Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 310–315, January 1996
Biochemistry

Antibodies against specific extracellular epitopes of the glucagon receptor block glucagon binding

(peptide/antigen/hormone receptor/G protein/signal transduction)

CECILIA G. UNSON*†, AARON M. CYPESS*, CUI-RONG WU*, PAUL K. GOLDSMITH‡, R. B. MERRIFIELD*, AND THOMAS P. SAKMAR*§

*The Rockefeller University and §Howard Hughes Medical Institute, New York, NY 10021; and ‡Molecular Pathophysiology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20814

Contributed by R. B. Merrifield, September 14, 1995

ABSTRACT **Polyclonal antibodies were prepared against synthetic peptides corresponding to four different extramembrane segments of the rat glucagon receptor. The antibodies bound specifically to native glucagon receptor as judged by immunofluorescence microscopy of cultured cells expressing a synthetic gene for the receptor. Antibodies to peptides designated PR-15 and DK-12 were directed against amino acid residues 103–117 and 126–137, respectively, of the extracellular N-terminal tail. Antibody to peptide KD-14 was directed against residues 206–219 of the first extracellular loop, and antibody to peptide ST-18, against the intracellular C-terminal tail, residues 468–485. The DK-12 and KD-14 antibodies, but not the PR-15 and ST-18 antibodies, could effectively block binding of ^{125}I-labeled glucagon to its receptor in liver membranes. Incubation of these antibodies with rat liver membranes resulted in both a decrease in the maximal hormonal binding capacity and an apparent decrease in glucagon affinity for its receptor. These effects were abolished in the presence of excess specific peptide antigen. In addition, DK-12 and KD-14 antibodies, but not PR-15 and ST-18 antibodies, interfered with glucagon-induced adenylyl cyclase activation in rat liver membranes and behaved as functional glucagon antagonists. These results demonstrate that DK-12 and KD-14 antibodies are pharmacologically active glucagon antagonists and strongly suggest that residues 126–137 of the N-terminal tail and residues 206–219 of the first extracellular loop contain determinants of ligand binding and may comprise the primary ligand-binding site on the glucagon receptor.**

The glucagon receptor belongs to a unique group within a larger family of seven-helix transmembrane receptors that are coupled to one or more intracellular signaling pathways via heterotrimeric guanine nucleotide-binding proteins (G proteins) (1, 2). Members of the glucagon receptor group include receptors for secretin (3), glucagon-like peptide 1 (GLP-1) (4), vasoactive intestinal peptide (VIP) (5), and growth hormone-releasing factor (6). The receptors have a relatively large extracellular N-terminal tail thought to be involved in hormone–receptor interaction (Fig. 1). By mechanisms not yet understood, the signal of hormone binding is conveyed from the cell surface across the transmembrane domain to activate G proteins on the cytoplasmic surface.

Peptide antagonists of glucagon have been developed that effectively compete for receptor sites and have defined several chemical features of the peptide ligand that affect receptor binding and activation (7–9). However, little is known about the corresponding hormone interaction sites on the receptor that regulate recognition and specificity and trigger post-binding biochemical events leading to the biological effect.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

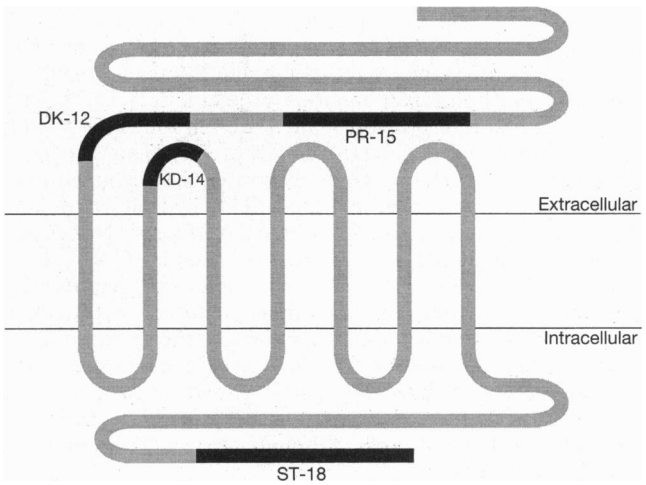

FIG. 1. Schematic representation of the rat glucagon receptor. Seven putative transmembrane helices based on previous models of G protein-coupled receptors are shown. The N terminus and extracellular surface are toward the top, and the C terminus and cytoplasmic surface are toward the bottom of the figure. The locations of the amino acid segments that were used to design the peptides for antibody production are labeled PR-15, DK-12, KD-14, and ST-18 and are not drawn to scale. The peptide sequences are given in Table 1.

