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Identification of Cholecystokinin-B/Gastrin Receptor Domains that Confer High Gastrin Affinity: Utilization of a Novel *Xenopus laevis* Cholecystokinin Receptor

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SUMMARY

A hallmark of the mammalian brain cholecystokinin (CCK) receptor, CCK-B/gastrin (CCK-BR), is its high affinity for two structurally related peptides, CCK and gastrin. Previous radioligand binding experiments suggested that the predominant CCK receptor from *Xenopus laevis* brain shares high affinity for sulfated cholecystokinin octapeptide but has ≥1000-fold lower affinity for gastrin. To determine the molecular basis for this pharmacological divergence between mammalian and lower vertebrate receptors, we isolated a cDNA encoding the *X. laevis* brain CCK receptor (CCK-XLR). CCK-XLR shares ~50% homology at the amino acid level with both the human CCK-BR and the peripheral CCK-A receptor subtypes. The recombinant *X. laevis* receptor has a distinct pharmacological profile of agonist and antagonist affinities and as such offers a useful tool for structure-function studies. We used CCK-XLR to map the human CCK-BR domains that confer high affinity for gastrin. A series of chimeric CCK-BR/CCK-XLR constructs was generated and pharmacologically characterized. While maintaining wild-type affinity for sulfated cholecystokinin octapeptide, receptors with increasing amino-terminal contributions from CCK-BR demonstrated a stepwise increase in gastrin affinity. Further dissection of the amino-terminal third of the human receptor, a domain that confers a >250-fold increase in gastrin affinity, revealed the importance of interactions among at least three subdomains. Additional structural requirements for gastrin affinity mapped to a segment spanning transmembrane domains IV and V.

The neuropeptide CCK modulates a wide range of physiological functions in the gastrointestinal tract and in the brain. Nanomolar concentrations of CCK trigger intracellular signaling events through two known G protein-coupled receptors, CCK-AR and CCK-BR. Each subtype has been extensively characterized in mammals. In the gastrointestinal tract, CCK-AR stimulates pancreatic enzyme secretion and gallbladder contraction, whereas CCK-BR mediates acid secretion by gastric parietal cells and enterochromaffin-like cell proliferation in the stomach (1, 2). CCK-BR is the predominant receptor subtype in mammalian brain and has been implicated in modulating anxiety and the perception of pain. In contrast, CCK-AR has a more limited central distribution and is postulated to play an important role in regulating satiety (3).

CCK receptor cDNAs have been isolated from dog, rat, *Mastomys natalensis*, guinea pig, and human (4–11). All CCK receptors identified to date share high affinity for the endogenous neuroendocrine peptide CCK-8. A distinctive pattern of affinities for other peptide and nonpeptide ligands has provided the basis for the classification of CCK receptor subtypes (12). A distinguishing hallmark of CCK-BRs is their high affinity for the endogenous peptide agonist gastrin (gastrin-17-I). The pharmacological classification of cloned mammalian CCK receptors is further supported by deduced amino acid sequence comparisons. Species homologs of either

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ABBREVIATIONS: CCK, cholecystokinin; CCK-AR, cholecystokinin-A receptor; CCK-BR, cholecystokinin-B/gastrin receptor; CCK-XLR, *Xenopus laevis* cholecystokinin receptor; WT, wild-type; CCK-4, cholecystokinin tetrapeptide; CCK-8, sulfated cholecystokinin octapeptide; gastrin-17-I, unsulfated gastrin heptadecapeptide; TM, transmembrane domain; L364,718, 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide; L365,260, 3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-*N*′-(3-methylphenyl)urea; CAM 1028, *N*(1)-[α-methyl-*N*-[[(1S-endobornyl)oxy]carbonyl]-*S*(L)-tryptophyl-2*R*-phenyl-*N*(2)-succinoylethane-1,2-diamine; CAM 1714, *N*-[α-methyl-*N*-[[(1R-*trans*-2-methylcyclohexyl)oxy]carbonyl]-*S*(L)-tryptophyl-*R*(D)-3-(phenylmethyl)-β-alanine; RT-PCR, reverse transcriptase polymerase chain reaction; SSC, standard sodium citrate; bp, base pair(s); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

CCK-AR or CCK-BR share ~90% identity. In contrast, within a given species, identity between these two subtypes is only ~50%.

It is thus well established that in mammals at least two different CCK receptors exist. In contrast, radioligand binding studies in ectotherms (e.g., amphibia, fish, and reptiles) suggest a single receptor subtype that is expressed in both brain and pancreas (13, 14). The latter studies demonstrated that the ectotherm receptor, like the CCK-BR subtype in mammals, is abundant in brain. The mammalian brain receptor has high affinity for both CCK-8 and gastrin, but the ectotherm brain receptor shares high affinity for only CCK-8. To understand the molecular basis for the pharmacological differences between the predominant *Xenopus laevis* and mammalian brain CCK receptors, we cloned an ectotherm CCK receptor from *X. laevis* brain (CCK-XLR) and used the cDNA for structure-function studies.

