Roles of Specific Extracellular Domains of the Glucagon Receptor in Ligand Binding and Signaling†

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ABSTRACT: To identify structural determinants of ligand binding in the glucagon receptor, eight receptor chimeras and additional receptor point mutants were prepared and studied. Amino acid residues 103−117 and 126−137 in the extracellular N-terminal tail and residues 206−219 and 220−231 in the first extracellular loop of the glucagon receptor were replaced with the corresponding segments of the glucagon-like peptide-1 receptor or the secretin receptor. Specific segments of both the N-terminal tail and the first extracellular loop of the glucagon receptor are required for hormone binding. The 206−219 segment of the first loop appears to be important for both glucagon binding and receptor activation. Functional studies with a synthetic chimeric peptide consisting of the N-terminal 14 residues of glucagon and the C-terminal 17 residues of glucagon-like peptide 1 suggest that hormone binding specificity may involve this segment of the first loop. The binding selectivity may arise in part from aspartic acid residues in this segment. Mutation of R-202 located at the junction between the second transmembrane helix and the first loop resulted in a mutant receptor that failed to bind glucagon or signal. We conclude that high-affinity glucagon binding requires multiple contacts with residues in the N-terminal tail and first extracellular loop domain of the glucagon receptor, with hormone specificity arising primarily from the amino acid 206−219 segment. The data suggest a model whereby glucagon first interacts with the N-terminal domain of the receptor followed by more specific interactions between the N-terminal half of the peptide and the first extracellular loop of the receptor, leading to activation.

The glucagon receptor (GR)[1] is a prototype of family B receptors in the GPCR superfamily and includes receptors for related hormones: glucagon, glucagon-like peptide-1 (GLP1), secretin, vasoactive intestinal peptide (VIP), vasoactive intestinal peptide-2 (VIP-2), calcitonin, growth-hormone-releasing hormone (GHRH or GRF), parathyroid hormone (PTH), PTH-related peptide (PTHrP), pituitary adenylyl cyclase-activating peptide (PACAP), gastric inhibitory peptide (GIP), and corticotrophin-releasing factor (CRF) (*1*, *2*). Cross-linking experiments and structure−activity relationship analyses of several family B receptors have mapped the peptide ligand binding site to the extracellular domain of the receptor (*3*−*9*). A common structural feature of the family B GPCRs is a long N-terminal domain of 100−200 amino acids, which contains six conserved cysteines that are presumed to form three disulfide bonds. Expressed and purified N-terminal domains of several receptors, including parathyroid hormone receptor (PTHR), PACAP receptor, and GLP1R, show low-affinity hormone binding, suggesting that the N-terminal domain alone cannot account for the binding affinity of intact receptors (*10*−*12*). In addition, when the entire N-terminal domains of the GR and the GLP1R were exchanged, neither glucagon nor GLP1 bound to the chimeric receptors (*13*). Taken together, existing data suggest that the peptide binding pocket is a discontinuous region consisting of contributions from the long N-terminal extension, the extracellular loops, and some residues at the membrane interface of the transmembrane helices. However, the molecular basis by which these receptors selectively recognize and bind their ligands is still largely unknown.

Extensive studies of glucagon, a hormone peptide of 29 amino acids, primarily using synthetic approaches, have identified several key amino acid residues that are required for GR binding and activation. For example, amino acid residues H-1, D-9, D-15, S-16, and D-21 in glucagon are important for either binding or signal transduction (*14*−*18*). In addition, a full-length peptide is required for full activity. The corresponding complementary sites on the GR, which mediate specificity of hormone recognition and constitute the bimolecular binding interface, have not been identified. A detailed understanding of how glucagon interacts with its receptor to regulate a variety of cellular metabolic pathways

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[1] Abbreviations: GPCR, G-protein-coupled receptor; GR, glucagon receptor; GLP1, glucagon-like peptide-1; GLP1R, glucagon-like peptide-1 receptor; 1e loop, first extracellular loop; 2e loop, second extracellular loop; SR, secretin receptor; PTHR, parathyroid hormone receptor; PACAP, pituitary adenylyl cyclase-activating peptide.

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Table 1: Pharmacological Parameters of GR Chimeras

chimeric receptor mutant[b]	glucagon			chimeric peptide ligand[a]	
	IC_{50}[c] (ligand binding) (nM)	max activity[d] (cAMP increase) (%)	EC_{50}[e] (cAMP increase) (nM)	max activity[d] (cAMP increase) (%)	EC_{50}[e] (cAMP increase) (nM)
wt GR	32 ± 4.7	100	3.2 ± 0.7	100	251 ± 33
C1, GR−GLP1R(103−117)	158 ± 44	67.1 ± 17	16 ± 4.9	80.3 ± 16	199 ± 31
C2, GR−SR(126−137)	794 ± 281	92.3 ± 17	21 ± 3.9	nd[f]	nd
C3, GR−GLP1R(126−137)	141 ± 52	84.7 ± 8.4	18 ± 3.3	62.5 ± 8.5	708 ± 249
C4, GR−SR(206−219)	>1000	8.5 ± 1.7	>1000	nd	nd
C5, GR−GLP1R(206−219)	631 ± 144	50.6 ± 12.3	42 ± 12	135 ± 12	112 ± 39
C6, GR−GLP1R(220−231)	50 ± 14	27.5 ± 2.4	55 ± 20	nd	nd
C7, GR−GLP1R(227−231)	63 ± 8.5	34.3 ± 6.6	59 ± 15	nd	nd
C8, GR−GLP1R(126−137/ 206−219)	>1000	16.6 ± 8.4	501 ± 161	51 ± 16	200 ± 52
wt GLP1R	>1000	23 ± 3.7	>1000	188 ± 36	12.6 ± 1.2

[a] A 31-residue glucagon−GLP1 peptide ligand chimera was synthesized comprising residues 1−14 of glucagon linked to residues 15−31 of GLP1. [b] All chimeric receptors were constructed using GR as the template and replacing the amino acid residues indicated with the corresponding residues from GLP1R or SR as noted. See Figure 1 for a sequence alignment of relevant family B GPCRs. [c] Concentration of unlabeled glucagon required to displace 50% of the receptor-bound [^{125}I]glucagon. Values given are the means ± SEM of at least three independent determinations. [d] Ratio (×100) of the maximum adenylyl cyclase stimulation of the receptor mutant to that of wild-type GR. Values given are the means ± SEM of at least three independent determinations. [e] Effective ligand concentration at 50% stimulation of adenylyl cyclase. Values given are the means ± SEM of at least three independent determinations. [f] nd = not determined.

might lead to the development of pharmacological agents useful in treating diabetes mellitus or other metabolic disorders.