A gene encoding the rat glucagon receptor was synthesized and expressed (10, 11). By use of this expression system, an N-terminal deletion mutant and five truncation mutants were prepared to delineate the protein domains required for binding and subsequent transmission of the hormonal message to intracellular effectors (12). Amino acid residues 20–115 were deleted from the N-terminal tail to provide a mutant receptor without asparagine-linked glycosylated sites. In addition, portions of the C terminus of the receptor were progressively truncated to provide two forms of the receptor protein with a shortened cytoplasmic tail and three smaller mutants that consisted of the N terminus followed by one, three, or five transmembrane helices. Results from binding competition studies of these mutants were consistent with the notion that an intact N terminus and contributions from the extracellular loops connecting the transmembrane helices were required for glucagon binding. In contrast, most of the C-terminal tail was not important for ligand binding or for the activation of adenylyl cyclase.

Four polyclonal antibodies to specific sequences from the rat glucagon receptor were prepared and affinity purified to complement mutagenesis studies and to gain further insight

Abbreviations: BSA, bovine serum albumin; GLP-1, glucagon-like peptide 1; VIP, vasoactive intestinal peptide.

†To whom reprint requests should be addressed at: Box 294, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

into glucagon receptor structure and function. The PR-15 and DK-12 antibodies were directed against synthetic peptides with sequences corresponding to amino acid residues 103–117 and 126–137, respectively, of the N-terminal tail. The KD-14 antibody was directed against a synthetic peptide corresponding to residues 206–219 of the first extracellular loop, and the ST-18 antibody was directed against a peptide corresponding to residues 468–485 of the intracellular C terminus (Fig. 1). The DK-12 and KD-14 antibodies effectively blocked ^{125}I-glucagon binding to receptors but did not cause an increase in adenylyl cyclase activity. Moreover, DK-12 and KD-14 antibodies caused an attenuation of the hormone-stimulated adenylyl cyclase response with inhibition indices comparable to those of glucagon antagonists. PR-15 and ST-18 antibodies had no effect on either glucagon binding to receptor or adenylyl cyclase activation. The antibodies detected the products of expression in plasma membranes of monkey kidney cells (COS-1) transfected with a synthetic glucagon receptor gene. These results indicate that residues 126–137 and 206–219—in the N-terminal tail and first extracellular loop, respectively—may play a direct role in glucagon binding.

MATERIALS AND METHODS

Preparation of Antibodies to Glucagon Receptor Peptides. Pentadecapeptide PR-15 (receptor residues 103–117), dodecapeptide DK-12 (receptor residues 126–137), and tetradecapeptide KD-14 (receptor residues 206–219) were assembled stepwise by the Merrifield solid-phase method (13) as previously described for peptide ST-18 (receptor residues 468–485) (11). The peptides were cleaved from the polymer support, analyzed by HPLC and by amino acid analysis, and used without further purification. Rabbits were immunized following peptide conjugation to keyhole limpet hemocyanin (11, 14). Antibody titer was initially monitored by immunoblot analysis of liver plasma membranes subjected to SDS/PAGE. All crude anti-peptide antisera were affinity purified on a column of peptide antigen coupled to agarose (Affi-Gel 15; Bio-Rad), and the affinity-purified antibodies were desalted and concentrated in phosphate-buffered saline, pH 7.4 (Microcon-10; Amicon), before storage at −20°C. Antibody protein concentration was determined by UV absorbance at 278 nm, with rabbit IgG as a standard.

Displacement of ^{125}I-Glucagon from Liver Membranes by Anti-Receptor Antibody. Monoiodinated ^{125}I-glucagon was obtained from NEN/DuPont. Glucagon and rabbit IgG were from Sigma. Plasma membranes were prepared from rat livers (15) and aliquots were stored in liquid nitrogen until use. Membrane protein concentration was determined by a modified Lowry method using bovine serum albumin (BSA) as standard (16). Aliquots of liver cell membrane suspension containing 15 μg of protein in 25 mM Tris·HCl/0.1% BSA, pH 7.4, were preincubated in the absence or presence of various concentrations of PR-15, DK-12, KD-14 or ST-18 antibody, or rabbit IgG, for 2 hr at 4°C. Following the addition of 0.5 nM ^{125}I-glucagon, incubation was continued for 60 min at 37°C in a final assay volume of 200 μl. Membrane-bound radioactivity was separated from free hormone by filtration on Durapore membrane filters (0.45 μm) in a vacuum sampling manifold (Millipore) and the filters were washed three times with 1 ml of cold Tris/BSA buffer. Radioactivity retained on the filters was measured on a 5221 Autogamma scintillation counter (Packard). Nonspecific binding measured in the presence of 4 μM unlabeled glucagon amounted to <10% of total counts bound. Membrane aliquots were tested for sequence-specific reactivity by preincubation for 2 hr at 4°C with various concentrations of antibody in the absence or presence of 25 μM peptide immunogen, followed by competitive binding with ^{125}I-glucagon as described above. As a standard for each assay, a parallel set of membrane samples was also preincubated under the same conditions, followed by continued incubation after the addition of radioligand (0.5 nM) and serial dilutions of unlabeled glucagon. Results were expressed as a percentage of the total ^{125}I-labeled glucagon bound to membranes alone versus the logarithm of glucagon or antibody concentration. Under analogous conditions, inhibition of ^{125}I-glucagon binding by DK-12 and KD-14 in membranes of COS-1 cells after transfection with a synthetic glucagon receptor gene was also assayed (11). All experiments were performed at least three times with duplicate determinations.