The current study demonstrates that the brain CCK-XLR is unique in sharing equal amino acid identity (~50%) with both mammalian CCK receptors, CCK-AR and CCK-BR. The novelty of this receptor is further supported by pharmacological characterization of the recombinant protein. Agonist and antagonist affinities of CCK-XLR fall outside the range expected for both mammalian CCK receptor subtypes. In light of its unique pharmacological profile and its relatively high degree of amino acid identity with both CCK-AR and CCK-BR, CCK-XLR provides a useful tool for structure-function studies. We therefore used CCK-XLR to define the CCK-BR domains that confer gastrin affinity. A series of chimeric CCK-BR/CCK-XLR constructs was pharmacologically characterized. We demonstrated that multiple domains of the human CCK-BR interact to confer high affinity for gastrin.

Experimental Procedures

Materials. Restriction endonucleases were obtained from New England Biolabs (Beverly, MA) or American Allied Biochemicals (Aurora, CO); [^{125}I]CCK-8 (2200 Ci/mmol) was obtained from New England Nuclear (Boston, MA); cell culture media were obtained from GIBCO-BRL (Gaithersburg, MD); fetal calf serum was obtained from Intergen (Purchase, NY); and CCK and gastrin peptides were obtained from Peninsula Laboratories (Belmont, CA) and Bachem California (Torrance, CA), respectively. L365,260 and L364,718 were generously provided by Merck, Sharp and Dohme Laboratories (Harlow, UK) and CAM 1028 and CAM 1714 were generously provided by Parke-Davis Neuroscience Research Center (Cambridge, UK).

Cloning and sequence analysis of a *X. laevis* CCK receptor cDNA. A 2.0-kb cDNA fragment encoding the human CCK-BR (10) and a 1.7-kb cDNA fragment corresponding to the protein coding region of the human CCK-AR (11) were combined in equimolar amounts and radiolabeled with [α-^{32}P]dCTP by priming with random hexamers (15). The mixture of radiolabeled CCK-AR and CCK-BR cDNAs was used as a hybridization probe to screen an *X. laevis* brain λgt 10 cDNA library (generous gift of Dr. Ben G. Szaro, State University of New York, Albany, NY). Filters were hybridized overnight at 55° in SSC (1× SSC consists of 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), containing 0.1% SDS and 100 μg/ml sheared denatured salmon sperm DNA. Filters were washed in 2× SSC /0.1% SDS for 60 min at 55°. Of 1.5×10^6 primary recombinants screened, six positive clones were identified; two of these were plaque purified. The corresponding cDNA inserts (1.4 and 2.6 kb) were subcloned into the expression vector pcDNA I (Invitrogen, San Diego, CA) and sequenced by the chain termination method (16) using a modified T7 DNA polymerase (United States Biochemical Corp.). The 2.6-kb clone, pcDNAI/CCK-XLR, was shown to be full length by deduced amino acid sequence comparison with known mammalian CCK receptors and was used for all subsequent steps. The protein coding region was fully sequenced on both strands. Nucleotide sequence was analyzed by the University of Wisconsin Genetics Computer Group program GAP (16).

Northern blot hybridization assay. Poly(A)$^+$ RNA from *X. laevis* tissues was separated on a 1.2% agarose/0.66 M formaldehyde gel and transferred to a Nytran membrane by capillary blotting. A 2600-bp cDNA corresponding to CCK-XLR was radiolabeled with [α-^{32}P]dCTP by priming with random hexamers. The blot was hybridized overnight at 42° in 50% formamide (v/v) containing 5× SSC, 20 mM sodium phosphate, pH 6.6, 1× Denhardt's solution, 0.5% SDS (w/v), 10% dextran sulfate (w/v), and 100 μg/ml salmon sperm DNA. The blot was washed for 40 min in 0.2× SSC/0.1% SDS at 65° and exposed in a Molecular Dynamics PhosphorImager for 48 hr. The blot was reprobed with an EF1-α cDNA (17) (generous gift of Dr. D. A. Melton, Harvard University, Cambridge, MA) under similar conditions.

RT-PCR. Tissue-specific expression of CCK-XLR mRNA was examined by RT-PCR using the GeneAmp amplification system (Perkin-Elmer, Norwalk, CT). In a total volume of 20 μl, 100 ng of total RNA from brain, pancreas, or stomach was incubated with random hexamers (2.5 μM) and transcribed into cDNA using 2.5 units of Moloney murine leukemia virus RT. PCR was carried out in a 100-μl reaction volume that included the entire 20 μl of template cDNA and 150 pmol of each of the following CCK-XLR cDNA primers: 5′-GATGGACCTCAACAAAGAAGCAA-3′ (sense, nucleotides 810–833) and 5′-GCTCCTGTGGCTGCAATGTCTTC-3′ (antisense, nucleotides 1265–1288).

Each of 35 cycles of PCR included DNA denaturation for 1 min at 94°, primer annealing for 1 min at 62°, and DNA extension for 1 min at 72°. After cycle 35, DNA synthesis was completed with a 7-min extension step at 72°. Twenty microliters of the reaction volume was examined on an ethidium bromide-stained agarose gel. In tissues expressing CCK-XLR mRNA, a 478-bp fragment was clearly visible.

