plasmid containing a 2.1 kb cDNA insert encoding a human CCK receptor. Open reading frame analysis of this cDNA predicts an encoded protein of 428 amino acids, with a calculated molecular mass of 47,841 daltons (Fig. 1). Based on the proximity of an in frame termination codon, the predicted initiator codon in this cDNA lies 45 nucleotides downstream relative to that reported for the rat $CCK_A$ receptor (10).

As expected, this protein sequence shares many of the characteristic structural features of the G protein-coupled family of receptors. Hydropathy analysis reveals seven hydrophobic segments, likely corresponding to transmembrane domains. Four potential sites of N-linked glycosylation are present (residues 10, 13, 24, and 190), and the vicinal cysteines (residues 387, 388) in the carboxyl tail represent a possible site of palmitoylation (17). Four cysteines (residues 18, 29, 114, and 196) are predicted to reside in the ectodomain, with residues 114 and 196 in analogous positions to the cysteines involved in a

```
                 # #      .  #     .          .
   1  MDVVDSLLVNGSNITPPCELGLENETLFCLDQPRPSKEWQPAVQILLYSL   50

          I      .        .          .          II
  51  IFLLSVLGNTLVITVLIRNKRMRTVTNIFLLSLAVSDLMLCLFCMPFNLI  100

          .      .        .  III  .          .
 101  PNLLKDFIFGSAVCKTTTYFMGTSVSVSTFNLVAISLERYGAICKPLQSR  150

          .        IV .          .          #
 151  VWQTKSHALKVIAATWCLSFTIMTPYPIYSNLVPFTKNNNQTANMCRFLL  200

          .          . V        .          .    ♦    ♦
 201  PNDVMQQSWHTFLLLILFLIPGIVMMVAYGLISLELYQGIKFEASQKKSA  250

       *  ♦          .          .          ♦
 251  KERKPSTTSSGKYEDSDGCYLQKTRPPRKLELRQLSTGSSSRANRIRSNS  300

          .        .  VI       .          .          _
 301  SAANLMAKKRVIRMLIVIVVLFFLCWMPIFSANAWRAYDTASAERRLSGT  350

          VII        .          .          .
 351  PISFILLLSYTSSCVNPIIYCFMNKRFRLGFMATFPCCPNPGPPGARGEV  400

          .          .
 401  GEEEEGGTTGASLSRFSYSHMSASVPPQ  428
```

Figure 1.
Deduced amino acid sequence of the human gallbladder $CCK_A$ receptor. Bars with Roman numerals are positioned over putative transmembrane domains. Potential N-linked glycosylation sites are indicated by #. Consensus sites for protein kinase C (♦) and protein kinase A (*), as predicted by patterns found in the Prosite database, are illustrated.

disulfide bond in rhodopsin (18). In addition, consensus sites for both protein kinase C (residues 245, 249, 260, and 290) and protein kinase A (residue 256) are present in predicted cytosolic domains.

This receptor shares significant amino acid homology with reported CCK binding proteins (Fig. 2). When compared to the predicted amino acid sequence of the rat $CCK_A$ receptor (10), it is 92% identical and 95% similar. Comparison with the predicted sequences of the human (19) and rat $CCK_B$ receptors (20), reveals 50% identity and 66% similarity to both. As noted previously (11), this family of receptors also shares significant amino acid identity with a number of other peptide hormone and biogenic amine receptors.

Pharmacologic characterization of recombinant receptor was performed with membranes from transfected COS cells. Radioiodinated CCK analogue bound specifically and with high affinity to membranes from COS cells transfected with pcDNA1 containing the cDNA insert of interest. Sham transfected COS cells expressed no saturable binding. Competition binding studies demonstrated inhibition of specific binding in a concentration-dependent manner by CCK-8, gastrin, L-364,718, and L-365,260 (Fig. 3). The concentrations required to inhibit 50% of specific binding ($IC_{50}$) were 0.02 nM CCK-8, 0.08 $\mu$M gastrin, 0.1 nM L-364,718, and 9 $\mu$M L-365,260. These relative affinities are consistent with reports characterizing $CCK_A$ receptors on gallbladder and pancreas from multiple species (5,7,21).