We prepared a series of chimeric receptors in which segments of the extracellular domain of GR were replaced by the corresponding sequences of GLP1R or SR. The pharmacological properties of these receptors were determined using native peptide hormones and a synthetic chimeric hormone that consisted of the N-terminal half of glucagon fused to the C-terminal half of GLP1. Additional GR receptor mutants with single-site or multiple-site replacements were constructed to elucidate the roles of specific binding site residues. Replacement of residues 103−117 and 126−137 in the N-terminal segment, and residues 206−219 in the first extracellular loop (1e loop) of GR, resulted in a significant attenuation of both agonist binding affinity and potency, suggesting that these receptor sequences are important in ligand−receptor interaction. Mutations of the 1e loop region had a more profound effect on both binding and activity than the replacement of either the 103−117 or 126−137 residues in the N-terminal tail. Consistent with tentative models for the binding of other family B receptor ligands (*13*, *19*), the results suggest that glucagon initially engages the N-terminal domain of GR and subsequently interacts with the 1e loop. In addition, a predominantly ionic binding pocket involving charged residues of the 1e loop provides additional stabilization of ligand−receptor interaction that leads to receptor activation.

EXPERIMENTAL PROCEDURES

Materials. The cDNA for the rat GLP1R was kindly provided by Dr. Svetlana Mojsov. [^{125}I]glucagon (507 Ci/mmol) and [^{125}I]GLP1 (2200 Ci/mmol) were from NEN Life Science Products. N-Glycosidase F and endoglycosidase H were from Roche Molecular Biochemicals.

Construction of GR Mutants. Site-directed mutagenesis was carried out using restriction fragment replacement on the synthetic gene for rat GR (GenBank/EMBL U14012) in a modified pGEM2 (Invitrogen) cloning vector (*20*). Oligonucleotides were synthesized on an Applied Biosystems model 392 synthesizer. Nucleotide sequences were confirmed by fluorescence-based dideoxy sequencing (Perkin-Elmer/Applied Biosystems model 377A DNA sequencer). Eight chimeric receptor mutants (C1−C8) were constructed (Table 1). Chimera C1 (GR−GLP1R(103−117)) was prepared by replacing the 75-bp AgeI−MluI restriction fragment of GR with a synthetic duplex encoding amino acid residues 103−117 of the GLP1R (numbering based on GR). Chimera C2 and chimera C3 were constructed by replacing 111-bp MluI−BssHII restriction fragments of GR with synthetic duplexes encoding amino acid residues 126−137 of the rat SR and the GLP1R, respectively. Chimera C4 and chimera C5 were constructed by replacing 81-bp AflII−PstI restriction fragments with synthetic duplexes encoding amino acid residues 206−219 of SR and GLP1R, respectively. Chimera C6 and chimera C7 were constructed by replacing restriction fragments BstXI−PstI and BstXI−SpeI, respectively, with synthetic duplexes encoding amino acid residues 220−231 and 227−231 of GLP1R. Chimera C8 was constructed by replacing both the MluI−BssHII and AflII−PstI restriction fragments with synthetic duplexes encoding amino acid residues 126−137 and 206−219, respectively, of GLP1R. A clone for each mutant with the correct DNA sequence was transferred into the eukaryotic expression vector pMT (*21*).

Construction of Point Mutants. GR mutants K206A, K206D, D210A, D210H, D219Y, D209A/D210A, D209A/D210A/D219A, and D209Q/D210H/D219Y were constructed by replacing restriction fragment BstXI−PstI with synthetic duplexes containing the desired codon alteration(s). R202A and R202D were likewise prepared by substituting an AflII−BstXI fragment with synthetic duplexes containing the desired codon change. A clone for each mutant with the correct DNA sequence was transferred into pMT.

Synthesis of Chimeric Peptide Hormone. A 31-mer glucagon−GLP1 peptide chimera (HSQGTFTSDYSKY-LEGQAAKEFIAWLVKGRG-NH2) was synthesized by solid-phase methods (*22*, *23*).

Characterization of Receptor Mutants. Wild-type GR and mutants were expressed in COS-1 cells by transient transfection with LipofectAMINE (Life Technologies, Inc.). After 48−72 h, the cells were washed with phosphate-buffered

Table 2: Pharmacological Parameters of GR Mutants of the 1e Loop

	glucagon			chimeric peptide ligand[a]	
receptor mutant	IC_{50}[b] (ligand binding) (nM)	max activity[c] (cAMP increase) (%)	EC_{50}[d] (cAMP increase) (nM)	max activity[c] (cAMP increase) (%)	EC_{50}[d] (cAMP increase) (nM)
wt GR	32 ± 4.7	100	3.2 ± 0.7	100	251 ± 33
K206A	126 ± 17	91.6 ± 4.3	4.7 ± 0.2	nd[e]	nd
K206D	35 ± 14.7	99.3 ± 6.8	10 ± 3.9	nd	nd
D210A	251 ± 34	84.5 ± 14.7	2.5 ± 0.7	nd	nd
D210H	20 ± 0.2	92.7 ± 4.3	2.9 ± 1.4	nd	nd
D219Y	158 ± 27	81 ± 8.1	11.2 ± 2.3	nd	nd
R202A	501 ± 116	62 ± 5	22.0 ± 3.1	nd	nd
R202D	>1000	39 ± 4.3	251 ± 40	nd	nd
D209A/D210A	126 ± 17	76.5 ± 7.5	25 ± 7	nd	nd
D209A/D210A/D219A	40 ± 5.3	62 ± 4.7	20 ± 4.6	nd	nd
D209Q/D210H/D219Y	437 ± 85	64.1 ± 8.6	42.6 ± 8.8	133 ± 16	94 ± 5.1
wt GLP1R	>1000	23 ± 3.7	>1000	188 ± 36	12.6 ± 1.2

