

# Mutational and Computational Analysis of the $\alpha_{1b}$ -Adrenergic Receptor

INVOLVEMENT OF BASIC AND HYDROPHOBIC RESIDUES IN RECEPTOR ACTIVATION AND G PROTEIN COUPLING\*<sup>§</sup>

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To investigate their role in receptor coupling to G<sub>q</sub>, we mutated all basic amino acids and some conserved hydrophobic residues of the cytosolic surface of the  $\alpha_{1b}$ -adrenergic receptor (AR). The wild type and mutated receptors were expressed in COS-7 cells and characterized for their ligand binding properties and ability to increase inositol phosphate accumulation. The experimental results have been interpreted in the context of both an *ab initio* model of the  $\alpha_{1b}$ -AR and of a new homology model built on the recently solved crystal structure of rhodopsin. Among the twenty-three basic amino acids mutated only mutations of three, Arg<sup>254</sup> and Lys<sup>258</sup> in the third intracellular loop and Lys<sup>291</sup> at the cytosolic extension of helix 6, markedly impaired the receptor-mediated inositol phosphate production. Additionally, mutations of two conserved hydrophobic residues, Val<sup>147</sup> and Leu<sup>151</sup> in the second intracellular loop had significant effects on receptor function. The functional analysis of the receptor mutants in conjunction with the predictions of molecular modeling supports the hypothesis that Arg<sup>254</sup>, Lys<sup>258</sup>, as well as Leu<sup>151</sup> are directly involved in receptor-G protein interaction and/or receptor-mediated activation of the G protein. In contrast, the residues belonging to the cytosolic extensions of helices 3 and 6 play a predominant role in the activation process of the  $\alpha_{1b}$ -AR. These findings contribute to the delineation of the molecular determinants of the  $\alpha_{1b}$ -AR/G<sub>q</sub> interface.

The  $\alpha_{1b}$ -adrenergic receptor ( $\alpha_{1b}$ -AR)<sup>1</sup> belongs to the superfamily of G protein-coupled receptors (GPCRs) that transmit a

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains Table II.

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<sup>1</sup> The abbreviations used are: AR, adrenergic receptor(s); G protein, guanyl nucleotide binding regulatory protein; GPCR, G protein-coupled receptor; IP, inositol phosphate; i2 and i3, second and third intracellular loop; DMEM, Dulbecco's modified Eagle's medium; [<sup>125</sup>I]HEAT, [<sup>125</sup>I]iodo-2-[ $\beta$ -(4-hydroxyphenyl)ethylaminomethyl]tetralone; MD, molecular dynamics; PLC, phospholipase C; PCR, polymerase chain reac-

tion; r.ms.d., root mean square deviation; CAM, constitutively active mutant; R and R\*, inactive and active states, respectively.

variety of signals across the cell membrane. Stimulation of the  $\alpha_{1b}$ -AR by catecholamines activates proteins of the G<sub>q</sub> family, resulting in the production of inositol phosphates (IP) via the activation of phospholipase C (PLC) (1).

GPCRs are structurally characterized by seven transmembrane  $\alpha$ -helices connected by alternating extracellular (e) and intracellular (i) loops. While the extracellular portion of these receptors is primarily involved in ligand binding, the cytosolic loops mediate the interaction of the receptors with a number of signaling and regulatory proteins, including G proteins, arrestins, and G protein-coupled receptor kinases (reviewed in Ref. 2).

Evidence suggests that a conformational adjustment within the helical bundle of the receptor underlies the process of agonist-induced activation of GPCRs (reviewed in Ref. 3). The current hypothesis is that the transition from the inactive (R) to active (R\*) state of a GPCR results in receptor interaction with, and activation of, a G protein. Thus a GPCR-mediated biological response involves a series of events (*i.e.* receptor activation, receptor-G protein interaction, and receptor-induced G protein activation) for which a detailed mechanism still remains elusive at the molecular level. Although residues located in the helical bundle and at the boundary between the membrane and the cytosol may play a role in the "conformational switch" underlying receptor activation, amino acids in the intracellular loops are believed to be more directly involved in receptor-G protein interaction and/or receptor-induced G protein activation. The combination of these two latter events, which cannot be unequivocally separated experimentally, is generally indicated with the term of receptor-G protein coupling.

