Selective Stabilization of the High Affinity Binding Conformation of Glucagon Receptor by the Long Splice Variant of Go\textsubscript{\alpha}\text{S}\textsuperscript{*}

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To analyze functional differences in the interactions of the glucagon receptor (GR) with the two predominant splice variants of Go\textsubscript{\alpha}\text{s}, GR was covalently linked to the short and the long forms Go\textsubscript{\alpha}\text{S} and Go\textsubscript{\alpha}\text{L} to produce the fusion proteins GR-Go\textsubscript{\alpha}\text{S} and GR-Go\textsubscript{\alpha}\text{L}. GR-Go\textsubscript{\alpha}\text{S} bound glucagon with an affinity similar to that of GR, while GR-Go\textsubscript{\alpha}\text{L} showed a 10-fold higher affinity for glucagon. In the presence of GTP\textgamma{S}, GR-Go\textsubscript{\alpha}\text{L} reverted to the low affinity glucagon binding conformation. Both GR-Go\textsubscript{\alpha}\text{S} and GR-Go\textsubscript{\alpha}\text{L} were constitutively active, causing elevated basal levels of cAMP even in the absence of glucagon. A mutant GR that failed to activate Go\textsubscript{\alpha}\text{S} (G23D1R) was fused to Go\textsubscript{\alpha}\text{L}. G23D1R-Go\textsubscript{\alpha}\text{L} bound glucagon with high affinity, but failed to elevate cAMP levels, suggesting that the mechanisms of GR-mediated Go\textsubscript{\alpha}\text{L} activation and Go\textsubscript{\alpha}\text{S}-induced high affinity glucagon binding are independent. Both GR-Go\textsubscript{\alpha}\text{S} and GR-Go\textsubscript{\alpha}\text{L} bound the antagonist desHis\textsuperscript{Nle\textsuperscript{9},Ala\textsuperscript{11},Ala\textsuperscript{16}}-glucagon amide with affinities similar to GR. The antagonist displayed partial agonist activity with Go\textsubscript{\alpha}\text{S}, but not with GR-Go\textsubscript{\alpha}\text{L}. Therefore, the partial agonist activity of the antagonist observed in intact cells appears to be due to GRs coupled to Go\textsubscript{\alpha}\text{L}. We conclude that Go\textsubscript{\alpha}\text{S} and Go\textsubscript{\alpha}\text{L} interact differently with GR and that specific coupling of GR to Go\textsubscript{\alpha}\text{L} may account for GTP-sensitive high affinity glucagon binding.

The biological effects of glucagon are initiated by high affinity binding to its membrane-bound receptor, a member of a distinct class within the superfamily of G protein-coupled receptors (GPCRs)\textsuperscript{1} (1–3). Upon glucagon binding, the glucagon receptor (GR) interacts with the heterotrimeric G protein, Go\textsubscript{\alpha}\text{s}, to catalyze GDP/GTP exchange. Two predominant splice variants of Go\textsubscript{\alpha}\text{s}, a short and a long form (Go\textsubscript{\alpha}\text{S} and Go\textsubscript{\alpha}\text{L}) result from differential splicing of a single precursor mRNA (4, 5). Go\textsubscript{\alpha}\text{S} and Go\textsubscript{\alpha}\text{L} are present in most cells; however, their relative proportions vary (6). The functional significance of the multiple forms of Go\textsubscript{\alpha}\text{s} is unclear, but the conservation of two predominant isoforms suggests that the efficiency of interac-

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\textsuperscript{‡} The abbreviations used are: GPCR, G protein-coupled receptor; GR, glucagon receptor; \beta\textsubscript{2}-AR, \beta\textsubscript{2}-adrenoreceptor; Go\textsubscript{\alpha}\text{s}, \alpha subunit of the G protein; Go\textsubscript{\alpha}\text{S}, long splice variant of the \alpha subunit of Go\textsubscript{\alpha}; Go\textsubscript{\alpha}\text{L}, short splice variant of the \alpha subunit of Go\textsubscript{\alpha}; transducin; PCR, polymerase chain reaction; loop i\textsubscript{2}, second intracellular loop; loop i\textsubscript{3}, third intracellular loop; GTP\textgamma{S}, guanosine 5’-O-(3-thiotriphosphate).