Inhibition of Hormone-Stimulated Adenylyl Cyclase. The effect of the antibodies on glucagon-stimulated cAMP production was determined by allowing various concentrations of each antibody to compete with a constant concentration of glucagon. Aliquots of liver membrane suspension containing 10 μg of protein were preincubated for 2 hr at 4°C with various concentrations of antibody in 25 mM Tris·HCl/0.1% BSA, pH 7.4. The mixtures were then incubated for 10 min at 37°C with a constant glucagon concentration (23 nM) in a final assay volume of 100 μl that contained, in addition, 25 mM $MgCl_2$, 0.025 mM GTP, 5 mM ATP, 0.9 mM theophylline, 17.2 mM creatine phosphate, and creatine kinase at 1 mg/ml. The reaction was terminated by immersing the samples in a boiling water bath for 3 min. The membrane protein was pelleted by centrifugation, and 50 μl aliquots of the supernatant fractions were assayed for the presence of cAMP (Amersham). In a parallel experiment, the ability of DK-12 or KD-14 antibody to mimic glucagon-induced adenylyl cyclase activation was also tested and compared with the natural glucagon-dose-dependent cyclase response in liver membranes. Plasma membrane aliquots were incubated with various amounts of DK-12 or KD-14 antibody, and the cAMP produced was determined.

Data for the stimulation of adenylyl cyclase were expressed as percent of maximal cAMP accumulation per milligram of membrane protein and plotted against the logarithm of antibody concentration. The ratio of the concentration of antibody to glucagon when the response was reduced to 50% of the response to 23 nM glucagon in the absence of antibody is the inhibition index, I/A_{50}. The pA_2 value (17), calculated from the dose–response curve, is the negative logarithm of the concentration of ligand that reduces the response of 1 unit of agonist to that of 0.5 unit of agonist.

Enzyme-Linked Immunosorbent Assay (ELISA) of Anti-peptide Antibodies. Reactivity of the purified antibodies with the synthetic peptide antigens and with glucagon was determined by ELISA (18).

Immunofluorescence Microscopy of Transfected COS-1 Cells Expressing Glucagon Receptor. The method used for the imaging of transiently transfected COS-1 cells was previously described (12). All images shown in Fig. 5 were photographed at 1000-fold magnification.

RESULTS

Preparation of Anti-peptide Antibodies. Peptides corresponding to preselected sequences from the rat glucagon receptor were synthesized. PR-15 and DK-12 were derived from amino acid positions 103–117 and 126–137 of the N-terminal tail, KD-14 from positions 206–219 of the first extracellular loop, and ST-18, from the extreme C-terminal tail, positions 468–485. Fig. 1 illustrates the topological location of these peptide epitopes on the receptor. Antibodies were raised in rabbits against each conjugated peptide and affinity purified. The antibodies are designated PR-15, DK-12, KD-14, and ST-18. The DK-12 and ST-18 antibodies were previously shown by immunoblot analysis to bind specifically to the glucagon receptor expressed in COS-1 cells (11, 12). The DK-12 antibody was also used in an immunofluorescence microscopy study of site-directed receptor mutants (12).