Chimeric constructs. The cDNAs encoding the human CCK-BR and CCK-XLR were subcloned into pcDNA I (Invitrogen). CCK-BR and CCK-XLR chimeras were constructed by exchanging cDNA fragments using restriction sites that occur in the native cDNAs or were introduced by oligonucleotide-directed mutagenesis. In corresponding segments of chimeric receptors, WT CCK-BR or WT CCK-XLR amino acid sequence was conserved. All receptor constructs were confirmed by restriction enzyme as well as by dideoxy-sequence analysis (Sequenase Version 2.0; USB-Amersham Life Science, Cleveland, OH).

Binding experiments. COS-7 cells (1.5×10^6) were plated onto 10-cm culture dishes (Nunc) and grown overnight in Dulbecco's minimal essential medium/10% fetal calf serum at 37°. The cells were transfected (18) with 5–7 μg of the eukaryotic expression vector pcDNA I containing the cDNAs encoding either CCK-XLR, human CCK-AR, human CCK-BR, or CCK-BR/CCK-XLR chimeras. On the next day, cells were split into 24-well dishes ($0.5–5.0 \times 10^4$ cells/well) (Costar, Cambridge, MA). After an additional 24 hr, competition binding experiments were performed in Hanks' balanced salt solution supplemented with 25 mM HEPES, pH 7.3, 0.2% bovine serum albumin, and 0.15 mM phenylmethylsulfonyl fluoride. Then, 20 pM [^{125}I]CCK-8 was used as radioligand. After incubation for 80 min at 37°, cell monolayers were washed three times with Hanks' balanced salt solution and hydrolyzed in 1 N NaOH, and bound radioactivity was quantified. Unlabeled competitors that were tested included the peptide agonists CCK-8, unsulfated CCK-8, gastrin-17-I, CCK-4 (Peninsula), and sulfated gastrin heptadecapeptide (Bachem); the nonpeptide antagonists L364,718 (19) and L365,260 (20); and the CCK dipeptoid antagonists CAM 1028 (PD135,158; see Ref. 25) and CAM 1714 (described as compound 28 l in Ref. 21). All IC_{50} values reported represent data obtained from at least three independent experiments, analyzed by computerized nonlinear curve fitting (Inplot 4.0; GraphPad, San Diego, CA).

Results and Discussion

CCK-XLR cDNA (Fig. 1) encodes a 453-amino acid protein with seven putative TMs. Comparison with known CCK receptor subtypes reveals ~50% amino acid identity with both the human CCK-AR and CCK-BRs (Fig. 2) (7, 9, 10). CCK-XLR shares a number of structural features with most other G protein-coupled receptors. Cysteine residues, one in the first extracellular loop (Cys141) and one in the second extracellular loop (Cys223), confer the potential to form an intrachain disulfide bond similar to that found in rhodopsin (22). Serine and threonine residues, clustered in the third cytoplasmic loop and at the carboxyl terminus, may serve as sites of phosphorylation, analogous to those found in the β-adrenergic receptor and rhodopsin (23). The amino terminus of the *X. laevis* receptor includes six potential asparagine-linked glycosylation sites (N-X-S/T), which is in contrast to the three found in mammalian CCK receptors.

The tissue distribution of CCK-XLR was assessed by high stringency Northern blot analysis of mRNA isolated from *X. laevis* brain, stomach, and pancreas (Fig. 3). Two predominant hybridizing transcripts (~7.5 kb) were found in brain, possibly indicating the existence of an alternatively spliced receptor variant. Only the larger of the transcripts was observed in stomach. Neither of the CCK-XLR signals was detected in pancreatic mRNA. To detect low level expression of CCK-XLR in pancreas, the tissue distribution of the CCK-XRL transcript was further examined by RT-PCR; the CCK-XRL transcript was detectable in brain and stomach but not in pancreatic RNA (not shown). Consistent with this result, an earlier study reported insufficient displaceable [^{125}I]CCK-33 binding to establish the existence of CCK receptors in *X. laevis* pancreas (14). The apparent absence of a pancreatic CCK receptor in *X. laevis* is in contrast with findings in other ectotherm pancreata (e.g., bullfrog, ratfish, garter snake) in which a clear profile of agonist binding, closely resembling the affinities of the CCK-XLR (see below), has been demonstrated (13, 14).

Pharmacological characterization of the *X. laevis* brain receptor expressed in COS-7 cells (Table 1) revealed agonist affinities resembling the mammalian CCK-AR subtype (5, 7, 9) more than the CCK-BR (10, 11, 24). CCK-XLR has high affinity for CCK-8 and low affinities for gastrin-17-I, CCK-4, and unsulfated CCK-8. In contrast to CCK-AR, CCK-XLR has moderately high affinity for sulfated gastrin heptadecapeptide, with an IC_{50} (50 nM) between the values determined for CCK-BR (0.19 nM) and CCK-AR (~1000 nM). The agonist affinities of the recombinant CCK-XLR closely resemble the values determined by radioligand binding experiments for CCK receptors in native ectotherm brain (13, 14).

The antagonist affinity profile of the CCK-XLR differs in several respects from the known mammalian CCK receptor subtypes (Table 1). The structurally related nonpeptide benzodiazepine-derived antagonists L365,260 and L364,718 have been extensively used to differentiate mammalian CCK-ARs from CCK-BRs. CCK-XLR binds the CCK-AR-specific compound L364,718 with greater affinity than the CCK-BR-specific compound L,365,260. However, when compared with mammalian CCK-A receptors (5, 7, 9), the CCK-XLR has an affinity for L364,718, which is ~1 log unit lower.

