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Involvement of Specific Hydrophobic, but not Hydrophilic, Amino Acids in the Third Intracellular Loop of the β-Adrenergic Receptor in the Activation of G_s

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SUMMARY

Mutagenesis and biochemical analysis have indicated that amino acid residues at the amino terminus of the third intracellular loops of guanine nucleotide-binding protein (G protein)-coupled receptors are important in mediating the coupling of the receptors to G proteins. Because the primary sequence of this region is not conserved among all receptors that couple to the same G protein, it has been suggested that some other physicochemical property of this domain may determine G protein activation. To determine the relative contributions of charge distribution and amino acid side chain interactions within this domain of the β-adrenergic receptor (βAR) to the activation of the G protein G_s, point mutations were introduced into this region of the βAR. Replacement of all four of the basic amino acid residues within this region (amino acids 222–236) with serine residues had a negligible effect on the ability of the βAR to activate G_s. In contrast, replacement of the hydrophobic amino acids within this same region with leucine residues resulted in a mutant receptor that was poorly coupled to G_s. These results suggest that specific hydrophobic interactions within this region of the receptor may play a more significant role than ionic or hydrophilic interactions in mediating G protein activation.

Many hormone receptors promote their cellular responses by interacting with members of the family of G proteins. Receptor activation of G proteins is agonist dependent and mediates the exchange of complexed GDP for GTP on the α subunit of the G protein. The GTP-bound G proteins interact with specific effector systems in the plasma membrane, leading to the propagation of intracellular signals. Of the G protein-coupled receptors, the βAR, which activates the G protein G_s to stimulate adenylyl cyclase, is perhaps the most well characterized, both pharmacologically and biochemically. The cloning of the mammalian βAR has revealed that the receptor consists of seven transmembrane hydrophobic helices connected by alternating extracellular and intracellular hydrophilic loops (1). Genetic and biochemical analyses of this receptor have revealed that the binding of agonists and antagonists involves amino acid residues within the transmembrane core of the protein (1). Deletion mutagenesis and molecular replacement studies, as well as peptide mapping experiments, have implicated loop i3 of G protein-coupled receptors as one of the major determinants of G protein activation (2–10). The deletion of short stretches of amino acids at the amino and carboxyl termini of this domain of the βAR diminishes the ability of the mutant receptor to activate G_s. In contrast, deletions of similar size from the central portion of the loop do not affect G protein coupling. These experiments implicated the terminal portions of this region of the receptor in G protein activation (3, 6). Molecular substitution studies, in which regions from loop i3 of the M1 muscarinic receptor were exchanged with the analogous region of the M2 receptor, revealed this region of the receptor to be important for G protein selectivity, as well (7–9). Additional studies on chimeric muscarinic and muscarinic/adrenergic receptors have suggested that the intracellular loops of the receptors contain several distinct determinants of G protein selectivity and activation (8–10). In several reports (9, 10), hybrid receptors were observed to couple to both of the signaling pathways of the parent receptors, suggesting that the critical determinants for effector selectivity are not contained within a single intracellular loop of the receptor. In fact, interactions between these loop regions may be important in G protein activation. Therefore, it has been suggested that some physicochemical feature of the intracellular region of the receptor, rather than the primary sequence of an individual loop, might play an important role in mediating the interaction between the receptor and the G protein. The sequence at the amino terminus of loop i3 of the hamster βAR and analogous sequences of other G protein-coupled receptors are predicted to form amphiphilic α-helices, according to the helical wheel model of Schifler and Edmunson and by the method of Chou

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; βAR, β-adrenergic receptor; loop i3, third intracellular loop; ^{125}I-CYP, ^{125}I-iodocyanopindolol; CHO, Chinese hamster ovary; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

	220				225					230					235
βAR	S	R	V	F	Q	V	A	K	R	Q	L	Q	K	I	D	K	S
(i3-1)βAR	.	.	.	.	.	.	.	S	S	.	.	.	S	.	.	S	.
(i3-2)βAR	.	.	.	.	.	.	.	S	.	.	.	.	S	.	.	S	.
(i3-3)βAR	.	.	L	L	.	L	L	.	.	.	.	.	.	L	.	.	.
(i3-4)βAR	.	K	A	L	A	A	L	A	K	K	.	.	.	.	.	.	.