[a] A 31-residue glucagon−GLP1 peptide ligand chimera was synthesized comprising residues 1−14 of glucagon linked to residues 15−31 of GLP1. [b] Concentration of unlabeled glucagon required to displace 50% of the receptor-bound [^{125}I]glucagon. Values given are the means ± SEM of at least three independent determinations. [c] Ratio (×100) of the maximum adenylyl cyclase stimulation of the receptor mutant to that of wild-type GR. Values given are the means ± SEM of at least three independent determinations. [d] Effective ligand concentration at 50% stimulation of adenylyl cyclase. Values given are the means ± SEM of at least three independent determinations. [e] nd = not determined.

saline, pH 7.4, and harvested. The preparation of membranes was carried out as previously described (*20*, *24*). The membrane protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). N-Glycosidase F digestion to remove N-linked carbohydrates, endoglycosidase H digestion to cleave immature, high mannose N-linked carbohydrates, and immunoblot analyses of expressed GR mutant proteins were carried out essentially as described (*20*, *24*). Immunoblots were scanned, and the intensities of the bands corresponding to deglycosylated receptor protein were analyzed using Scion IMAGE software. Competition binding experiments with [^{125}I]glucagon and adenylyl cyclase activity assays to determine intracellular cAMP levels as a function of glucagon concentration were performed as reported (*20*). Competitive binding studies with [^{125}I]GLP1 were performed in membranes from cells expressing GR−GLP1R chimeric receptors and compared with those in membranes from cells expressing GR or GLP1R. Binding affinity and adenylyl cyclase response to the chimeric glucagon−GLP1 peptide were also assayed in COS-1 cells transfected with GR−GLP1R chimeric receptor genes. All ligand binding and adenylyl cyclase studies were performed at least three times to verify reproducibility on each construct. The data were fit to logistic functions and the IC_{50} and EC_{50} values calculated from the inflection points of the best-fit curves with Sigma Plot version 4 (Jandel Scientific Software).

Immunofluorescence Assays. Circular microscope coverglasses (12 mm diameter) were flame-sterilized and placed in six-well tissue culture dishes. COS-1 cells were plated and transfected with wild-type and mutant receptor genes. After transfection, the cells were fixed, and blocked as previously described (*24*). Following incubation with DK-12 as the primary antibody, the transfected cells were incubated with a secondary antibody, fluorescein isothiocyanate (FITC)-conjugated mouse anti-rabbit IgG (Santa Cruz Biotechnology). The coverslips were mounted on microscope slides, and the cells were viewed with a Zeiss Axiophot fluorescence microscope.

In another experiment, COS-1 cells transiently transfected in tissue culture dishes (100 mm) were harvested with PBS and 1 mM EDTA and treated with primary and secondary antibodies incubated at 1 mg/mL as described above. The cells were washed with PBS and then analyzed using a Becton−Dickinson FACSort flow cytometer. For negative control samples, nonspecific control rabbit antiserum (Sigma) was used as primary antibodies.

RESULTS

Eight chimeric receptors and 10 point mutants of GR were constructed (Tables 1 and 2). In creating the chimeric receptors C1−C7, portions of the N-terminal tail (amino acids 103−117 or 126−137) or the first extracellular loop (1e loop) (amino acids 206−219, 220−231, or 227−231) of GR were replaced by the corresponding amino acid sequences from either GLP1R or SR. An amino acid sequence alignment of the relevant regions is presented in Figure 1A,B. Chimera C8 contained replacements derived from the GLP1R sequence of both regions. These segments were chosen in part on the basis of a previous study using anti-GR antibodies, which blocked glucagon binding and receptor activation (*25*). To examine the importance of the charged residues in the 1e loop, mutant GRs with single or multiple amino acid substitutions were constructed. The locations of the regions in GR that were altered are shown in Figure 1C. All of the receptor chimeras and point mutants were prepared by restriction fragment replacement using a synthetic rat GR gene and heterologously expressed in COS-1 cells following transient transfection (*20*).

Immunoblot analysis of membrane preparations from cells expressing the GR mutants was carried out using ST-18 antibody, which recognizes the extreme C-terminus of GR (*20*). Each GR mutant migrated as a broad band with an apparent molecular mass of 55−75 kDa that collapsed to a band at about 48 kDa after N-glycosidase F treatment (Figure 2). The mutant receptors were insensitive to endoglycosidase H digestion, which confirmed that they were correctly folded and transported to the cell surface (not shown) (*24*). Equal amounts of plasmid DNA (3.5 μg) were used in the transfections, and equal amounts of membrane protein (10 μg) were subjected to glycosidase digestion and immunoblot analysis. The intensities of the immunoreactive 48 kDa band corresponding to the expressed wild-type GR and mutant

