Vol. 212, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 6, 1995 Pages 204-211

MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF A HUMAN SECRETIN RECEPTOR+

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Received May 11, 1995

Secretin is a gastrointestinal hormone responsible for the regulation of bicarbonate, potassium ion and enzyme secretion from the pancreas. A cDNA encoding the human secretin receptor was isolated from a human pancreatic adenocarcinoma cell-line cDNA library using polymerase chain reaction and library screening techniques. The cDNA isolated is 1717 bp in length encoding a 440 amino acid long polypeptide. Computer analysis of the receptor indicated that it is a member of the glucagon-VIP-secretin receptor family and is a G-protein coupled receptor containing seven hydrophobic transmembrane domains. The receptor was subsequently expressed in COS-7 cells and was able to bind specifically to human secretin with high affinity as indicated by the competitive displacement assay. The human secretin receptor was found to be functionally coupled to the stimulation of adenylyl cyclase resulting in the accumulation of intracellular cAMP in a dose-dependent manner. By Northern blot analysis, a 1.8 Kb mRNA was detected in human pancreas and intestine, while weak hybridization signals were detected in human colon, kidney and lung. Functional characterization of this receptor should enhance our understanding of the physiology and pathophysiology of human secretin, its structure-function, receptor interaction and receptor tissue distribution.

Secretin is a 27 amino acid peptide hormone discovered in 1902 (1) which was found to be one of the most potent physiological regulators of bicarbonate, water, potassium ion and enzyme secretion from the pancreas. Other pharmacological actions of secretin had also been reported with effector tissues all over the body including brain, heart, stomach, intestine and kidney (2-6).

The rat secretin precursor polypeptide is 134 amino acid long and is synthesized and released from endocrine S cells located in the mucosa of the small intestine (7). Recent report indicated that rat secretin transcripts are also detected in heart, lung, kidney, testis, gastrointestinal tract and brain (8). Within the rat brain, specific regions such as the cortex, hypothalamus, medulla oblongata, pons, and hypophysis were found to contain the secretin transcripts, suggesting that secretin may also act as a neuromodulator (7,9-11). Amino acid sequence alignment of secretin with other gut-brain peptides revealed that it belongs to a family of peptides including vasoactive intestinal peptide (VIP), glucagon, pituitary adenylate cyclase activating

+ The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession number: U28281.

0006-291X/95 $12.00

peptide (PACAP), growth hormone releasing hormone (GHRH), peptide histidine isoleucine (PHI) and glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP).

The function of the secretin family of peptides are mediated via their interaction with specific surface receptors. This peptide family was found to interact with a distinct family of G-protein coupled receptors which stimulate adenylyl cyclase resulting in the accumulation of intracellular cAMP (12). Secretin receptors have been reported in rat gastric glands, pancreatic acini and a neuroglioma hybrid cell-line (13-15). In this report, a human secretin receptor (HSR) was isolated, characterized and functionally expressed. Functional characterization of this receptor should enhance our understanding of the human physiology and pathophysiology of secretin, its structure-function and receptor interaction in the future.

METHODS AND MATERIALS

Cloning of the Human Secretin Receptor cDNA. A partial cDNA clone corresponding to the HSR transmembrane domains 2 to 7 was obtained by PCR using the human pancreatic adenocarcinoma cell-line cDNA library (Stratagene) as the template DNA. The primers for PCR were designed according to the consensus regions among the secretin receptor family. The sequence of the primers are G2 (5'-TGCAYTGYACNMGNAAYTAYATYCA -3') and G7 (5'- TGSACCTCNCCRTTNASRAARCARTA -3'). The PCR typically contained 50 pmole of each primers, 200 μM of dNTPs and 5 units of Taq polymerase (GIBCO/BRL). The reaction times were 1 min. at 94°C, 58°C and 72°C, respectively, for 30 cycles. The PCR fragment was subcloned into pUC 18 for DNA sequence analysis from both ends using the T7 sequencing kit (Pharmacia). The partial cDNA clone corresponding to the HSR was used as a probe to screen the human pancreatic adenocarcinoma cell-line cDNA library (0.5 million) (Stratagene). The probe was labeled by Megaprime DNA labeling kit (Amersham) and [α-^{32}P]-dATP (Amersham). A full length cDNA encoding the HSR was isolated from the library and sequenced from both strands using synthetic primers. The DNA sequences obtained were analysis by DNASIS (Hitachi).

