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CLONING AND EXPRESSION OF A HUMAN GLUCAGON RECEPTOR[1]

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SUMMARY: A human glucagon receptor has been cloned from human liver tissue. The 1578-bp cDNA clone encodes a protein of 477 amino acids with 82% identity to the rat glucagon receptor. The predicted secondary structure and homology to known proteins places this receptor within the superfamily of seven transmembrane domain G protein coupled receptors. Transfection of the human glucagon receptor into COS-7 cells confers upon them high affinity binding for [^{125}I] glucagon. In membranes prepared from COS-7 cells transfected with the human glucagon receptor, the binding of [^{125}I] glucagon is inhibited with the rank order of potency glucagon > oxyntomodulin > glucagon-like peptide 1 (7-36) amide >> glucagon-like peptide 2 = gastric inhibitory peptide = secretin.

Glucagon is a 29 amino acid peptide proteolytically cleaved from the 180 amino acid proglucagon peptide in the pancreatic α cells. The proglucagon peptide is processed differently in the stomach to yield other peptides such as glicentin, oxyntomodulin, GLP-1 and GLP-2 (1). Glucagon is released by the pancreas in response to low levels of blood glucose and acts on hepatocytes via a specific receptor to activate adenylate cyclase, increase cAMP levels, and activate a cAMP-dependent protein kinase (1). The resulting increase in liver glycogenolysis and gluconeogenesis leads to a net release of glucose by the liver (1). During high blood glucose levels, insulin acts to deactivate the cAMP-dependent protein kinase reversing the glucagon mediated enhancement of glycogenolysis and gluconeogenesis (1). Hyperglycemia results when insulin is unavailable (IDDM) or when insulin is not fully effective (NIDDM). Although treatments for diabetes have traditionally focused on increasing insulin levels, antagonism of glucagon function may provide an alternative therapy. Indeed, the hyperglycemia of diabetic rats has been reduced by infusions of a glucagon antagonist (2), and both the hyperglycemia and ketoacidosis of IDDM in humans has been reduced by suppression of glucagon secretion (3). Thus,

[1] The sequence for the human glucagon receptor gene has been deposited in the GenBank library (accession no. U03469).

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*ABBREVIATIONS: BSA, bovine serum albumin; DTT, dithiothreitol; GLP, glucagon-like peptide; GIP, gastric inhibitory peptide; GHRH, growth hormone releasing-hormone; GPCR, G protein coupled receptor; IDDM, insulin-dependent diabetes mellitus; NIDDM; non-insulin-dependent diabetes mellitus; PACAP, pituitary adenylate cyclase activating polypeptide; PCR, polymerase chain reaction; PTH, parathyroid hormone; VIP, vasoactive intestinal peptide.

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a possible approach to reducing the effect of glucagon is to develop a safe and effective glucagon receptor antagonist.

Hepatic glucagon receptors have been solubilized and partially characterized (4, 5, 6). In addition, receptors for glucagon have been described in adipocytes (7), brain (8), kidney (9), lymphocytes (10), retina (11), and intestine (12). The rat intestinal glucagon receptor, located in the gastric fundus, has a 20-fold lower affinity for glucagon than oxyntomodulin, while the hepatic receptor has a 10-fold higher affinity for glucagon (12). Recently, the rat hepatic glucagon receptor was cloned (13, 14). This receptor has seven putative transmembrane domains and belongs to a subfamily of the GPCR that bind peptides related to glucagon such as calcitonin, GHRH, GLP-1, PACAP, PTH, secretin, and VIP. In this report, we describe the cloning and characterization of a cDNA encoding the full length human glucagon receptor.

MATERIALS AND METHODS

Culture of COS-7 cells. COS-7 cells were cultured in Dulbecco's modified Eagle medium (Gibco-BRL) with 10% heat inactivated fetal calf serum.