Fig. 1. Schematic representation of βAR mutations. Amino acid sequence of the wild-type βAR is shown on the *top*. *Dotted line*, unmutated amino acid residues.

and Fasman (11) (Fig. 1). One face of this predicted α-helix consists of hydrophilic and positively charged residues, whereas the other face is composed of hydrophobic amino acids. It has been hypothesized that it is the amphiphilic α-helical nature of these regions that promotes the interaction of the receptor with the G protein. In support of this hypothesis, the bee venom peptide mastoparan, which forms an amphiphilic α-helix in the presence of phospholipid vesicles, has been determined to activate the G protein G_o (11). Moreover, the synthetic peptide corresponding to amino acids 218–232 of the βAR was observed to activate a recombinant preparation of G_s but not G_i when reconstituted into phospholipid vesicles (12). These results suggest that the structural information encoded within the region corresponding to the amino-terminal portion of loop i3 is sufficient to promote some activation of G proteins.

The present study was undertaken to determine whether the physical properties of this region, the specific chemical nature of the amino acid side chains, or the overall charge distribution within this domain is the most important determinant of the interaction of the receptor with the G protein. Amino acid substitutions have been introduced into this region of the βAR in such a way as to maintain both the predicted α-helical structure and the amphiphilic side chain distribution within this domain, while changing the amino acid side chains at different positions. The data suggest that the positively charged residues that are aligned along one face of the amphipathic helix in the receptor are not important in mediating G protein coupling. In contrast, the specific hydrophobic side chains aligned along the other face of the helix appear to be critical determinants of G protein activation.

Experimental Procedures

Mutagenesis and expression. The nucleotide sequence for the hamster βAR gene has been published (13). Oligonucleotide-directed *in vitro* mutagenesis was used to create the mutations, and the identities of the mutations were confirmed by dideoxy sequencing of the mutant plasmids, as previously described. The expression of the gene in simian COS-7 cells was performed as previously described, using the pSVL vector (14). Stably transformed CHO cell lines were obtained by co-transfection of the pSVβAR plasmid with pNeo, and individual transformed clones were selected by resistance to G-418. Cells were grown in Dulbecco's modified Eagle's medium, in an atmosphere of 5% CO_2, at 37°.

Membrane preparation and receptor assays. Cell membranes were prepared by hypotonic lysis of cell monolayers in 1 mM Tris, pH 7.5, as previously described (14). Membranes were resuspended at a protein concentration of 1–2 mg/ml, in TME buffer (75 mM Tris, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EDTA). Saturation binding was performed with 10–400 pM ^{125}I-CYP, for 1 hr at room temperature, in TME buffer. Competition binding was performed with 35 pM ^{125}I-CYP for 1 hr at room temperature, as previously described, and the data were analyzed using the iterative program LIGAND (15). Adenylyl cyclase activity was determined by the method of Salomon *et al.* (16), using the βAR agonist isoproterenol for 30 min at 30°, as previously described (14). Maximal adenylyl cyclase activity was determined in the presence of 1 mM NaF, and the data were analyzed by nonlinear regression.

Protein immunoblotting. Immunoblot analysis was carried out as previously described, using an antibody prepared against the peptide sequence corresponding to the hamster βAR-404–418 (14). Immunoreactivity was assessed by autoradiography after incubation with ^{125}I-Protein A.

Results

In order to ascertain the contribution of specific amino acid side chains to the interaction of the receptor with G proteins, mutant βARs were created in which individual amino acids within the region 222–236, at the amino terminus of loop i3, were replaced. The amino acid substitutions were chosen such that both the hydrophobicity profile and the predicted α-helical nature of this region of the receptor would be maintained while the chemical nature of individual amino acid side chains was altered (Fig. 1). The hydrophilic face of the putative α-helix formed by the amino terminus of βAR loop i3 is highly charged, containing an arginine and three lysine residues. In mutant (i3–1)βAR, all of these charged residues were replaced with the neutral hydrophilic amino acid serine. In the related mutant (i3–2)βAR, the three lysine residues at positions 227, 232, and 235 were replaced with serines, leaving the arginine at position 228 intact. The hydrophobic residue leucine was substituted for each of the hydrophobic residues within the region 222–236 in mutant (i3–3)βAR, to allow determination of the contribution of specific hydrophobic amino acid side chains in this region to G_s activation. Finally, the mutant (i3–4)βAR incorporated the sequence of the bee venom peptide mastoparan into this region of the receptor.