Functional Expression of the Cloned Receptor and Binding Studies. A 1.8 Kb Not1-Apa1 DNA fragment corresponding to the full-length HSR was subcloned into the expression vector pRC-CMV (Stratagene). The expression plasmid pRC-CMV-HSR (2 μg) was used to transfect 0.2 million of COS-7 cells in 6-well plates (Costar) by DEAE-Dextran transient expression method (16). Three days after transfection, the cells were washed once with MEM with 1 mg/ml of bovine serum albumin and then incubated with the same medium containing 0.2 mM of 1-methyl-3-isobutylxanthine (RBI) and the desired concentrations of peptide for 45 min. at 37°C. After incubation, the medium was removed and the cells were lyzed by the addition of 1 ml of iced ethanol. The cell debris was pelleted by centrifugation (10,000xg for 10 min.) and the supernatant was dried by a vacuum concentrator. The cAMP levels were then quantified by a radioimmunoassay kit (Amersham). The receptor binding and competitive assays were performed as described previously (16). Permanent cell-line expressing HSR was produced by transfecting COS-7 cells using Lipofectin reagent (BRL/GIBCO) followed by G418 selection at 500 μg/ml (BRL/GIBCO). After 2 weeks of selection, 0.2 million of the cells were seeded onto a 6-well plate 2 days before binding assays. The cells were washed twice with a buffer containing 50 mM Tris·HCl (pH 7.4), 200 mM sucrose, 5 mM $MgCl_2$ and 10 mg/ml bovine serum albumin. For saturation assays, the cells were incubated in triplicate with 0.5 ml of the same buffer supplemented with 0.1 mM PMSF and 1 mg/ml of bacitracin (incubation buffer), and various concentration of ^{125}I-secretin in the presence and in the absence of 1 μM cold secretin. After incubation at 22°C for 60 min., the cells were washed twice with ice-cold incubation buffer and solubilized with 1 ml of 1 M NaOH, and the amount of bound radioactive ligand was determined by a γ-counter (Pharmacia). For competition assays, the cells were incubated with 100,000 cpm of ^{125}I-secretin alone or in the presence of various concentrations of cold peptide. Human secretin was labeled using ^{125}I according to Chang and Chey, 1980 (17). All the peptides used in this study were purchased from Bachem Fine Chemicals, Inc. (California).

Northern Blot Analysis. The full-length HSR cDNA was used as a probe for hybridization. The human tissue RNA blots were purchased from Clontech. Hybridization was

carried out overnight at 65°C using standard condition and was washed in 0.1X SSPE, 0.1% SDS at 65°C for 1 hour. As an internal control, the same blot was re-probed with a 2.0 Kb human ß-actin cDNA. The blots were analyzed by autoradiography and also by a Phosphoimager (Molecular Dynamics) to determine the relative expression levels of the receptor in different tissues.