EXPERIMENTAL PROCEDURES

Materials—The cDNA for the rat Go\textsubscript{\alpha}, long splice variant (Go\textsubscript{\alpha}\text{L}) was kindly provided by Dr. Randall R. Reed (17). The construction of the rat
GR synthetic gene (GenBankTM/EMBL Data Bank accession no. U14012) was reported previously (18). Affinity-purified anti-Ga antibody RM, raised against the C-terminal decapeptide RMLHRQYELL of Ga, was kindly provided by Dr. Allen M. Spiegel (19). The preparation and characterization of the anti-Ga antibody DK-12 was reported previously (20, 21). The synthesis by solid phase methods and characterization of the glucagon antagonist desHis1[Ala8, Ala11, Ala16]glucagon amide have been described (22, 23). 125I-Glucagon (507 C/mmol) was from NEN Life Science Products. N-Glycosidase F, endoglycosidase H, and GTP-S were from Roche Molecular Biochemicals.

Construction of the GR-Ga Fusion DNAs—The GR-Ga fusion DNAs were generated using a combination of restriction fragment replacement and PCR-based techniques in a modified pGEM-2 (Invitrogen) cloning vector. All nucleotide sequences were verified by fluorescence-based sequencing (Perkin Elmer/Applied Biosystems model 377A DNA sequencing). GR-Ga-L was assembled in a four-part ligation consisting of: (i) the large AvrII-NoI restriction fragment from the GR synthetic gene in pGEM-2, which deletes the last eight codons (position 478–485) and the termination codon from the C terminus of GR; (ii) a BseRI-NoI restriction fragment encoding residues 20–385 of Ga-L; (iii) a 78-base pair synthetic duplex with cohesive ends for AvrII and BseRI encoding the C-terminal residues 478–485 of GR and the N-terminal residues 2–19 of Ga-L; and (iv) a 30-base pair synthetic duplex with cohesive ends for NosI and NoI encoding the C-terminal 386–393 residues of Ga-L, two successive stop codons, and a NoI site at the 3′-end of the open frame, GR-Ga-L was generated by the overlap extension PCR protocol using Fmu polymerase (24) and the GR-Ga-L construct as template. A set of primers was synthesized to remove codons for amino acids 72–86 and to replace Glu 71 by Asp. In the first round of PCR, two overlapping DNA sequences were amplified independently. The sense primer at the deletion site and an antisense primer from the NosI site of Ga-L were used in one PCR reaction, and the antisense deletion primer and an extension primer from the SacII site of GR were used in another reaction. In a second round of PCR, the two overlapping sequences were spliced together to form a single product using the terminal extension primers from the complementary strands. The resulting PCR-amplified fragment was digested with AccI and BglII and ligated into GR-Ga-L in pGEM-2. After DNA sequencing, a SpeI-NoI fragment with the correct Ga-S sequence was introduced in place of the analogous restriction fragment in GR-Ga-L in the eukaryotic expression vector pMT (25). In GR mutant G23D1R, the 12 loop (Ser252–Glu261) and the i3 loop (Leu334–Ala349) were replaced by an eukaryotic expression vector pMT (25). In GR mutant G23D1R, the i2 loop (Ser252–Glu261) and the i3 loop (Leu334–Ala349) were replaced by an

RESULTS

Characterization of Expressed GR-Ga Fusions—The rat GR was fused with the short and long forms of Ga (Ga5S and Ga5-L) to construct GR-Ga5-S and GR-Ga5-L. The 3′-terminal codon of the open reading frame of the synthetic gene was joined directly to codon 2 of the cDNAs encoding the two Ga5 variants. The nonsense codon of the GR gene and the initiator Met codon of the Ga5 cDNAs were removed. Ga5-L differs from Ga5-S by 16 amino acids. Ga5-L contains a Glu in place of Asp at position 71 and an insert of 15 amino acids starting with position 72 (17). The 15-amino acid insert in Ga5-L is located within the first of two linkers connecting the Ras and helical domains in the structure of Ga5 (Fig. 1). Ga5-L was also fused to GR mutant G23D1R, in which the i2 and i3 loops were both replaced with the i1 loop of the D4 dopamine receptor, to form G23D1R-Ga5-L. G23D1R did not couple to cellular Ga as described previously (26). In addition, experiments were carried out in which GR and Ga5-L were co-expressed from separate vectors in COS-1 cells.