Characterization of mutant receptors expressed in COS-7 cells. The wild-type and mutant βAR genes were transfected into COS-7 cells, and the structures of the expressed proteins were assessed by immunoblotting. As previously observed, transfection of COS-7 cells with the wild-type βAR plasmid resulted in expression of an immunoreactive protein with an apparent molecular mass of 67 kDa, similar to the βAR purified from hamster lung (14). Transfection of COS-7 cells with each of the mutant βAR plasmids resulted in similar levels of expression of the same 67-kDa immunoreactive protein (data

TABLE 1

Agonist binding by wild-type and mutant βARs expressed in COS cells

Saturation binding of ^{125}I-CYP was performed with 225 pM ^{125}I-CYP, with wild-type and mutant βARs in COS cell membranes, as described in Experimental Procedures. Isoproterenol binding was measured in competition with 35 pM ^{125}I-CYP, in the presence of increasing concentrations of isoproterenol, as described. Results were analyzed using the LIGAND program (15) and represent the average of two to four separate experiments.

Receptor	B_{max}	K_d (^{125}I-CYP)	K_d (isoproterenol)
	pmol/mg	*pM*	*M*
βAR	3.0 ± 1.5	86 ± 9	$1.9 \pm 0.7 \times 10^{-7}$
(i3-1)βAR	4.1 ± 3.2	122 ± 17	$1.4 \pm 0.1 \times 10^{-7}$
(i3-2)βAR	4.2 ± 3.3	154 ± 38	$2.0 \pm 0.1 \times 10^{-7}$
(i3-3)βAR	2.1 ± 0.6	202 ± 4	$2.8 \pm 1.5 \times 10^{-9}$ (67%) $1.8 \pm 2.3 \times 10^{-7}$ (33%)
(i3-4)βAR	3.1 ± 3.3	111 ± 50	$2.4 \pm 3.2 \times 10^{-6}$

TABLE 2

Agonist binding and adenylyl cyclase stimulation by wild-type and mutant βARs expressed in CHO cells

Competition binding of isoproterenol with 35 pM ^{125}I-CYP, in the presence or absence of 100 μM Gpp(NH)p, was performed as described in Experimental Procedures. Data were analyzed using the LIGAND program (15). Results are reported for two affinity states only in cases where a two-site fit was significantly better than a one-site fit ($p < 0.02$). K_1 and K_2 represent the K_d values of the high and low affinity states, respectively, and the numbers in parentheses represent the percentage of the total receptor population that is in the high affinity state. Adenylyl cyclase parameters were determined by nonlinear regression analysis, from curves such as those shown in Fig. 2. Percentage of maximal stimulation refers to the maximal stimulation by 10 mM NaF. Data shown are the mean ± standard deviation, averaged from two to four independent experiments.

Receptor	B_{max} (^{125}I-CYP)	K_d (isoproterenol)				Adenylyl cyclase	
		−Gpp(NH)p			+Gpp(NH)p	K_{act}	Stimulation
		K_1	K_2				
	fmol/mg	M				M	% of maximal
βAR	270	$3 \pm 3 \times 10^{-9}$	$8 \pm 9 \times 10^{-7}$	(59)[a]	$1 \pm 0.6 \times 10^{-7}$	3×10^{-8}	97 ± 3
(i3-1)βAR	292	$1 \pm 0.1 \times 10^{-7}$		(100)	$4 \pm 2 \times 10^{-7}$	7×10^{-8}	75 ± 6
(i3-2)βAR	240	$1 \pm 0.3 \times 10^{-7}$		(100)	$7 \pm 4 \times 10^{-7}$	1×10^{-7}	84 ± 5
(i3-3)βAR	198	$3 \pm 0.7 \times 10^{-8}$		(100)	$4 \pm 1 \times 10^{-8}$	9×10^{-8}	29 ± 10
(i3-4)βAR	69	$4 \pm 1 \times 10^{-9}$	$2 \pm 0.7 \times 10^{-7}$	(50)	$6 \pm 3 \times 10^{-7}$	8×10^{-8}	19 ± 4

