Synthesis and Expression of a Gene for the Rat Glucagon Receptor

REPLACEMENT OF AN ASPARTIC ACID IN THE EXTRACELLULAR DOMAIN PREVENTS GLUCAGON BINDING*

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Cynthia J. L. Carruthers†, Cecilia G. Unson§, Hyang Nina Kim∥, and Thomas P. Sakmar¶
From the †Howard Hughes Medical Institute, Laboratory of Molecular Biology and Biochemistry, §Rockefeller University, New York, New York 10021

In order to facilitate structure-function studies of the glucagon receptor by site-directed mutagenesis, we have designed and synthesized a gene for the rat glucagon receptor. The gene codes for the native 485-amino-acid protein but contains 91 unique restriction sites. To characterize gene expression, a highly specific, high affinity antipeptide antibody was prepared against the receptor. The synthetic gene was expressed in transiently transfected monkey kidney (COS-1) cells. COS cells expressing the synthetic receptor gene bound glucagon with affinity and specificity similar to that of hepatocytes containing native receptor. The transfected COS cells also showed increased intracellular cAMP levels in response to glucagon. The functional role of an aspartic acid residue in the NH$_2$-terminal tail of the receptor was tested by site-directed mutagenesis. This site in the related growth hormone releasing factor receptor was shown to be responsible for the little mouse (lit) genetic defect that results in mice of small size with hypoplastic pituitary glands. Mutant glucagon receptors with amino acid replacements of Asp$^{64}$ were expressed at normal levels in COS cells but failed to bind glucagon. These results indicate that amino acid Asp$^{64}$ may play a key role in glucagon binding to receptor.

Glucagon, a 29-residue peptide hormone, is secreted by the α-cells of the islet tissue of the pancreas. Glucagon plays a central role in the physiology of blood glucose regulation. It acts as a counter regulatory hormone of insulin to increase hepatic glucose production and decrease glucose utilization in non-hepatic tissues. Diabetes mellitus is the most common endocrine disease. The major metabolic complications of diabetes mellitus, hyperglycemia, and ketosis are accompanied by a relative or absolute increase in the ratio of glucagon to insulin (Unger, 1976). A complete understanding of the pathophysiology of both insulin-dependent diabetes and non-insulin-dependent diabetes will require characterization of both the insulin and glucagon cellular signaling pathways at a molecular level.

Extensive structure-function studies have focused primarily on the design and synthesis of peptide analogs of glucagon to investigate its mechanism of action and to identify clinically useful hormone antagonists (Unson et al., 1987, 1994a). These efforts have identified specific residues in glucagon that are responsible for either receptor binding or signaling. Ser$^{13}$ and Asp$^{25}$ were shown to be important determinants of receptor binding (Unson and Merrifield, 1994; Unson et al., 1994b). His$^{1}$, Asp$^{9}$, and Ser$^{16}$ constitute a putative triad responsible for activation of the receptor and subsequent biological effect (Unson and Merrifield, 1994).

The recent isolation of glucagon receptor cDNA clones from rat and human liver has confirmed that the receptor is a member of the superfAMILY of seven-transmembrane domain G protein-coupled receptors (Jelinkè et al., 1993). According to tentative structural models of related receptors, the hormone-binding site probably consists of a contribution from the large extracellular domain of the receptor (Fig. 1), which includes the NH$_2$-terminal tail and loops connecting transmembrane helices. However, transmembrane signaling must involve ligand-mediated communication between the extracellular domain and the intracellular domain where heterotrimeric G proteins are activated by the receptor.

To investigate the molecular mechanism of hormone-receptor interaction and of receptor activation, we have designed and synthesized a gene for the rat glucagon receptor. COS cells expressing the synthetic receptor gene and purified COS cell membranes bound glucagon with high affinity and displayed the appropriate peptide hormone specificity. The transfected COS cells also showed increased intracellular cAMP levels in response to glucagon. Site-directed mutant glucagon receptors with amino acid replacements of Asp$^{24}$ were expressed at normal levels in COS cells but failed to bind glucagon. These results indicate that Asp$^{24}$ may play a key role in glucagon binding to receptor.

**EXPERIMENTAL PROCEDURES**

*Design of the Synthetic Rat Glucagon Receptor Gene—Synthetic gene design was carried out using strategies that have been described elsewhere (Ferretti et al., 1998; Sakmar and Khorana, 1998; Carruthers and Sakmar, 1995). The nucleotide sequence was determined with the aid of sequence analysis software (MacVector) with a reverse translation algorithm. The Khorana (1976, 1979) method was employed for gene synthesis. Thus, both upper and lower DNA strands were totally chemically synthesized. The synthetic gene was flanked by EcoRI and Ncol restriction sites and was assembled from three fragments, A, B, and C. Fragment A (EcoRI to MluI) was 362 bp, fragment B (MluI to BamHI) was 564 bp, and fragment C (BamHI to Ncol) was 546 bp. Duplexes within a fragment were designed to have four or five base unique nonpalindromic 5'-overhangs. Fragments A-C were constructed from 8, 14, and 14 oligonucleotides, respectively. The oligonucleotides ranged in size from 72 to 92 bases.

†Assistant Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Box 284, Rockefeller University, 1230 York Ave., New York, NY 10021. Tel.: 212-327-8288; Fax: 212-327-8570.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U14012.

