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CLONING AND FUNCTIONAL EXPRESSION OF A HUMAN GLUCAGON-LIKE PEPTIDE-1 RECEPTOR

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A human glucagon- like 1 peptide receptor has been cloned from the gastric tumor cell line HGT-1. The cDNA clone encodes a protein of 463 amino acids and is a member of the superfamily of seven transmembrane domain G protein coupled receptors. Transfection of the human GLP-1 receptor into COS-7 cells confers upon them high affinity binding for [^{125}I] GLP-1 (7-36) amide. In membranes prepared from COS-7 cells transfected with the human GLP-1 receptor, the binding of [^{125}I] GLP-1 (7-36) amide is inhibited with the rank order of potency GLP-1 (7-36) amide > glucagon > secretin, characteristic of a GLP-1 receptor. The human GLP-1 receptor is functionally coupled to increases in intracellular cAMP in these cells: incubation of COS-7 cells expressing the human GLP-1 receptor with GLP-1 (7-36) amide gives rise to a 4-fold increase in cyclic AMP over basal levels, with an EC_{50} of 25pM. Glucagon is also a full agonist but is 200-fold less potent than GLP-1 (7-36) amide in stimulating the human GLP-1 receptor.

GLP-1 is one of several hormones shown to potentiate glucose-induced insulin secretion. Such hormones, known as incretins, are produced in the gut, released in response to a meal, and interact with specific receptors on pancreatic islets to cause glucose-dependent insulin secretion (1). GLP-1 is produced by postranslational processing of the proglucagon gene product in intestinal L cells, through a biologically inactive 37 amino acid form [GLP-1 (1-37)] to either of two biologically active forms, GLP-1 (7-37) and GLP-1 (7-36) amide. These biologically active forms of GLP-1 are the most potent incretins known, stimulating glucose-mediated insulin secretion at concentrations as low as 10pM. Infusion of GLP-1 (7-36) amide into patients with type II diabetes leads to increased levels of insulin secretion which occurs in an glucose-dependent fashion (2, 3). These data suggest that compounds that act via the GLP-1 receptor may be therapeutic in the treatment of type II diabetes.

GLP-1 receptors have also been described in lung (4) adipose (5), brain (6) and the gastric tumor cell line HGT-1 (7). A cDNA encoding a GLP-1 receptor has recently been cloned from rat

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The following abbreviations are used: GLP-1, glucagon-like peptide-1; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; BSA, bovine serum albumin.

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pancreatic islets (8). This receptor has seven putative transmembrane domains and belongs to the superfamily of G protein coupled receptors. The GLP-1 receptor is most homologous to other members of a recently defined subclass of G protein coupled receptors that includes, amongst others, the receptors for glucagon and secretin. The GLP-1 receptor acts via stimulation of adenylyl cyclase to raise intracellular levels of cAMP (1). We now report the cloning, expression, and pharmacological characterization of a human GLP-1 receptor from the gastric tumor cell line HGT-1.

Materials and Methods

Culture of HGT-1 and COS-7 cells. HGT-1 cells (cell line Cl.19A, a human gastric carcinoma cell line) were obtained from Dr. C.L. Laboisse and cultured as described (9). COS-7 cells were cultured in DMEM with 10% heat inactivated fetal calf serum.

Cloning of the human GLP-1 receptor. Poly A^+ RNA was isolated from HGT-1 cells using the Fast-Track system (Invitrogen). cDNA was prepared from 5μg of HGT-1 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). For the PCR-RACE protocols the cDNA was ligated into plasmid pcDNA I (Invitrogen) after restriction with BST XI.

HGT-1 cDNA was incubated with 2U of *Amplitaq* DNA polymerase (Perkin-Elmer Cetus) and degenerate primers based on the rat GLP-1 receptor cDNA (8). Both the forward primer [5' ATG CA(AG) TA(CT) TG(CT) GTN GC 3'] and the reverse primer [5' AT(AG) TCN GT(AC) TT(AG) CAC AT 3'] are 32-fold degenerate 17-mers. PCR was performed in a 50µl reaction volume for 35 cycles (1 min. at 95°C, 0.5 min. at 42°C, 1 min. at 72°C) followed by 7 min. at 72°C. The 337 bp product was subcloned into the pCR II plasmid using the TA cloning method (Invitrogen).