Cloning a segment of the human glucagon receptor. Poly A^+ RNA was isolated from human liver using the Fast-Track system (Invitrogen). cDNA was prepared from 5 µg of human liver poly A^+ RNA by simultaneous priming with random hexanucleotides and oligo dT primers using the Riboclone cDNA synthesis system (Promega). The cDNA was ligated with non-palindromic *Bst*XI linkers (Invitrogen). Excess linkers were removed by gel-filtration over a cDNA sizing column (Gibco-BRL). The cDNA was ligated into plasmid pcDNAI (Invitrogen) after digestion with restriction enzyme *Bst*XI. Except where noted, all PCR reactions were performed with 2U of *Pfu*I DNA polymerase (Stratagene), 1 µg of each primer, and 10 to 500 ng of template in a 55 µL reaction volume in 1x *Pfu*I buffer #3 (Stratagene) with 10% dimethyl sulfoxide in an OmniGene (Hybraid) thermal cycler. PCR products were purified through 1% agarose gel, isolated using a Qiaex beads (Qiagen), and cloned into various vectors using T4 DNA ligase (IBI). The human liver cDNA was incubated with degenerate primers based on the rat glucagon receptor cDNA (13). To facilitate the cloning of PCR products the forward primers [5' CCG GAA TTC CAT CCA (T/C)GG GAA (T/C)CT (A/C/G/T)TT (T/C)GC 3'] contained a *Hin*dIII site and the reverse primers [5' GGG GAA GCT TCG TGG AC(A/C/G/T) CCC AG(A/C/G/T) AG(A/C/G/T) GG(A/G/T) AT 3'] contained an *Xba*I site. After 40 cycles of PCR (1 min 96°C, 1 min 50°C, 1 min 72°C) in a PH-6 thermal cycler (Techne), a 602-bp product was subcloned into pCRII using the TA cloning method (Invitrogen), and designated pVE2640. The DNA sequence of alkali denatured double strand templates was determined using Sequenase (US Biochemicals). The sequence of an additional 154-bp of cDNA, located 3' to the end of the cDNA represented in pVE2640, was obtained from another PCR product. A forward primer [5' TGG GCA GTG GTC AAG TGT CTG TTC 3'] internal to the 602-bp clone and a reverse primer complementary to pcDNAI [5' CTT CAC AAA GAT CCT CTA GCA TTT AGG T 3'] and were used in a 45 cycle PCR reaction (1 min 96°C, 1 min 55°C, 1.5 min 72°C). The products of the reaction were cloned into pCRII and screened by colony hybridization (15) with the 602-bp fragment of pVE2640 labeled by random priming (Stratagene). A clone, pVE2660, containing a 440-bp product was isolated and sequenced.

Isolation of a genomic cosmid clone containing the human glucagon receptor gene. A cosmid library of human foreskin fibroblast DNA prepared by partial digestion with *Mbo*I and ligation into sCos-1 vector (16) was obtained from J. Mudgett (Merck Research Labs.) The 602-bp fragment of pVE2640 was labeled by random priming (Stratagene) and used to probe 4 x 10^5 library clones by colony hybridization (15). Cosmid pVE2671 (37 kb) was isolated and confirmed by Southern analysis (15) to contain two *Bam*HI fragments (2.0 kb and 4.3 kb) that hybridized to the 602-bp probe. Each *Bam*HI fragment had an end sequence that matched the DNA sequence on either side of the unique *Bam*HI site in the 602-bp fragment. These two *Bam*HI fragments were cloned into pVE2036 (17). A sequencing primer [5' CGC CTG GGC AAA GTG CTA 3'] complementary to the 3' end of the insert in pVE2660 was used to obtain sequence information from the 2.0-kb *Bam*HI fragment which extended 250 bp beyond that on pVE2660, past the apparent TGA stop codon for the gene. The sequence near the ATG start codon for the gene was localized by sequencing the end of a PCR product from pVE2671. A forward primer complementary to DNA 5' to the rat cDNA ATG [5' TGG CTA CCC AGA GGC ATG 3'] and a primer internal to the 602-bp insert on pVE2640 [5' AGC AGC CCA TCA ATG ACC 3'] were

used in a 20 cycle, low stringency PCR reaction (20 sec 95°C, 20 sec 45°C, 90 sec 72°C). The resulting 2.7-kb fragment was blunt-end cloned into pCR-Script SK(+) (Stratagene), the end sequenced, and a primer complementary to the region past the ATG [5' AGG CCA GCA GCA GCA GCA AC 3'] used to sequence 200 bp 5' to the ATG start codon on the 4.0-kb *Bam*HI fragment from the pVE2671.