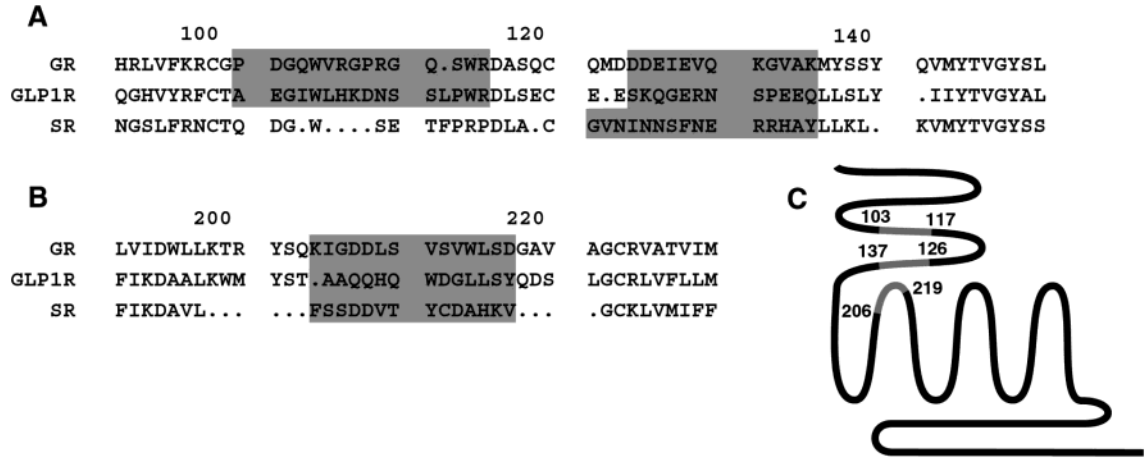

FIGURE 1: Alignment of the N-terminal and 1e loop domains of family B GPCRs GR, GLP1R, and SR and the location of sequences in GR that were replaced with the corresponding GLP1R and SR sequences. (A) Partial sequences from the N-terminal tail of GR, GLP1R, and SR were aligned. The shaded area shows residues 103−117 and 126−137 of GR that were replaced by corresponding residues from GLP1R and SR. (B) The shaded area shows residues 206−219 of the 1e loop that were replaced by sequences from GLP1R and SR. (C) Schematic representation of the rat glucagon receptor secondary structure showing the extracellular domains that were altered by mutagenesis to produce receptor chimeras. Sequences in the N-terminus and in the 1e loop were replaced with sequences from GLP1R or SR to give receptor chimeras (Table 1).

receptors were compared and normalized to the maximal GR value. In general, the GR chimeras were expressed at similar levels with slightly lower efficiency compared with wild-type GR, and on the basis of band intensities consistently varied from GR with a less than 10% difference (Figure 2).

To confirm that the receptor mutants that either fail to bind glucagon or exhibit attenuated binding affinites are expressed on the cell surface, we used DK-12 antibody to detect expression by indirect immunofluorescence. DK-12 is an antibody generated against the peptide sequence 126−137 of the extracellular region of rat GR and has been used to detect correct processing of mutant GRs to the plasma membrane of transfected cells by fluorescence microscopy (*24*, *25*). Cells expressing mutant receptors with the extracellular epitope (C4, C5, R202D, and D209Q/D210H/D219Y) showed a fluorescence staining pattern similar to that of cells expressing wild-type receptor (not shown), consistent with the results obtained from glycosidase digestion and immunoblot analysis. Fluorescence staining was not detected in nontransfected cells. In addition, flow cytometry analysis was performed on similarly stained transfected cells. Plots of relative fluorescence intensity against cell number showed that the percent of positively-fluorescent-stained cells expressing receptor mutant was within 10% of that of the wild-type receptor.

Pharmacological Characterization of Chimeric Receptors. In competitive binding experiments with [^{125}I]glucagon (0.13 nM), the expressed GR gave an IC_{50} value of 32 nM. The EC_{50} value for glucagon-dependent adenylyl cyclase stimulation was 3.2 nM. Both values were consistent with previous reports for expressed GR (Table 1) (*26*, *27*). For each of the mutant receptors there was good correlation between the relative affinity of glucagon binding and the relative potency of glucagon-dependent adenylyl cyclase stimulation. As expected, a decrease in glucagon binding affinity was accompanied by a decrease in the relative ability to activate adenylyl cyclase, indicating that the effect of the mutation on glucagon binding was functionally linked to receptor activation. In general, the chimeric receptor mutants bound glucagon and retained the ability to stimulate adenylyl cyclase activity, suggesting that the tertiary structures of the mutants were maintained despite the sequence modifications. However, certain regions appeared to be more functionally sensitive to alteration than others. Within a range of receptor expression, the maximum response and potency of cAMP production have been shown to be unaffected by the amount of DNA used over a wide range, except when very low amounts of DNA (0.25 μg) were used for transfection (*28*, *29*).

Replacements of the N-terminal region of GR with the analogous residues of the GLP1R in chimera C1 and chimera C3 led to 4−5-fold decreases in glucagon binding affinities and concomitant decreases in the potencies of adenylyl cyclase activation (Figures 3 and 4, Table 1). More significantly, replacement of residues 206−219 of the 1e loop of GR with the corresponding residues in GLP1R in chimera C5 resulted in an 18-fold reduction of glucagon binding affinity and a comparable decrease in the potency of activation (Figures 3 and 4, Table 1). Not surprisingly, the combined substitution of both the 126−137 and 206−219 segments with residues from the GLP1R in the chimera C8 completely abolished its ability to bind glucagon and severely diminished cAMP production (Figures 3 and 4, Table 1). The mutation of both regions led to a greater loss of function than the additive effect of each single mutation (chimera C3 and chimera C5), suggesting a cooperative effect of both regions in glucagon binding.