RESULTS AND DISCUSSIONS

Isolation of the Human Secretin Receptor cDNA. A partial cDNA clone corresponding to the transmembrane domain 2 to 7 was obtained from a human pancreatic adenocarcinoma cell-line cDNA library by PCR using a pair of degenerate primers G2 and G7. After DNA sequence analysis, this partial cDNA clone was then used as a probe to screen the same library and a full length cDNA of 1717 bp encoding the HSR was obtained. Nucleotide sequencing of the secretin receptor cDNA clone revealed a single open reading frame of 1320 bp encoding a protein of 440 amino acids with a predicted molecular weight of 50.2 KD (Fig. 1). The cDNA and amino acid sequence homology between human and rat secretin receptor were found to be 76% and 80%, respectively. A Kyte-Doolittle hydrophobicity analysis of the receptor indicates that the protein belongs to the G-protein coupled receptor family with seven segments of hydrophobic amino acids presumably forming the transmembrane spanning regions (Fig. 2). In addition, the receptor also contains a putative hydrophobic leader peptide (22 amino acid) followed by a large extracellular hydrophilic region (122 amino acid) and a hydrophilic cytoplasmic domain at the C-terminal (42 amino acid) (Fig. 1). There are four conserved putative N-link glycosylation sites (residues 72, 100, 106 and 128) and seven conserved cysteine residues (position 32, 45, 66, 75, 89, 107 and 123) within the N-terminal extracellular domain when compared to rat secretin receptor (18). These conserved sequences may be essential for proper folding of the receptor and for ligand binding. It is also found that three of the cysteine residues (position 66, 75, 89) are conserved among all the receptors within the glucagon-VIP-secretin receptor super-family; these residues are likely to be important for receptor function by formation of disulfide bonds. Pretreatment of cell lines expressing rat secretin receptor with the reducing agent dithiothreitol also reduced the binding capacity, suggesting that integrity of the disulfide bridges are needed for secretin and receptor interaction (19). The aspartic acid residue at position 71 is also conserved among all receptors; a missense mutation of this residue in the GHRH receptor in mouse was found to be responsible for the little mouse phenotype (20), again indicating the importance of this residue in receptor function.

Functional Expression of Human Secretin Receptor. To demonstrate that the isolated HSR could transduce a physiological signal, cAMP dose-response curves in the presence of various related peptides were measured (Fig. 3). This was done by transient transfection of the COS-7 cells with the expression vector pRC-CMV-HSR carrying the full length cDNA encoding the HSR. Incubation of COS-7 cells expressing the HSR with secretin lead to a 10-fold increase in cAMP over basal levels and the EC_{50} was determined to be 0.2 nM. VIP and PACAP could also stimulate the production of cAMP in the transfected cells but were 100-fold less potent; presumably due to the structural similarities between the peptides. Under identical assay condition, glucagon, GLP-1 and GHRH showed no significant increase in cAMP over basal levels. These results agree