Immunoblot analysis of the membrane preparations from cells expressing GR-Ga5-L was carried out with DK-12 anti-GR antibody (Fig. 2A) and RM anti-Ga5 antibody (Fig. 2B). The fusion protein GR-Ga5-L immunoreacted with DK-12 to yield a band with an apparent molecular mass of 102 kDa after N-glycosidase treatment (Fig. 2A) (20, 21). This result was expected since the apparent molecular mass of Ga5-L is 52 kDa (4), and the GR migrates as a broad band with an apparent molecular mass of 55–75 kDa that collapsed to a band at 50 kDa after N-glycosidase F treatment. GR-Ga5-S migrated with an apparent molecular mass of 95 kDa, which is consistent with the apparent molecular mass of Ga5-S (45 kDa) plus GR (Fig. 2A) (4).

The RM antibody, which is directed against a C-terminal peptide of Ga5, did not react with GR. However, as expected it detected in COS-1 cells the endogenous short and long forms of Ga5, which migrate with apparent molecular masses of 45 and 52 kDa, respectively (Fig. 2B, lanes 1 and 2). The immunoblots consistently showed that the COS-1 cell membranes contained a greater proportion of the short form relative to the long form

FIG. 1. Schematic representation of the GR-Ga fusion proteins. The extracellular N-terminal tail of the GR-Ga fusion protein is toward the top of the figure. Seven putative transmembrane helices are depicted within the plasma membrane (PM). The intracellular C-terminal tail of the GR is covalently joined to the N terminus of Ga5. Ga5 consists of two structural domains - a helical domain and a Ras domain. The guanine-nucleotide binding site lies in a pocket between these domains, which are connected by two linker sequences. The predominant splice variants of Ga5 differ in the amino acid sequence of linker 1. Linker 1 of Ga5-L contains a 15-amino acid insert at position 72 and a glutamic acid instead of an aspartic acid at position 71. The C-terminal tail of GR-Ga5 is oriented toward the cytoplasmic domain of GR. Agonist-dependent guanine-nucleotide exchange is thought to require the interaction of the C-terminal tail of Ga5 with intracellular loops i2 and i3 of activated GR.
of Gaα, Bands at apparent molecular masses of 102 and 95 kDa corresponding to deglycosylated GR-Gaα-L and GR-Gaα-S (Fig. 2B, lanes 4 and 6), respectively, were also detected by RM antibody. The membrane preparations also contained minor bands that were immunoreactive with RM antibody at 35, 40, and 60 kDa that could be degradation products derived from the fusion proteins. The fusion proteins were not recognized by anti-Ga antibody ST-18, which was generated against a C-terminal peptide of GR. This suggests that the ST-18 epitope is eclipsed in the fusion or that the free C-terminal carboxylic acid group is required for immunoreactivity (data not shown) (18). Both GR and GR-Gaα-L were insensitive to endoglycosidase H digestion, indicating that they were properly processed and transported to the cell surface (20) (Fig. 3A).

**Competitive Ligand Binding Experiments**—Increasing concentrations of glucagon were used to compete with 125I-glucagon for binding to membranes from COS-1 cells containing the expressed fusion proteins. GR bound glucagon with the expected average IC50 value of 46 nM (18, 20, 26) (Table I). GR-Gaα-L displayed an affinity for glucagon that was essentially the same as non-fused GR (Fig. 4). In contrast, GR-Gaα-L bound glucagon with an IC50 value of 3.2 nM (Fig. 4, Table I). GR and Gaα-L were co-expressed in COS-1 cells to determine if simply increasing the proportion of Gaα-L to GR increased glucagon-binding affinity. The overexpression of Gaα-L was confirmed by immunoblot analysis using RM antibody to detect an increase in the intensity of the 52-kDa band, which repre-