The abbreviations used are: bp, base pairs; G protein, guanine nucleotide-binding regulatory protein; GLP-1, glucagon-like peptide 1; GRF, growth hormone releasing factor; VIP, vasoactive intestinal peptide; PAGES, polyacrylamide gel electrophoresis.
Oligonucleotide Preparation—Oligonucleotide synthesis was carried out on an Applied Biosystems model 392 synthesizer. Purification and characterization of synthetic DNA was carried out essentially as described (Ferretti et al., 1986; Sakmar and Khorana, 1988).

Oligonucleotide 5'-End Phosphorylation—Synthetic oligonucleotides isolated by preparative 1% agarose gel electrophoresis were phosphorylated batchwise. The 5'-terminal oligonucleotide on the upper strand and the 5'-terminal oligonucleotide on the lower strand of each fragment were not phosphorylated. A mixture of 100 pmol of each oligonucleotide in 50 mM Tris-HCl, pH 8.0, was heated for 3 min at 90 °C and quick chilled on ice. Solutions were added to give final concentrations of 10 mM MgCl₂, 2 mM spermidine, 10 mM dithiotreitol, 1 mM ATP, and 4 units of T4-polynucleotide kinase (New England Biolabs). The mixture was incubated at 37 °C for 45 min, heated at 90 °C for 3 min, then quick chilled on ice. The phosphorylation reaction was repeated a second time after readdition of dithiotreitol and kinase.

Annealing and T4 DNA Ligase-Catalyzed Duplex Joining Reactions—Complementary oligonucleotides for an entire gene fragment were annealed by adding 100 pmol each of the 5'-terminal upper strand and 5'-terminal lower strand oligonucleotides to the phosphorylated oligonucleotide mix in a final volume of 100 μl in 50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂. The annealed oligonucleotides were joined by incubation for 16 h at 14 °C in 66 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithiotreitol, 1 mM ATP, and 25 units of T4 DNA ligase (Boehringer Mannheim) in a final volume of 100 μl.

Purification and Cloning of Synthetic Gene Fragments—The products of the ligation reaction were separated on a preparative 1.5% agarose gel. The full-length synthetic DNA fragment bands were excised from the gel and purified using the Qiagen Gel Extraction Kit (Qiagen). Each fragment was cloned into a modified pGEM-2 vector. XLI-Blue Electrocompetent Escherichia coli cells (Stratagene) were transformed by electroporation. DNA mini-preps were prepared for restriction analysis and subsequent dideoxy sequencing on double-stranded plasmid DNA sequence. United States Biochemicals Co., Cleveland, OH (Sanger et al., 1977). A clone for each fragment with the correct DNA sequence was selected for assembly of the full-length synthetic gene into an expression vector.

Assembly and Characterization of Full-length Synthetic Genes—The assembled synthetic fragments were purified, the full-length gene was assembled into the cloning vector. The cloned full-length gene was resequenced in both directions using synthetic internal primers. The gene was transferred into the eukaryotic expression vector pMT3 (Ernst et al., 1988). The pMT3 vector containing the synthetic rat glucagon receptor gene is referred to as pMT5.

Construction of Glucagon Receptor Mutant Genes—The glucagon receptor mutant genes D64E, D64G, D64K, and D64N were prepared by restriction fragment replacement of the synthetic gene in the pGEM expression vector (10 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 1 mM ATP, and 25 units of T4 DNA ligase (Boehringer Mannheim) in a final volume of 100 μl. Purification and Cloning of Synthetic Gene Fragments—The products of the ligation reaction were separated on a preparative 1.5% agarose gel. The full-length synthetic DNA fragment bands were excised from the gel and purified using the Qiagen Gel Extraction Kit (Qiagen). Each fragment was cloned into a modified pGEM-2 vector. XLI-Blue Electrocompetent Escherichia coli cells (Stratagene) were transformed by electroporation. DNA mini-preps were prepared for restriction analysis and subsequent dideoxy sequencing on double-stranded plasmid DNA sequence. United States Biochemicals Co., Cleveland, OH (Sanger et al., 1977). A clone for each fragment with the correct DNA sequence was selected for assembly of the full-length synthetic gene into an expression vector.

Assembly and Characterization of Full-length Synthetic Genes—The assembled synthetic fragments were purified, the full-length gene was assembled into the cloning vector. The cloned full-length gene was resequenced in both directions using synthetic internal primers. The gene was transferred into the eukaryotic expression vector pMT3 (Ernst et al., 1988). The pMT3 vector containing the synthetic rat glucagon receptor gene is referred to as pMT5.

Construction of Glucagon Receptor Mutant Genes—The glucagon receptor mutant genes D64E, D64G, D64K, and D64N were prepared by restriction fragment replacement of the synthetic gene in the pGEM cloning vector (Lo et al., 1984). Each mutant was prepared by replacement of the 74-bp BsmBI-KpnI restriction fragment of the desired codon alteration. The four mutant genes were constructed by adding 100 pmol each of the 5' terminal upper strand and lower strand oligonucleotides in 50 mM Tris-HCl, pH 8.0, was heated for 3 min at 90 °C and quick chilled on ice. Solutions were added to give final concentrations of 10 mM MgCl₂, 2 mM spermidine, 10 mM dithiotreitol, 1 mM ATP, and 4 units of T4-polynucleotide kinase (New England Biolabs). The mixture was incubated at 37 °C for 16 h at 14 °C in 66 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 1 mM dithiotreitol, 1 mM ATP, and 25 units of T4 DNA ligase (Boehringer Mannheim) in a final volume of 100 μl.