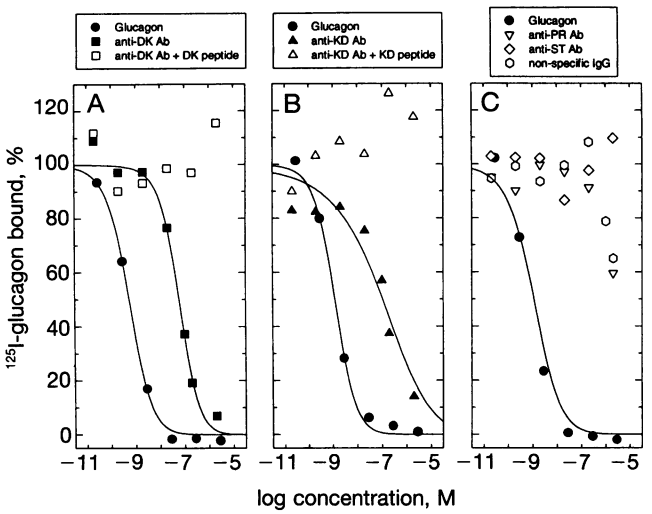

FIG. 2. Inhibition of ^{125}I-glucagon binding by anti-glucagon receptor antibodies. Competitive displacement of ^{125}I-labeled glucagon bound to liver membranes was determined by incubation with ^{125}I-glucagon alone and with the indicated concentrations of unlabeled glucagon or anti-receptor antibody. Data are presented as percent of total binding of the radiolabeled hormone versus the logarithm of glucagon or antibody concentration. Each symbol represents the mean of duplicate determinations and was curve-fitted, where appropriate, according to a single-ligand-binding-site model. Average IC_{50} values from multiple experiments are given in Table 1. (*A*) Inhibition of ^{125}I-glucagon binding by increasing concentrations of unlabeled glucagon (●), DK-12 antibody (■), and DK-12 antibody in the presence of 25 μM DK-12 peptide antigen (□). The results of a single representative experiment are presented. The IC_{50} value for glucagon in this experiment was 0.54 nM. (*B*) Inhibition of ^{125}I-glucagon binding by increasing concentrations of unlabeled glucagon (●), KD-14 antibody (▲), and KD-14 antibody in the presence of 25 μM KD-14 peptide antigen (△). The results of a single representative experiment are presented. The IC_{50} value for glucagon in this experiment was 1.2 nM. (*C*) Inhibition of ^{125}I-glucagon binding by increasing concentrations of unlabeled glucagon (IC_{50} = 1.1 nM) (●), PR-15 antibody (▽), ST-18 antibody (◇), and preimmune IgG (○).

Effect of Antibodies on ^{125}I-Glucagon Binding to Receptors in Liver Membranes. Preincubation of rat liver membranes with each of the affinity-purified antibody preparations was followed by continued incubation with ^{125}I-glucagon. Inhibition of ^{125}I-labeled glucagon binding to liver membrane receptors in the presence of increasing concentrations of DK-12, KD-14, PR-15, or ST-18 antibody or rabbit IgG is shown in Fig. 2. The DK-12 and KD-14 antibodies blocked glucagon binding in a concentration-dependent manner. The binding–displacement curves for both DK-12 and KD-14 fit well to the same logistic function that described glucagon binding (Fig. 2). The concentration of antibody required to inhibit 50% of ^{125}I-glucagon binding (IC_{50} value) was determined from curve fits of multiple independent experiments to be 37 ± 10 nM (mean ± SE, n = 5) for DK-12 and 45 ± 23 nM (n = 4) for KD-14 (Table 1).

Under the same conditions, PR-15 or ST-18 antibody or purified rabbit IgG did not compete significantly with ^{125}I-glucagon binding to receptor sites in liver membranes. Sequence specificity of the inhibitory effect was demonstrated by the observation that the inhibition of ^{125}I-glucagon binding by both DK-12 and KD-14 antibodies was completely reversed in the presence of their respective peptide immunogen (Fig. 2). No reversal was observed in the presence of the other peptide immunogens (data not shown). With the same procedure, DK-12 and KD-14 antibodies also blocked binding of ^{125}I-glucagon to membranes of COS-1 cells expressing receptor after transient transfection with a synthetic glucagon receptor gene (data not shown).

In addition, the effect of DK-12 and KD-14 antibodies on the competition profile for glucagon binding to its receptors in liver membranes was assessed. Competition of ^{125}I-glucagon binding by incubation of liver membranes with unlabeled glucagon in the absence or presence of the indicated concentrations of DK-12 or KD-14 antibodies is shown in Fig. 3. The presence of 31 nM antibody resulted in a decrease in the maximal binding capacity of ^{125}I-glucagon to 30% of the total specific binding measured in the absence of antibody. An increase of the antibody concentration to 125 nM further decreased the maximal glucagon binding to <10% of the total specific binding.

Moreover, when each set of determinations was expressed as the fraction of total radiolabeled ligand bound and plotted against the logarithm of glucagon concentration, the IC_{50} value for glucagon binding increased from about 1 nM in the absence of antibody to >10 nM in the presence of 125 nM DK-12 or KD-14 antibody (data not shown). The apparent shift in the displacement concentration for half-maximal binding of ^{125}I-glucagon by unlabeled hormone in the presence of antibody is consistent with a competitive mechanism of inhibition by the antibody. IC_{50} values were not altered when the same experiments were carried out in the presence of peptide antigen or in the presence of PR-15 or ST-18 antibody (data not shown).