Replacements of the N-terminal region or 1e loop of GR with the analogous residues of the SR in chimera C2 and chimera C4, respectively, caused more severe loss of function. Chimera C2 displayed a 19-fold decrease in glucagon binding affinity and a 6-fold decrease in potency of adenylyl cyclase activation. Moreover, parallel to the effect of GLP1R-derived replacements of the 1e loop, the chimera C4, bearing the analogous residues from the 1e loop of SR, showed almost a complete loss of glucagon binding and glucagon-dependent adenylyl cyclase stimulation (Table 1). Replacements of portions of the 1e loop of GR with the

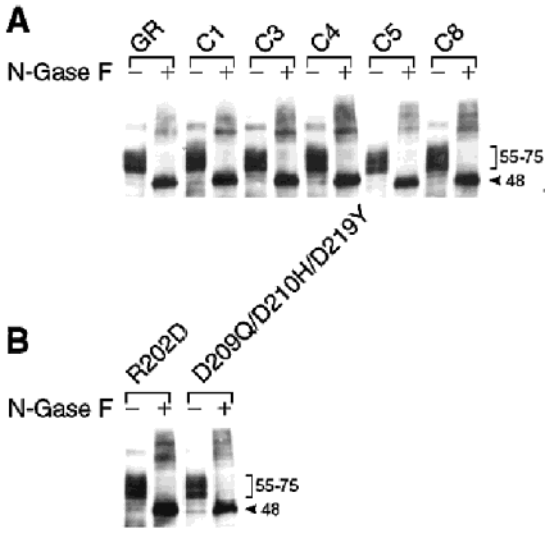

FIGURE 2: Immunoblot analysis of receptor mutants expressed in transiently transfected COS-1 cells. Membranes from COS-1 cells that had been transfected with GR, chimeric mutants, and point mutants were treated with N-glycosidase F (N-Gase F) to remove N-linked carbohydrates. Samples (10 μg of protein per lane) were separated by SDS−PAGE, transferred to Immobilon-P membranes, and probed with anti-GR ST-18 as described in the Experimental Procedures. Immunoreactive bands were visualized by chemiluminescence (ECL). Lanes labeled "−" and "+" correspond to samples untreated and treated with N-Gase F. (A) shows immunoblots of GR and mutant chimeras C1, C3, C4, C5, and C8. (B) shows immunoblots of point mutants R202D and D209Q/D210H/D219Y. In all the lanes labeled "−" GR and GR mutants were visualized as a broad band at an apparent molecular mass of 55−75 kDa. In lanes labeled "+", GR and GR mutants deglycosylated by treatment with N-Gase F migrated with an apparent molecular mass of about 48 kDa. The intensities of the 48 kDa bands corresponding to mutant proteins were analyzed and compared as percentages of the wild-type GR band intensity. Higher molecular weight bands appeared to be dimers of the GR or GR mutants. Dimerization of GR was described previously (*20*). The arrows labeled "48" indicate unglycosylated receptor, and the brackets labeled "55−75" indicate the broad band arising from glycosylated forms of the receptor, the numbers 55−75 being apparent molecular mass indicators in kilodaltons.

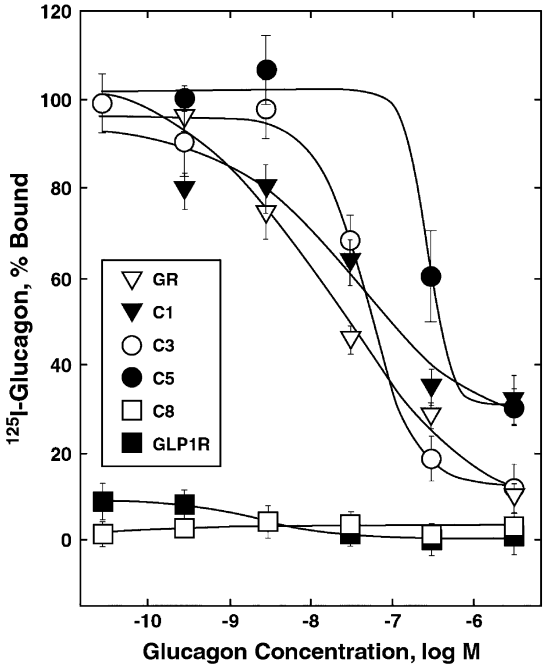

FIGURE 3: Competitive displacement assay of [^{125}I]glucagon binding to COS-1 cell membranes expressing GR and GR mutant chimeras. Membranes from COS-1 cells expressing GR and GR mutant chimeras were incubated with [^{125}I]glucagon and the indicated concentrations of unlabeled glucagon as described in the Experimental Procedures. Data are presented as percentages of the total binding of radiolabeled hormone versus the log of glucagon concentration. Each symbol represents the mean of duplicate determinations. The concentration of unlabeled glucagon required to displace 50% of the receptor-bound [^{125}I]glucagon (IC_{50}) was calculated from the curve (Table 1) and represents the average of at least three independent determinations. COS-1 cells transfected with GLP1R and the double chimeric receptor C8 failed to bind glucagon.

analogous residues of the GLP1R in chimera C6 and chimera C7 did not cause significant effects on glucagon binding. However, the mutations caused a 15−17-fold loss in the potency of glucagon-dependent adenylyl cyclase stimulation, indicating that glucagon binding and receptor activation were uncoupled by these mutations (Table 1). Together these results confirm that the glucagon binding pocket comprises at least portions of amino acid residues 103−117 and 126−137 of the N-terminal tail and residues 206−219 of the 1e loop. However, mutation of the 206−219 segment in the 1e loop region had more of an adverse effect on both glucagon binding and glucagon-dependent signaling than replacement of either the 103−117 or the 126−137 sequence in the N-terminal tail, implying that the 1e loop may contain specific determinants of ligand binding.

Pharmacological Characterization of Point Mutants of GR. The first half of the 1e loop sequence of GR contains five charged residues: R-202, K-206, D-209, D-210, and D-219. To investigate the possibility that these residues might interact with glucagon, 10 point mutants of GR were constructed to define the role of charged residues in the putative binding pocket: R202A, R202D, K206A, K206D, D210A, D210H, D219Y, D209A/D210A, D209A/D210A/D219A, and D209Q/D210H/D219Y. The pharmacological parameters of these expressed GR mutants are listed in Table 2.