Expression of Glucagon Receptor and Mutant Receptor Genes in COS-1 Cells—Receptor genes were expressed transiently in COS-1 cells according to the DEAE-dextran procedure previously reported for the expression of rhodopsin (Oprin et al., 1987; Sakmar et al., 1988). Detergent Lysis of COS-1 Cells—Transfected COS-1 cells from a single clone were harvested in a single 15-ml Falcon tube in suspension. The cells were resuspended in 15 ml of cold hypotonic buffer (1 mM Tris-HCl, pH 6.8, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.7 μg/ml pepstatin, 10 μM EDTA) and forced through a 26-gauge needle three times. The total volume was increased to 20 ml with hypotonic buffer to give a hypotonic cell lysate fraction. The hypotonic cell lysate (10 ml) was layered onto 15 ml of a 38% (w/v) sucrose solution in buffer A (150 mM NaCl, 1 mM MgCl₂, 10 mM EDTA, 20 mM Tris-HCl, pH 6.8, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.7 μg/ml pepstatin) in a 1 x 3.5 inch SW-28 ultra centrifuge tube. After centrifugation for 15,000 revolutions/min at 4 °C, the interface band was collected in a 10-ml syringe with an 18-gauge needle. The volume was brought to 50 ml with buffer A, transferred to a Ti-45 tube, and spun at 40,000 revolutions/min for 30 min at 4 °C. The membrane pellet was resuspended in 20 ml of buffer A and spun a second time. The washed pellet was resuspended in 0.6 ml of buffer A, frozen on dry ice, and stored in 0.1-ml aliquots at -80 °C.

Treatment of Expressed Glucagon Receptor and Mutants with N-Glycosidase F—Detergent cell lysate (30 μl or approximately one-tenth of a plate of transfected COS cells) was treated with 0.3 units of N-glycosidase F (Boehringer Mannheim). The digest was shown to be complete after 2 h of incubation at 37 °C. Plasma membrane prepara tion (10 μl, approximately one-tenth of a plate of transfected COS cells) was mixed with 10 μl of buffer A and treated with 0.3 units of N-glycosidase F. The digest was shown to be complete after 3 h of incubation at 37 °C.

Immunoblot Analysis of Expressed Glucagon Receptor and Mutants—Cell lysate samples were loaded without boiling onto a 1-mm thick 4% stacking, 10% separating SDS-polyacrylamide gel, and electrophoretically separated. Rainbow prestained molecular weight standards (Amersham Corp.) or Kaleidoscope prestained standards (Bio-Rad) were included on some gels. After electrophoresis, proteins were electro-phototransferred to Immobilon-P transfer membrane (Millipore) with a Trans-Blot.S.D. Semi-Dry Transfer Cell (Bio-rad) set at 15 V for 30 min at 4 °C. The membrane was blocked in 10 ml of TTBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) for 14 h at 4 °C in a glass bottle rotating in a hybridization chamber (Robbins Scientific). A 1:20,000 dilution of the ST-18 anti-peptide glucagon receptor antibody in 10 ml of TTBS (0.05% (v/v) Tween 20) was incubated with the membrane for 20 min at room temperature in a hybridization chamber._after three washes of 5 min in 10 ml of TTBS, the membrane was incubated with a 1:10,000 dilution of goat anti-rabbit IgG peroxidase in 10 ml of TTBS for 20 min. After an additional three washes in 10 ml of TTBS, immunoreactive bands were visualized by enhanced chemiluminescence treatment (ECL, Amersham Corp.) and exposure to X-OMAT AR film (Kodak).

Binding of [125I]Glucagon to Transfected Cells—Moniodinated [125I]glucagon was obtained from NEN Dupont. Secretin, vasoactive intestinal peptide (VIP), and glucagon were from Sigma and glucagon-like peptide 1 (GLP-1, residues 7–37) was kindly provided by Dr. S. Mojsov. On day 4 after transfection, COS cells were transfected with pMT5 or pMT3 and the Asp⁴ mutant gene constructs were washed once with sodium phosphate-buffered saline, pH 7.4, detached with phosphate-buffered saline containing 1 mM EDTA, centrifuged (400 x g), and resuspended at 4 °C in RPMI 1640 buffer containing 25 μM HEPES, pH 7.4, 1 mg/ml bovine serum albumin, and 1 mg/ml bacitracin. After use, unlabeled glucagon binding was terminated with 100 μM unlabeled glucagon and incubation containing 10⁶ cells were incubated for 60 min at 30 °C with [125I]glucagon (0.5 nM) in the absence or presence of increasing concentrations of unlabeled glucagon in a final assay volume of 200 μl. Cells were subsequently washed three times with 1 ml of cold buffer by filtration on Durapore polycarbonate membrane filters (0.45 μm) and membrane (Millipore). Radioactivity retained on the filters was counted on a Wizard 1470 gamma-counter (Wallac, Inc.). Non-specific binding measured in
the presence of 10 μM glucagon amounted to less than 10% of total counts bound. To test for specificity, increasing concentrations of the peptide hormones secretin, VIP, and GLP-1 were allowed to compete with 125I-labeled glucagon for binding to transfected cells.