![Fig. 2. Immunoblot analysis of the GR-Gaα fusions expressed in transiently transfected COS-1 cells. Membranes from COS-1 cells that had been transfected with GR, GR-Gaα-S, and GR-Gaα-L were treated with N-glycosidase F (N-Gase F) to remove N-linked carbohydrates. Samples (10 μg of protein/lane) were separated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes, and probed with anti-GR DK-12 antibody (A) or anti-Gaα antibody RM (B) as described under "Experimental Procedures." Immunoreactive bands were visualized by chemiluminescence. Lanes labeled (−) and (+) correspond to samples untreated and treated with N-glycosidase F, respectively. GR was visualized as a broad band at an apparent molecular mass of 55–75 kDa (lane 1, −). The deglycosylated GR migrated with an apparent molecular mass of about 48 kDa (lane 2, +). The glycosylated (lanes 3 (−) and 5 (−)) and deglycosylated (lanes 4 (+) and 6 (+)) forms of GR-Gaα-L and GR-Gaα-S migrated with the expected molecular masses. Higher molecular weight bands appeared to be dimers of the GR or fusion proteins. Dimerization of GR was described previously (18). B, the identical sample as in panel A was probed with anti-Gaα antibody RM. In COS-1 membranes expressing GR (lanes 1 and 2), the endogenous Gaα splice variants, Gaα-S and Gaα-L were visualized at apparent molecular masses of 46 and 52 kDa, respectively. Gaα-S was more abundant than Gaα-L. GR-Gaα-L and GR-Gaα-S (lanes 3 and 5) migrated as broad bands that collapsed to single bands with apparent molecular masses of 100 and 93 kDa, respectively, after digestion with N-glycosidase F (lanes 4 and 6). Probable fusion protein dimers were visible with molecular masses above 115 kDa. The endogenous Gaα splice variants were also visible in lanes 3–6. Other RM immunoreactive bands were presumably derived from proteolysis of the fusion proteins.

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**Table I**

| Glucagon | Antagonist* |
|----------|------------|
| GR-Gaα  | Competitive binding (IC50) | Adenylyl cyclase activity (EC50) | Competitive binding (IC50) | Adenylyl cyclase activity (EC50) |
| GR       | 46.5       | 5.8       | 71         | Partial agonist |
| GR-Gaα-L | 3.2        | 5.1       | 78         | Partial agonist |
| GR-Gaα-S | 44.6       | 22.4      | 71         | No activation |
| G23D1R   | 50.1       | No activation | 112        | ND*         |
| G23D1R-Gaα-L | 6.3        | No activation | 63         | ND*         |
| GR + Gaα-L | 16        | 2.0       | ND*        | ND*         |

*The glucagon antagonist, desHis1[Nle5,Ala11,Ala16]glucagon amide, showed <0.001% adenylyl cyclase activity relative to glucagon when assayed in liver membranes (22) and weak partial agonist activity (0.01% relative to glucagon) when assayed in COS-1 cells.

*The concentration of unlabeled glucagon or antagonist required to displace 50% of receptor-bound 125I-glucagon. Values given are the means of at least three independent determinations.

*Effective glucagon concentration at 50% stimulation of adenylyl cyclase. Values given are the means of at least three independent determinations.

*Not determined.

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...ents total combined endogenous and recombinant Gaα-L expression (data not shown). The expression of GR in combination with overexpression of Gaα-L resulted in an increase in glucagon binding affinity. However, the IC50 value obtained for the fusion protein GR-Gaα-L (IC50 value = 3.2 nM) was still about 5-fold lower than that obtained for GR in the presence of excess Gaα-L (IC50 value = 16 nM) (Table I).

Mutation of the loops i2 and i3 of GR has little effect on the...
ligand-binding pocket of the receptor. GR mutant G23D1R bound glucagon with an affinity similar to that of the wild-type GR (26). However, G23D1R was unable to activate endogenous Go in COS-1 cells as measured by cAMP accumulation, or in HEK-293T cells as measured by calcium flux (26). Despite the inability of G23D1R to activate Go, tethering G23D1R to Go-L in the fusion protein G23D1R-Go-L led to a 10-fold increase in affinity for glucagon. The IC50 value for competitive displacement of labeled glucagon from G23D1R-Go-L (IC50 value = 6.3 nM) was similar to that of the wild-type GR (Table I).

Competitive binding of glucagon by the fusion proteins GR-Goa-S and GR-Goa-L was also measured in the presence of GTPγS, a non-hydrolyzable GTP analogue. The affinity of glucagon for GR and for GR-Goa-S was not affected by the presence of 100 μM GTPγS. This result suggests that GR-Goa-S displayed a single low affinity state that was independent of GTP. However, GR-Goa-L displayed two glucagon-binding affinities. In the presence of 100 μM GTPγS, the binding affinity of glucagon for GR-Goa-L shifted to an IC50 value about 10-fold higher than that observed in the absence of GTPγS (Fig. 5).