We have previously provided evidence that the negatively and positively charged amino acids of the conserved DRY motif at the cytosolic end of helix 3 play a key role in the activation process of the  $\alpha_{1b}$ -AR (4–6). Following a combination of experimental and computer-simulated mutagenesis of the  $\alpha_{1b}$ -AR, we have hypothesized that protonation of the aspartate (Asp<sup>142</sup>) and a shift of the arginine (Arg<sup>143</sup>) out of a conserved "polar pocket" are crucial steps in the transition of the receptor from the inactive (R) to active (R\*) state (4–6).

Several studies have tried to identify the amino acids of different GPCRs involved in G protein coupling at both experimental (as reviewed in Ref. 2) and theoretical levels (7–10). The majority of these studies indicate that sequences in the i2 loop as well as in the N and C termini of the i3 loop play an

tion; r.ms.d., root mean square deviation; CAM, constitutively active mutant; R and R\*, inactive and active states, respectively.

important role in the efficiency of receptor-G protein coupling and/or in the selectivity of receptor-G protein recognition. BBXXB or BBXB motifs located in different cytosolic loops (where B is any basic amino acid and X is any residue) have been implicated in the coupling of a number of GPCRs to G proteins (11–13). However, this motif has not been found to be universally important for all GPCRs. Other studies have identified hydrophobic amino acids as contributing to the receptor-G protein interface (14–16). In conclusion, what has become abundantly clear is that there is no simple sequence determinant that can be attributed to receptor-G protein coupling.

In a recent modeling study (8), docking simulations between active forms of the  $\alpha_{1b}$ -AR and a  $G_q$  heterotrimer led us to suggest that the positive surface of the cytosolic portion of GPCRs could complement a negative surface found on different G protein  $\alpha$  subunits and thereby play a role in receptor-G protein coupling. However, the docking simulations also suggested that, despite the large number of cationic amino acids, only some might interact with anionic residues in the  $G_q$   $\alpha$ -subunit. To investigate the role of cationic residues in receptor-G protein coupling, we have mutated all the basic amino acids located in the i1, i2, and i3 loops of the  $\alpha_{1b}$ -AR and investigated the effect of these mutations on receptor-mediated production of IP. In addition, we have also characterized the effects resulting from mutations of conserved hydrophobic residues in the cytosolic portion of the receptor.

Our findings demonstrate that mutations of the basic residues in the cytosolic portion of the receptor have wide ranging phenotypes. Only mutations of three (Arg<sup>254</sup>, Lys<sup>258</sup>, and Lys<sup>291</sup>) out of the twenty-three basic amino acids studied impaired the receptor-mediated signaling response. We also demonstrate an important role for two highly conserved hydrophobic residues in receptor function. The effect of these mutations has been evaluated in the context of both the *ab initio* model previously described (17) and a new model of the  $\alpha_{1b}$ -AR built on the recently solved 2.8-Å crystal structure of rhodopsin (18).

#### EXPERIMENTAL PROCEDURES

**Materials**—COS-7 cells were from the American Type Culture Collection (Rockville, MD); DMEM, gentamicin, fetal bovine serum, and restriction enzymes from Life Technologies, Inc. (Grand Island, NY); *Pwo* polymerase was from Roche Molecular Biochemicals (Mannheim, Germany); [<sup>125</sup>I]HEAT and [<sup>3</sup>H]inositol from PerkinElmer Life Sciences (Boston, MA); epinephrine was from Sigma Chemical Co. (St. Louis, MO), and prazosin from Research Biochemical International.

**Mutagenesis of the  $\alpha_{1b}$ -AR**—The cDNA of the hamster  $\alpha_{1b}$ -AR (19) was mutated using PCR-mediated mutagenesis and *Pwo* DNA polymerase. The constructs were subcloned in the pRK5 expression vector, and mutations were confirmed by automated DNA sequencing (Microsynth GmbH, Switzerland).