The pharmacological difference between CCK-XLR and the mammalian receptor subtypes is further illustrated by affinities for the CCK receptor peptoid antagonists CAM 1714 and CAM 1028. These compounds were designed to include key chemical groups of CCK-4 (25). CAM 1714, a CCK-A-specific

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   1  ATGGAGTCTC TCAGGTCCCT CAGTAACATC AGTGCCCTCC ATGAACTGCT
  51  CTGTCGCTAT AGCAACCTGT CTGGCACCCT AACATGGAAC CTTTCCAGCA
 101  CCAATGGCAC CCACAACCTC ACAACTGCCA ACTGGCCCCC CTGGAACCTC
 151  AACTGCACCC CCATATTGGA TCGCAAGAAG CCCAGTCCCT CAGACCTGAA
 201  CCTGTGGGTG CGGATTGTGA TGTACAGCGT CATCTTCCTG CTGAGCGTTT
 251  TCGGCAACAC CCTGATCATT ATTGTGTTAG TGATGAACAA ACGTCTCCGC
 301  ACCATCACCA ACTCCTTCCT CCTGTCTCTG GCCCTCAGTG ACCTCATGGT
 351  GGCCGTACTC TGCATGCCCT TCACCCTCAT CCCAAACCTC ATGGAGAACT
 401  TCATCTTCGG GGAGGTCATA TGCCGGGCAG CCGCCTACTT CATGGGCCTT
 451  TCTGTCAGCG TCTCCACATT CAACCTTGTG GCCATCTCCA TTGAGCGCTA
 501  CAGTGCCATC TGTAACCCAC TGAAATCCCG GGTCTGGCAG ACTCGATCCC
 551  ACGCCTACCG GGTGATTGCC GCTACCTGGG TCCTGTCCAG CATTATAATG
 601  ATCCCATATC TGGTCTACAA CAAGACTGTA ACGTTTCCCA TGAAGGACCG
 651  GAGAGTCGGA CACCAGTGTC GCCTGGTTTG GCCCAGTAAG CAGGTGCAGC
 701  AGGCATGGTA CGTCCTACTC CTTACTATCC TCTTCTTCAT ACCTGGGGTG
 751  GTGATGATCG TGGCCTATGG ACTTATCTCC CGGGAGCTGT ACAGGGGCAT
 801  CCAGTTTGAG ATGGACCTCA ACAAAGAAGC AAAAGCTCAC AAAAATGGAG
 851  TCTCAACTCC AACCACCATT CCGAGCGGTG ATGAGGGAGA CGGCTGTTAC
 901  ATTCAGGTCA CCAAGAGGCG CAACACCATG GAGATGTCCA CATTGACTCC
 951  AAGTGTCTGC ACCAAGATGG ATCGAGCACG GATCAACAAC TCAGAAGCCA
1001  AACTCATGGC TAAAAAGCGT GTGATTCGAA TGCTTATTGT CATCGTGGCC
1051  ATGTTCTTCA TATGCTGGAT GCCCATTTTC GTAGCCAACA CTTGGAAGGC
1101  CTTCGATGAA TTATCTGCTT TCAACACCCT GACGGGAGCA CCCATCTCTT
1151  TTATCCACCT CCTGAGTTAC ACATCAGCTT GCGTCAACCC TCTCATCTAC
1201  TGCTTCATGA ACAAGCGTTT CCGTAAGGCC TTTCTGGGCA CGTTTTCCAG
1251  CTGTATCAAG CCCTGCCGCA ATTTCCGTGA TACAGATGAA GACATTGCAG
1301  CCACAGGAGC ATCTCTCTCT AAATTCAGTT ATACTACTGT TAGCAGCTTG
1351  GGCCCTGCTT GA*
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Fig. 1. Nucleotide sequence encoding the brain CCK-XLR. The cDNA encoding CCK-XLR was isolated by low stringency hybridization screening (for details, see Experimental Procedures). The protein coding sequence of the cDNA comprises 1362 nucleotides. The GenBank accession number for the CCK-XLR nucleotide sequence is U49258.