R-202 and K-206 in GR do not align with positively charged residues in the GLP1R or SR. Neutralization of the charge in mutant K206A reduced the glucagon binding affinity 3-fold, but the receptor was still capable of maximal glucagon-dependent signaling. The inverse effect was observed when the charge was reversed in mutant K206D. In contrast to K-206, the positive charge at R-202 appeared to be critical for both glucagon binding and receptor activation. The mutation R202A caused a 15-fold reduction of glucagon binding affinity and a 6-fold loss of potency relative to those of GR (Table 2). Reversal of charge in the mutant R202D resulted in a complete loss in ligand binding affinity and a greatly attenuated adenylyl cyclase activity. Both immunoblot analysis (Figure 2B) and immunofluorescence microscopy showed that R202D was fully processed to the cell membrane surface.

The mutant D210A displayed an 8-fold decrease in glucagon binding affinity, but was still able to cause full glucagon-dependent adenylyl cyclase stimulation. The phenotype of mutant D210H was indistinguishable from that of wild-type GR. D219Y displayed a 3−5-fold decrease in relative potencies of glucagon binding and glucagon-dependent adenylyl cyclase activation. The receptor mutant D209A/D210A/D219A, in which all three aspartic acid residues were

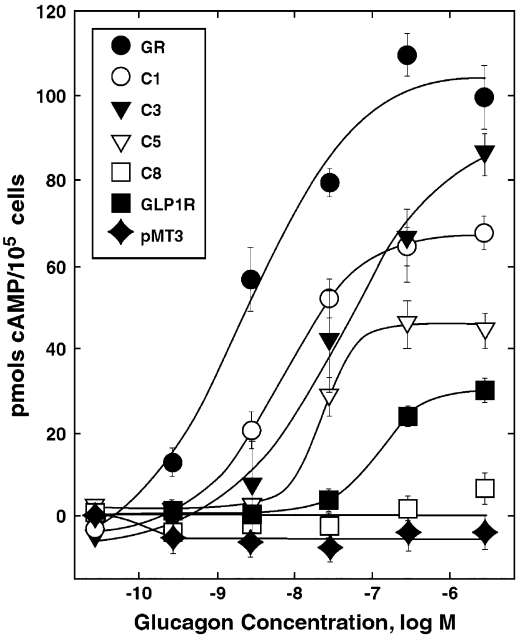

FIGURE 4: Adenylyl cyclase activity of COS-1 cells expressing GR and receptor chimeras. COS-1 cells were transiently transfected with GR, receptor chimeric genes, or vector and incubated with increasing concentrations of glucagon as described in the Experimental Procedures. Cell extracts were assayed for cAMP using a method that measures the ability of cAMP in each sample to compete with [8-^{3}H]cAMP for a high-affinity cAMP binding protein. The number of picomoles of cAMP produced by 10^5 transfected COS-1 cells is plotted as a percentage of the maximum response versus the log of peptide concentration. Each symbol represents the mean from duplicate determinations, and the curves represent at least three independent experiments. Experiments were repeated at least three times to verify reproducibility. COS-1 cells transfected with vector and chimera C8 failed to activate adenylyl cyclase upon treatment with glucagon.

substituted with neutral alanines, displayed near normal affinity for glucagon, but signaled with a 6-fold lower potency when compared with GR (Table 2). Alanine substitution of two of the three aspartic acids in mutant D209A/D210A caused a 4-fold loss in binding and a 7-fold decrease in signaling potency (Table 2). Because the negatively charged residues of the 1e loop are not conserved between GR and GLP1R, a triple point receptor mutant, D209Q/D210H/D219Y, in which each aspartic acid of the 1e loop of GR was replaced by its counterpart in GLP1R was constructed. Mutant D209Q/D210H/D219Y displayed a 13-fold reduction of both glucagon binding affinity and potency of glucagon-dependent adenylyl cyclase stimulation, which was comparable to the loss of activity displayed by the receptor chimera C5 (Table 1). Flow cytometry analysis and immunoblot analysis (Figure 2) indicated that both receptor mutants were expressed at similar levels. Immunofluorescence microscopy revealed similar staining patterns.

Studies with the Glucagon−GLP1 Peptide Chimera. In general, receptor chimeras containing 1e loop sequences from GLP1R remained responsive to glucagon, but chimera C4 containing the 1e loop sequence from SR did not. This result suggested that glucagon might recognize structural patterns in the sequence from the 1e loop of GLP1R that were not contained in the analogous SR sequence. However, none of the GR−GLP1R chimeras bound [^{125}I]GLP1, nor did they stimulate increased cAMP production in the presence of GLP1 (data not shown). A dual-chimeric approach was employed to address the possibility that the homologous peptide hormones glucagon and GLP1 might use the same regions in their respective receptors. A peptide ligand chimera was synthesized comprising the N-terminal 1−14 residues of glucagon linked to the C-terminal 15−31 residues of GLP1. This chimeric hormone was reported to possess the chemical features necessary for selective recognition by both receptors (*23*). The chimeric glucagon−GLP1 peptide ligand activated expressed recombinant GLP1R with an EC_{50} value that was 20 times better than that for the activation of expressed GR (Tables 1 and 2). This result correlated well with values previously reported for the affinity of the chimeric hormone to both receptors in hepatocyte membranes (*23*). The glucagon−GLP1 chimeric hormone activated the GR−GLP1R 1e loop chimera, C5, with potency about 2-fold higher than that of wild-type GR (Table 1). The triple mutant D209Q/D210H/D219Y, in which the three aspartic acids from the GR 1e loop were substituted by their GLP1R counterparts, was activated by the peptide chimera with efficacy similar to that of chimera C5 (Table 2). Interestingly, both receptor mutants also exhibited a higher maximum response to glucagon−GLP1 hybrid-dependent stimulation of adenylyl cyclase than wild-type GR (Tables 1 and 2). While receptor chimera C1 containing residues 103−117 from the N-terminal tail of GLP1R showed a slight increase in potency, chimera C3 containing residues 126−137 from the N-terminal tail of GLP1R did not display an increase in the potency of chimeric hormone-mediated activation (Table 1). These results support the notion that both glucagon and GLP1 must associate with specific residues in the 1e loop of their respective receptors during binding and activation.

