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Determination of Structural Domains for G Protein Coupling and Ligand Binding in β_3-Adrenergic Receptor

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SUMMARY

The β_3-adrenergic receptor (β_3AR) is a member of the superfamily of G protein-coupled receptors that are characterized by seven putative transmembrane helices connected by hydrophilic loops. The mechanism by which the activated βARs transmit the signals across the plasma membrane involves the stimulation of G_s, which in turn activates adenylyl cyclase, yielding the second messenger cAMP. In the present study, we created a series of mutant β_3ARs to explore the structural basis for the subtype-specific binding of BRL 37344, a β_3-selective agonist, and for the coupling of the receptor to G_s. To study the mechanism of subtype-specific binding of BRL 37344, chimeric β_2/β_3ARs were constructed and expressed in Raji cells. Binding studies suggest that the transmembrane segment 5 region of the β_3AR contains critical determinants for observed high affinity for BRL 37344. Previous studies of β_2ARs have demonstrated a role for the third intracellular loop in activating G_s. To investigate the role of this region in the β_3AR, we constructed mutant β_3ARs lacking a small segment of the amino- or carboxyl-terminal domain of the third intracellular loop. Expression of these mutant receptors in mouse L cells and Raji cells reveals that although both mutants are capable of binding the antagonist [^{125}I]iodocyanopindolol, the agonist-stimulated cAMP production mediated by these mutant receptors is markedly attenuated or abolished. In addition, both mutant β_3ARs exhibit an approximately 10-fold increase in affinity for agonist binding, whereas the affinity for antagonists is not affected. This increased agonist affinity is not altered by treatment with 100 μM 5′quanylyl-imidodiphosphate, suggesting that these mutant receptors are uncoupled from G proteins. The results of the present study demonstrate that these regions of the third intracellular loop of β_3AR are critical for coupling to G proteins and suggest a role for these regions in maintaining the resting state of the unliganded receptor.

The β-adrenergic receptors (βARs) belong to the superfamily of G protein-coupled receptors that are characterized by seven putative hydrophobic transmembrane helices connected by hydrophilic loops. Three subtypes of βARs (β_1, β_2, and β_3) have been characterized by pharmacological as well as molecular cloning approaches (for a recent review, see Ref. 1). These receptors share the same endogenous catecholamine ligands (norepinephrine and epinephrine) and activate a common signal transduction pathway. On stimulation by agonists, the receptors undergo conformational changes, which in turn activate the stimulatory G_s, thereby leading to the activation of adenylyl cyclase and the production of intracellular cAMP. The second messenger cAMP interacts with various effectors within the cell, eliciting the ultimate biological activities. The three receptor subtypes can be characterized by their relative affinities for epinephrine and norepinephrine, with the β_1 subtype having equal affinities for the two agonists, the β_2 subtype having higher affinity for epinephrine, and the β_3 subtype having higher affinity for norepinephrine. The three receptor subtypes also have different tissue distributions, with the β_1AR expressed predominantly in the heart, where it is the target for the β blocker class of drugs, and the β_2AR expressed in skeletal muscle and in the lungs, where it mediates the bronchorelaxor properties of the β agonists. The β_3AR is a more recently defined class of βARs expressed primarily in adipose tissue (2, 3). At present, the physiological function of β_3AR is only partially appreciated and is believed to be related to energy metabolism and thermogenesis (4, 5). Pharmacologically, these receptors have a number of distinct features and were originally called "atypical" βARs. For example, β_3ARs display high affinity for atypical βAR-selective agonists such as BRL 37344 but a reduced affinity for several classic β antagonists such as propranolol, and they show only a limited degree of stereoselectivity for ligand binding (4, 6). Moreover, the β_3AR appears to be more weakly coupled to G_s than are the β_1AR or β_2AR, and in contrast to other members of the βAR family, β_3AR does not appear to be desensitized on receptor activation (7, 8).

The receptor-mediated cascade of signal transduction begins with the binding of the ligand to its receptor. During the past decade, the molecular mechanisms underlying such ligand/receptor interactions have been studied most extensively for the β_2AR system. Several amino acid residues in

ABBREVIATIONS: AR, adrenergic receptor; [^{125}I]CYP, [^{125}I]iodocyanopindolol; PCR, polymerase chain reaction; PKA, protein kinase A, TM, transmembrane segment.

the β_2AR, including Asp-113, Ser-204, Ser-207, Phe-290, and Asn-312, have been identified as forming the putative binding pocket for nonselective β agonists and antagonists (9). All of these amino acid residues are completely conserved in β_3AR and are likely to play a similar role in binding to the common ligands. More recently, the successful development of β_3-selective ligands, represented by BRL 37344, suggests that these new compounds interact with β_3ARs differently than with β_1ARs and β_2ARs and that additional structural determinants (amino acid residues) must exist within β_3ARs to account for the enhanced binding affinity for BRL 37344 compared with β_1ARs and β_2ARs. At present, however, the molecular mechanisms for such subtype specificity remain undetermined.