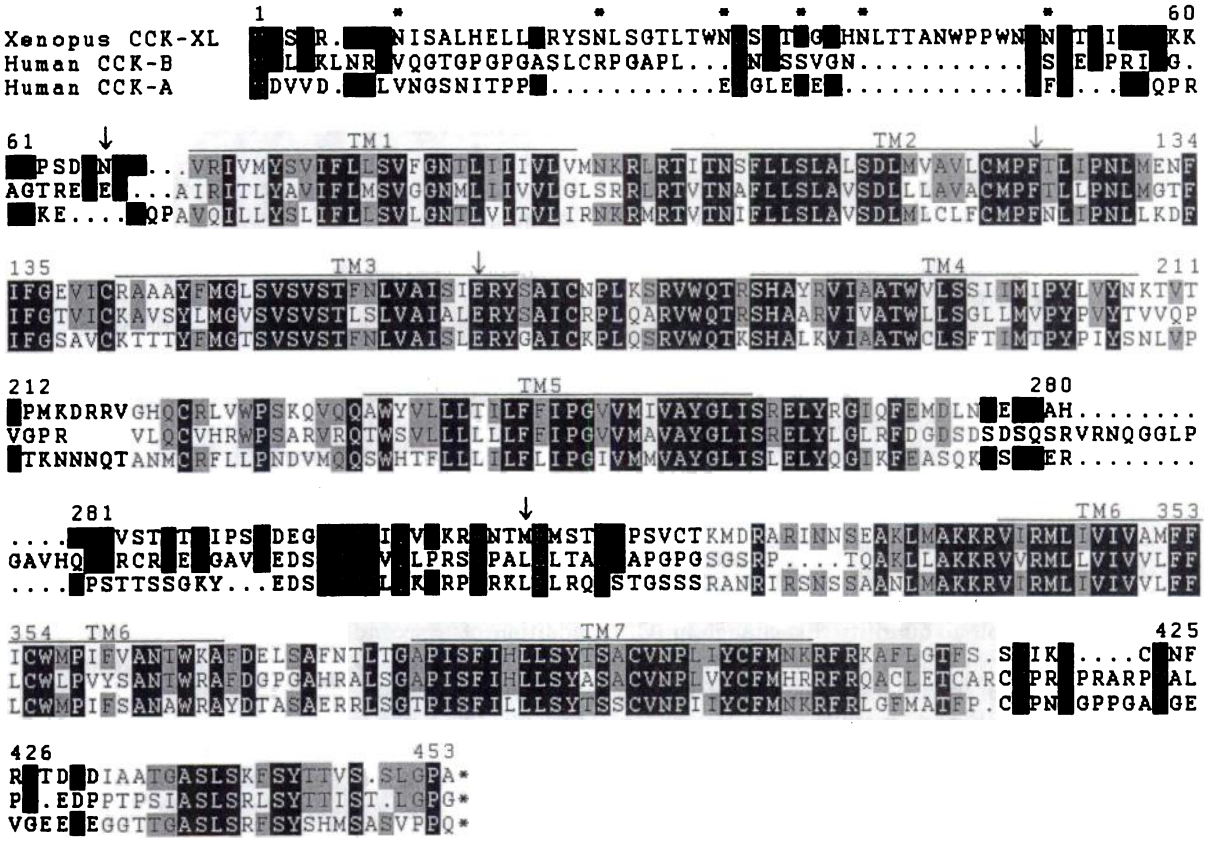

Fig. 2. Amino acid sequence comparison among CCK-XLR, human CCK-AR, and human CCK-BR. *Blackened residues*, identical in all receptors; *shaded amino acids*, identical in at least two of the three receptors; *bars over sequences*, TMs as predicted by a rhodopsin-based model of G protein-coupled receptor structure (28). *Numbering*, CCK-XLR amino acids. *, Potential asparagine-linked glycosylation sites. *Arrows*, positions corresponding to cDNA restriction sites used for chimeric receptor construction (see Experimental Procedures for details).

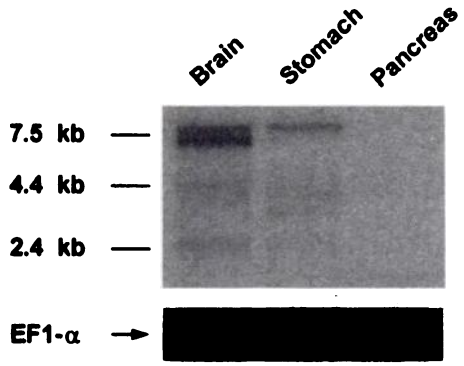

Fig. 3. Northern blot analysis of CCK-XLR receptor transcripts in *X. laevis* tissues. Two micrograms of *X. laevis* mRNA from respective tissues were transferred to a Nytran membrane and probed with the full-length CCK-XLR cDNA (see Experimental Procedures for details). Digital autoradiographs were obtained after a 48-hr exposure using a PhosphorImager. *Horizontal bars*, molecular weight markers for RNA. Equal loading of all RNA samples was confirmed by hybridization with an EF1-α cDNA.

TABLE 1

Pharmacological characterization of the *X. laevis* brain receptor expressed in COS-7 cells

	IC_{50} ± standard error		
	CCK-XLR	CCK-BR	CCK-AR
		nM	
Agonists			
CCK-8	2.4 ± 0.6	0.13 ± 0.02	1.9 ± 0.2
Gastrin-17-I	>1,000	0.62 ± 0.14	>1,000
Sulfated gastrin hepta-decapeptide	50 ± 9	0.19 ± 0.02	~1,000
Unsulfated CCK-8	>2,000	1.9 ± 0.6	483 ± 163
CCK-4	>20,000	12.5 ± 1.5	>20,000
Antagonists			
L364,718	8.4 ± 2.4	116 ± 34	1.4 ± 0.4
L365,260	550 ± 58	9.3 ± 1.2	~1,000
CAM 1028	>2,000	2.4 ± 0.5	~1,000
CAM 1714	>2,000	>2,000	34 ± 1

receptor antagonist, shows no detectable affinity for CCK-XLR. In addition, CCK-XLR does not recognize the related peptoid antagonist CAM 1028, a compound with high affinity for CCK-BR (Table 1).