HGT-1 cDNA ligated into pcDNA I was used as the starting material in a modification of the PCR-RACE protocol (10). Forward and reverse primers complimentary to the resulting 337 bp fragment were used in conjunction with appropriate primers made to pcDNA I to amplify in a 3' or 5' direction, respectively. PCR reactions were screened by Southern blot analysis (11) using a GLP-1 receptor specific probe. Reactions containing hybridizing products were subcloned using the TA cloning method and identified by colony hybridization (12).

HGT-1 cDNA, forward primer 5' TGGTGGATTCCTGAACTCC 3', reverse primer 5' CCTGTGGTTTCACAAGAAGC 3', and cloned *Pfu* polymerase (Stratagene) were used to amplify the entire GLP-1 cDNA. Reactions were heated at 96°C for 5 min. and then 35 thermal cycles performed (1 min. at 95°C, 0.5 min. at 55°C, 2 min. at 72°C). Reactions were performed in the presence of 10% dimethylsulfoxide. Products were subcloned into pCR-Script SK^+ (Stratagene) and identified by colony hybridization. DNA sequencing was performed on plasmids using dye-primer chemistry on an Applied Biosystems Model 373A Sequencer. DNA sequences were analyzed using the GCG software package (Genetic Computer Group).

COS-7 cell transfection and pharmacological characterization. COS-7 cells were transfected by electroporation with the human GLP-1 receptor cDNA subcloned into pcDNA I/neo (Invitrogen). Cells were harvested after 60-72h. For the measurement of cAMP, cells were resuspended in ACC buffer (75mM Tris-HCl pH 7.4, 250mM sucrose, 12.5 mM $MgCl_2$, 1.5mM EDTA, 0.2mM $Na_2S_2O_3$, 0.1mM Ro-201724) and incubated with ligand for 45 min. at 22°C as described (13). cAMP was quantified by radioimmunoassay using an Attoflow automated radioimmunoassay machine (ATTO Instruments Inc.).

[^{125}I] GLP-1 (7-36) amide was prepared using a modification of the Iodo-Gen method (14). Carrier-free monoiodinated [^{125}I] GLP-1 (7-36) amide was purified by HPLC using a Vydac C_{18} column. Membranes were prepared by hypotonic lysis, frozen in liquid N_2 and stored at

-80°C as described (15). Binding studies were carried out in phosphate buffered saline containing 0.1% BSA, 2μg/ml leupeptin, 15μg/ml benzamidine, 40μg/ml bacitracin, 5μg/ml soybean trypsin inhibitor, 25μM PMSF, 3μM o-phenathroline, and the indicated concentration of [^{125}I] GLP-1 (7-36) amide for 1h at 22°C. Membranes were harvested on GF/C filters (Whatman) that had been presoaked in 0.5% polyethylenimine/0.1% BSA. Data were analyzed using the Inplot program (Graphpad Software).

Results and Discussion

A cDNA encoding a human GLP-1 receptor was isolated using the polymerase chain reaction. Degenerate PCR employing primers based on the rat GLP-1 receptor cDNA sequence (8) was used to isolate a 337 bp fragment of the human GLP-1 receptor from HGT-1 cell cDNA (Fig. 1). Following subcloning of HGT-1 cell cDNA into pcDNA I, a modification of the PCR-RACE