[a] Percentage in high affinity state.

not shown), indicating that all of the mutant receptors were properly processed and expressed in the plasma membrane of the cell. The affinities of these mutant receptors for the βAR agonist isoproterenol and the radiolabeled antagonist ^{125}I-CYP were determined in binding studies using COS-7 cell membranes (Table 1). All of the mutant receptors bound ^{125}I-CYP with high affinity. With the exception of (i3–3)βAR, which showed a 2-fold decrease in affinity for ^{125}I-CYP, the mutant receptors bound this antagonist with an affinity similar to that of the wild-type protein. The wild-type βAR bound the agonist isoproterenol with a single class of low affinity binding sites, as has previously been described for transient expression of the βAR in this expression system. Mutant receptors (i3–1)βAR, (i3–2)βAR, and (i3–4)βAR bound isoproterenol with affinities similar to that of the wild-type receptor. The receptor in membranes from cells expressing (i3–3)βAR exhibited slightly higher affinity binding for isoproterenol than did the wild-type receptor. This binding was best fit to two classes of sites, with the majority of the receptors having a high affinity for isoproterenol. The increased affinity of this mutant receptor for isoproterenol is reminiscent of the intermediate affinity state previously observed for mutant βARs with deletions within loop i3 that coupled poorly to G proteins (6, 12).

Characterization of mutant receptors expressed in CHO cells. In order to examine the effect of the mutations on the ability of the receptor to couple to G_s and activate adenylyl cyclase, the mutant cDNAs were transfected into CHO cells and clones stably expressing the mutant receptors were selected. The agonist affinities for the various mutant βARs were determined, both in the presence and in the absence of the nonhydrolyzable GTP analog Gpp(NH)p, with the results shown in Table 2 and Fig. 2. The wild-type βAR bound the agonist isoproterenol with two classes of binding sites, similar to those previously reported for the βAR stably expressed in CHO cells or in mouse L cells (14). Upon addition of Gpp(NH)p, all of these receptors were converted to the low affinity state, and the high affinity binding site was no longer observed, indicating that the βAR is coupled to a G protein in these cells. As previously reported, isoproterenol is a full agonist at the wild-type βAR, stimulating adenylyl cyclase activation to 100% of the level seen in the presence of 10 mM NaF, with a K_{act} of 3×10^{-8} M (Table 2; Fig. 3).

All of the mutant receptors showed some degree of G protein coupling, as assessed by the sensitivity of the agonist affinity to Gpp(NH)p (Table 2; Fig. 2). However, the mutants varied in their abilities to activate adenylyl cyclase. Mutants (i3–1)βAR and (i3–2)βAR, in which the charged amino acids were substituted with serine residues, showed intact adenylyl cyclase activities. Isoproterenol-stimulated adenylyl cyclase activation by (i3–1)βAR and (i3–2)βAR reached 75% and 84% of the maximal stimulation by NaF, respectively. In contrast, isoproterenol-mediated activation of adenylyl cyclase activity in membranes prepared from cells expressing (i3–3)βAR was attenuated, reaching only 29% of the maximal stimulation observed with NaF.

The mutant (i3–4)βAR, which incorporated the amino acid sequence of the bee venom peptide mastoparan, also bound isoproterenol in a manner similar to that of the wild-type βAR. Approximately half of the total receptor population was in the high affinity binding state, and this population was shifted mostly to the low affinity state upon addition of Gpp(NH)p (Table 2). However, isoproterenol-mediated adenylyl cyclase activity was markedly reduced for this mutant receptor, with a maximal stimulation of only 19% of the level obtained in the presence of NaF. This reduced level of adenylyl cyclase activation was not due to the lower number of receptors present in the (i3–4)βAR cell line analyzed in the present study, because previous experiments on cells expressing the wild-type receptor at levels of 30–300 fmol/mg showed these levels to be sufficient to activate adenylyl cyclase maximally in CHO cells (3, 6).[1] Because mastoparan itself has been shown to activate the G proteins G_i and G_o when reconstituted into phospholipid vesicles, the functional coupling of (i3–4)βAR to other G proteins was also investigated in these cells. Neither isoproterenol-mediated inhibition of forskolin-stimulated adenylyl cyclase activity nor isoproterenol-stimulated accumulation of inositol phosphates could be detected in the cells expressing this mutant receptor, however (data not shown), suggesting that, if (i3–4)βAR couples to a G protein in CHO cells, it is neither G_i nor G_p.