The significant differences in agonist and antagonist binding between CCK-XLR and both known mammalian CCK receptor subtypes makes it difficult to consider CCK-XLR as either CCK-AR or CCK-BR. The problem in applying the established subtype classification to the *X. laevis* protein is further illustrated by amino acid sequence comparisons between CCK-XLR and the mammalian receptors. Because CCK-XLR shares equal identity (~50%) with both CCK-AR and CCK-BR, it is unlikely that the *X. laevis* protein is a species homolog of either mammalian subtype. Nevertheless, the high degree of amino acid homology between CCK-XLR and its mammalian counterparts, its high affinity for CCK-8, and its pattern of expression justify classification of this protein as a member of the CCK receptor family. Furthermore, the similarities between CCK-XLR and the mamma-

lian CCK receptors, CCK-A and CCK-BR, (Fig. 2) suggest that these three proteins arose from a common ancestral precursor.

The unique pharmacological profile of CCK-XLR, as well as its structural similarities with the CCK-AR and CCK-BR, make the *X. laevis* receptor a particularly useful tool for structure-function studies. One of the most striking distinguishing features between the human CCK-BR and the *X. laevis* brain receptor is the marked difference in gastrin affinity. To determine the molecular basis for this difference, a series of chimeric CCK-BR/CCK-XLR constructs were generated. In these chimeras, increases in amino-terminal segments from CCK-BR sequentially replaced the corresponding sequence of CCK-XLR. Pharmacological analysis revealed that high affinity for the common radioligand [^{125}I]CCK-8 was conserved in each of these recombinant proteins (Fig. 4), suggesting that the tertiary structure of these receptors remained intact.

Transfer of the extracellular amino terminus (Fig. 4, *receptor 2*) from the human CCK-BR to the CCK-XLR increased gastrin affinity into the measurable range (IC_{50} = 6000 nM). However, it was not possible to quantify this change in IC_{50} because the affinity of the WT CCK-XLR for gastrin-17-I is too low to assign an accurate value. Extension of human sequence through TM 2 (Fig. 4, *receptor 3*) conferred an additional 9-fold increase in affinity for gastrin-17-I (IC_{50} = 680 nM). Introduction of the amino-terminal third of the human CCK-BR into the CCK-XLR (Fig. 4, *receptor 4*), resulted in a >250-fold increase in gastrin-17-I affinity (IC_{50} = 21 nM). This limited region of human sequence was therefore sufficient to convert the frog receptor to a protein with relatively high affinity for gastrin. The addition of human sequence through TM 5 (Fig. 4, *receptor 5*) further increased gastrin-17-I affinity to 2.1 nM, within 4-fold of the value obtained with the human WT CCK-BR.

These results suggest that gastrin-17-I affinity is primarily conferred by receptor domains located in the amino-terminal two thirds of the human CCK-BR. Within this segment, the majority of the affinity increase can be attributed to the amino-terminal third of the receptor (>250-fold). The region spanning TMs IV and V contributes an additional 10-fold increase in affinity, whereas only minor contributions are conferred by the regions spanning TMs VI and VII (<4-fold) and the extracellular amino-terminal end.

To further characterize and map the domains within the amino-terminal third of the human CCK-BR that convey high affinity for gastrin-17-I, this region was further divided into three subdomains (A, B, and C; Fig. 5). Transfer of the human A subdomain (the extracellular amino terminus) resulted in a low but quantifiable IC_{50} value for gastrin-17-I (6000 nM) as shown earlier (see also Fig. 4, *receptor 2*). The introduction of the B subdomain, a segment spanning TM I and most of TM II, increased gastrin-17-I affinity to 1600 nM. The A + B, A + C, and B + C chimeras illustrate that the addition of a second subdomain further increased gastrin-17-I affinity, to 680, 590, and 77 nM, respectively. These double-block substitutions suggest that an interaction between subdomains is required for high affinity gastrin-17-I binding. This conclusion is further supported by the observation that none of the double-block substitutions confers affinity as high as when all three blocks (A, B, and C) are combined.

Our findings with chimeric CCK-BR/CCK-XLR constructs conform with the current view that binding sites conferring agonist selectivity for peptide hormone receptors are localized to multiple sites within both the extracellular and TMs