Binding of 125I-Glucagon to Transfected COS Cell Membranes—
Plasma membranes were prepared from transfected COS-1 cells as described above. Membrane protein content was determined by a modified Lowry procedure (Markwell et al., 1978). Competitive binding with radiolabeled glucagon on COS cell membranes was performed as described above in 25 mM Tris-HCl, pH 7.4, 1 mg/ml bovine serum albumin, and 1 mg/ml bacitracin. Binding was initiated in a final volume of 200 μl by the addition of membrane suspension containing 40 μg of protein.

| S | G | S | G | L | K | E | C | E | P | S | A | K | T | L | S | A | L | S | L | P | R | L | A | D | S | P | T |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| G | A | S | M | L | Q | L | K | E | C | P | D | G | H | C | T | G | A | P | A | M | S | G | H | S | G | S | A | M | R | E | Q | L | K |
| S | S | G | T | G | C | E | P | S | A | K | T | S | L | S | A | L | S | L | P | R | L | A | D | S | P | T |

Intracellular cAMP Assay—cAMP levels in 100-μl aliquots of transfected COS cell suspensions were determined in triplicate in final assay volumes of 200 μl in RPMI 1640 containing 25 mM HEPES, pH 7.4, 1 mg/ml bovine serum albumin, 1 mg/ml bacitracin. In addition, samples contained 5 mM theophylline (Sigma) either alone or with the indicated concentration of glucagon. The mixtures were incubated at 37 °C for 45 min. The reaction was terminated by the addition of 300 μl of cold ethanol and evaporated to dryness. The residue was resuspended in 100 μl of distilled water and centrifuged. Aliquots (50 μl) of the supernatant fraction were assayed for the presence of cAMP using a method (Amersham Corp.) that measures the ability of cAMP in each sample to compete with [8-3H]cAMP for a high affinity cAMP-binding protein.

Analysis for Competition Binding Assays and cAMP Assay—
Symbols in all figures represent data plotted as the mean of triplicate samples from single experiments. Experiments were repeated on identical frozen samples to verify reproducibility. When appropriate, curve-fitting was carried out to a 4 parameter logistic function (sigmoid) of the form f(x) = [(a - d)/(1 + ([x/c]p) + d)] + a. Values for IC50, EC20, and Kd were determined from the inflection point (c) of the best-fit curve.

RESULTS

Design and Synthesis of a Gene Encoding the Rat Glucagon Receptor—The synthetic rat glucagon receptor gene was designed to encode the reported amino acid sequence (Fig. 1). However, because of codon degeneracy it was possible to introduce a total of 91 unique restriction endonuclease cleavage sites that would facilitate site-directed mutagenesis by restriction fragment replacement. The reported cDNA sequence contained 30 potentially useful unique restriction sites (Jelinek et al., 1993). The gene was synthesized according to a strategy devised by Khorana and co-workers (1978, 1979), which involves the total chemical synthesis of both upper and lower DNA strands. The completed gene was 1472 bp in length and was constructed from three fragments which were cloned independently. The correct nucleotide sequences were confirmed by dideoxy DNA sequencing, and the three fragments were subcloned to assemble the complete gene.

Preparation of Anti-peptide Antibody Directed against the COOH-terminal Tail of the Rat Glucagon Receptor—A rabbit antibody (ST-18) against a synthetic peptide was prepared and purified. The peptide sequence was derived from the COOH-terminal 18 amino acid residues of the rat glucagon receptor. The antibody detected the presence of an immunoreactive band of the appropriate molecular weight in rat hepatocyte prepara-

Expression of the Synthetic Rat Glucagon Receptor Gene in COS Cells—The synthetic rat glucagon receptor gene was expressed in COS-1 cells following transient transfection by a DEAE-dextran procedure. Immunoblot analysis of the synthetic rat glucagon receptor gene expressed in transfected COS cells is shown in Fig. 2. Detergent cell lysates, hypotonic cell lysates, and plasma membrane preparations of transfected cells were prepared. Detergent cell lysates were performed in the presence or absence of protease inhibitors. Lysates of cells transfected with vector containing the synthetic glucagon receptor gene (pMT3) showed a prominent broad band migrating with an apparent molecular mass of 55-75 kDa. A potential glucagon dimer band migrated at about 110 kDa. An additional band migrating at about 35 kDa not seen in the pMT3 lane was...
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**Fig. 2.** Immunoblot analysis of the synthetic rat glucagon receptor gene expressed in transiently transfected COS-1 cells. Detergent cell lysates, hypotonic cell lysates, and plasma membrane preparations were separated by 10% SDS-PAGE, transferred to a membrane, and probed with ST-18 anti-peptide glucagon receptor antibody. Immunoreactive bands were visualized by chemiluminescence. Cells were transfected with vector alone (pMT3) or with vector containing the synthetic glucagon receptor gene (pMT5). The glucagon receptor is visualized as a broad band migrating at an apparent molecular mass of 55–75 kDa. A potential receptor dimer migrates at about 110 kDa. The band at 25 kDa is likely to be due to a cross-reacting COS cell protein. Plasma membrane preparations also contain a broad 55–75 kDa glucagon receptor band, an apparent dimer band at 110 kDa, and the 35 kDa band. However, the 35 kDa band appears relatively less intense. The lanes labeled pMT5 contain plasma membrane from one-tenth and three-tenths of a 100-mm plate of transfected COS cells was loaded in each lane. Lanes containing samples not subjected to N-glycosidase F digestion (−) resulted in the glucagon receptor migrating as shown in Fig. 2. Samples digested with N-glycosidase F (+) resulted in predominantly a single monomer band migrating with an apparent molecular mass of 48 kDa as well as an apparent dimer band. The migration of Kaleidoscope prestained standards (Bio-Rad) are shown to the right of the immunoblot.