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1    tggtggaattcctgaactccccgccATGGCCGGCGCCCCCGGCCCGCTGCGCCTTGCGCTGCTGCTGCTCGGGATGGTGGGCAG
                           M  A  G  A  P  G  P  L  R  L  A  L  L  L  L  G  M  V  G  R
85   GGCCGGCCCCCGCCCCCAGGGTGCCACTGTGTCCCTCTGGGAGACGGTGCAGAAATGGCGAGAATACCGACGCCAGTGCCAGCG
      A  G  P  R  P  Q  G  A  T  V  S  L  W  E  T  V  Q  K  W  R  E  Y  R  R  Q  C  Q  R
169  CTCCCTGACTGAGGATCCACCTCCTGCCACAGACTTGTTCTGCAACCGGACCTTCGATGAATACGCCTGCTGGCCAGATGGGGA
      S  L  T  E  D  P  P  P  A  T  D  L  F  C  N  R  T  F  D  E  Y  A  C  W  P  D  G  E
253  GCCAGGCTCGTTCGTGAATGTCAGCTGCCCCTGGTACCTGCCCTGGGCCAGCAGTGTGCCGCAGGGCCACGTGTACCGGTTCTG
      P  G  S  F  V  N  V  S  C  P  W  Y  L  P  W  A  S  S  V  P  Q  G  H  V  Y  R  F  C
337  CACAGCTGAAGGCCTCTGGCTGCAGAAGGACAACTCCAGCCTGCCCTGGAGGGACTTGTCGGAGTGCGAGGAGTCCAAGCGAGG
      T  A  E  G  L  W  L  Q  K  D  N  S  S  L  P  W  R  D  L  S  E  C  E  E  S  K  R  G
421  GGAGAGAAGCTCCCCGGAGGAGCAGCTCCTGTTCCTCTACATCATCTACACGGTGGGCTACGCACTCTCCTTCTCTGCTCTGGT
      E  R  S  S  P  E  E  Q  L  L  F  L  Y  I  I  Y  T  V  G  Y  A  L  S  F  S  A  L  V
505  TATCGCCTCTGCGATCCTCCTCGGCTTCAGACACCTGCACTGCACCAGGAACTACATCCACCTGAACCTGTTTGCATCCTTCAT
      I  A  S  A  I  L  L  G  F  R  H  L  H  C  T  R  N  Y  I  H  L  N  L  F  A  S  F  I
589  CCTGCGAGCATTGTCCGTCTTCATCAAGGACGCAGCCCTGAAGTGGATGTATAGCACAGCCGCCCAGCAGCACCAGTGGGATGG
      L  R  A  L  S  V  F  I  K  D  A  A  L  K  W  M  Y  S  T  A  A  Q  Q  H  Q  W  D  G
673  GCTCCTCTCCTACCAGGACTCTCTGAGCTGCCGCCTGGTGTTTCTGCTCATGCAGTACTGTGTGGCGGCCAATTACTACTGGCT
      L  L  S  Y  Q  D  S  L  S  C  R  L  V  F  L  L  M  Q  Y  C  V  A  A  N  Y  Y  W  L
757  CTTGGTGGAGGGCGTGTACCTGTACACACTGCTGGCCTTCTCGGTCTTATCTGAGCAATGGATCTTCAGGCTCTACGTGAGCAT
      L  V  E  G  V  Y  L  Y  T  L  L  A  F  S  V  L  S  E  Q  W  I  F  R  L  Y  V  S  I
841  AGGCTGGGGTGTTCCCCTGCTGTTTGTTGTCCCCTGGGGCATTGTCAAGTACCTCTATGAGGACGAGGGCTGCTGGACCAGGAA
      G  W  G  V  P  L  L  F  V  V  P  W  G  I  V  K  Y  L  Y  E  D  E  G  C  W  T  R  N
925  CTCCAACATGAACTACTGGCTCATTATCCGGCTGCCCATTCTCTTTGCCATTGGGGTGAACTTCCTCATCTTTGTTCGGGTCAT
      S  N  M  N  Y  W  L  I  I  R  L  P  I  L  F  A  I  G  V  N  F  L  I  F  V  R  V  I
1009 CTGCATCGTGGTATCCAAACTGAAGGCCAATCTCATGTGCAAGACAGACATCAAATGCAGACTTGCCAAGTCCACGCTGACACT
      C  I  V  V  S  K  L  K  A  N  L  M  C  K  T  D  I  K  C  R  L  A  K  S  T  L  T  L
1093 CATCCCCCTGCTGGGGACTCATGAGGTCATCTTTGCCTTTGTGATGGACGAGCACGCCCGGGGGACCCTGCGCTTCATCAAGCT
      I  P  L  L  G  T  H  E  V  I  F  A  F  V  M  D  E  H  A  R  G  T  L  R  F  I  K  L
1177 GTTTACAGAGCTCTCCTTCACCTCCTTCCAGGGGCTGATGGTGGCCATATTATACTGCTTTGTCAACAATGAGGTCCAGCTGGA
      F  T  E  L  S  F  T  S  F  Q  G  L  M  V  A  I  L  Y  C  F  V  N  N  E  V  Q  L  E
1261 ATTTCGGAAGAGCTGGGAGCGCTGGCGGCTTGAGCACTTGCACATCCAGAGGGACAGCAGCATGAAGCCCCTCAAGTGTCCCAC
      F  R  K  S  W  E  R  W  R  L  E  H  L  H  I  Q  R  D  S  S  M  K  P  L  K  C  P  T
1345 CAGCAGCCTGAGCAGTGGAGCCACGGCGGGCAGCAGCATGTACACAGCCACTTGCCAGGCCTCCTGCAGCtgagactccagcgc
      S  S  L  S  S  G  A  T  A  G  S  S  M  Y  T  A  T  C  Q  A  S  C  S  *
1429 ctgccctccctggggtccttgctgcaggccgggtggccaatccaggagaagcagcctcctaatttgatcacagtggcgagagga
1513 gaggaaaaacgatcgctgtgaaaatgaggaggattgcttcttgtgaaaccacagg
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Figure 1. Sequence of the human GLP-1 receptor cDNA. The 463 residue amino acid sequence deduced from the cDNA sequence is shown below in single letter code. The 337 bp fragment isolated by degenerate PCR is underlined. Three consensus sequences for N-linked glycoslylation are marked with diamonds.