Discussion

In the present study, we have used substitution mutagenesis to analyze the physical properties of the amino-terminal por-

[1] C. D. S., A. H. C., and R. R. C. H., unpublished observations.

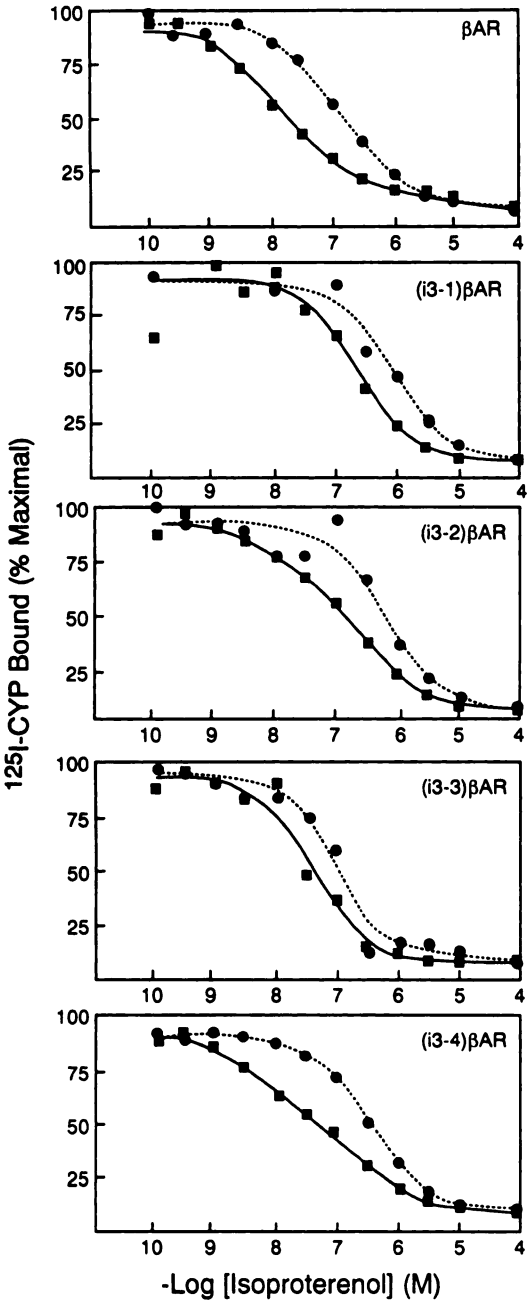

Fig. 2. Competition binding to wild-type and mutant βARs. Competition binding of isoproterenol with 35 pM ^{125}I-CYP, in the presence (●) or absence (■) of 100 μM Gpp(NH)p, was performed as described in Experimental Procedures. Data were analyzed by the LIGAND program (15). The data shown are representative of two or three separate experiments, with each point done in duplicate.

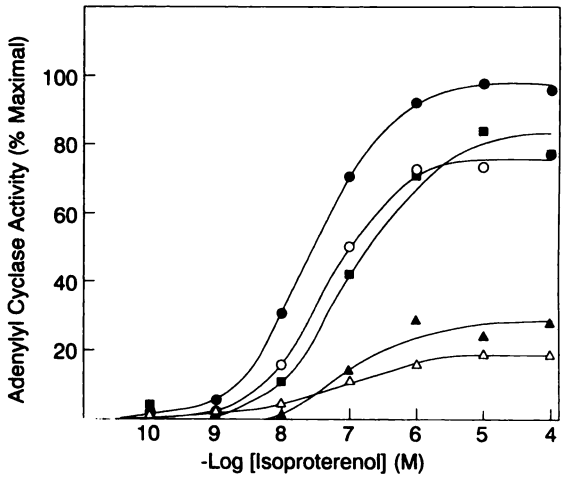

Fig. 3. Adenylyl cyclase stimulation by wild-type and mutant βARs. Membranes were prepared from CHO cell lines stably expressing the mutant receptors and adenylyl cyclase activity was measured in the presence of increasing concentrations of isoproterenol, as described in Experimental Procedures. This activity is expressed as a percentage of the maximal stimulation by 10 mM NaF. For the experiment shown, the adenylyl cyclase activities for the various mutant receptors, expressed as basal/10^{-5} M isoproterenol/10 mM NaF, in pmol of [^{32}P]cAMP/min/mg of protein, were as follows: wild-type βAR (●), 34/183/183; (i3–1)βAR (○), 21/111/144; (i3–2)βAR (■), 11/92/106; (i3–3)βAR (▲), 7/25/66; (i3–4)βAR (△), 13/34/123. The data shown are representative of two separate experiments. Similar levels of adenylyl cyclase stimulation were observed in five independent clonal cell lines for each mutant receptor.