Second-messenger signalling through this recombinant protein was studied in transfected COS cells using fura-2 to measure changes in $[Ca^{2+}]_i$ in response to CCK-8 (16). One nM CCK-8 triggered a marked increase in $[Ca^{2+}]_i$, with no response in sham transfected cells (Fig. 4). This agrees with observations in rat pancreatic acinar cells (22). The combination of this biological response, the functional binding data, and the structural homology of the predicted sequence with that reported for the rat $CCK_A$ receptor, confirms its identity as the human $CCK_A$ receptor.

While animal models provide insights into processes occurring in man, knowledge of the precise molecular structure of human receptors provides additional opportunities. It provides a mechanism to study aspects of receptor physiology which might be specific to this species, and provides a comparison for potential receptor mutations or polymorphisms. There is precedent for a change in a single amino acid in a G protein-coupled receptor to result in marked alteration in signalling, and even to result in oncogenicity (23). A potential clinical setting for CCK receptor dysfunction might include subsets of patients with gallstones and acalculous cholecystitis who appear to have impaired responsiveness to CCK (24). Further, CCK has been implicated in the development and progression of certain pancreatic adenocarcinomas (3). The molecular characterization of this receptor may provide

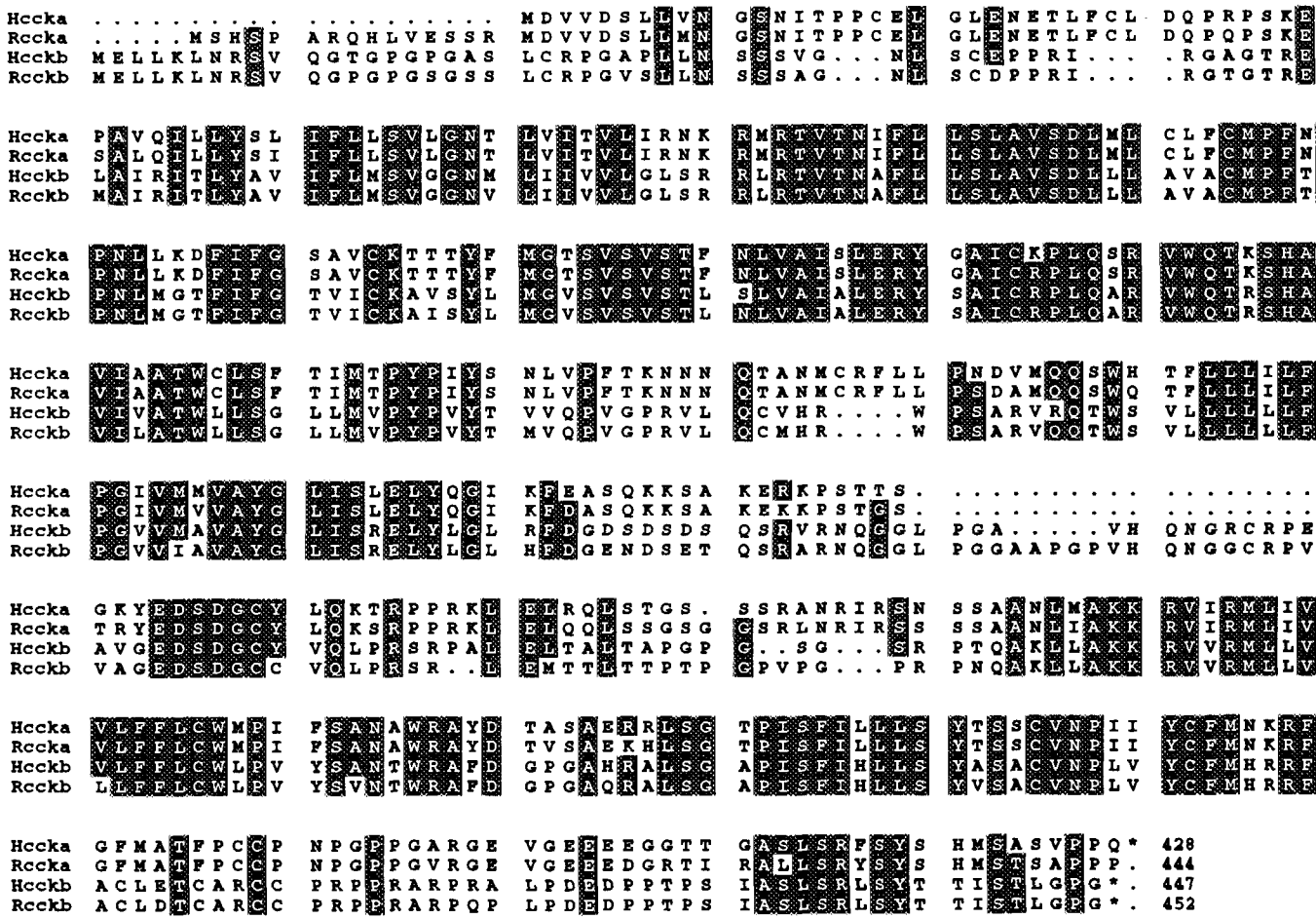


Figure 2.

Predicted primary structure of the human $CCK_A$ receptor and alignment with established receptors in this family. Shaded amino acids are identical in at least three of the four receptors. Amino acids are numbered on the right side. Hccka, human $CCK_A$ receptor, Rccka, rat $CCK_A$ receptor, Hcckb, human $CCK_B$/gastrin receptor, Rcckb, rat $CCK_B$/gastrin receptor.

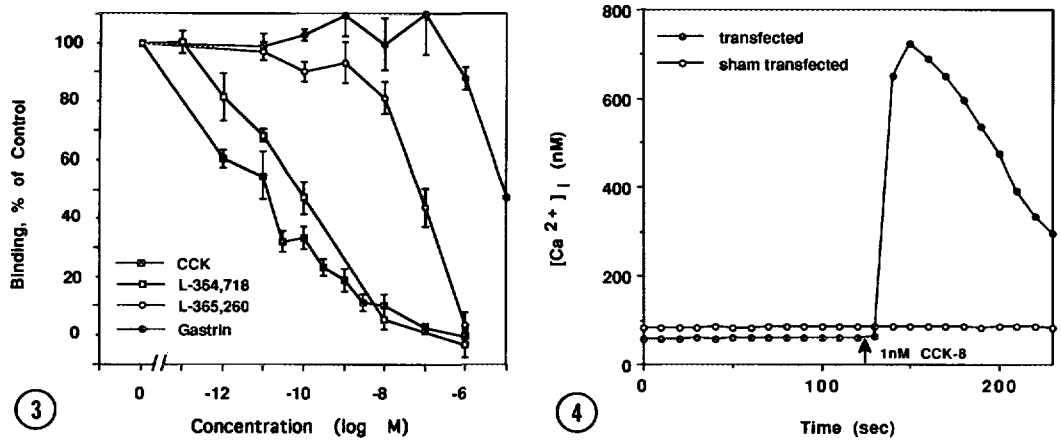


Figure 3.
Competition for binding of radioiodinated D-Tyr-Gly-[(Nle$^{28,31}$)CCK-26-33] to COS cell membranes containing recombinant receptor. Membranes were incubated with radioligand plus varied concentrations of CCK-8, gastrin, L-364,718, and L-365,260. Specific binding is plotted as a percentage of that observed in the absence of competition. Each value represents the mean±SEM of three separate experiments performed in duplicate. Binding was high affinity, saturable, and specific, with these agents inhibiting binding with relative affinities expected for binding to a $CCK_A$ receptor. Sham transfected cells showed no saturable binding.

Figure 4.
Second-messenger signalling in transfected COS cells. Fura-2-loaded COS cells were stimulated with 1 nM CCK-8 (indicated with arrow), and $[Ca^{2+}]_i$ was determined from fluorescence emission ratios at 340/380 nm. Data shown are representative of three experiments.

information vital to our understanding of these and other disease processes in which CCK may be implicated.