DISCUSSION

Glucagon is a prototypical ligand for family B GPCRs (*2*). Extensive structure−activity relationship studies have singled out specific chemical features of glucagon that affect receptor binding and activation, but the entire full-length molecule appears to be required for full agonist activity. Since glucagon consists of 29 amino acids, its contact site in GR has been presumed to be a discontinuous domain on the extracellular surface of GR, with contributions from its long N-terminal extension and extracellular loops connecting the transmembrane helices. The glucagon binding domain was more precisely mapped using anti-GR antibodies generated against synthetic peptides corresponding to specific receptor sequences. Antibodies DK-12 and KD-14 generated against synthetic peptides with amino acid sequences corresponding to residues 126−137 of the N-terminal tail and 206−219 of the first extracellular loop, respectively, competed with glucagon for receptor binding sites (*25*). In contrast, ST-18 antibody raised against an 18-residue peptide from the C-terminal end of GR located in the cytoplasmic domains had no effect on the binding of glucagon. DK-12 and KD-14 also behaved like glucagon antagonists, preventing glucagon-dependent activation of adenylyl cyclase (*25*). These results suggested that the amino acid residues 126−137 and 206−219 of GR might be components of the glucagon binding pocket (*25*). Since peptide ligands of family B GPCRs share significant structural and conformational homology, this suggestion has been supported by photo-

labeling experiments and receptor mutagenesis analyses of various family B members (*4*−*9*, *30*−*32*).

To investigate the functions of specific regions of the extracellular domain of GR, we prepared a series of chimeric receptors in which portions of GR were replaced by the corresponding amino acid residues from GLP1R or SR. GLP1R and SR were chosen because they are most closely related to GR among family B GPCRs, sharing 48% and 43% sequence identity with GR, respectively. Residues 103−117 and 126−137 in the N-terminal tail and 206−219 in the 1e loop of GR were targeted for study. Despite their overall degrees of sequence similarity, the receptors are highly divergent in these regions (Figure 1A,B). Therefore, we hypothesized that the chimeras might provide information related to the determinants of peptide ligand binding selectivity. The chimeras exhibited a decrease in affinity for glucagon, which was paralleled by a loss in potency of activation (Table 1).

The putative 145-residue N-terminal domain of GR contains six cysteine residues, which are strictly conserved within family B members and are presumed to form three specific disulfide bonds. The precise disulfide connectivity was determined for the N-terminal fragment of human PTHR (*11*). Mutagenesis studies of family B GPCRs have shown direct involvement of this domain in peptide ligand binding. For example, we reported that a GR mutant with a 96-residue deletion from the N-terminal tail, including five of the six conserved cysteine residues, was expressed on the cell surface but failed to bind glucagon, emphasizing that the integrity of the tertiary structure was crucial for high-affinity glucagon binding (*24*). An antibody raised against a peptide corresponding to residues 103−117 did not block glucagon binding, but this sequence may be positioned in close proximity to residues 126−137 as a consequence of tertiary structure and disulfide bond formation (*25*). This may account for the observation that replacement of sequences 103−117 and 126−137 in chimeric receptors C1, C2, and C3 produced decreases in ligand binding affinities and potencies of activation. Because the six cysteine residues are conserved, the general tertiary structure of the N-terminal domain might be maintained despite other differences in primary structure. Although replacement of residues 126−137 with sequences from SR (C2) was much less tolerated than replacement of either residues 103−117 (C1) or residues 126−137 (C3) with sequences from GLP1R, glucagon binding was not completely blocked in any of the three chimeric receptors (Table 1).

The N-terminal fragments of PTHR, PACAP receptor, and GLP1R have been expressed either in E. coli in the form of inclusion bodies or in secreted form from COS-7 or Sf9 cells (*11*, *12*, *33*). In all cases, although the purified domains bound peptide hormone, the binding affinity was sufficiently weak to suggest that the N-terminal domain of family B receptors alone cannot account for the binding affinity of the intact receptor. Our observation that the 206−219 region of the 1e loop of GR was far more sensitive to alteration than the sequences from the N-terminus further supports these findings. In contrast to the chimeras involving the N-terminal domain, greater than 95% of the receptor function was lost in C5 (GR−GLP1R(206−219)), and an almost complete loss was observed in C4 (GR−SR(206−219)). The combined replacement of the 126−137 and 206−219 segments in receptor chimera C8 also led essentially to a complete loss of function.

Residues 206−219 are most likely located in the central portion of the 1e loop (Figure 1C). Replacement of the adjacent 220−231 residues that extend to the border of transmembrane helix 3 with the corresponding sequence from GLP1R in chimeras C6 and C7 did not seriously impair high-affinity ligand binding. Thus, the central portion of the 1e loop is important in glucagon binding, but residues 220−231 near helix 3 are not. A conserved cysteine in this segment (C-225) is likely to be involved in a disulfide bridge with C-295 of the second extracellular loop (2e loop). A disulfide bond appears to be conserved at this position in both family A and family B GPCRs (*34*). In the crystal structure of rhodopsin, the analogous disulfide bridge moves the 2e loop away from the surface and specifically stretches that segment of the 2e loop over a crevice between transmembrane helices (*35*, *36*). Similarly, a putative disulfide bridge in GR between C-225 of the 1e loop and C-295 of the 2e loop constrains the 220−231 segment of the 1e loop and might pull it toward the closely packed region above the helix bundle, an area not easily accessible to a peptide ligand the size of glucagon. However, the resulting receptor conformation could define a specific path of access to a binding site in the bilayer.