Another important step in the signal transduction pathway for all G protein-coupled receptors is the direct interaction between the receptor and the G protein. Several studies with mutagenesis and synthetic peptide mapping have demonstrated that in ARs as well as other G protein-coupled receptors, the structural determinants for G protein coupling usually reside within the intracellular loops, especially the third intracellular loop and carboxyl-terminal regions (10–16). In the β_2AR, for example, it has been shown that the specificity of receptor/G protein interaction is dictated by the third intracellular loop (13) and that a critical region for G protein coupling can be further localized to a small segment of amino acid residues at the amino- and carboxyl-terminal portions of this loop (10, 11). The structural domain(s) involved in G protein coupling of the β_3AR have not been explored. Because of the apparently weaker coupling of the β_3AR to G_s compared with the β_2AR, the present study was undertaken to examine whether the amino- and carboxyl-terminal regions of the third cytoplasmic loop are also critical for G protein coupling to β_3ARs. In addition, the molecular basis for receptor subtype-selective binding of BRL 37344 was investigated.

Materials and Methods

Construction and expression of mutant β_3ARs. Three deletion mutants of human β_3AR were constructed with the use of PCR and synthetic oligonucleotide linker adapter methods (Fig. 1). D(227–234) was made by digesting the wild-type human β_3AR cDNA (provided by J. G. Granneman, Wayne State University, MI) with *Acc*I and *Pvu*II, followed by re-ligation with a linker adaptor, 5′CTACGCGCGG3′/3′TGCGCGCC5′. The mutant encodes a β_3AR lacking eight amino acid residues (VFVVATRQ) at the amino-terminal portion of the third intracellular loop. D(277–289) was constructed by ligating two PCR products, which were obtained by using the following primer pairs: (a) 5′ CTAGAGTTGGCGCCCTGTGCACCTTGGGT3′/5′ CTTCTGAAGCTTCAGG CCTAAGAAACTCCCCAA GAAGCCCCGTGCAGCCGTTGGCAAAGCCTGGGC TGCGCTGGG CTGCTCCGGG 3′) and (b) 5′AGTGCACAGGGCGCCGCAGGC GGGCACCCC3′/5′ GTCATCGTGGCCATCGCCTGGA 3′. After the digestion of product a with *Nar*I/*Bse*AI and of product b with *Nar*I/*Acc*I, the two PCR fragments were then ligated with the β_3 DNA fragment (*Acc*I/*Bse*AI). This mutant receptor lacks 13 amino acid residues (RRPARLLPLREHR) at the carboxyl-terminal portion of the third intracellular loop. D(244–291) was created by ligating the partially digested *Pvu*II/*Apa*LI fragment of β_3AR with a linker adapter, 5′ CTGCGCTTGCTGCGCGGGGAGCTGGGC3′/5′TGCAGCCCAGCTCCCCGCGCAGCAAGCGCAG3′, thereby generating a receptor lacking a major portion of the middle and carboxyl-terminal regions of the third intracellular loop (RFPPEESPPAPSRSLAPA-

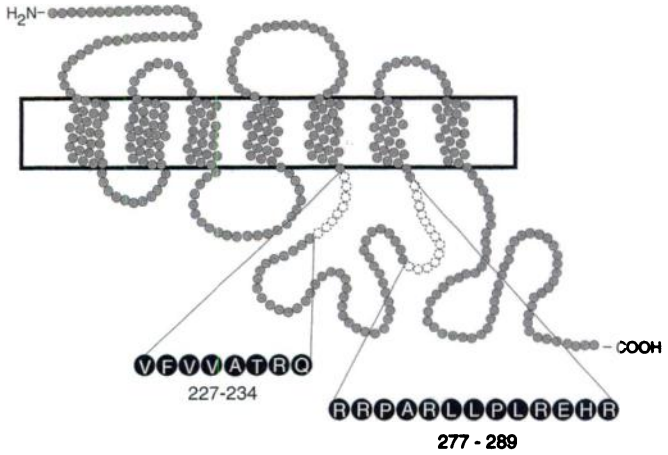


Fig. 1. Schematic illustration of deletion mutants of β_3AR. *Shaded circles*, amino acid residues, illustrating the seven transmembrane helix topology of the receptor with its amino terminus on the extracellular side and carboxyl terminus on the cytoplasmic side of the membrane. *Dotted circles*, relative positions of the deleted amino acid residues.

PVGTCAPPEGCPACGRRP AAR LLPLREHRAL). The nucleotide sequences of the mutant receptors were confirmed by DNA sequencing.