The binding affinity of the glucagon analogue desHis1-[Nle9,Ala11,Ala16]glucagon amide for the GR-Goa fusion proteins was also evaluated. The glucagon analogue binds to native GRs in rat liver membranes with an affinity similar to that of glucagon and effectively inhibits glucagon action (22). In COS-1 cell membranes expressing recombinant GRs, desHis1-[Nle9,Ala11,Ala16]glucagon amide displayed a slightly lower binding affinity (IC50 = 71 nM) than glucagon. The glucagon analogue had the same binding affinity for GR-Goa-S, GR-Goa-L, and GR (Table I). Bringing the GR and Goa signaling partners in close proximity in the fusion proteins did not lead to higher binding affinities for the antagonist even in the absence of GTP.

Glucagon-dependent Adenylyl Cyclase Activity—The fusion proteins were assayed for the ability to mediate an increase in intracellular cAMP upon stimulation with glucagon. The fusion proteins GR-Goa-S and GR-Goa-L caused glucagon-dependent adenylyl cyclase activation, which led to an increase in intracellular cAMP concentrations in transfected COS-1 cells. The EC50 value of glucagon-dependent adenylyl cyclase activation in COS-1 cells expressing GR was 5.8 nM (Table I). Essentially the same EC50 value was obtained for GR-Goa-S (5.1 nM). Consistent with a lower glucagon binding affinity, GR-Goa-S exhibited an EC50 value that was 4 times higher than that measured for GR-Goa-L or non-fused GR (22.4 nM).

In addition, expression of both fusion proteins GR-Goa-S and GR-Goa-L produced elevated basal levels of cAMP in the absence of glucagon, which is characteristic of constitutive receptor signaling (Fig. 6). The constitutive activity of the fusion proteins GR-Goa-S and GR-Goa-L was not suppressed by desHis1-[Nle9,Ala11,Ala16]glucagon amide. The fact that desHis1-[Nle9,Ala11,Ala16]glucagon amide had no effect on the constitutive activity of the fusion proteins confirms that the elevated basal activity is not caused by endogenous glucagon. In the classification of antagonists, this result would mean that desHis1-[Nle9,Ala11,Ala16]glucagon amide is a neutral antagonist, not a negative antagonist or inverse agonist (27, 28). DesHis1-[Nle9,Ala11,Ala16]glucagon amide showed no measurable adenylyl cyclase activity (<0.001% relative to glucagon), when assayed in liver membrane preparations. However, in COS-1 cells expressing recombinant GRs, a small CAMP elevation, 0.01% relative to glucagon, was observed upon treatment with desHis1-[Nle9,Ala11,Ala16]glucagon amide, consistent with very weak partial agonist activity (Fig. 7). Interestingly, the glucagon analogue induced adenylyl cyclase activity above baseline only in cells expressing GR-Goa-L, but not in cells expressing GR-Goa-S (Fig. 7). This observation suggests that the partial agonist activity in intact cells induced by desHis1-[Nle9,Ala11,Ala16]glucagon amide may be attributed to receptors that are coupled to Goa-L, and not Goa-S.

Glucagon-dependent cyclase activity was also assayed in...
COS-1 cells expressing the mutant receptor-Gasa fusion, G23D1R-Gasa-L. G23D1R was shown to be incapable of signaling due to the replacement of its second and third intracellular loops with the first intracellular loop of the unrelated D4 dopamine receptor (26). G23D1R-Gasa-L bound glucagon with a higher affinity than G23D1R and displayed an IC50 value (6.3 nM) close to that of GR-Gasa-L. However, unlike GR-Gasa-L, G23D1R-Gasa-L was not constitutively active in the absence of glucagon, and treatment with glucagon did not cause adenyl cyclase activation (Fig. 6).

**DISCUSSION**

The effect of GTP to lower the affinity of certain hormones for membrane receptors was described in the pioneering work of M. Rodbell (29). The molecular basis of the “GTP effect” is now generally understood. Heterotrimeric G proteins bind to GPCRs to stabilize a high affinity agonist-binding conformation. In the presence of agonist, GTP induces the dissociation of G protein from its receptor, which then assumes a low affinity agonist-binding conformation. GRs in hepatocyte membranes display the classical GTP effect, but they are also known to exist in two interconverting ligand affinity conformations that are modulated by Gs even prior to glucagon binding (30). Gs exists in two predominant splice variants, Gs-S and Gs-L. We aimed to test whether differences in GR coupling to these splice variants may account for the observed heterogeneity in GR ligand-binding affinity.