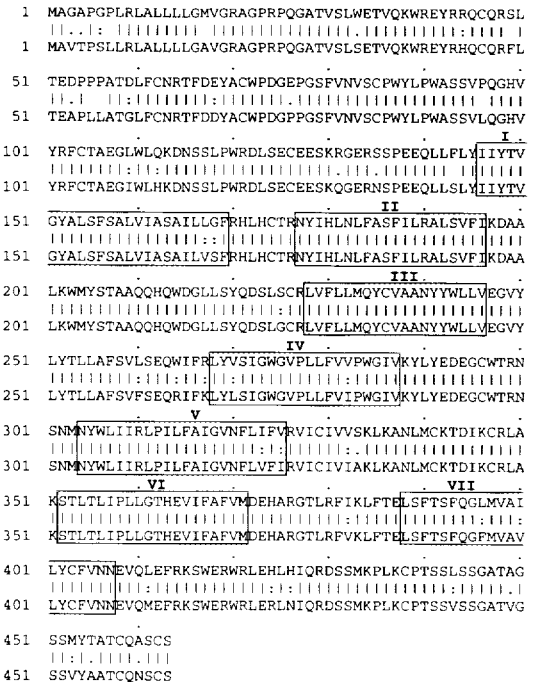

Figure 2. Comparison of the human and rat GLP-1 receptor cDNA sequences. Alignment of the human (top) and rat GLP-1 receptors was made using the GCG Gap program. The seven putative transmembrane domains are boxed.

protocol allowed cloning of the 5' and 3' ends of the GLP-1 receptor coding sequence. Utilizing primers annealing to sequences within the 337 bp fragment in combination with primers complimentary to the pcDNA I vector, a series of cDNA fragments extending in a 5' or 3' direction were cloned. A series of four overlapping cDNA fragments comprising the coding region and both 5' and 3' untranslated regions were cloned by this method. Synthetic oligonucleotides that anneal to the 5' and 3' untranslated regions were then used to amplify a single cDNA encompassing the entire GLP-1 receptor coding sequence. This cDNA encodes a 463 amino acid protein that is identical in length to the rat GLP-1 receptor. The human and rat GLP-1 receptors are 91% identical at the amino acid level (Fig. 2), and 87% identical at the nucleotide level, within the coding region .

The human GLP-1 receptor was subcloned into the eukaryotic expression plasmid pcDNA I/neo, placing expression of the receptor under the control of the cytomegalovirus promoter. Transfection of the human GLP-1 receptor expression construct into COS-7 cells confers upon them high affinity [^{125}I] GLP-1 (7-36) amide binding (Fig. 3). Under identical assay conditions membranes prepared from mock transfected COS-7 cells displayed no specific [^{125}I] GLP-1 (7-36) amide binding (data not shown). For pharmacological characterization of the expressed receptor, the ability of GLP-1 (7-36) amide and related peptides to displace the binding of [^{125}I] GLP-1 (7-36) amide was determined. As shown in Figure 3, GLP-1 (7-36) amide inhibits the