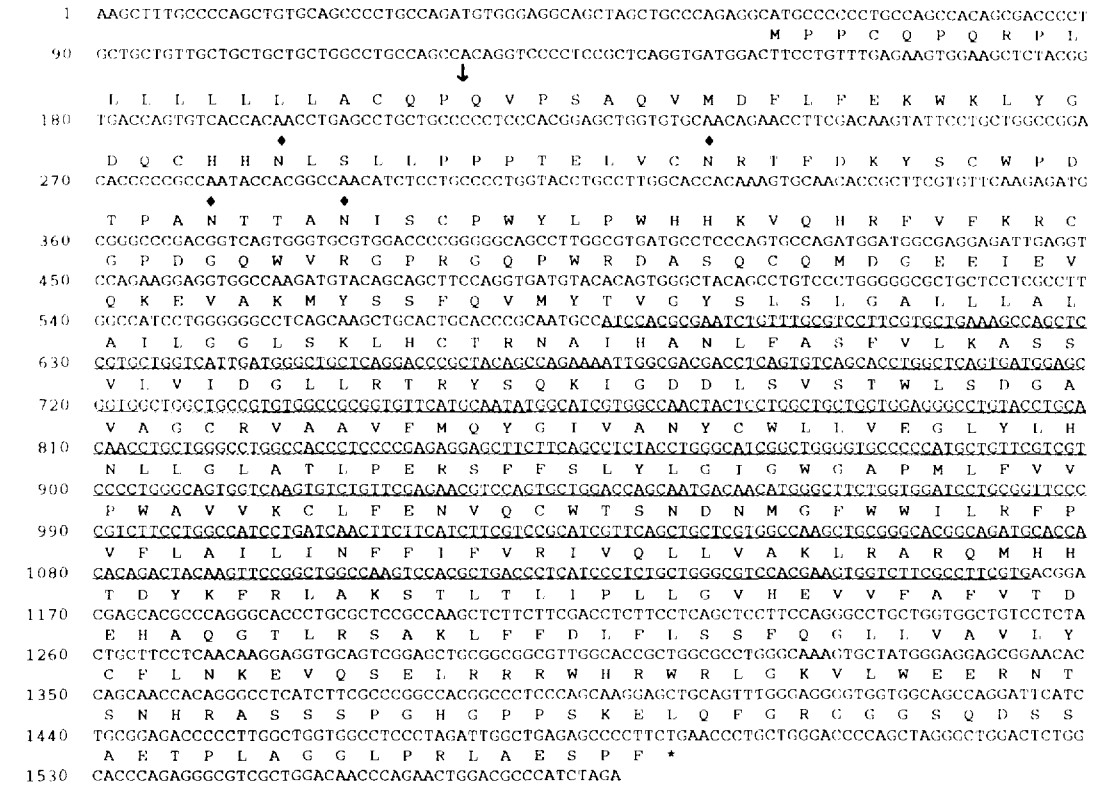
Isolation of a PCR clone encoding the full length human glucagon receptor. DNA was prepared from a fetal liver cDNA library in λgt10 (Clontech, #HL1064a). 1.2 x10^8 phage were infected into 5 ml of an overnight culture of C600 (Clontech) in LB (13) plus 0.2% maltose and 10 mM $MgSO_4$, diluted into 500 ml of LB plus 10 mM $MgSO_4$, grown overnight, and the DNA isolated from the phage (Qiagen). This DNA was used in two 35 cycle PCR reactions (20 sec 94°C, 20 sec 60°C, 90 sec 72°C). One reaction contained a forward primer located 5' to the ATG start, which contained an *Hin*dIII site, [5' AGG AAA GCT TTG CCC CAG CTG TG 3'] and a reverse primer [5' TG ATC AGG ATG GCC AGG AAG 3'], located 3' to the *Bam*HI site in the 602-bp fragment, which yielded a 1040-bp PCR product. The second reaction contained a primer 5' to the *Bam*HI site [5' CCC TGG GCA GTG GTC AAG 3'] and a primer 3' to the TGA stop codon [5' CCA GCT CTA GAT GGG CGT CCA GTT CTG 3'], which contained a *Xba*I site, and yielded a 670-bp PCR product. The *Hin*dIII-*Bam*HI and *Bam*HI-*Xba*I fragments were cloned into pcDNA I/Neo (Invitrogen) to yield pVE2702. The sequence of the cDNA in pVE2702 was determined and analyzed using the GCG software package (Genetic Computer Group).