The β_2/β_3 chimeric receptors were generated by combining different portions of human β_2ARs and β_3ARs with PCR and cassette mutagenesis approaches (Table 2). C1 and C2 were constructed by exchanging, between β_2ARs and β_3ARs, the *Acc*I/*Bam*HI fragment composing the third intracellular loop to the carboxyl terminus. To make C8, a PCR fragment constituting the amino terminus to TM 4 of β_3AR was generated by using a T7 primer (present in the vector sequence upstream from the β_3 coding region) and a β_3 reverse primer containing a *Kpn*I site at the 5′ end (5′AGTACGGTACCACTGGCTCATGATGG3′). The PCR product was then digested with *Hind*III/*Kpn*I, and the resulting β_3 fragment was subcloned into the corresponding *Hind*III/*Kpn*I sites of β_2AR construct. For C11, a β_2 PCR fragment covering the amino terminus to TM 2 was amplified by using a T7 primer and a β_2 reverse primer containing an *Nar*I site (5′TGCCATGGCGGCAAAAGTCCACATTTTC3′). After digestion with *Hind*III/*Nar*I, the β_2 sequence was subcloned into the equivalent *Hind*III/*Nar*I sites of the β_3 construct. Finally, C14 was constructed by replacing the *Acc*I/*Bam*HI β_2 fragment of C8 with the corresponding β_3 sequences.

The wild-type and mutant receptors were either subcloned into the expression vector pRC/CMV (Invitrogen, San Diego, CA) for transient expression in mouse L cells using a standard DEAE-Dextran transfection method or subcloned into expression vector p636 (a generous gift from Bill Sugden, University of Wisconsin, Madison, WI) for expression in Raji cells by electroporation, as previously described (17).

Receptor binding. The membranes were prepared 3 days (in L cells) or 7–10 days (in Raji cells) after the transfection by harvesting the cells in ice-cold lysis buffer (5 mM Tris, pH 7.4, 2 mM EDTA), followed by a 15-min centrifugation at 38,000 × *g*. The membrane pellet was then resuspended in TME buffer (75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 1.5 mM EDTA). Equilibrium binding to the β_3AR was performed in a final volume of 0.25 ml TME buffer, including membranes, 240 pM [^{125}I]CYP (Du Pont-NEN, Boston, MA), and serial dilutions of the competing ligands. Nonspecific binding was determined in the presence of 10^{-4} M propranolol. Binding reactions were incubated for 90 min at 23° and terminated by rapid filtration over GF/C filters presoaked in 0.1% polyethylenimine. The radioactivity was quantified with a Packard gamma counter. The binding data were analyzed with nonlinear curve-fitting programs of GraphPad software (San Diego, CA).

cAMP production. L cells were harvested in phosphate-buffered saline with 5 mM EDTA, pelleted, and then resuspended in ACC buffer (75 mM Tris, pH 7.4, 250 mM sucrose, 12.5 mM $MgCl_2$, 1.5 mM EDTA, 1 μM ascorbic acid, and 0.6 mM 3-isobutyl-1-methylxanthine). The cells were incubated with various concentrations of isoproterenol for 45 min at room temperature, and the reaction was terminated by boiling for 3 min. The concentration of cAMP in the lysate was determined with a modified PKA binding assay (18) or an automated cAMP radioimmunoassay (Atto Instruments, Rockville, MD). For the PKA binding assay, the lysate was incubated with 3.6 nM ^{3}H-cAMP (DuPont-NEN, Boston, MA) and 5 μg PKA (Sigma Chemical Co., St. Louis, MO) in a final volume of 185 μl for 2–24 hr at 4°, followed by rapid filtration over GF/C filters with cold washing buffer solution (20 mM potassium phosphate, pH 6.0); the radioactivity on the filter was then quantified with a beta counter. For the automated radioimmunoassay, the cell lysate was diluted 5-fold in 0.1 N HCl and then acetylated by the mixture of 150 μl of acid-diluted sample with 6 μl of acetylation mixture (acetic anhydride/triethylamine, 1:2.5). The acetylated samples were assayed with an Attoflow instrument (Rockville, MD) according to the manufacturer's protocol, and the final concentration of cAMP was determined by comparison with a cAMP standard curve.

Results

In a study of the interactions between a receptor and G proteins in a heterologous expression system, the choice of an appropriate host cell line is important. Mouse L cells were used for functional analysis in the present study because (a) the parental L cell line does not have detectable βAR binding or receptor-mediated adenylyl cyclase activity and (b) this cell line had previously been shown to be capable of expressing functionally active β_2AR (10). When wild-type β_3AR was expressed in L cells, a basal level of cAMP production of 300 ± 170 fmol/mg protein/min (four experiments) was measured, and this level is essentially identical to the basal cAMP level in nontransfected L cells (data not shown). As shown in Fig. 2, the βAR agonist isoproterenol caused a dose-dependent 3.5-fold stimulation of cAMP production with an EC_{50} value of 26 nM. The expression of D(227–234) and D(277–289) in L cells did not significantly alter the basal cAMP level (310 ± 100 pmol/mg protein/min, four experiments) compared with cells expressing the wild-type receptors. However, the isoproterenol-mediated production of cAMP was markedly attenuated or abolished in cells expressing these mutant receptors, with a maximal stimulation of 1.3-fold and 1.8-fold, respectively (Fig. 2 and Table 1). The overall level of expression of the wild-type β_3AR in L cells was similar to or slightly higher than that of the deletion mutants. However, it was observed that the isoproterenol-stimulated cAMP responses (-fold stimulation over basal) by the wild-type β_3AR did not vary over this range of receptor expression, indicating that the decreased ability of the mutants to stimulate cAMP accumulation was not due to the slightly decreased expression of the mutant receptors.