In summary, we have cloned the cDNA encoding a G protein-coupled human CCK receptor from a human gallbladder library. Based on its extensive amino acid identity with the rat $CCK_A$ receptor, and pharmacologic and functional characterization of the recombinant receptor in COS cells, this cDNA almost certainly encodes the human $CCK_A$ receptor. The successful cDNA cloning and expression of this receptor will allow studies critical to our understanding of its role in human physiology and pathophysiology.

## ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (DK32878) and Glaxo Pharmaceutical Company. Special thanks to Sara Erickson for her secretarial assistance and Delia Pinon and Steve Bronk for their technical assistance.

## REFERENCES

1. Miller, L.J. (1991) In CCK antagonists in gastroenterology. Basic and clinical status (G. Adler and C. Beglinger, Eds.), pp. 27-34. Springer-Verlag, Berlin.

2. Mutt, V. (1988) Adv. Metab. Disord. 11, 251-320.
3. Axelson, J., Ihse, I., and Håkanson, R. (1992) Scand. J. Gastroenterol. 27, 993-998.
4. Crawley, J.N. (1991) Trends. Pharmacol. Sci. 12, 232-236.
5. Miller, L.J., Rosenzweig, S.A., and Jamieson, J.D. (1981) J. Biol. Chem. 256, 12417-12423.
6. Shaw, M.J., Hadac, E.M., and Miller, L.J. (1987) J. Biol. Chem. 262, 14313-14318.
7. von Schrenck, T., Moran, T.H., Heinz Erian, P., Gardner, J.D., and Jensen, R.T. (1988) Am. J. Physiol. 255, G512-G521.
8. Pearson, R.K. and Miller, L.J. (1987) J. Biol. Chem. 262, 869-876.
9. Klueppelberg, U.G., Molero, X., Barrett, R.W., and Miller, L.J. (1990) Mol. Pharmacol. 38, 159-163.
10. Wank, S.A., Harkins, R., Jensen, R.T., Shapira, H., De Weerth, A., and Slattery, T. (1992) Proc. Natl. Acad. Sci. USA 89, 3125-3129.
11. Kopin, A.S., Lee, Y.M., McBride, E.W., Miller, L.J., Lu, M., Lin, H.Y., Kolakowski, L.F., and Beinborn, M. (1992) Proc. Natl. Acad. Sci. USA 89, 3605-3609.
12. Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156-159.
13. Schowalter, D.B. and Sommer, S.S. (1989) Anal. Biochem. 177, 90-94.
14. Lopata, M.A., Cleveland, D.W., and Sollner-Webb, B. (1984) Nucleic Acids Res. 12, 5707-5717.
15. Pearson, R.K., Powers, S.P., Hadac, E.M., Gaisano, H., and Miller, L.J. (1987) Biochem. Biophys. Res. Commun. 147, 346-353.
16. Grynkiewicz, G., Poenie, M., and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440-3450.
17. St. Jules, R.S. and O'Brien, P.J. (1986) Exp. Eye Res. 43, 929-940.
18. Karnik, S.S. and Khorana, H.G. (1990) J. Biol. Chem. 265, 17520-17524.
19. Pisegna, J.R., De Weerth, A., Huppi, K., and Wank, S.A. (1992) Biochem. Biophys. Res. Commun. 189, 296-303.
20. Wank, S.A., Pisegna, J.R., and De Weerth, A. (1992) Proc. Natl. Acad. Sci. USA 89, 8691-8695.
21. Schjoldager, B., Molero, X., and Miller, L.J. (1989) Gastroenterology 96, 1119-1125.
22. Matozaki, T., Goke, B., Tsunoda, Y., Rodriguez, M., Martinez, J., and Williams, J.A. (1990) J. Biol. Chem. 265, 6247-6254.
23. Allen, L.F., Lefkowitz, R.J., Caron, M.G., and Cotecchia, S. (1991) Proc. Natl. Acad. Sci. USA 88, 11354-11358.
24. Behar, J., Lee, K.Y., Thompson, W.R., and Biancani, P. (1989) Gastroenterology 97, 1479-1484.