**Cell Culture and Transfection**—COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum and gentamicin (100  $\mu$ g/ml) and transfected using the DEAE-dextran method. For inositol phosphate determination, COS-7 cells ( $0.15 \times 10^6$ ) were seeded in 12-well plates. The quantity of transfected receptor encoding DNA was 0.3–3  $\mu$ g/ $10^6$  cells.

**Ligand Binding**—Membrane preparations derived from cells expressing the  $\alpha_{1b}$ -AR or its mutants and ligand binding assays using [<sup>125</sup>I]HEAT were performed as previously described (19). Prazosin ( $10^{-6}$  M) was used to determine nonspecific binding. [<sup>125</sup>I]HEAT at a concentration of 250 pM was used for measuring receptor expression at a single concentration and 80 pM for competition binding analysis. Saturation analysis and competition curves were analyzed using Prism 3.02 (GraphPad Software Inc., San Diego, CA).

**Inositol Phosphate Measurements**—Transfected cells were labeled for 12 h with *myo*-[<sup>3</sup>H]inositol at 4  $\mu$ Ci/ml in inositol-free DMEM supplemented with 1% fetal bovine serum. Cells were preincubated for 10 min in phosphate-buffered saline containing 20 mM LiCl and then stimulated for 45 min with different concentrations of epinephrine from  $10^{-10}$  to  $10^{-4}$  M. Total inositol phosphates were extracted and separated as described previously (19).

**Ab Initio Modeling of the  $\alpha_{1b}$ -AR and Its Mutants**—*Ab initio* modeling of the  $\alpha_{1b}$ -AR receptor was achieved following the iterative procedure previously described (17). The wild type  $\alpha_{1b}$ -AR input structure was selected from among over 200 tested input arrangements according to both internal and external consistency criteria and was used to produce the input structures for the receptor mutants. These structures were obtained by substituting the mutated residue in the wild type input structure by means of the molecular graphics package QUANTA (release 98; Molecular Simulations Inc., Waltham, MA). Minimization and molecular dynamics (MD) simulations of the receptor models were performed using the program CHARMM (Molecular Simulations Inc), following the computational protocol previously described (17). In a previous study MD runs of 1050 ps were performed to compare the dynamic features of the wild type receptor with those of the constitutively active mutants (8). Because the first 100 ps of the equilibrated trajectory were sufficiently representative of the whole trajectory and given the high number of mutants considered in this study, MD runs of 150 ps were generally performed following the same heating and equilibration set-up as that employed for the longer MD simulations. The results reported were collected every 0.5 ps during the last 100 ps of the equilibrated MD trajectory. Finally, for each mutant the structure averaged over the 200 structures stored during the production phase were used for the comparative analysis.

The average minimized structure of the wild type  $\alpha_{1b}$ -AR showed a root mean square deviation (r.m.s.d.) of 3.94 Å from the rhodopsin structure, the deviation being larger (r.m.s.d. = 4.85 Å) before calculations. r.m.s.d. levels were computed by superimposing the main chain atoms of segments 37–62, 74–100, 111–133, 152–171, 202–225, 253–276, and 286–306, representing the seven transmembrane helices of rhodopsin, with those in the homologous segments 45–70, 82–107, 119–141, 161–180, 202–225, 295–318, and 328–348 of the average minimized structure of the wild type  $\alpha_{1b}$ -AR.