**Fig. 3.** Immunoblot analysis of deglycosylated synthetic glucagon receptor in plasma membrane and cell lysate preparations. Plasma membrane and cell lysate preparations from COS cells transfected with pMT5 and pMT3 were digested with N-glycosidase F. Samples were separated by 10% SDS-PAGE, transferred to a membrane, and probed with ST-18 anti-peptide glucagon receptor antibody. Immunoreactive bands were visualized by chemiluminescence. Material from one-tenth of a 100-mm plate of transfected COS cells was loaded in each lane. Lanes containing samples not subjected to N-glycosidase F digestion (−) resulted in the glucagon receptor migrating as shown in Fig. 2. Samples digested with N-glycosidase F (+) resulted in predominantly a single monomer band migrating with an apparent molecular mass of 48 kDa as well as an apparent dimer band. The migration of Kaleidoscope prestained standards (Bio-Rad) are shown to the right of the immunoblot.

Also apparent, the presence of protease inhibitors in the detergent lysis buffer (lanes labeled +I) greatly increased the yield of immunoreactive material relative to the yield in the absence of inhibitors (lanes labeled −I). Lysates of cells transfected with vector alone (pMT3) showed a single band migrating with an apparent molecular mass of 25 kDa. The immunoreactive band at 25 kDa is likely to be due to a cross-reacting COS cell protein. Plasma membrane preparations also contain a broad 55–75 kDa glucagon receptor band, an apparent dimer band at 110 kDa, and the 35 kDa band. However, the 35 kDa band appears relatively less intense. The lanes labeled pMT5 contain plasma membrane from one-tenth and three-tenths of a 100-mm culture plate, respectively. The 25 kDa band is not visualized in the plasma membrane preparations. The molecular weight estimates of the immunoreactive bands were determined relative to the mobility of prescained Rainbow protein molecular weight markers.

Propylene glycol was used to solubilize and store COS-1 cells. Plasma membrane and cell lysate preparations from COS cells transfected with pMT5 or pMT3 were digested with N-glycosidase F. Immunoblot analysis of deglycosylated synthetic glucagon receptor in the plasma membrane and cell lysate preparations is shown in Fig. 3. Lanes containing samples not subjected to N-glycosidase F digestion resulted in the glucagon receptor migrating as shown in Fig. 2. Digestion with N-glycosidase F caused the broad band migrating with an apparent molecular mass of 55–75 kDa to collapse to a single monomer band migrating with an apparent molecular mass of about 48 kDa. The slight difference between the migrations of the plasma membrane and cell lysate bands results from the expression of the cell lysate sample to dodecyl maltoside detergent before treatment with SDS gel-loading buffer. The apparent dimer band also migrated more rapidly after N-glycosidase F treatment. The faint band at 35 kDa was not affected by N-glycosidase F treatment.

Estimation of Level of Expression of Glucagon Receptor in Transiently Transfected COS Cells—The amount of glucagon receptor expressed in COS cells was estimated. A chimeric receptor tagged at the 3'-end with a nucleotide sequence encoding the epitope of an anti-rhodopsin monoclonal antibody, ID4, was constructed (Molday and MacKenzie, 1983). Quantitative immunoblot analysis was carried out on cell lysates transfected with the chimeric receptor using purified bovine rhodopsin as an internal standard. The most precise results were obtained from samples where both the chimeric glucagon receptor and rhodopsin were deglycosylated using N-glycosidase F so that each lane contained essentially only a distinct monomer and dimer band (not shown). The expression level of the glucagon receptor was estimated to be an average of 3.5 x 10^5 receptors/cell, assuming that 100% of the cells were expressing receptor.