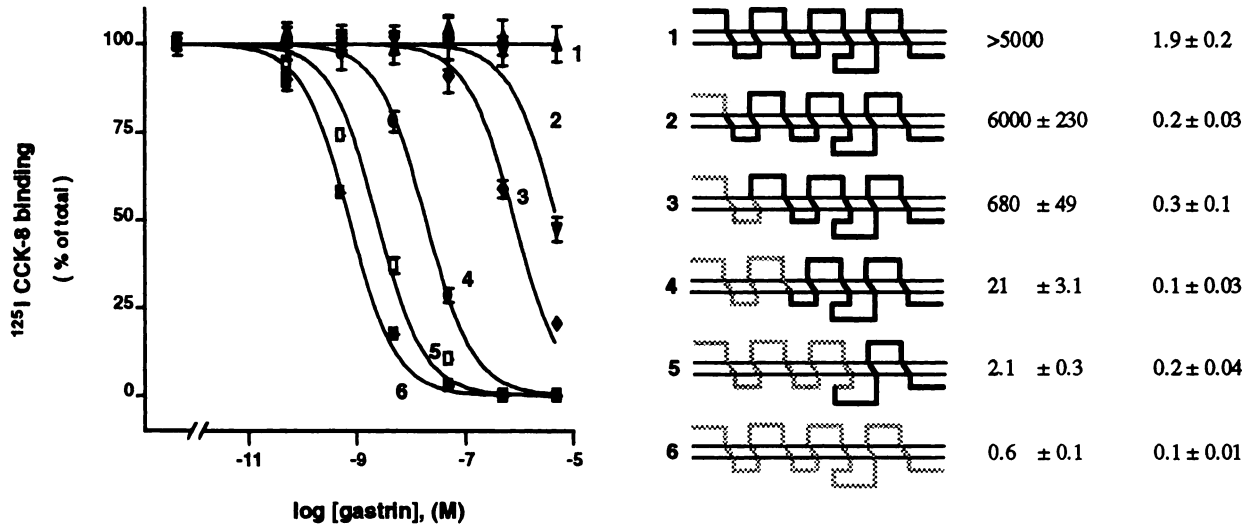

Fig. 4. Human CCK/CCK-XLR chimeras reveal domains conferring gastrin-17-I affinity. WT and chimeric receptors were transiently expressed in COS-7 cells. Gastrin-17-I and CCK-8 affinities were determined by [^{125}I]CCK-8 competition binding experiments. *Left*, [^{125}I]CCK-8 binding is plotted as a function of increasing concentration of unlabeled gastrin-17-I. Numbering of competition curves corresponds with the respective receptor drawing. *Gray line*, human CCK-BR contributions; *black line*, CCK-XLR segments. *Right*, IC_{50} values (in nM) for gastrin-17-I and CCK-8. Data represent mean ± standard error of at least three independent experiments.

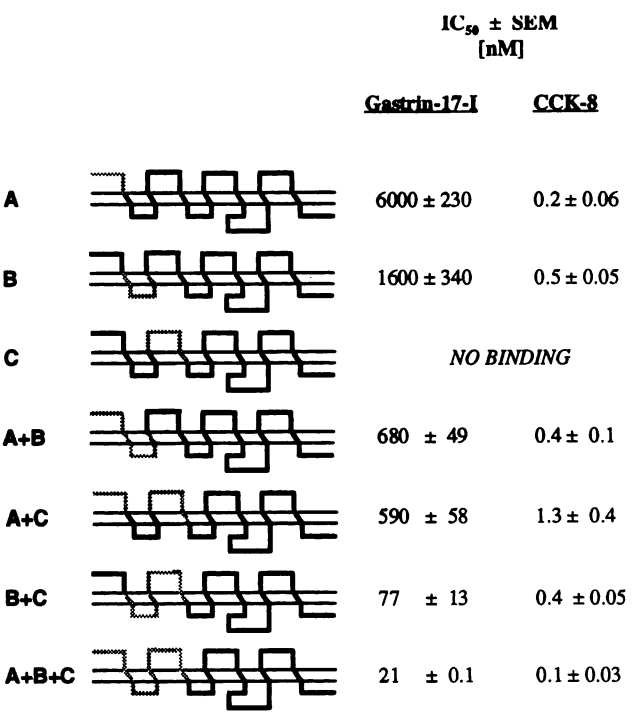

Fig. 5. Gastrin-17-I affinity is conferred by multiple interacting subdomains within the amino-terminal third of the human CCK-BR. Chimeric receptors were transiently expressed in COS-7 cells. Gastrin-17-I and CCK-8 affinities were determined by [^{125}I]CCK-8 competition binding. *Gray line*, human CCK-BR contributions; *black line*, CCK-XLR receptor segments. *Right*, IC_{50} values (in nM) for gastrin-17-I and CCK-8. Data represent mean ± standard error of at least three independent experiments. Chimera C had no displaceable [^{125}I]CCK-8 binding.

of the respective proteins. This general concept, best established for the neurokinin and angiotensin receptors (26, 27), seems to apply to CCK-BR as well. Using CCK-XLR as a novel tool, we demonstrated that multiple interacting subdomains, clustered in large part in the amino-terminal third of the CCK-BR, confer high affinity for the endogenous peptide agonist gastrin-17-I.