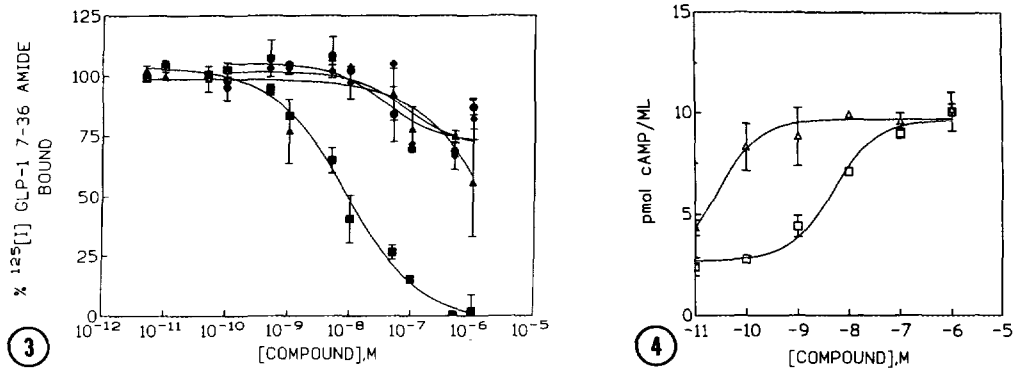

Figure 3. Displacement of [^{125}I] GLP-1 (7-36) amide binding to transfected COS-7 cells. COS-7 cells (7x10^6 cells) were transfected with 20μg of human GLP-1 receptor cDNA in pcDNAI/neo and membranes prepared and frozen as detailed in "Materials and Methods". 27μg of membrane protein was incubated with 50pM [^{125}I] GLP-1 (7-36) amide and the indicated concentrations of ligand. Data shown are means +/- S.E.M. of duplicate determinations and are representative of 2 such experiments. **Symbols:** squares, GLP-1 (7-36) amide; triangles, glucagon; diamonds, gastric inhibitory peptide; circles, secretin.

Figure 4. cAMP accumulation in transfected COS-7 cells. COS-7 cells (7x10^6 cells) were transfected with 100μg of human GLP-1 receptor cDNA. Cells were harvested and cAMP accumulation determined as outlined in "Material and Methods". Data shown are the mean +/- S.E.M. from triplicate determinations from a single experiment. **Symbols:** triangles, GLP-1 (7-36) amide; squares, glucagon.

binding of [^{125}I] GLP-1 (7-36) amide to the receptor with an IC_{50} of 4 nM. Glucagon, gastric inhibitory peptide, and secretin inhibit [^{125}I] GLP-1 (7-36) amide binding with a potency at least a 100-fold lower, consistent with the identification of the receptor as a GLP-1 receptor.

The human GLP-1 receptor is functionally coupled to adenylyl cyclase in transiently transfected COS-7 cells (Fig. 4). Incubation of COS-7 cells expressing the human GLP-1 receptor with GLP-1 (7-36) amide leads to a 4-fold increase in cAMP over basal levels. Under identical assay conditions mock transfected COS-7 cells show no significant increase in cAMP over basal levels (data not shown). GLP-1 (7-36) amide stimulates cAMP accumulation with an EC_{50} of 25pM. Glucagon also stimulates cAMP accumulation in COS-7 cells transfected with the human GLP-1 receptor but with a 200-fold decrease in potency compared with GLP-1 (7-36) amide (Fig. 4). The decreased potency for glucagon is consistent with its acting via the human GLP-1 receptor.

In summary we have cloned, expressed and characterized a human GLP-1 receptor. The high degree of homology between the cDNA described in this report and that cloned from rat pancreatic islets suggests that these DNAs encode species variants of the same receptor. This GLP-1 receptor shows significant (58%) sequence homology to the recently cloned rat glucagon receptor (16). The expressed human GLP-1 receptor also binds and is activated by glucagon, albeit with a lower affinity than GLP-1 (7-36) amide. Thus the GLP-1 and glucagon receptors appear by functional as well as structural criteria to be closely related receptor subtypes.

Whether other receptor subtypes exist within this family remains to be explored. The isolation of the human GLP-1 receptor cDNA will aid in the discovery of compounds that act via this receptor for potential use in the treatment of diabetes.

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