Effect of Antibodies on Activation of Adenylyl Cyclase. Since DK-12 and KD-14 antibodies bound to the glucagon receptor with relatively high affinities, the ability of the antibodies to mimic agonist-stimulated adenylyl cyclase activation, which is triggered upon agonist-ligand binding to receptors, was tested. Concentrations of up to 1.2 μM DK-12 antibody or 52.0 μM KD-14 antibody did not produce a measurable accumulation of cAMP in liver membranes, compared with that produced by increasing amounts of glucagon (Fig. 4).

Table 1. Pharmacological parameters of glucagon receptor antibodies

Antibody		Receptor binding	Inhibition of adenylyl cyclase	
Name	Peptide epitope (location)*	IC_{50},† nM	I/A_{50}‡	pA_2§
PR-15	PDGQWVRGPRGQSWR (103–117)	—	—	—
DK-12	DDEIEVQKGVAK (126–137)	37	34	6.9
KD-14	KIGDDLSVSVWLSD (206–219)	45	61	6.8
ST-18	SAKTSLASSLPRLADSPT (468–485)	—	—	—

*Numbers refer to the amino acid position in the rat glucagon receptor (see Fig. 1).
†Concentration of antibody required to displace 50% of receptor-bound glucagon (see Fig. 2). For DK-12 antibody, 37 nM was the mean of five independent determinations with a standard error of 10 nM. For KD-14, 45 nM was the mean of four determinations with a standard error of 23 nM. The PR-15 and ST-18 antibodies did not inhibit glucagon binding.
‡The inhibition index is the ratio of inhibitor concentration to agonist concentration when the response is reduced to 50% of the response of agonist in the absence of inhibitor.
§Defined in ref. 17.

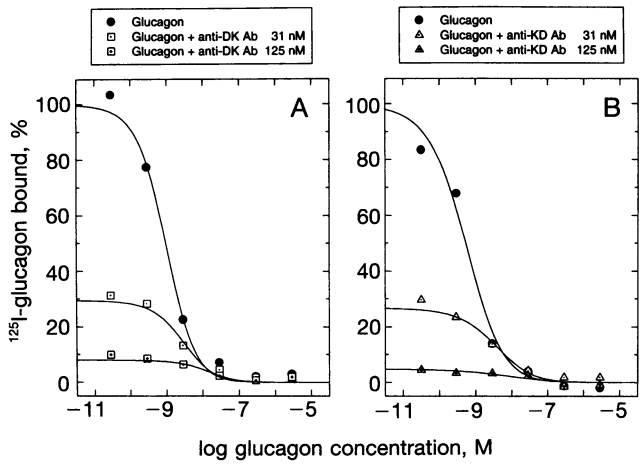

FIG. 3. Competitive displacement of ^{125}I-glucagon in the presence of anti-receptor antibody. Liver membranes were incubated with radiolabeled glucagon and various concentrations of unlabeled glucagon, with or without anti-receptor antibody. The percent of total binding of the radiolabeled hormone is plotted versus the logarithm of glucagon concentration. Each symbol represents the mean of duplicate measurements. (*A*) The maximum binding of ^{125}I-glucagon to liver membranes decreased to 25% in the presence of 31 nM DK-12 antibody and to 6% in the presence of 125 nM DK-12 antibody. The IC_{50} value for inhibition of ^{125}I-glucagon binding (0.98 nM) was increased to 2.9 nM in the presence of 31 nM DK-12 antibody and to 14.4 nM in the presence of 125 nM DK-12 antibody, as discussed in the text. (*B*) The maximum binding of ^{125}I-glucagon to liver membranes decreased to 30% in the presence of 31 nM KD-14 antibody and to 7% in the presence of 125 nM KD-14 antibody. The IC_{50} value for inhibition of ^{125}I-glucagon binding (0.53 nM) was correspondingly increased, as discussed in the text, to 3.6 nM in the presence of 31 nM KD-14 antibody and to 6.7 nM in the presence of 125 nM KD-14 antibody.