One useful approach to study the interactions of GPCRs and G proteins has been to join a Gs subunit directly to the C terminus of a GPCR and express a single fusion protein signaling complex (31–37). This strategy allows unambiguous assignment of the functional consequences of agonist binding and subsequent receptor activation and signaling. Ideally, the fusion proteins ensure a defined 1:1 stoichiometry of GPCR to a specific Ga subtype and enhance the efficiency and specificity of activation by constraining two predetermined signaling partners in relatively close proximity. Early attempts to find differences between the long and the short forms of Gs in its interaction with the β2-adrenoreceptor (β2-AR) led to conflicting observations (38, 39). However, fusion of the β2-AR with each of the two forms of Gs demonstrated that the properties of either protein in the fusion did not change, and revealed that the subtle structural differences in the two variants of Gs had significant effects on β2-AR-mediated signaling (33). When expressed in S9 cells, the β2-AR in β2-AR-Gs-S, but not in β2-AR-Gs-S, possessed characteristics of a constitutively active receptor that could be inhibited by an inverse agonist of β2-AR (33).

The GR belongs to a unique class of receptors within the GPCR family and bears little sequence resemblance to the β2-AR, which falls within the rhodopsin-like group of receptors (1–3). We previously demonstrated that the 12 and 13 loops of the GR are essential for G protein coupling (26). Replacement of both cytosolic domains with the 11 loop sequence of the D4 dopamine receptor in the receptor-mutant, G23D1R, resulted in complete loss of G protein signaling, as measured by cAMP accumulation and calcium flux assays (26). The expressed GR can mediate both cAMP elevation and calcium flux by coupling to Gs (26).

Fusion of GR to Gs-S and to Gs-L allowed efficient coupling between both of the tethered partners, as demonstrated by the ability of transfected cells expressing the fusion to undergo...
glucagon-stimulated adenylyl cyclase activity (Fig. 6). This established that the intrinsic properties of either protein were preserved within the fusion construct, and that the physical connection did not hinder or restrict the conformational changes and domain movements that must accompany the activation process in both proteins. In the case of the β2-AR-Gαs fusion, it was shown that downstream post-activation events such as desensitization and the re-palmitoylation mechanisms were compromised, presumably because complete separation of receptor from G protein was not possible in the fusion protein (32, 36). Moreover, another study reported that restricting the mobility of Gαs relative to receptor in β2-AR-Gαs affected its GTPase activity (40).

The C-terminal tail of GR is relatively long compared with that of β2-AR. We preserved the long C-terminal tail of GR in the design of the fusion, even though mutant receptors lacking most of the C-terminal extension still coupled efficiently to Gαs (20). Since signaling requires Gαs to contact determinants within the i2 and i3 loops of GR simultaneously, the Gαs portion of the fusion requires enough flexibility and mobility to align its C-terminal close to the cytosolic loops of GR, to dissociate after GDP/GTP exchange, and to interact with adenylyl cyclase. The long C-terminal extension presumably serves as a flexible tether to minimize steric hindrance during intraprotein contact.

Transfected non-fused wild-type GR bound glucagon with the expected average dissociation constant (IC50 value) of 46 nm (18, 20, 26) (Table I). The IC50 was significantly higher than that of GRs in liver membranes, but was typical of recombinant receptors expressed transiently in COS cells or stably in HEK-293 cells (18, 41). This low affinity binding is presumably caused by inefficient coupling to G proteins, or a lack of G proteins relative to overexpressed receptors (41, 42). Fig. 4 shows that the GR in GR-Gαs-L bound glucagon with a higher affinity than non-fused GR. The IC50 for glucagon binding shifted more than 10-fold closer to values observed for high affinity native receptors in hepatocytes (Table I). Consistent with this observation, co-expression of GR and Gαs-L also resulted in improved ligand-binding affinity (Table I). While these results supported the assumption that an increase in the proportion of Gαs-L to GR shifted the binding affinity, the IC50 value measured for GR-Gαs-L was still 5-fold lower than that obtained for co-expressed GR and Gαs-L, suggesting that the physical proximity of the signaling partners in the fusion also contributed to the improved ligand-binding affinity. In marked contrast to GR-Gαs-L, GR-Gαs-S had an affinity for glucagon that was essentially the same as non-fused GR, with IC50 values at least 10-fold higher than that measured for GR-Gαs-L (Fig. 4, Table I).