Characterization of Binding of Peptide Hormones to the Expressed Glucagon Receptor—Competitive binding specificity of COS cells transfected with the synthetic glucagon receptor gene is shown in Fig. 4. COS cells were transiently transfected with an expression vector containing the synthetic glucagon receptor gene (pMT5). Competitive displacement of 125I-labeled glucagon bound to transfected cells was determined by incubation with 125I-glucagon (0.5 nM) alone and with the indicated concentrations of unlabeled glucagon, and the related peptide hormones GLP-1 (residues 7–37), secretin, and VIP. Cells transfected with control vector pMT3 showed insignificant binding of 125I-glucagon. The displacement curves for both glucagon and GLP-1 fit well to ideal sigmoid curves with four parameters. The concentration of unlabeled glucagon required to displace 50% of receptor-bound 125I-glucagon, the IC50 value, was determined from the curve fit to be 10.8 nM for glucagon and 9.9 μM for GLP-1. Secretin and VIP did not compete with 125I-glucagon for receptor-binding sites.
Indicated concentrations of unlabeled glucagon and the related peptide hormones GLP-1 (residues 7-37), secretin, and VIP. Data are presented as percent of total binding of the radiolabeled hormone versus the log of peptide concentration. Maximum binding (100% on the y axis) was less than 10% of total added radioactivity. Each symbol represents the mean of triplicate determinations and was curve-fitted where appropriate based on a single ligand-binding site model as described under "Experimental Procedures." Cells transfected with control vector pMT3 were transfected with the synthetic glucagon receptor gene. The increase in intracellular cAMP level was determined when cells were incubated with increasing concentrations of glucagon in the presence of 5 mM theophylline. cAMP was quantitated using an assay method that measured the ability of cAMP in each sample to displace \(^{3}H\)cAMP from a cAMP-binding protein. Each symbol represents the mean of triplicate determinations and is plotted as picomoles cAMP/10^6 cells for glucagon and 9.9 \(\mu\)M for GLP-1 based on the fits of the curves shown. Secretin and VIP did not compete with \(^{125}\)I-glucagon for receptor-binding sites.

**Glucagon-dependent Stimulation of Adenylyl Cyclase in Transfected COS Cells**—Adenylyl cyclase activity of COS cells expressing the synthetic glucagon receptor gene is shown in Fig. 5. COS cells were transfected with vector containing the synthetic glucagon receptor gene (pMT5) or with vector alone (pMT3). The increase in intracellular cAMP level was determined when cells were incubated with increasing concentrations of glucagon in the presence of 5 mM theophylline. cAMP was quantitated using an assay method that measured the ability of cAMP in each sample to displace \(^{3}H\)cAMP from a cAMP-binding protein. COS cells expressing the glucagon receptor responded to glucagon with a maximal increase in cAMP levels of approximately six times that in cells transfected with control vector pMT3. The effective concentration at 50% stimulation of adenylyl cyclase (EC_{50}) for cells expressing the glucagon receptor was 0.22 nM as determined by the fit of the curve shown.

**Preparation and Expression of Mutant Glucagon Receptor Genes**—The glucagon receptor mutant genes D64E, D64G, D64K, and D64N were prepared by site-directed mutagenesis of the synthetic gene for the rat glucagon receptor as described under "Experimental Procedures." The location of Asp^64 on the NH_{2}-terminal tail of the receptor is shown schematically in Fig. 1 and in a primary structure alignment in Fig. 6. The genes were expressed in COS cells in parallel with the native gene following transient transfection by a DEAE-dextran procedure.

**Characterization of the Mutant Glucagon Receptors**—Immunoblot analysis of cell lysates of transiently transfected COS cells expressing native or mutant glucagon receptor genes is shown in Fig. 7. Detergent cell lysates were prepared using doceyl maltoside hypotonic buffer from COS cells expressing native glucagon receptor (pMTS), D64E, D64G, D64K, and D64N. Samples were divided and one fraction was treated with N-glycosidase F to remove N-linked carbohydrates. The pattern of immunoreactive bands for the native glucagon receptor (pMT5) is similar to that seen in Figs. 2 and 3. Digestion with N-glycosidase F caused the broad band migrating with an apparent molecular mass of ~55-75 kDa to collapse to a single monomer band migrating with an apparent molecular mass of ~45 kDa. The band at 35 kDa was generally less apparent in this particular preparation. The band patterns of the undigested mutant receptors were similar to each other but differed somewhat from that of the native receptor. The mutant receptors showed four immunoreactive bands that might represent heterogeneity of glycosylation at the four putative N-linked glycosylation sites on the N-terminal tail of the receptor. After N-glycosidase F treatment, each of the mutant receptors showed a single distinct monomer band with the same apparent molecular weight as that of the native receptor. The levels of expression of the mutant receptors were also similar to that of the native receptor as judged by the immunoblot analysis.

Immunoblot analysis of plasma membrane preparations of COS cells expressing native or mutant glucagon receptor genes is shown in Fig. 8. Samples were divided, and one fraction was treated with N-glycosidase F to remove N-linked carbohydrates. The pattern of immunoreactive bands for the native glucagon receptor (pMT5), with or without N-glycosidase F treatment, is similar to that seen in Fig. 3. The band patterns of the mutant receptors were similar to those seen in Fig. 7.
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Fig. 6. Primary structure alignment of related G protein-coupled receptors. The amino-terminal region of the rat glucagon receptor centered around Asp$^6$ is aligned with other related G protein-coupled receptors. Abbreviations are as follows: rGR (rat glucagon receptor) (Jelinek et al., 1993), hGR (human glucagon receptor) (unpublished data; GenBank accession No. L20316), rSRR (rat secretin receptor) (Ishihara et al., 1991), rGLPR (rat glucagon-like peptide 1 receptor) (Thorens, 1992), hVPR (human vasoactive intestinal peptide receptor) (Shreedharan et al., 1993), rCPT (porcine calcitonin receptor) (Lin et al., 1991), oPHTR (opossum parathyroid hormone receptor) (Jappner et al., 1991), mGRFR (mouse growth hormone-releasing factor receptor) (Lin et al., 1993). The numbering is from the deduced amino acid sequence of each reported receptor clone. Amino acid residues Cys$^6$, Asp$^6$, and Trp$^6$ (numbering based on the rat glucagon receptor) are conserved in all receptors listed. ATrp residue is found in all but one receptor at position 70. An Asp to Gly mutation at position 64 was shown to be essential in each case as shown in Fig. 8. Each of the four replacement mutations at Asp$^6$ resulted in the complete inability of the mutant receptor to bind glucagon. The IC$_{50}$ value for inhibition of $^{125}$l-glucagon binding of native receptor in COS cell membranes was 19.7 nM based on the fit of the curve shown.