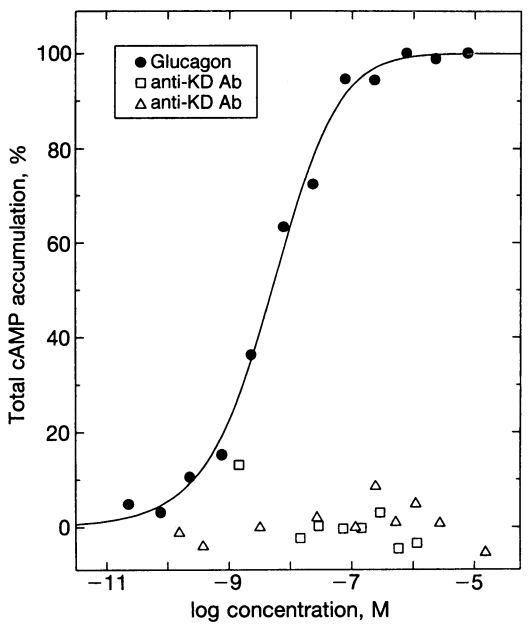

FIG. 4. Glucagon-induced adenylyl cyclase activation. The increase in intracellular cAMP when liver membranes were incubated with increasing concentrations of glucagon was determined. Each symbol represents the mean of duplicate determinations and is plotted as the percent of total cAMP accumulation versus the logarithm of glucagon or antibody concentration. The effective concentration at 50% stimulation of adenylyl cyclase (EC_{50} value) was 5.0 nM. The DK-12 and KD-14 antibodies, which bind to the glucagon receptor and prevent glucagon binding, did not stimulate adenylyl cyclase activity. These antibodies displayed properties of competitive inhibitors of glucagon.

Inhibition of Glucagon-Stimulated Adenylyl Cyclase Activity. Each antibody was also assayed for its ability to inhibit glucagon-dependent adenylyl cyclase activity. The increase in intracellular cAMP produced in liver membranes was determined when a constant amount of glucagon (23 nM) was incubated alone or with increasing concentrations of antibody. The cAMP increase induced by 23 nM glucagon was progressively reduced to near-basal levels by both DK-12 and KD-14 antibodies (data not shown). The concentration of inhibitor required to decrease the response of 23 nM glucagon to 50% was 0.8 μM DK-12 antibody and 1.4 μM KD-14 antibody, which corresponded to inhibition indices (I/A_{50}) of 34 and 61, and pA_2 values of 6.9 and 6.8, respectively (Table 1). The presence of PR-15 or ST-18 antibody did not alter the agonist response elicited by glucagon (data not shown).

ELISA. To test the antibodies for reactivity to their peptide antigens as well as for crossreactivity toward glucagon, an ELISA was performed. The purified antipeptide antibodies bound to their respective peptide antigens, and none of the four antibodies reacted with glucagon (data not shown).

Immunofluorescence Microscopy of Transfected COS-1 Cells. Evidence for the specificity of the antibodies to the native glucagon receptor was demonstrated by immunofluorescence microscopy of transiently transfected COS-1 cells. PR-15, DK-12, and KD-14 antibodies, produced against extracellular epitopes, generated a positive fluorescent signal when incubated with cells transfected with an expression vector containing the synthetic receptor gene under nonpermeabilizing and permeabilizing conditions (Fig. 5). In contrast, ST-18 antibody, which was raised against an intracellular epitope, gave a positive signal only under permeabilizing conditions which permit entry of the primary and secondary antibodies into the cells. Cells transfected with the expression vector not containing the glucagon receptor gene were not stained (data not shown). These results show that the antibodies recognize their specific epitopes, since the expressed receptors are known to be transported to the cell surface (12).

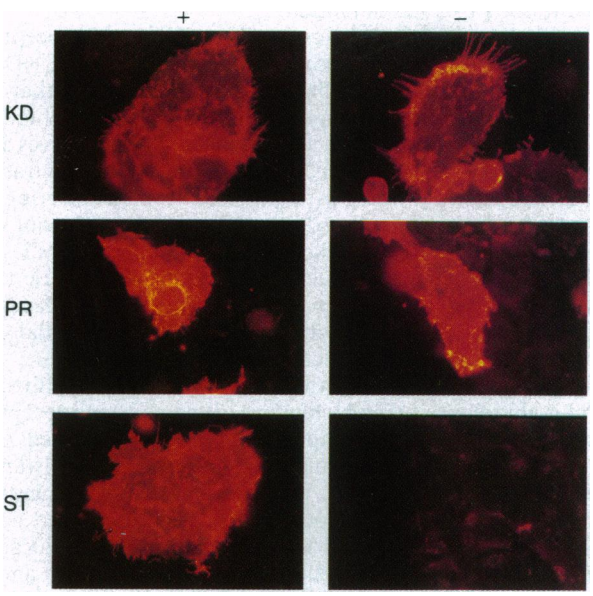

FIG. 5. Immunofluorescence microscopy of COS-1 cells transfected with the glucagon receptor gene. Antibody to receptor peptide KD-14, PR-15, or ST-18 was used as the primary antibody as indicated. A rhodamine-conjugated antibody was used as the secondary antibody. For each primary antibody, permeabilized cells (+) are compared with nonpermeabilized cells (−). The epitopes of KD-14 and PR-15 antibodies are extracellular and the epitope of ST-18 antibody is intracellular (Fig. 1). Immunofluorescence microscopy using DK-12 antibody was previously reported (12). (×1000.)