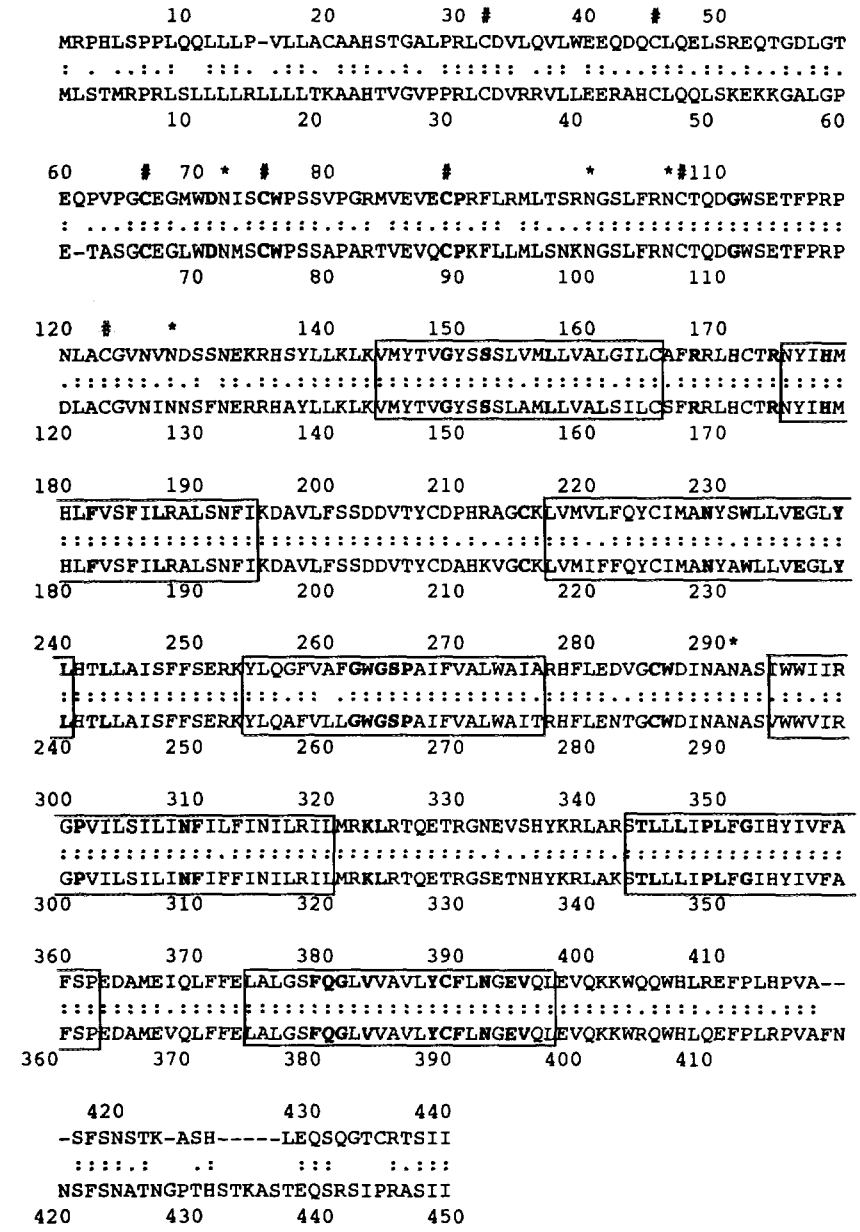

Figure 1. Comparison of the human and rat secretin receptor. Alignment of the human (top) and rat receptor was performed by the DNASIS (Hitachi) software. The seven putative transmembrane domains are boxed. Identical and conservative changes are indicated by the symbols ":" and ".", respectively. The consensus sequences for N-linked glycosylation sites (Asn-X-Thr/Ser) and conserved cysteine residues are labeled "*" and "#", respectively. Conserved residues within all the receptors in the secretin receptor super-family including calcitonin, GHRH, VIP, secretin, glucagon, GLP-1, PTH and GIP receptors are typed in bold.

with similar study of the rat secretin receptor (18). The binding specificity of the receptor was examined by competitive displacement of the ^{125}I-secretin using different concentrations of secretin, VIP and glucagon (Fig. 4). The relative potency of these three peptides in displacing ^{125}I-secretin are secretin >> VIP >> glucagon. Expression studies of HSR indicated that the

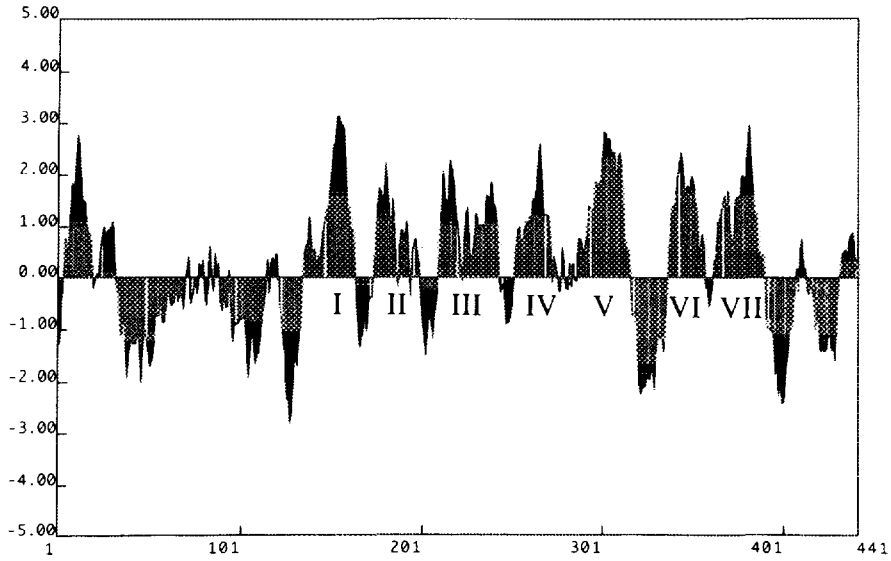

Figure 2. Hydropathy plot of the predicted amino acid sequence of HSR. The plot was obtained by the method of Kyte and Doolittle using the DNASIS (Hitachi) software. The X-axis indicates position of the amino acid residues of the precursor protein. The putative transmembrane regions are labeled from I to VII as indicated in the graph.

cloned receptor is functional; it interacts specifically with secretin and is coupled to cAMP intracellular messenger system.