Fig. 8. Immunoblot analysis of plasma membranes of transiently transfected COS-1 cells expressing native or mutant glucagon receptor genes. Plasma membranes were prepared from COS cells expressing native glucagon receptor (pMT5), D64E, D64G, D64K, and D64N. Samples were divided and one fraction was treated with N-glycosidase F to remove N-linked carbohydrates. Paired samples were subjected to 10% SDS-PAGE, transferred to membrane, and probed with ST-18 antibody. Lanes labeled + were treated with N-glycosidase F and lanes labeled − were untreated. Each lane contains material from one-tenth of a 100-mm culture plate.

Fig. 9. Competition for $^{125}$l-glucagon binding to COS cell membranes expressing native or mutant glucagon receptor genes. Membranes from COS cells transiently transfected with the synthetic glucagon receptor gene (pMT5), D64E, D64N, D64K, and D64G, or vector alone (pMT3) were incubated with radiolabeled glucagon and increasing concentrations of unlabeled glucagon. Immunoblot analysis of each of the samples assayed is shown in Fig. 8. Total radioactivity bound (counts/min) is plotted versus log of glucagon concentration where each symbol represents the mean of triplicate measurements. Each of the four replacement mutations at Asp$^6$ resulted in the complete inability of the mutant receptor to bind glucagon. The IC$_{50}$ value for inhibition of $^{125}$l-glucagon binding of native receptor in COS cell membranes was 19.7 nM based on the fit of the curve shown.

FIG. 7. Immunoblot analysis of cell lysates of transiently transfected COS-1 cells expressing native or mutant glucagon receptor genes. Cell lysates were prepared from COS cells expressing native glucagon receptor (pMT5), D64E, D64G, D64K, and D64N. Samples were divided and one fraction was treated with N-glycosidase F to remove N-linked carbohydrates. Paired samples were subjected to 10% SDS-PAGE, transferred to membrane, and probed with ST-18 antibody. Lanes labeled + were treated with N-glycosidase F, and lanes labeled − were untreated. Each lane contains material from one-tenth of a 100-mm culture plate.

However, the plasma membrane preparations for each of the mutants showed three prominent immunoreactive bands rather than the four bands apparent in the detergent cell lysates (Fig. 7). In addition, the band with the highest electrophoretic mobility was enriched relative to the other two bands.

**Ligand Binding of Mutant Glucagon Receptors—Competition for $^{125}$l-glucagon binding to COS cell membranes expressing native or mutant glucagon receptor genes**

Membranes from COS cells transiently transfected with the synthetic glucagon receptor gene (pMT5), mutant D64E, D64N, D64K, D64G, or vector alone (pMT3) were incubated with radiolabeled glucagon and increasing concentrations of unlabeled glucagon. The levels of receptor were essentially identical in each case as shown in Fig. 8. Each of the four replacement mutants at Asp$^6$ resulted in the complete loss of the mutant receptor ability to bind glucagon. The IC$_{50}$ value for inhibition of $^{125}$l-glucagon binding of native receptor in COS cell membranes was 19.7 nM as determined from the fit of the sigmoid curve plotted in Fig. 9.

**DISCUSSION**

The aim of this work was to develop a system to facilitate structure-function studies of the glucagon receptor and related peptide hormone receptors. The first phase of the work involved the design and synthesis of a gene for the rat glucagon receptor. The synthetic gene consists of a nucleotide sequence that encodes the proper amino acid sequence but contains a relatively large number of unique restriction endonuclease recognition
sites. A synthetic gene is generally useful to facilitate site-directed mutagenesis by restriction fragment replacement and to optimize for a particular expression system based on codon usage or base composition considerations. Synthetic genes have been used successfully for the study of other G protein-coupled receptors, particularly those of the opsin family of receptors (Ferretti et al., 1986; Oprian et al., 1991).

A tentative schematic representation of the rat glucagon receptor primary and secondary structure is shown in Fig. 1. Seven putative transmembrane helices (helix A through helix G) based on previous models of G protein-coupled receptors are shown (Dratz and Hargrave, 1983; Sakmar et al., 1989; Sanderly, 1993). Four sites of potential N-linked glycosylation on the amino terminus are labeled with asterisks (*) in Fig. 1. The COOH-terminal 18 amino acids of the rat glucagon receptor were used to design a peptide, ST-18, for anti-peptide antibody production. A highly specific, high affinity antibody was obtained, which was used for immunoblot analysis of the expression of the glucagon receptor gene and site-directed mutant genes. The immunoblot in Fig. 2 shows that the antibody reacted with the products of expression of the vector containing the synthetic rat glucagon receptor. A band with an apparent molecular mass of 25 kDa was the only background visible in cells transfected with vector alone. The antibody showed affinity for the monomer and dimer forms of the receptor (Fig. 2). As expected, the extent of receptor glycosylation did not affect the affinity of the antibody since the antibody epitope was the COOH-terminal tail of the receptor (Fig. 3). The affinity of the antibody was not precisely quantitated, but we estimate the affinity to be similar to that of the anti-rodopsin monoclonal antibody ID4 for rhodopsin based on immunoblot analyses of a mutant glucagon receptor containing the ID4 epitope sequence added as a tag to the COOH-terminal tail of the glucagon receptor. A more complete description of the properties of this antibody and other antiglucagon receptor antibodies will be reported separately.