DISCUSSION

Four polyclonal antibodies were produced against peptides with unique sequences preselected from the rat glucagon receptor protein (Fig. 1). The DK-12 and KD-14 antibodies, directed against the membrane-proximal portion of the extracellular N-terminal tail and the first extracellular loop, respectively, were found to compete with glucagon for binding to liver membrane receptors and to reduce glucagon-mediated production of cAMP to near basal levels. Antibody binding to the receptor, however, did not induce agonist-like activation of adenylyl cyclase. The PR-15 and ST-18 antibodies, directed against a more central segment of the extracellular N-terminal tail and the distal end of the intracellular C-terminal tail, respectively, did not have significant effects on glucagon binding or signal transduction by the receptor.

The peptide epitopes were chosen to fulfill several criteria. First, the secondary structure profiles for the sequences predicted that they should be hydrophilic, surface-exposed, and antigenic (19–21). Second, comparison with the primary structures of other members of the glucagon receptor subclass revealed that these epitopes were unique and had no sequence similarity to equivalent sites in the closely related secretin or GLP-1 receptors. These segments are, however, well conserved in the glucagon receptors of rat (1), human (22), and mouse (23). Finally, the epitope locations in the glucagon receptor were selected on the basis of evidence from several mutagenesis studies of other G protein-coupled receptors that implicated these regions in ligand binding and receptor activation (24–26).

The DK-12 and KD-14 antibodies blocked the binding of ^{125}I-glucagon to membrane-bound receptors in a concentration-dependent manner, exhibiting high affinities for the receptor, with IC_{50} values of 37 nM and 45 nM, respectively (Table 1). The observed inhibition was completely reversed in the presence of peptide antigen. Each antibody recognized only its peptide antigen and no detectable crossreactivity of the antibodies with glucagon was found in an ELISA, thus ruling out the possibility that antibody binding to glucagon contributed to the observed inhibition.

When quantitative glucagon binding affinity to its receptors was determined in the presence of either DK-12 or KD-14 antibody, total specific binding decreased, suggesting that the antibody and glucagon competed for the same ligand-binding site on the receptor (Fig. 3). The glucagon-binding curves also shifted to the right with increasing antibody concentration (data not shown), which is characteristic of a lowering of agonist-binding affinity. In addition, competitive behavior was shown by the antagonist-like response of the antibodies in causing attenuation of glucagon-induced accumulation of cAMP in liver membranes. Indeed, like typical glucagon antagonists (7, 9), the antibody–receptor complex did not stimulate adenylyl cyclase activity (Fig. 4).

Although it is clear that the DK-12 and KD-14 antibodies inhibit glucagon binding by binding to the glucagon receptor, the exact nature of the competition cannot be specified. It is possible that antibody binding causes an effect at a distance, such as steric interference, that prevents glucagon binding. However, the PR-15 antibody, which binds to amino acids 103–117 of the N-terminal tail, had no effect on glucagon binding, whereas the DK-12 antibody, which binds to amino acids 126–137, inhibited glucagon binding. Thus, the PR-15 and DK-12 antibody epitopes seem to delimit a putative glucagon-binding domain that includes at least the N-terminal tail of the receptor distal to position 117. The KD-14 antibody results indicate that the region spanning residues 206–219 in the first extracellular loop is also a component of the glucagon-binding domain.

A composite of recent findings utilizing glucagon/GLP-1 receptor chimeras (27), secretin/VIP receptor chimeras (28), and glucagon receptor mutants (12) also suggests that the glucagon-binding domain may consist primarily of a discontinuous domain that encompasses residues proximal to the point at which the first transmembrane segment begins and includes the first extracellular loop. Other interhelical loops and transmembrane domains are required for proper membrane transport and are likely to provide intraprotein or protein–ligand contacts that stabilize the agonist-bound conformation of the receptor (12).

The DK-12, KD-14, and PR-15 antibodies, raised against extracellular epitopes, gave a positive signal upon immunofluorescence microscopy of COS-1 cells transfected with a synthetic glucagon receptor gene, under both nonpermeabilizing and permeabilizing conditions, but not on untransfected cells (Fig. 5) (12). As expected, the ST-18 antibody, raised against an intracellular epitope, produced a positive signal only under permeabilizing conditions (Fig. 5) (12). Although proteins may be partially denatured during the mild fixation procedure, the results indicate that the extracellular epitopes are exposed on the cell surface and are therefore likely to be accessible to antibody or hormone in membrane preparations or *in vivo*.

Systematic structure–function analysis through the chemical synthesis of peptide analogues has led to potent glucagon antagonists (9, 29). The next step in the rational design of antagonists is the identification of residues in the glucagon receptor that participate directly in ligand binding and dictate strict binding specificity. Further studies employing molecular biology and chemical synthesis are required to verify these potential ligand–receptor interactions.