In the classical view, the GR exists in the “off” state, R, until glucagon docks into its binding site and induces a change of conformation in R to the active form R*, which initiates the signaling cascade by interacting with G proteins. The extended ternary model of receptor activation considers that receptors are in close contact with G proteins to form a complex with some basal activity even in the absence of agonist (43–45). In the fusion, the signaling partners were constrained to pre-couple, and should be in the activated form R*G even prior to ligand binding. It is reasonable to assume that the conformational change in the receptor induced by interaction with Gαs-S in GR-Gαs-S, must be different from that induced by association with Gαs-L in GR-Gαs-L. Our results show that glucagon was able to discriminate between GR-Gαs-S and GR-Gαs-L and suggest that glucagon stabilized the active state of Gαs-L-coupled GRs more than Gαs-S-coupled GRs.

The high affinity state of GPCRs is known to be sensitive to guanine nucleotides, and only agonist binding to high affinity receptors are expected to be GTP-sensitive. Upon GDP/GTP exchange, GTP-occupied Gαs dissociates from GR, and the affinity of receptor for hormone is decreased. The binding experiments were carried out using membranes isolated from cells expressing the fusion proteins and all Gαs domains would be expected to be either GDP-bound or nucleotide-free and primed for activation by glucagon binding to GR. As shown in Fig. 5, in the presence of 100 μM GTPγS, the displacement curve for glucagon binding to GR-Gαs-L shifted to the right to a higher IC50 value. In contrast, the “GTP shift” was not observed with glucagon binding to membranes expressing GR-Gαs-S or non-fused GR (Fig. 5).

Following transient transfection in COS-1 cells, the GR in both fusion proteins GR-Gαs-S and GR-Gαs-L activated adenylyl cyclase in response to glucagon (Fig. 6). In addition, both fusion proteins displayed 3-fold elevations in the basal levels of cAMP over that of expressed GR in the absence of glucagon. Glucagon treatment caused a further increase in adenylyl cyclase stimulation. Certain mutations in GPCRs have been shown to increase the likelihood of agonist-independent signal transduction and are associated with specific familial diseases (46, 47). A His178→Arg mutation in the i1 loop of GR was also reported to cause constitutive activity (48). Constitutive activity of the fusion proteins suggests that pre-coupling of the signaling partners stabilizes the active conformation to cause GDP/GTP exchange and alter the basal activity even in the absence of agonist. In contrast, G23D1R-Gαs-L was not constitutively active even though it displayed high affinity glucagon binding. In addition, it did not support additional cAMP production in response to glucagon.

Interestingly, glucagon bound to the fusion G23D1R-Gαs-L with an IC50 value similar to that obtained for GR-Gαs-L, a 10-fold increase in affinity from that obtained for G23D1R (26). This result shows that the high affinity glucagon-binding conformation in the G23D1R-Gαs-L complex does not require the involvement of the i2 and i3 loops of GR. This observation is striking because the G23D1R receptor itself does not display high affinity glucagon binding and does not couple to Gαs. The result with G23D1R-Gαs-L suggests that the GR mutant G23D1R is analogous to rhodopsin mutants that bind, but fail to activate, the retinal G protein, transducin (Gt) (49). Certain rhodopsin mutants with alterations of their i2 and i3 loops bound Gt in the GDP form, but failed to induce nucleotide exchange or Gt release in the presence of GTP (49, 50). Even though these mutants did not activate Gt, they were stabilized in the metarhodopsin II conformation, which is analogous to the high affinity agonist-binding state of GPCRs with diffusible ligands. To our knowledge, the GR mutant G23D1R in the context of the G23D1R-Gαs-L fusion is the first example of such a phenotype in a group II GPCR.

Extensive structure-activity analysis allowed us to identify residues in glucagon that contribute a specific structural determinant to either binding or transduction (51–53). Residues at positions 1, 9, and 16 of glucagon are essential for receptor activation, but less important for binding. Replacement of these residues led to the analogue desHis[1]Nle[9,Ala[11,Ala[16]]glucagon amide, which has been shown to be a potent competitive glucagon antagonist (22, 54). In the present study, desHis[1]-[Nle[9,Ala[11,Ala[16]]glucagon amide displayed similar binding affinity for GR and the fusion proteins. This property is characteristic of antagonists that are unable to distinguish between different receptor agonist-affinity conformations. The fact that both fusion proteins exhibited elevated basal levels of adenylyl cyclase activation even in the presence of increasing concentrations of desHis[1]-[Nle[9,Ala[11,Ala[16]]glucagon amide confirms that
the elevated basal cAMP levels are not caused by glucagon (Fig. 7). In the classification of antagonists, desHis1[Nle9,Ala11,Ala16]-glucagon amide would be referred to as a neutral antagonist and not a negative antagonist or inverse agonist (27, 28). Although the antagonist showed no measurable adenylyl cyclase activation when assayed in liver membrane preparations, very weak partial agonist activity was detectable in COS-1 cells expressing recombinant GRs (Fig. 7). Taken together, the results indicate that the adenylyl cyclase activation observed in intact cells when stimulated by desHis1[Nle9,Ala11,Ala16]-glucagon amide may be attributable to Goα1-L coupled GRs, since partial agonist activity was observed only in cells expressing GR-Goα1-L, and not in cells expressing GR-Goα1-S.