COS-7 cell transfection and pharmacological characterization. A suspension of 7 x 10^6 COS-7 cells were transfected with 25 μg of pVE2702 by electroporation (18). Cells were harvested after 60-72 h. Membranes were prepared by hypotonic lysis, frozen in liquid nitrogen, and stored at -20°C as described (16). Competition binding studies were carried out in 200 μl of 20 mM Tris pH 7.0 buffer containing 0.1% BSA, 1 mM DTT, 100 μM aprotinin, 25 μM phenylmethylsulfonyl fluoride, 3 μM o-phenathroline, 100 pM [^{125}I] glucagon (1:1 mix of (3-^{125}I] iodotyrosyl^{10})glucagon:(3-^{125}I] iodotyrosyl^{13})glucagon, ≈ 81 TBq/mmol, (NEN)) and the indicated concentration of peptide for 20 min at 22°C. Membranes were harvested on GF/C filters (Whatman) that had been presoaked in 0.5% polyethylenimine. Data were analyzed using the Inplot program (Graphpad Software).

RESULTS AND DISCUSSION

A cDNA encoding a human glucagon receptor was isolated using a combination of techniques including degenerate PCR, genomic cosmid screening, and PCR. Degenerate PCR employing primers based on the rat glucagon receptor cDNA sequence (13) was used to isolate a 602-bp fragment of the human glucagon receptor from liver cDNA (Fig. 1). A cosmid clone, pVE2671, was isolated by screening a genomic cosmid library with the 602-bp fragment from pVE2640. The 4.3-kb and 2.0-kb *Bam*HI fragments which cross hybridized to the 602-bp fragment were cloned into pVE2036 to yield pVE2678 and pVE2681, respectively. The region 3' to the TGA stop codon of the human glucagon receptor gene was located by partial determination of the nucleotide sequence of pVE2681. The region 5' to the ATG start codon of the receptor gene was located after isolating a fragment containing the ATG region by PCR as described in "Materials and Methods". The nucleotide sequence from the regions encoding the ATG and TGA codons of the genomic clone were then used to design PCR primers to amplify two overlapping cDNA segments. These segments were ligated together and cloned into the expression vector pcDNA I/Neo to yield pVE2702, a cDNA clone which encoded the entire human glucagon receptor. The nucleotide sequence of the cDNA in pVE2702 and in an independent clone were identical, indicating that PCR induced mutations did not occur in the nucleotide sequence. The 1578-bp cDNA in pVE2702 encodes a 477 amino acid protein (Fig. 1) with a predicted molecular weight of 54-kDa and a pI of 8.7. Hydropathy analysis (19) indicates that the protein has eight hydrophobic domains (Fig. 1). The amino terminal hydrophobic domain (amino acids 1 to 21) is followed by a putative signal peptide cleavage site (20), and presumably represents a signal peptide which is cleaved to yield a mature protein of 52-kDa molecular weight. The other seven hydrophobic regions place this protein in the class of GPCR. The amino terminal extracellular region contains four

A



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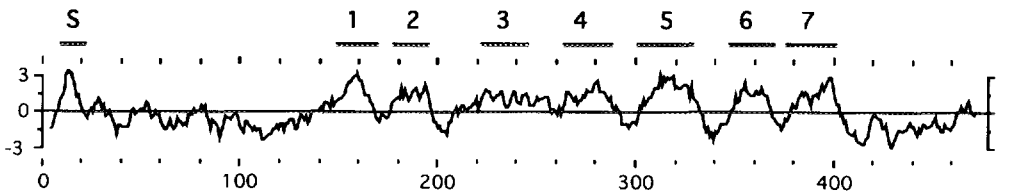

Figure 1. A. Nucleotide sequence of the human glucagon receptor cDNA. The 477 amino acid sequence deduced from the cDNA nucleotide sequence is shown below the nucleotide sequence in single letter code. The arrow indicates the predicted cleavage site occurring after the putative signal sequence. The 602-bp fragment isolated by degenerate PCR is underlined. Four consensus sequences for N-linked glycosylation (Asn-X-Thr/Ser) are marked with diamonds.
B. Hydropathy plot (19) of the glucagon receptor with the predicted signal peptide (labeled S) and seven transmembrane regions indicated.

consensus N-linked glycosylation sites (marked with diamonds in Fig. 1), in agreement with the four N-linked oligosaccharide chains identified in cross-linking studies and monoclonal antibody analysis of the rat glucagon receptor (5, 6).