We wish to thank C. Cheung, B. Yoo, S. Gravina, A. Spiegel, and T. Zvyaga. This work was supported in part by U.S. Public Health Service Grant DK24039. T.P.S. is an Associate Investigator of the Howard Hughes Medical Institute.

1. Jelinek, L. J., Lok, S., Rosenberg, G. B., Smith, R. A., Grant, F. J., Biggs, S., Bensch, P. A., Kuifper, J. L., Sheppard, P. O., Sprecher, C. A., O'Hara, P. J., Foster, D., Walker, K. M., Chen, L. H. J., McKernan, P. A. & Kindsvogel, W. (1993) *Science* **259,** 1614–1616.
2. Segre, G. V. & Goldring, S. R. (1993) *Trends Endocrinol. Metab.* **4,** 309–314.
3. Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K. & Nagata, S. (1991) *EMBO J.* **10,** 1635–1641.
4. Thorens, B. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8641–8646.
5. Ishihara, T., Shigemoto, R., Mori, K. & Nagata, S. (1992) *Neuron* **8,** 811–819.
6. Mayo, K. E. (1992) *Mol. Endocrinol.* **6,** 1734–1744.
7. Unson, C. G., MacDonald, D., Ray, K., Durrah, T. L. & Merrifield, R. B. (1991) *J. Biol. Chem.* **266,** 2763–2766.
8. Unson, C. G., MacDonald, D. & Merrifield, R. B. (1993) *Arch. Biochem. Biophys.* **300,** 747–750.
9. Unson, C. G., Wu, C. R., Fitzpatrick, K. J. & Merrifield, R. B. (1994) *J. Biol. Chem.* **269,** 12548–12551.
10. Carruthers, C. J. L. & Sakmar, T. P. (1995) *Methods Neurosci.* **25,** 322–344.
11. Carruthers, C. J. L., Unson, C. G., Kim, H. N. & Sakmar, T. P. (1994) *J. Biol. Chem.* **269,** 29321–29328.
12. Unson, C. G., Cypess, A. M., Kim, H. N., Goldsmith, P. K., Carruthers, C. J. L., Merrifield, R. B. & Sakmar, T. P. (1995) *J. Biol. Chem.* **270,** 27720–27727.
13. Barany, G. & Merrifield, R. B. (1979) in *The Peptides*, eds. Gross, E. & Meinhofer, J. (Academic, New York), Vol. 2, pp. 1–284.
14. Goldsmith, P. K., Gierschik, P., Milligan, G., Unson, C. G., Vinitsky, R., Malech, H. L. & Spiegel, A. M. (1987) *J. Biol. Chem.* **262,** 14683–14688.
15. Pohl, S. L. (1976) in *Methods in Receptor Research*, ed. Blecher, M. (Dekker, New York), pp. 160–164.
16. Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87,** 206–210.
17. Arunlakshana, O. & Schild, H. O. (1959) *Br. J. Pharmacol.* **14,** 48–58.
18. Harlow, E. & Lane, D. (1988) in *Antibodies: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 553–612.

19. Hopp, T. P. & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3824–3828.
20. Emini, E. A., Hughes, J. V., Perlow, D. S. & Boger, J. (1985) *J. Virol.* **55,** 836–839.
21. Jameson, B. A. & Wolf, H. (1988) *Comput. Appl. Biosci.* **4,** 181–186.
22. MacNeil, D. J., Occi, J. L., Hey, P. J., Strader, C. D. & Graziano, M. P. (1994) *Biochem. Biophys. Res. Commun.* **198,** 328–334.
23. Burcelin, R., Li, J. & Charron, M. J. (1995) *Gene* **164,** 305–310.
24. Hjorth, S. A., Schambye, H. T., Greenlee, W. J. & Schwartz, T. W. (1994) *J. Biol. Chem.* **269,** 30953–30959.
25. Siciliano, S. J., Rollins, T. E., DeMartino, J., Konteatis, Z., Malkowitz, L., Riper, G. V., Bondy, S., Rosen, H. & Springer, M. S. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1212–1218.
26. Jüppner, H., Schipani, E., Bringhurst, F. R., McClure, I., Keutmann, H. T., Potts, J. T., Jr., Kronenberg, H. M., Abou-Samra, A. B., Segre, G. V. & Gardella, T. J. (1994) *Endocrinology* **134,** 879–884.
27. Buggy, J. J., Livingston, J. N., Rabin, D. U. & Heeja, Y.-W. (1995) *J. Biol. Chem.* **270,** 7474–7478.
28. Holtmann, M. H., Hadac, E. M. & Miller, L. J. (1995) *J. Biol. Chem.* **270,** 14394–14398.
29. Unson, C. G., Gurzenda, E. M. & Merrifield, R. B. (1989) *Peptides* **10,** 1171–1177.