Acknowledgments

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References

1. Liddle, R. A. Cholecystokinin, in *Gut Peptides* (J. H. Walsh and G. J. Dockray, eds.). Raven Press, New York, 175–216 (1994).
2. Walsh, J. H. Gastrin, in *Gut Peptides* (J. H. Walsh and G. J. Dockray, eds.). Raven Press, New York, 75–121 (1994).
3. Woodruff, G. N., D. R. Hill, P. Boden, R. Pinnock, L. Singh, and J. Hughes. Functional role of brain CCK receptors. *Neuropeptides* **19** (suppl.):45–56 (1991).
4. Kopin, A. S., Y. M. Lee, E. W. McBride, L. J. Miller, M. Lu, H. Y. Lin, L. F. Kolakowski, Jr., and M. Beinborn. Expression cloning and characterization of the canine parietal cell gastrin receptor. *Proc. Natl. Acad. Sci. USA* **89**:3605–3609 (1992).
5. Wank, S. A., J. R. Pisegna, and A. de Weerth. Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. *Proc. Natl. Acad. Sci. USA* **89**:8691–8695 (1992).
6. Nakata, H., T. Matsui, M. Ito, T. Taniguchi, Y. Naribayashi, N. Arima, A. Nakamura, Y. Kinoshita, K. Chihara, S. Hosoda, and T. Chiba. Cloning and characterization of gastrin receptor from ECL carcinoid tumor of *Mastomys natalensis*. *Biochem. Biophys. Res. Commun.* **187**:1151–1157 (1992).
7. de Weerth, A., J. R. Pisegna, K. Huppi, and S. A. Wank. Molecular cloning, functional expression and chromosomal localization of the human cholecystokinin type A receptor. *Biochem. Biophys. Res. Commun.* **194**:811–818 (1993).
8. de Weerth, A., J. R. Pisegna, and S. A. Wank. Guinea pig gallbladder and pancreas possess identical CCK-A receptor subtypes: receptor cloning and expression. *Am. J. Physiol.* **265**:G1116–G1121 (1993).
9. Ulrich, C. D., I. Ferber, E. Holicky, E. Hadac, G. Buell, and L. J. Miller. Molecular cloning and functional expression of the human gallbladder cholecystokinin A receptor. *Biochem. Biophys. Res. Commun.* **193**:204–211 (1993).
10. Lee, Y. M., M. Beinborn, E. W. McBride, M. Lu, L. F. Kolakowski, Jr., and A. S. Kopin. The human brain cholecystokinin-B/gastrin receptor: cloning and characterization. *J. Biol. Chem.* **268**:8164–8169 (1993).
11. Pisegna, J. R., A. de Weerth, K. Huppi, and S. A. Wank. Molecular cloning of the human brain and gastric cholecystokinin receptor: structure, functional expression and chromosomal localization. *Biochem. Biophys. Res. Commun.* **189**:296–303 (1992).
12. Watson, S. P., and D. Girdlestone: 1995 Receptor and ion channel nomenclature. *Trends Pharmacol. Sci.* **16** (suppl.):24–25 (1995).
13. Vigna, S. R., R. W. Steigerwalt, and J. A. Williams. Characterization of cholecystokinin receptors in bullfrog (*Rana catesbeiana*) brain and pancreas. *Regul. Peptides* **9**:199–212 (1984).
14. Vigna, S. R., M. C. Thorndyke, and J. A. Williams. Evidence for a common evolutionary origin of brain and pancreas cholecystokinin receptors. *Proc. Natl. Acad. Sci. USA* **83**:4355–4359 (1986).
15. Feinberg, A. P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analy. Biochem.* **132**:6–13 (1983).
16. Devereux, J., P. Haeberli, and O. Smithies. A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res.* **12**:387–395 (1984).
17. Hemmati-Brivanlou, A., and D. A. Melton. A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. *Nature (Lond.)* **359**:609–614 (1992).
18. Pacholczyk, T., R. D. Blakely, and S. G. Amara. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. *Nature (Lond.)* **350**:350–354 (1991).
19. Chang, R. S., and V. J. Lotti. Biochemical and pharmacological characterization of an extremely potent and selective nonpeptide cholecystokinin antagonist. *Proc. Natl. Acad. Sci. USA* **83**:4923–4926 (1986).
20. Lotti, V. J., and R. S. Chang. A new potent and selective non-peptide gastrin antagonist and brain cholecystokinin receptor (CCK-B) ligand: L365,260. *Eur. J. Pharmacol.* **162**:273–280 (1989).
21. Boden, P. R., M. Higginbottom, D. R. Hill, D. C. Horwell, J. Hughes, D. C. Rees, E. Roberts, L. Singh, N. Suman-Chauhan, and G. N. Woodruff. Cholecystokinin dipeptoid antagonists: design, synthesis, and anxiolytic profile of some novel CCK-A and CCK-B selective and "mixed" CCK-A/CCK-B antagonists. *J. Med. Chem.* **36**:552–565 (1993).
22. Karnik, S. S., T. P. Sakmar, H. B. Chen, and H. G. Khorana. Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin. *Proc. Natl. Acad. Sci. USA* **85**:8459–8463 (1988).
23. Dohlman, H. G., J. Thorner, M. G. Caron, and R. J. Lefkowitz. Model systems for the study of seven-transmembrane-segment receptors. *Annu. Rev. Biochem.* **60**:653–688 (1991).
24. Kopin, A. S., E. W. McBride, S. M. Quinn, L. F. Kolakowski, Jr., and M. Beinborn. The role of the cholecystokinin-B/gastrin receptor transmembrane domains in determining affinity for subtype-selective ligands. *J. Biol. Chem.* **270**:5019–5023 (1995).
25. Horwell, D. C. Development of CCK-B antagonists. *Neuropeptides* **19** (suppl.):57–64 (1991).
26. Schwartz, T. W., U. Gether, H. T. Schambye, and S. A. Hjorth. Molecular mechanism of action of non-peptide ligands for peptide receptors. *Curr. Pharmaceut. Design* **1**:325–342 (1995).
27. Schwartz, T. W. Locating ligand-binding sites in 7TM receptors by protein engineering. *Curr. Opin. Biotechnol.* **5**:434–444 (1994).
28. Baldwin, J. M. The probable arrangement of the helices in G protein-coupled receptors. *EMBO J.* **12**:1693–1703 (1993).

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