Competition binding with [^{125}I]CYP indicated that wild-type β_3AR displayed two affinity sites for agonist isoproterenol: a high affinity site ($K_d = 5.0 \times 10^{-8}$ M), accounting for 28% of the receptor population, and a low affinity site ($K_d = 2.6 \times 10^{-6}$ M), accounting for 72% of the receptors. Deletion of either the amino- or carboxyl-terminal portion of the third intracellular loop of β_3AR resulted in a predominantly monophasic binding isotherm. The affinity of this single site was intermediate between the affinities of the two components of the wild-type receptor (Table 1). Neither detectable [^{125}I]CYP binding nor isoproterenol-stimulated cAMP production was observed for cells transfected with D(244–291). The reason for the absence of activity of D(244–291) is not known. It is conceivable that the deletion of a large portion of the third intracellular loop and/or the removal of two additional amino acid residues (290 and 291) close to TM 6 may disrupt the stability of the membrane spanning topology and/or cause aberrant folding of the receptor.

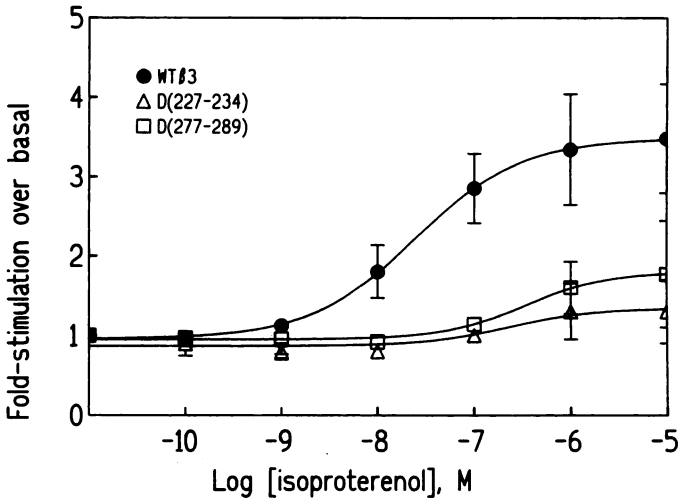


Fig. 2. Isoproterenol-stimulated production of cAMP in L cells expressing wild-type ARs and mutant β_3ARs. Receptors were expressed transiently in L cells. Receptor levels for both the wild-type and mutants usually ranged from 50 to 150 fmol/mg protein as estimated by single point binding assays with [^{125}I]CYP, and within any given experiment, the level of expression for the wild-type receptor was no more than 170 ± 47% (mean ± standard error, four experiments) higher than that of the mutants. Deletion mutations did not alter the receptor affinity to [^{125}I]CYP (K_d = 0.85, 1, and 1 nM for the wild-type β_3ARs, D(227–234) β_3ARs, and D(277–289) β_3ARs, respectively). At 72 hr after transfection, the cells were harvested and stimulated with various concentrations of isoproterenol, and the amount of cAMP in cell lysate was measured as described in Materials and Methods. Results are presented as -fold stimulation over the basal values of cAMP. Basal cAMP values varied among experiments, but in each experiment the basal value for the mutants was within 0.9–1.4-fold that for the wild-type receptor. Data are mean ± standard error of two to four independent determinations.

As shown, when the wild-type β_3AR is expressed in L cells, a considerable number of the receptors are in the high affinity state for isoproterenol binding, whereas the introduction of the deletion mutations resulted in receptors with a single affinity state that was only slightly lower than this high affinity state (Table 1). To further investigate the effects of the deletions on agonist binding affinity, we expressed the wild-type and D(277–289) β_3ARs in Raji cells, where the vast majority of receptors would be expected to exist in the low affinity state due to a high level of overexpression of the receptors relative to G_s proteins. The wild-type β_3ARs expressed in Raji cells displayed an affinity of 2.2 ± 0.3 × 10^{-6} M (four experiments) for isoproterenol. The affinity of the receptor for the agonist was not altered in the presence of 100 μM 5′quanylyl-imidodiphosphate (data not shown), an agent that binds to the guanine nucleotide binding site on G proteins and disrupts the coupling between receptors and G proteins. When D(277–289) was expressed in Raji cells, the competition binding curve for isoproterenol was shifted to the left by an order of magnitude, yielding an IC_{50} value of 3.8 ±

TABLE 1

Agonist binding and stimulation of cAMP production by wild-type and mutant β_3 ARs

The ARs were expressed transiently in L cells. K_H and K_L represent the affinity values of high and low affinity binding sites, respectively. $\%K_H$ and $\%K_L$ indicate the percentage of receptors in high and low affinity states, respectively. The maximal stimulation of cAMP production was measured in the presence of 10^{-5} M isoproterenol. Data are given as mean ± standard error of two to five independent determinations.