High level expression of the synthetic rat glucagon receptor gene in a vector (pMT5) where transcription was under the control of the human adenovirus major late promoter was obtained in transiently transfected COS cells. Immunoblots of COS cell detergent lysates, hypotonic lysates, and plasma membrane preparations are shown in Fig. 2. The major receptor band migrated as a broad band with an apparent molecular mass of 55–75 kDa. This range is consistent with the predicted molecular weight of the receptor (Jelinek et al., 1993) and with the electrophoretic mobility of native receptor labeled with a photoactivatable glucagon analog (Iyengar and Herberg, 1984; Iwanij and Hur, 1985). A second major immunoreactive band probably corresponds to a receptor dimer. The presence of a glucagon receptor dimer was also reported (Iwanij and Vincent, 1990). Some G protein-coupled receptors, such as rhodopsin, have been reported to dimerize readily even under denaturing and reducing conditions (Oprian et al., 1987).

The binding affinity and specificity of the expressed glucagon receptor was evaluated as shown in Fig. 4. COS cells transfected with the glucagon receptor gene bound labeled glucagon, which was competed with unlabeled glucagon according to an ideal four parameter logistic function with an apparent dissociation constant of 10.6 nM. This value is similar to those reported previously under a variety of conditions for native receptor in hepatocytes (Sonne et al., 1978; Bharucha and Tager, 1990) and for an expressed cDNA clone (Jelinek et al., 1993).

As expected, secretin and VIP did not compete with $^{125}$I-glucagon for receptor binding sites (Rodbell et al., 1971; Bataille et al., 1974).

A partial dose-response curve for glucagon-dependent adenyl cyclase stimulation in transfected COS cells is shown in Fig. 5. As expected, treatment of cells expressing the glucagon receptor with glucagon resulted in an increase in cAMP levels compared with control cells. The EC$_{50}$ value of this response was 0.22 nM. This value is similar to those reported previously and is less than the apparent glucagon dissociation constant (Jelinek et al., 1993). This result indicates that the heterologously expressed glucagon receptor gene can couple to endogenous Gs and adenyl cyclase to produce the expected cAMP response to glucagon. A calcium flux in transfected COS cells was also demonstrated in preliminary experiments (not shown), and the complete characterization of this response is the subject of ongoing work.

The molecular basis of the little (lit) mouse phenotype was recently demonstrated (Lin et al., 1993). An Asp to Gly mutation at position 60 in the hypothalamic growth hormone releasing factor (GRF) receptor was shown to correlate to a reduced hormone-dependent cAMP response in transfected cells (Lin et al., 1993). It was postulated that the mutation affected ligand binding properties of the mutant receptor. The GRF receptor is structurally related to the glucagon receptor as shown in a primary structure alignment of related G protein-coupled receptors (Lin et al., 1993) (Fig. 6). The amino-terminal region of the rat glucagon receptor centered around Asp$^{64}$ is aligned with other related G protein-coupled receptors. Asp$^{64}$ in the GRF receptor corresponds to Asp$^{64}$ in the glucagon receptor.

In order to test the role of Asp$^{64}$ in glucagon binding, 4-amino-acid replacement mutants (D64E, D64N, D64K, and D64G) in the synthetic rat glucagon receptor gene were prepared and characterized. The mutant receptor genes were expressed at levels similar to that of the native receptor (Fig. 7). The mutants were also found in the plasma membrane at levels similar to that of the native receptor (Fig. 8). The glycosylation pattern of each of the mutant receptors was somewhat altered as described above. This could be due to the fact that the Asp$^{64}$ residue is located between the four putative glycosylation sites and particularly close to Asn$^{66}$ (see Fig. 1).

As shown in Fig. 9, each of the glucagon receptor mutants with a single amino acid replacement at Asp$^{64}$ failed to bind glucagon. This ligand binding defect could indicate a direct interaction between Asp$^{64}$ and glucagon in the native receptor. However, the same result might be expected if a mutation caused a more general structural perturbation of the extracellular domain of the receptor. In any case, it is likely that the structure of the extracellular domain is important for glucagon binding. These results are also consistent with the explanation proposed for the molecular defect in the GRF receptor (Lin et al., 1993) and with mutagenesis studies of the ligand-binding domains of other related receptors (Braun et al., 1991).

The system described in this report should allow more detailed characterization of the pharmacology of the glucagon receptor using additional receptor mutants in conjunction with glucagon peptide analogs. In addition, it is expected that permanent cell lines expressing the synthetic gene for the rat glucagon receptor will facilitate the biochemical characterization of the dual G protein signaling systems coupled to the receptor.

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Glucagon Receptor Expression