tion of loop i3 of the βAR that might contribute to the activation of G_s by the receptor. All of these mutant βARs appear to fold normally, as assessed by the ability of the receptor proteins to be inserted into the cell membranes and undergo full glycosylation. However, small local conformational effects of these molecular replacements cannot be ruled out without more rigorous structural analysis of the mutant proteins. In (i3–1)βAR and (i3–2)βAR, the basic amino acids located near the amino terminus of loop i3 (three lysine residues and one arginine) were replaced with serine residues, in order to remove the positive charges from this region of the receptor without affecting the hydropathicity profile of the putative α-helical structure. These mutant receptors exhibit a slightly reduced affinity for isoproterenol and a small decrease in the maximal level of adenylyl cyclase stimulation by the agonist, indicating a slight perturbation of the interaction between the receptor and the G protein. However, these amino acid substitutions do not appear to affect substantially the ability of the receptor to interact with G_s, as determined both by the sensitivity of agonist binding to the nonhydrolyzable GTP analog Gpp(NH)p and by the ability of the agonist isoproterenol to stimulate adenylyl cyclase. In contrast, replacement of all of the hydrophobic amino acids in this region of the βAR with leucine residues, maintaining the amphipathic nature of this region of the receptor while altering all of the hydrophobic side chains, results in a mutant receptor [(i3–3)βAR] that couples poorly to G_s. Isoproterenol-mediated adenylyl cyclase stimulation by this mutant receptor reaches only 29% the level of the wild-type βAR.

These data suggest that the chemical nature of the amino acid side chains on the hydrophobic face of the amino terminus of loop i3 in the βAR forms a critical determinant of the ability of the receptor to activate G proteins. This may reflect either a general contribution of the hydrophobic face of this region to G protein activation or a more specific role for one or more of the hydrophobic amino acid side chains that were replaced by leucine residues in this study. The precise nature of the hydrophilic amino acids within this region does not appear to be as important, because the substitution of all of the charged amino acids with serine residues has only a minimal effect on the ability of the βAR to activate G_s. This observation is surprising, in light of the unusual concentration of charged amino acid side chains within this region of the receptor. However, an examination of this region in related G protein-coupled recep-

tors shows that the substitution of each of these individual basic amino acid residues with a neutral hydrophilic or even acidic residue occurs in other receptor proteins. The substitution of mastoparan for this region results in a mutant βAR that couples poorly to G proteins, although mastoparan itself has been demonstrated to activate G proteins at least as well as the peptide corresponding to the amino terminus of βAR loop i3 (11, 12). The substitution of mastoparan in this region of the βAR would result in a change in the orientation of the dipole of the amphipathic helix within the receptor, however, suggesting that the orientation, as well as the primary sequence, of the hydrophobic residues in this domain may be critical for activation of G proteins.

As discussed above, the region at the amino terminus of loop i3 would be predicted to fold as an amphipathic α-helix, essentially forming a cytosolic extension of the fifth transmembrane helix of the receptor. The activation of the βAR by catecholamine agonists requires the interaction of the catechol hydroxyl substituents of the ligand with the side chains of two serine residues in the fifth transmembrane domain of the receptor (17). Thus, it has been suggested that the conformational changes induced in the fifth transmembrane helix of the receptor by this interaction could lead to an alteration in the conformation of the cytosolic domain at the bottom of the helix, resulting in G protein activation. The results of the present study support this model for receptor activation and suggest that the hydrophobic rather than the hydrophilic face of this cytosolic α-helix is important in activating the G protein. Direct biophysical analysis of the interactions between the receptor and the G protein should allow further definition of specific points of contact between the agonist-occupied receptor and the G protein.

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