	Binding affinity				cAMP production	
	K_H	$\%K_H$	K_L	$\%K_L$	Maximum stimulation (-fold over basal)	K_{act}
	M		M			M
Wild-type β_3AR	$5.0 \pm 2.1 \times 10^{-8}$	28 ± 4	$2.6 \pm 0.7 \times 10^{-6}$	72 ± 4	3.5 ± 0.7	$2.6 \pm 0.3 \times 10^{-8}$
D(227-234)AR	$1.8 \pm 1.0 \times 10^{-7}$	100	ND		1.3 ± 0.4	ND
D(277-289)AR	$2.2 \pm 0.4 \times 10^{-7}$	87 ± 13	4.4×10^{-6}[a]	13 ± 13	1.8 ± 0.7	ND

ND = not detectable.
[a] The low affinity binding site was detected in only one experiment.

0.6×10^{-7} M (Fig. 3A, four experiments). This affinity is very similar to that observed for the mutant receptor expressed in L cells and was also determined to be insensitive to treatment with guanine nucleotide ($IC_{50} = 2.5 \pm 0.6 \times 10^{-7}$ M in the presence of 100 μM 5′guanylyl-imidodiphosphate). In contrast to the increase in affinity observed for the agonist isoproterenol, the binding to the antagonist propranolol was not affected by the deletion (Fig. 3B).

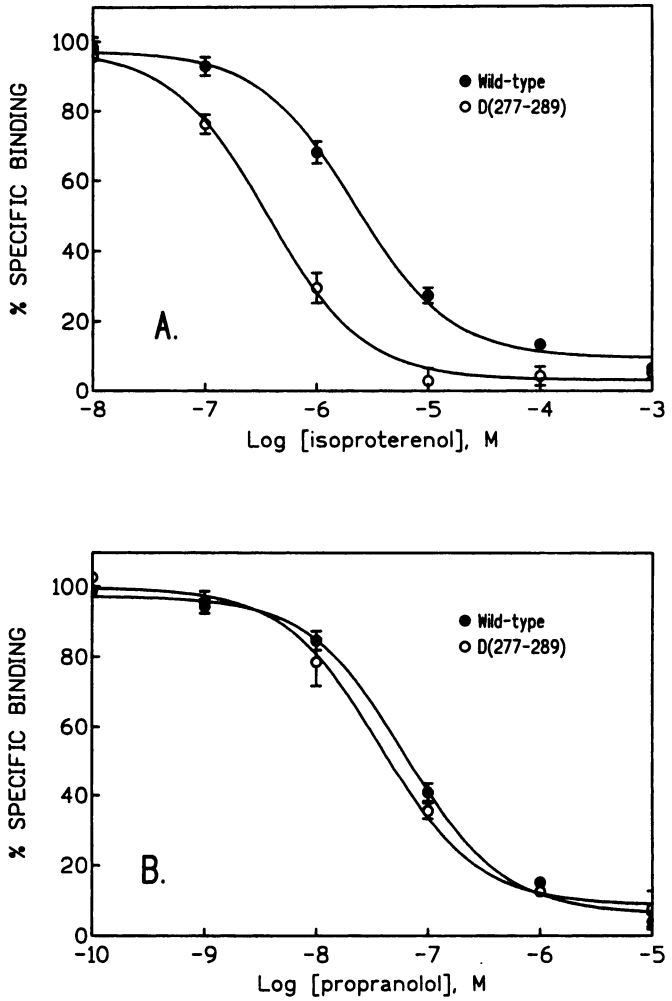


Fig. 3. Competition binding of [^{125}I]CYP to wild-type ARs and mutant β_3ARs expressed in Raji cells. In general, the expression levels of the receptors were in the range of 300-1000 fmol/mg protein. A, isoproterenol binding; B, propranolol binding. Data are mean ± standard error of four independent experiments.

To study the mechanism of subtype-specific binding of BRL 37344, a series of human β_2 and β_3 chimeric receptors were constructed and expressed in Raji cells (Table 2). The wild-type β_3AR displayed a 10-fold higher affinity for BRL 37344 and a 26-fold lower affinity for the β antagonist propranolol compared with the wild-type β_2AR (Table 2). When the amino terminus and TM 1–5 of the β_3AR were replaced with the analogous β_2 sequence (C1), the chimera displayed a binding profile similar to that of the wild-type β_2AR. However, when the converse chimera (C2) was tested, no detectable binding of [^{125}I]CYP was observed. The loss of ligand binding in C2 may result from aberrant folding or translocation of the chimeric protein. To assess the role of the first half of the β_3AR (TM 1–4) in high affinity binding to BRL 37344, C8 was constructed and tested. The binding of C8 revealed an approximately 10- and 100-fold decrease in affinity for BRL 37344 compared with β_2AR and β_3AR, respectively. However, the binding affinity of C8 for propranolol remained unaltered, suggesting that the overall structure of the receptor is preserved and that the binding of BRL 37344 is sensitive to relatively subtle conformational changes (Table 2). When TM 3–7 of the β_2AR was replaced with the corresponding β_3 domains (C11), the chimera exhibited an increased binding affinity for BRL 37344, indicating that these β_3 domains may carry the determinants for subtype specificity. Further sequence comparison of C1, C2, C8, and C11 revealed that the high affinity for BRL 37344 may be attributed to a much narrower domain comprising the second extracellular loop and TM 5 of the β_3AR. When this small segment of the β_3AR was replaced by the equivalent β_2 sequences (C14), the binding affinity for BRL 37344, but not propranolol, was reduced markedly to the level of wild-type β_2AR (Table 2). To further examine the role of the TM 5 region, the reciprocal chimera to C14 was constructed and tested. This chimera, C16, contains all of the sequences from β_2AR except for the second extracellular loop and TM 5, which are from β_3AR. The expression of C16 in Raji cells, however, failed to yield any detectable [^{125}I]CYP binding, a similar result to that described for C2.