In addition, membranes containing expressed GR-Goα1-L were able to support full adenylyl cyclase activation in response to increasing concentrations of glucagon, while membranes expressing only non-fused GR did not. This suggests that either the COS-1 membranes were stripped of most of the endogenous Goα, during membrane preparation or that, after one activation cycle, most of the endogenous Goα that dissociates from the membranes were unable to participate in ensuing rounds of reactivation (55, 56). Despite pertussis toxin pretreatment to eliminate potential interaction with endogenous Goα, there was no indication that the Goα-AR fusion Goα-AR-C351Goα1 had the capacity to activate both its fusion partner Goα1, and endogenous Goα in an agonist-dependent manner when expressed in COS cells (37).

Whether the 15-amino acid difference in linker 1 between the Ras-like and helical domains of Goα variants has direct influence on agonist efficacy is unclear since it is remote from the C terminus of Goα which is known to interact with receptor. However, linker 1 is situated near the switch 1 and switch 2 regions of Goα which border the nucleotide binding cleft and are known to change conformation upon GTP binding. The flexibility afforded by the 15-residue peptide insert in linker 1 of Goα-L may facilitate GDP/GTP exchange (11, 58, 59). The IC50 values of the antagonist show that there was essentially no difference in the ability of desHis1[Nle9,Ala11,Ala16]-glucagon amide to stabilize either GR-Goα1-S or GR-Goα1-L, but the subsequent signal transfer to effector via receptors coupled to Goα1-L appears to be favored.

Previous work with β2-AR-Gα fusion proteins showed that Goα-L had a lower affinity for GDP and was more easily activated, if not already nucleotide-free (33). Based on those observations, it appears that Goα-L-coupled GR is more primed for activation than GR coupled to Goα-S. Thus, it is likely that after the initial recognition, even small conformational changes in the receptor brought about by the interaction of desHis1-[Nle9,Ala11,Ala16]glucagon amide with activating residues on the extracellular face of the receptor might be sufficient to transduce a signal via the already empty Goα-L in the fusion, but not readily with GDP-bound Goα-S. Despite its partial activity on Goα-L-coupled receptors, desHis1[Nle9,Ala11,Ala16]-glucagon amide is a potent inhibitor of glucagon action and may not only compete for hormone binding sites, but also prevent the receptor from assuming the conformation required for full agonist activity (22, 54).

The chemical or conformational features of a glucagon analogue that determine whether it is an agonist, partial agonist, or antagonist remains the object of investigation in drug development (22, 51–53, 57, 60, 61). It is clear that the efficacy and potency of ligands are regulated not only by their recognition sites in the receptor, but also by the type and relative amounts of G proteins expressed within the cell. Evidence from the GR-Goα-L fusions shows that pre-coupling of GR to different Goα isoforms can generate multiple conformations with distinct ligand-binding affinities resulting in diverse cellular responses. Glucagon-binding affinity for the GR-Goα-L fusion is GTP-sensitive and simulates pharmacological responses of native receptors in hepatocytes. Thus, it may be a useful model for pharmacological and biophysical studies in recombinant systems. The enhanced basal activity of GR-Goα-L will be useful in assessing novel glucagon analogues for inverse agonist activity. More importantly, a GR mutant-Goα1-L fusion protein revealed the existence of an active intermediate in the glucagon signaling cascade analogous to the metarhodopsin II species of the rhodopsin pathway and should facilitate further analysis of GR interaction with Goα.
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Selective Stabilization of the High Affinity Binding Conformation of Glucagon Receptor by the Long Splice Variant of G \( \alpha_s \)

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