A search of the GenBank, EMBL, and SwissProt databases revealed that the human glucagon receptor has the highest homology to the rat glucagon receptor (14). The rat and human receptors are 82% identical at both the amino acid (Fig. 2), and the nucleotide sequence level. The amino terminal domain, from the signal peptide cleavage site to the first transmembrane domain, has the highest identity (91%) between the human and rat receptors (amino acids 22 to 142). This conserved segment

Figure 2. Alignment of the human (top) and rat glucagon receptor sequences. The seven transmembrane spanning domains are boxed. Six cysteines conserved among receptors related to the glucagon receptor are indicated by asterisks.

of the receptor, which is considerably longer than those in the biogenic amine family of GPCR, may be part of the peptide ligand binding domain. The transmembrane regions of the human and rat receptors, amino acids 143 to 404, are 90% identical. In contrast, the carboxy terminal tails, which consists of 60 amino acids in the human glucagon receptor and 53 amino acids in the rat glucagon receptor, are only 48% identical. The only other known proteins with significant similarity to the human glucagon receptor are other GPCR which bind peptides homologous to glucagon. Examples of these receptors, and their identity to the human glucagon receptor include: human GLP-1 receptor, 47% identical (21); rat secretin receptor, 42% identical (22); human VIP receptor, 38% identical (23); rat PACAP receptor, 36% identical (24); human PTH receptor, 35% identical (25); human GHRH receptor, 32% identical (26); and human calcitonin receptor, 30% identical (27). Interestingly, all of these receptors share six conserved cysteines within extracellular domains (marked with asterisks in Fig. 2) which could be important in determining the receptors' structure. Four of the conserved cysteines are located in the amino terminal domain, corresponding to human glucagon receptor amino acids 58, 67, 81 and 100. The other two conserved cysteines (amino acids 224 and 294) are also at locations conserved in the biogenic amine family of GPCR.

The expression of the glucagon receptor in pVE2702 is under the control of the cytomegalovirus promoter. Transfection of the human glucagon receptor expression construct into COS-7 cells confers upon them high affinity binding of [^{125}I] glucagon. Under identical assay conditions membranes prepared from mock transfected COS-7 cells displayed no specific [^{125}I] glucagon binding (data not shown). For pharmacological characterization of the expressed receptor, the ability of glucagon and related peptides to displace the binding of [^{125}I] glucagon was determined. As shown in Fig. 3, glucagon inhibits the binding of [^{125}I] glucagon to the receptor with an IC_{50} of 5 nM. Oxyntomodulin and GLP-1 (7-36) amide displace [^{125}I] glucagon binding with >10 fold lower affinity. Concentrations

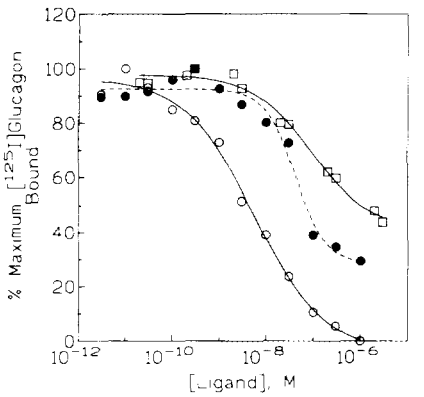

Figure 3. Displacement of [125I] glucagon binding to membranes prepared from transfected COS-7 cells. COS-7 cells ($7x10^6$ cells) were transfected with 25 μg of human pVE2702 DNA and membranes prepared and frozen as detailed in "Materials and Methods". 3 μg of membrane protein was incubated with 100 pM [125I] glucagon and the indicated concentrations of ligand. Data shown are means of duplicate determinations and are representative of two such experiments. Symbols: open circles, glucagon; filled circles, oxyntomodulin; open squares, GLP-1 (7-36) amide.

of GIP, GLP-2, and secretin up to 3 μM failed to displace [125I] glucagon binding (data not shown), consistent with the identification of the cloned receptor as a glucagon specific receptor.

In summary, we have cloned, expressed and characterized a human glucagon receptor. This receptor shows the highest sequence homologies to the rat glucagon receptor (14) and to the human GLP-1 receptor (21). The expressed glucagon receptor also binds oxyntomodulin and GLP-1 (7-36) amide, albeit with a lower affinity than glucagon. The isolation of the human glucagon receptor cDNA will aid in the discovery of compounds that act as antagonists of this receptor for potential use in the treatment of diabetes.

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