**Comparative Modeling of  $\alpha_{1b}$ -AR and Its Mutants**—Another model of the  $\alpha_{1b}$ -AR was built by comparative modeling (20) using the recently determined 2.8-Å x-ray structure of rhodopsin (18) as a template. Eight different chimeric  $\alpha_{1b}$ -AR/rhodopsin templates (shown in the supplementary material) were constructed in which the e2, the i3, and in some cases only the i2 loop were extracted from the input structure of the *ab initio* model of the  $\alpha_{1b}$ -AR. Furthermore, in the chimeras helix 5 has been elongated by 10 amino acids using the  $\alpha_{1b}$ -AR sequence after deleting the 226–235 rhodopsin segment. Finally, an  $\alpha$ -helical segment of 6 amino acids using the  $\alpha_{1b}$ -AR sequence has been added to the N terminus of the helix 6 of rhodopsin after deleting the 240–248 rhodopsin segment. For each of the eight different templates, MODELLER generated 25 models. Among the 200 models finally obtained, 20 models were selected showing low restraint violations and low numbers of main-chain and side-chain bad conformations or close contacts. These models were completed by the addition of the polar hydrogen's and subjected to automatic and manual rotation of the side-chain torsion angles when in bad conformations, as well as to energy minimization and MD simulations according to the computational protocol employed for simulating the *ab initio*  $\alpha_{1b}$ -AR model. Different combinations of intra-helix distance constraints were also probed. About 450 MD trial runs were done to select the proper input structure for the wild type  $\alpha_{1b}$ -AR. The final input structure selected that was obtained using the alignment (see the supplementary material) was then used for generating the input structures for the receptor mutants. The structures of the wild type receptor and its mutants averaged over the last 100 ps of the 150 ps MD trajectory were finally minimized and considered for the comparative analysis.

The input structure of the wild type  $\alpha_{1b}$ -AR showed an r.m.s.d. of 0.17 Å from the rhodopsin structure (r.m.s.d. was computed by employing the matching criteria described above for the *ab initio* model). This deviation increases to 2.07 Å upon energy minimization and MD simulations, becoming quite close to the value that would be expected given a sequence identity of 22.4% between the transmembrane segments of the  $\alpha_{1b}$ -AR and rhodopsin (21).

#### RESULTS

**Expression of Receptor Mutants**—The wild type and mutated  $\alpha_{1b}$ -ARs were expressed in COS-7 cells and tested for their ability to bind the radioligand [<sup>125</sup>I]HEAT and epinephrine. Saturation binding experiments indicated that the  $K_D$  of [<sup>125</sup>I]HEAT was  $\sim$ 80 pM for all the receptors studied (results not shown), whereas the  $IC_{50}$  values for epinephrine varied as indicated in Table I. The affinity of prazosin for the different

TABLE I  
Functional properties of the  $\alpha_{1b}$ -AR and its mutants

The wild type  $\alpha_{1b}$ -AR (WT) and its mutants were expressed in COS-7 cells. Receptor expression was measured using 250 pm of [<sup>125</sup>I]HEAT on membrane preparations derived from transfected cells from one well of a six-well dish (approximately 150  $\mu$ g of protein). Inositol phosphate (IP) accumulation was measured following incubation in the absence (Basal) or presence of 100  $\mu$ M epinephrine (Epi-stimulated) for 45 min. The IP accumulation is expressed as the percentage increase in IP levels above those of mock transfected cells. Results for receptor expression and IP accumulation are the mean  $\pm$  S.E. of at least three independent experiments. The IC<sub>50</sub> for epinephrine was assessed in competition binding experiments using 80 pm of [<sup>125</sup>I]HEAT. The IC<sub>50</sub> values are from thirty and three independent experiments for the wild type and mutated receptors, respectively. The EC<sub>50</sub> values are from fifteen and two independent experiments for the wild type and mutated receptors, respectively.