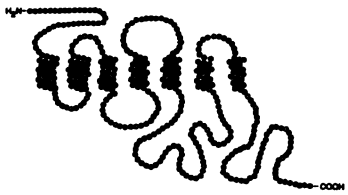
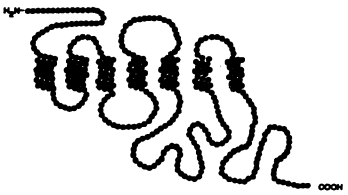
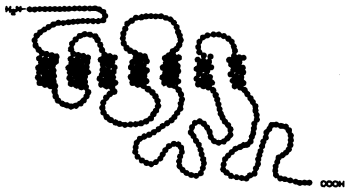
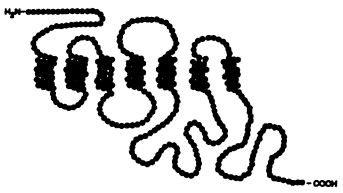
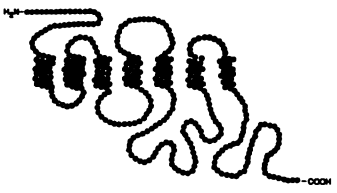
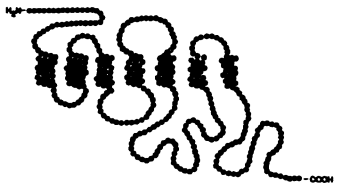
Discussion

The third intracellular loop has been implicated in G protein coupling for a number of receptors; perhaps the most thoroughly studied example is β_2AR (10–16). It has been shown that the specificity of G protein coupling can be altered by substituting the third intracellular loops between

TABLE 2

Ligand binding affinity for wild-type and chimeric β_2/β_3ARs expressed in Raji cells

The construction, expression, and ligand binding of the receptors are described in Materials and Methods. The schematics illustrate the junctions of chimeric receptors and their relative positions with respect to TM. The amino acid residues at the junctions are listed under each chimera, and their position numbers are indicated in parentheses. Data are given as mean ± standard error of three to nine experiments.

		IC_{50}	
		BRL 37344	Propranolol
		nM	
Wild-type β_2		3,400 ± 1,100	3 ± 1
Wild-type β_3		380 ± 43	80 ± 11
C1 β_2V(218)/β_3Y(224)		1,100 ± 260	7 ± 4
C8 β_3W(177)/β_2Y(174)		53,000 ± 4,800	9 ± 2
C11 β_2G(102)/β_3A(106)		97 ± 27	26 ± 6
C14 β_3W(177)/β_2Y(174) β_2V(218)/β_3Y(224)		3,300 ± 270	26 ± 7

different receptors (13, 19) and that deletion mutants of β_2AR that lack small segments of amino acid residues at the amino- or carboxyl-terminal regions of the third intracellular loop display impaired coupling to G_s and adenylyl cyclase activation. These latter mutants also display an increase in agonist affinity (10, 11), similar to that observed for the β_3AR in the present study. The results in the present study demonstrate that deletion of a small segment from either the amino-terminal (8 amino acid residues) or carboxyl-terminal (13 amino acid residues) region of the third intracellular loop of the β_3AR is sufficient to uncouple the receptors from adenylyl cyclase activity on agonist stimulation. In addition, both mutant β_3ARs exhibit an increased affinity for isoproterenol without affecting the affinity of antagonists. Although the interactions between the mutant β_3ARs and G proteins were not measured directly in the present study, the inability of the mutants to couple to adenylyl cyclase and the insensitivity of agonist binding to guanine nucleotide support the notion that the deletions prevent effective coupling between the receptors and G_s. Taken together, the data indicate that the structural domains at the amino- and carboxyl-terminal regions of the third intracellular loop of β_3ARs are critical for G protein coupling. Because there is only moderate sequence homology (63% at the amino-terminal and 31% at the carboxyl-terminal regions of the third intracellular loop) between β_2AR and β_3AR in these regions, the molecular determinants for G protein binding are most likely not based on amino acid side-chain specificity but rather may be governed by the conformational or physicochemical properties of this region of the receptor.