Tissue Expression of Human Secretin Receptor mRNA. A Northern blot analysis of polyA+ mRNA from various human tissues probed with the cDNA clone was performed (Fig. 5). A dominant 1.8 Kb transcript was detected in the pancreas and in the intestine, while weak

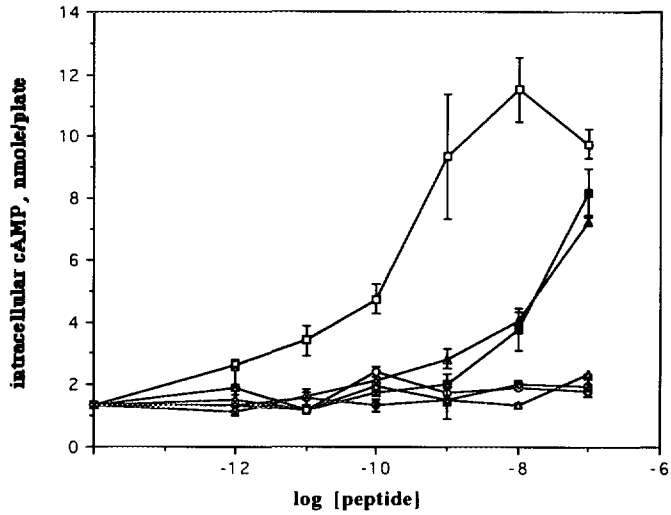

Figure 3. cAMP dose-response curve in transfected COS-7 cells. COS-7 cells were transfected with the expression vector pRC-CMV-HSR. The transfected cells were incubated with various concentrations of human peptides including secretin (open square), VIP (solid square), GHRH (open triangle), PACAP (solid triangle), GLP-1 (open circle) and glucagon (solid circle). The assays were done in triplicates, and the values are mean ± SEM.

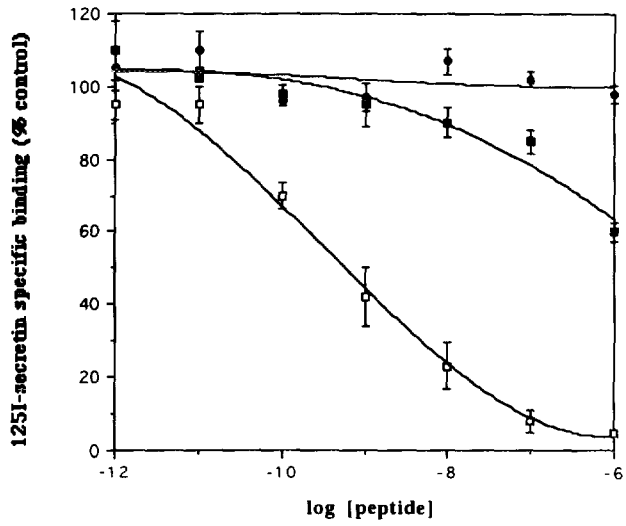

Figure 4. Displacement of binding to the transfected COS-7 cells. The permanently transfected cells were incubated with 100,000 cpm of ^{125}I-secretin alone or in the presence of various concentrations of cold peptides: human secretin (open square), VIP (solid square) and glucagon (solid circle). Specific binding was found to be around 4,000 cpm (with and without 1 μM of cold secretin). The assays were done in triplicates, and the values are mean $\pm$ SEM.