Interestingly, while chimeras C6 and C7 retained glucagon binding affinities similar to that of wild-type GR, both mutants exhibited a 15−18-fold loss in the ability to stimulate signal transduction (Table 1). A disproportionate change in agonist potency versus binding affinity indicates uncoupling of ligand binding and receptor activation. The 220−231 region might contribute less directly to ligand binding affinity while providing molecular contacts for glucagon-induced receptor activation. It is plausible that, following the initial binding event, the transmembrane helices and loops reorganize to accommodate the peptide. Movement of the extracellular flap might expose the 220−231 segment of the 1e loop, which either directly provides contact points or facilitates subsequent interactions that lead to signal transduction.

Five charged amino acid residues in the 1e loop are unique to GR and were studied in detail by site-directed mutagenesis. Aspartic acids at positions 209, 210, and 219 were simultaneously altered to the corresponding amino acids in GLP1R. The resulting mutant receptor D209Q/D210H/D219Y displayed a loss of function phenotype similar to that of 1e loop receptor chimera C5 (Table 2). A triple alanine mutant, D209A/D210A/D219A, retained good binding affinity but exhibited a 6-fold decrease in the potency of activation. Initial attempts to sort out the individual contributions of the D-209 and D-210 residues were not informative and netted mixed results since neither of the single amino acid substitutions caused a significant effect. D219Y and the double alanine mutant D209A/D210A showed definite effects. Therefore, the adjacent D-209 and D-210 residues appear to be functionally redundant. This observation further underscores the importance of the 206−219 segment of the 1e loop. Because replacement of the aspartic acid residues with neutral alanine residues was well tolerated, a negatively charged 1e loop may not be critical for high-affinity binding. However, the K206D mutant displayed a normal phenotype, which suggests that a net negative charge stabilizes the interaction of the ligand with this region.

In contrast, there is a critical requirement for a positive charge at the extracellular border of transmembrane helix 2 at a junction with the 1e loop. Replacement of R-202 with a negatively charged aspartic acid in R202D resulted in a complete loss of receptor function (Table 2). Although it is likely that an arginine at this position is required to define the correct receptor topology within the membrane bilayer, it is possible that replacement of R-202 prevented a critical association with negatively charged residues of glucagon. We and others have identified amino acid residues H-1, D-9, D-15, S-16, and D-21 in glucagon as important for either binding or transduction (*14*−*18*). Any of these amino acids could contribute to glucagon binding energy through ionic or H-bond interactions with R-202. In VIP, D-3 was shown to contact an arginine and a lysine residue at analogous positions at the extracellular end of transmembrane helix 2 of the VIP receptor (*37*). Alternatively, arginines can interact with aromatic side chains of phenylalanine, tyrosine, or tryptophan through cation−π-orbital interaction. This potent noncovalent binding force can occur at or near the surface of proteins and contribute to ligand receptor interactions (*38*−*40*). R-202 might fill a pocket formed by aromatic rings in a stabilizing cation−π-orbital interaction. A recent structural study of PACAP peptide bound to its receptor identified a unique β-coil in PACAP induced upon binding (*41*). This compact β-coil structure produces a hydrophobic patch composed of the side chains of I-5, F-6, and Y-10. On the basis of the high sequence homology between glucagon and PACAP, glucagon would be expected to present a similar conformation at the binding site that could also involve a neighboring Y-13. Indeed, earlier structure−activity studies proposed a role for a hydrophobic cluster in the structure of glucagon and emphasized that proper stacking of F-6, Y-10, and Y-13 was important for binding and transduction (*42*−*44*).

Tentative models have been proposed for the binding of family B receptors to their peptide ligands (*13*, *19*). The models advocate that binding of the peptide ligand to its receptor is kinetically favored when it occurs in two steps, presumably at two sites on the receptor. In the PACAP binding model, a largely helical C-terminus first associates nonspecifically with the membrane surface until the N-terminus finds its target receptor (*41*). Conformational changes restricted to the N-terminal 1−7 residues are initially induced by hydrophobic interactions between ligand and receptor. Study of the PTH receptor system has further corroborated a two-site mode of ligand recognition (*45*, *46*). The C-terminal portion of the ligand binds the extracellular N-terminal domain of the receptor, and the N-terminal ligand portion binds to the juxtamembrane receptor domain. Our results are consistent with these general two-site models for the binding of peptide ligands to family B GPCRs. The more hydrophobic C-terminal portion of glucagon first interacts with the N-terminus at the surface of the membrane bilayer and draws the peptide to the second site of interaction, the 1e loop, where additional favorable interactions provide the stabilization energy that leads to activation. In particular, the dual-chimeric approach in which receptor chimeras were assayed with a glucagon−GLP1 chimeric hormone suggests specific similarities between glucagon and GLP1 binding mechanisms. Glucagon apparently recognized a structural motif in the GLP1R 1e loop chimera that was not contained in an analogous sequence of SR. This result supports the idea that the 1e loop contains supplementary determinants for selective receptor−ligand recognition. Moreover, the apparent shifts to increased potency relative to that of wild-type GR of the chimeric peptide on the 1e loop chimeric receptors point to a requirement for both glucagon and GLP1 to associate with the 1e loop in the mechanism of activation. The binding affinities of the receptor mutants for the peptide chimera should closely parallel the activation data and are consistent with the observation that selective recognition of GR and GLP1R is determined by residues located at opposite ends of the homologous peptide (*23*). Recombinant GR may have lost affinity and potency for the peptide chimera which does not have the cognate C-terminal sequence. Tethering of the C-terminus is the putative first step in the activation mechanism of family B GPCRs (*13*, *47*). The 1e loop receptor chimera contains elements of both GR and GLP1R that bind to the amino terminus of the ligand and trigger the subsequent step of receptor stimulation.

In summary, our results suggest that glucagon initially engages the N-terminal domain of GR and subsequently interacts with a predominantly ionic binding pocket of the 1e loop. In particular, R-202 at the top of transmembrane helix 2 may be a strong determinant of specific glucagon binding by excluding similar ligands while positioning glucagon for additional interaction with sites that lead to receptor activation.

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