The precise mechanisms underlying the observed increase in agonist affinity for the mutant receptors remain unknown. G protein-coupled receptors have been observed to exist in two affinity states with respect to agonist binding. These two functional states are interconvertable depending on whether the receptor is coupled to a G protein: the receptor displays high affinity for agonist when it is coupled to a G protein and low affinity when it is uncoupled from a G protein. However, our observations that the deletion mutants show high affinity for agonist while being uncoupled from G proteins indicate that coupling to G proteins per se is not a prerequisite for formation of the high affinity state. Recently, several reports have shown that mutations within the carboxyl-terminal region of the third intracellular loop of α_1ARs, α_2ARs, and β_2ARs result in constitutively active receptors with increased affinity for agonist (20–23). Based on these findings, the authors hypothesized that G protein-coupled receptors can exist in an inactive state or in an active, high affinity state that is capable of binding to G proteins. The transition from the inactive to the active state is postulated to involve conformational changes that release the as-yet-undefined constraint imposed by structural domains within the receptor. The conformational change may be induced, under normal circumstances, by agonist binding or promoted by mutations that disrupt the constraining mechanisms and allow the receptor to "relax" into the high affinity conformation (24). The result from the present study is consistent with this hypothesis. However, these data as well as the previous results with the β_2AR (10) suggest that in addition to forming sites of interaction with G_s proteins, the amino- and carboxyl-terminal regions of the third intracellular loop may play a role in keeping the receptor in the inactive state. Deletion of either of these regions would remove such a constraint and drive the isomerization toward the high affinity conformation. It has been reported that certain rhodopsin mutants with deletions in the same cytoplasmic loop region are capable of binding to G protein (G_t) with high affinity but fail to activate G_t (25). Whether an analogous mechanism is responsible for the observed functional uncoupling in the β_3 mutants requires further investigation with purified proteins.

In contrast to the G protein-coupling domains, the ligand binding sites are believed to be located in the TMs of βARs. In β_2ARs, several amino acid residues in TMs 3 and 5–7 have been shown to be critical for binding of nonselective β agonists and antagonists (9). A similar mechanism may be operative in β_3ARs because all these important amino acid residues are also present in the β_3AR (2). BRL 37344 represents a new class of compounds that are relatively selective for the β_3AR. The structural determinants responsible for the enhanced affinity of the β_3AR for BRL 37344 have not yet been determined. In the present study, we demonstrate that a small stretch of amino acid residues comprising TM 5 and the second extracellular loop of the β_3AR may be responsible for the higher affinity of this receptor subtype for BRL 37344. Comparison of amino acid sequences in the TM 5 region reveals only few nonconservative changes among the three βAR subtypes. Point mutations at these positions from β_3 to β_2 residues (P203A and S210I) resulted in no change in BRL 37344 affinity (data not shown). Thus, it appears that the high affinity of the β_3AR for BRL 37344 may be determined by interactions with multiple residues in the transmembrane domains of the receptor. A similar pattern of multiple interactions has been postulated to explain the differential affinity of epinephrine and norepinephrine for the β_1ARs and β_2ARs, where single residue substitutions failed to account for subtype specificity (26, 27). In addition, the potential contribution by residues in the second extracellular loop region cannot be dismissed. In this regard, it is interesting to note that the acidic functionality on BRL 37344 plays a role in the subtype specificity of this agonist. The possibility that this polar substituent projects into the extracellular space requires further exploration with genetic and biophysical approaches.

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References

1. Strosberg, A. D. Structure, function and regulation of adrenergic receptors. *Protein Sci.* **2**:1198–1209 (1993).
2. Emorine, L. J., S. Marullo, M.-M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A. D. Strosberg. Molecular characterization of the human β3-adrenergic receptor. *Science (Washington D. C.)* **245**:1118–1121 (1989).
3. Granneman, J. G., K. N. Lahners, and D. Rao. Rodent and human β_3-adrenergic receptor genes contain an intron within the protein-coding block. *Mol. Pharmacol.* **42**:964–970 (1992).
4. Arch, J. R. S., A. T. Ainsworth, M. A. Cawthorge, V. Piercy, M. V. Sennitt, V. E. Thody, C. Wilson, and S. Wilson. Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature (Lond.)* **309**:163–165 (1984).
5. Emorine, L., N. Blin, and Strosberg, A. D. The human β3-adrenoceptor: the search for a physiological function. *Trends Pharmacol. Sci.* **15**:3–7 (1993).
6. Sillence, M. N., N. G. Moore, G. G. Pegg, and D. B. Lindsay. Ligand binding properties of putative *beta* 3-adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes. *Br. J. Pharmacol.* **109**:1157–1163 (1993).
7. Carpene, C., J. Galitzky, P. Collon, F. Esclapes, M. Dauzats, and M.