hybridization signals were observed in colon, kidney and lung tissues. The relative expression levels of HSR in these tissues are pancreas (100%), small intestine (15%), colon (2%), lung (2%) and kidney (4%). No hybridization signal was detected in heart, brain, placenta, liver, skeletal muscle, spleen, thymus, prostate, testis and leukocyte (Fig. 5). Secretin transcripts were found in various rat brain regions and it is likely that secretin receptor may also be present in the brain (7,9). However, there were controversial reports indicating the presence (21) and absence of this receptor in rat brain (18). In order to see whether the lack of hybridization signal in human brain was due to the relatively low abundance of secretin mRNA in whole brain mRNAs, a Northern blot using mRNAs prepared from various regions of human brain (amygdala, caudate nucleus, corpus callosum, hippocampus, whole brain, substantia nigra, subthalamic nucleus and thalamus) was performed and again no signal was detected (Fig. 5). This result suggested that HSR mRNA is not present in human brain within the detection limit of Northern blot analysis. The hypothesis that secretin may act as a neuromodulator remains to be clarified. It was previously reported that secretin was able to stimulate contraction of rat ventricular cardiomyocytes (3) and the presence of secretin receptor transcripts in rat heart (18); on the other hand, there was no hybridization signal detected in human heart tissue while the rat heart showed the most abundant signal. The apparaent discrepancies between the tissue distribution of secretin receptor in human and rat may reflect an interesting shift of secretin physiology. It was also noted that tissues expressing low levels of secretin receptor (gastrointestinal tract, kidney and lung) were expressing secretin as well(8), again, the physiological significance of this observation remains to be investigated.

In conclusion, a human secretin receptor has been cloned, characterized and functionally expressed in mammalian cells. The high degree of sequence homology between both the cDNA and amino

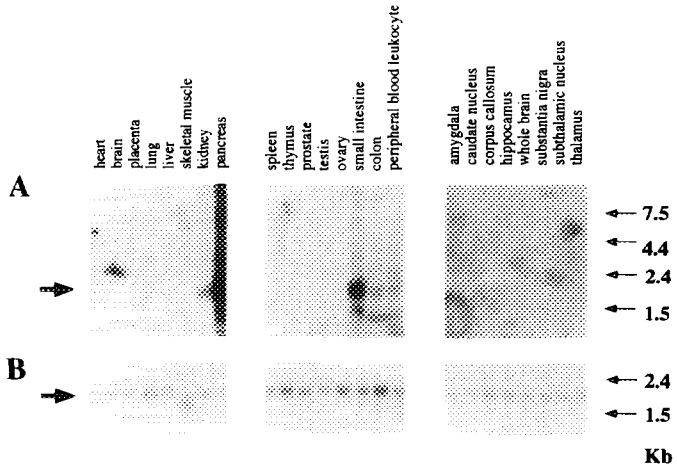

Figure 5. Northern blot analysis of HSR mRNA in various human tissues as indicated. Panel A was probed with ^{32}P-labeled HSR cDNA, panel B was probed with ^{32}P-labeled human ß-actin cDNA as control to indicate the integrity and relative quantity of the mRNA loaded on the gel. Note also that heart and skeletal muscle contained two forms of ß-actin, 2.0 Kb and 1.8 Kb,as shown. The relative expression of HSR mRNA as determined by a Phosphoimager (Molecular Dynamics) was as follows: pancreas (100%), small intestine (15%), colon (2%), lung (2%) and kidney (4%).

acid sequence reported here and that of the rat secretin receptor suggests that these receptors are species variants of the same protein. The expressed HSR interact specifically with human secretin resulting in the production of intracellular cAMP with an EC_{50} of around 0.2 nM. Northern blot analysis of the receptor revealed a 1.8 Kb transcript which is expressed mainly in pancreas and intestine and less in colon, kidney and lung; while no expression was detected in heart nor various regions of the brain.

ACKNOWLEDGMENTS

The author would like to thank Loretta Lam, Tony Yuen, Fred Leung and Kei Li Yu for their technical help in library screening, secretin labeling and critical review of the manuscript. The work was supported by HK government RGC and CRCG grants.

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