Lafontan. Desensitization of *beta*-1 and *beta*-2, but not *beta*-3, adrenoceptor-mediated lipolytic responses of adipocytes after long-term norepinephrine infusion. *J. Pharmacol. Exp. Ther.* **265**:237–247 (1993).
8. Liggett, S. B., N. J. Freedman, D. A. Schwinn, and R. J. Lefkowitz. Structural basis for receptor subtype-specific regulation revealed by a chimeric β3/β2-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **90**:3665–3669 (1993).
9. Strader, C. D., T. M. Fong, M. R. Tota, D. Underwood, and R. A. F. Dixon. Structure and function of G protein-coupled receptors. *Annu. Rev. Biochem.* **63**:101–132 (1994).
10. Strader, C. D, R. A. F. Dixon, A. H. Cheung, M. R. Candelore, A. D. Blake, and I. S. Sigal. Mutations that uncouple the β-adrenergic receptor from G_s and increase agonist affinity. *J. Biol. Chem.* **262**:16439–16443 (1987).
11. Dixon, R. A. F., I. S. Sigal, E. Rands, R. B. Register, M. R. Candelore, A. D. Blake, and C. D. Strader. Ligand binding to the β-adrenergic receptor involves its rhodopsin-like core. *Nature (Lond.)* **326**:73–77 (1987).
12. Dixon, R. A. F., I. S. Sigal, and C. D. Strader. Structure-function analysis of the β-adrenergic receptor. *Cold Spring Harbor Symp. Quant. Biol.* **53**:487–497 (1988).
13. Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Chimeric a2-, β2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Washington D. C.)* **240**:1310–1316 (1988).
14. Kubo, T., H. Bujo, I. Akiba, J. Nakai, M. Mishina, and S. Numa. Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. *FEBS Lett.* **241**:119–125 (1988).
15. Cheung, A. H., R.-R. C. Huang, M. P. Graziano, and C. D. Strader. Specific activation of G_s by synthetic peptides corresponding to an intracellular loop of the β-adrenergic receptor. *FEBS Lett.* **279**:277–280 (1991).
16. Dalman, H. M., and R. R. Neubig. Two peptides from the α2A-adrenergic receptor alter receptor G protein coupling by distinct mechanisms. *J. Biol. Chem.* **266**:11025–11029 (1991).
17. Suryanarayana, S., and B. M. Kobilka. Construction and expression of chimeric receptors to understand the structure-function relationships in adrenergic receptors. *Methods Comp. Methods Enzymol.* **3**:193–204 (1991).
18. Barton, A. C., L. Black, and D. R. Sibley. Agonist-induced desensitization of D2 dopamine receptors in human Y-79 retinoblastoma cells. *Mol. Pharmacol.* **39**:650–658 (1991).
19. Wong, S. K.-F., E. M. Parker, and E. M. Ross. Chimeric muscarinic cholinergic:β-adrenergic receptors that activate G_s in response to muscarinic agonists. *J. Biol. Chem.* **265**:6219–6224 (1990).
20. Cotecchia, S., A. Exum, M. G. Caron, and Lefkowitz, R. J. Regions of the α1-adrenergic receptor involved in coupling to phosphotidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc. Natl. Acad. Sci. USA* **87**:2896–2900 (1990).
21. Kjelsberg, M. A., S. Cotecchia, J. Ostrowski, M. G. Caron, and R. J. Lefkowitz. Constitutive activation of the a1b-adrenergic receptor by all amino acid substitutions at a single site: evidence for a region which constrains receptor activation. *J. Biol. Chem.* **267**:1430–1433 (1992).
22. Ren, Q., H. Kurose, R. J. Lefkowitz, and S. Cotecchia. Constitutively active mutants of the a2-adrenergic receptor. *J. Biol. Chem.* **268**:16483–16487 (1993).
23. Samama, P., S. Cotecchia, T. Costa, and R. J. Lefkowitz. A mutation-induced activated state of the β2-adrenergic receptor: extending the ternary complex model. *J. Biol. Chem.* **268**:4625–4636 (1993).
24. Lefkowitz, R. J., S. Cotecchia, P. Samama, and T. Costa. Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.* **14**:303–307 (1993).
25. Franke, R. R., B. Konig, T. P. Sakmar, H. G. Khorana, and K. P. Hofmann. Rhodopsin mutants that bind but fail to activate transducin. *Science (Washington D. C.)* **250**:123–125 (1990).
26. Frielle, T., K. W. Daniel, M. G. Caron, and R. J. Lefkowitz. Structural basis of *beta*-adrenergic receptor subtype specificity studied with chimeric *beta 1/beta* 2-adrenergic receptors. *Proc. Natl. Acad. Sci. U.S.A.* **85**:9494–9498 (1988).
27. Dixon, R. A. F., W. S. Hill, M. R. Candelore, E. Rands, R. E. Diehl, M. S. Marshall, I. S. Sigal, and C. D. Strader. Genetic analysis of the molecular basis for β-adrenergic receptor subtype specificity. *Proteins* **6**:267–274 (1989).

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