Receptor	Expression	Basal IP	Epi-stimulated IP	IC <sub>50</sub> Epi	EC <sub>50</sub> Epi
	<i>fmol/well</i>	%	%	$\mu$ M	nM
WT (high expression)	264 $\pm$ 36	36 $\pm$ 16	365 $\pm$ 12	5.9 $\pm$ 0.36	38 $\pm$ 6
WT (ave. expression)	158 $\pm$ 10	33 $\pm$ 16	303 $\pm$ 50		
WT (low expression)	63 $\pm$ 8	21 $\pm$ 6	148 $\pm$ 30		
D142A	110 $\pm$ 27	269 $\pm$ 89 <sup>a</sup>	634 $\pm$ 136	0.22 $\pm$ 0.06 <sup>a</sup>	ND <sup>b</sup>
A293E	121 $\pm$ 73	352 $\pm$ 43 <sup>a</sup>	852 $\pm$ 104 <sup>a</sup>	0.1 $\pm$ 0.02 <sup>a</sup>	ND
R74E	182 $\pm$ 22	10 $\pm$ 6	296 $\pm$ 23	7.3 $\pm$ 0.2	38 $\pm$ 3
H75E	180 $\pm$ 37	16 $\pm$ 7	267 $\pm$ 21	5.9 $\pm$ 0.9	56 $\pm$ 2
R77E	87 $\pm$ 15	29 $\pm$ 2	248 $\pm$ 33	5.7 $\pm$ 0.3	110 $\pm$ 25
R74E/R77E	28 $\pm$ 4	11 $\pm$ 8	84 $\pm$ 12	5.7 $\pm$ 0.5	14 $\pm$ 6
R74E/H75E	93 $\pm$ 10	24 $\pm$ 7	217 $\pm$ 83	4.9 $\pm$ 0.8	10 $\pm$ 6
R148E	198 $\pm$ 16	219 $\pm$ 42 <sup>a</sup>	537 $\pm$ 72 <sup>a</sup>	2.6 $\pm$ 0.8	48 $\pm$ 15
R159E	232 $\pm$ 39	38 $\pm$ 7	635 $\pm$ 210 <sup>a</sup>	2.7 $\pm$ 0.8	28 $\pm$ 10
R160E	285 $\pm$ 36	82 $\pm$ 40	528 $\pm$ 76 <sup>a</sup>	7.4 $\pm$ 0.7	28 $\pm$ 5
R148E/R159E/R160E	77 $\pm$ 21	180 $\pm$ 96 <sup>a</sup>	636 $\pm$ 170 <sup>a</sup>	7.4 $\pm$ 0.5	19 $\pm$ 2
K161E	79 $\pm$ 16	61 $\pm$ 31	1037 $\pm$ 475 <sup>a</sup>	3.1 $\pm$ 0.06	33 $\pm$ 7
K231E	182 $\pm$ 41	117 $\pm$ 24 <sup>a</sup>	678 $\pm$ 56 <sup>a</sup>	4.1 $\pm$ 0.3	16 $\pm$ 3
R232E	153 $\pm$ 35	53 $\pm$ 3	632 $\pm$ 70 <sup>a</sup>	2.4 $\pm$ 0.6	74 $\pm$ 20
K235E	121 $\pm$ 27	20 $\pm$ 4	360 $\pm$ 27	5 $\pm$ 0.2	23 $\pm$ 3
K243E	145 $\pm$ 52	14 $\pm$ 9	284 $\pm$ 83	6.4 $\pm$ 0.2	100 $\pm$ 15
K249E	147 $\pm$ 43	31 $\pm$ 9	881 $\pm$ 55 <sup>a</sup>	3.9 $\pm$ 1.1	32 $\pm$ 10
R254E	187 $\pm$ 35	8 $\pm$ 5	109 $\pm$ 16 <sup>a</sup>	2.9 $\pm$ 0.8	ND
R254A	118 $\pm$ 38	8 $\pm$ 4	125 $\pm$ 15 <sup>a</sup>	3.5 $\pm$ 0.1	21 $\pm$ 7
K258E	179 $\pm$ 39	14 $\pm$ 5	165 $\pm$ 79	7.3 $\pm$ 0.6	ND
K258A	103 $\pm$ 31	27 $\pm$ 20	356 $\pm$ 83	5 $\pm$ 0.03	48 $\pm$ 12
R254E/K258E	162 $\pm$ 42	15 $\pm$ 5	30 $\pm$ 12 <sup>a</sup>	10 $\pm$ 0.3	ND
R254A/K258A	125 $\pm$ 37	11 $\pm$ 7	81 $\pm$ 20 <sup>a</sup>	4.4 $\pm$ 0.5	ND
R254E/K258E/D142A	79 $\pm$ 3	14 $\pm$ 3	25 $\pm$ 5 <sup>a</sup>	0.22 $\pm$ 0.06 <sup>a</sup>	ND
R254E/K258E/A293E	177 $\pm$ 27	16 $\pm$ 4	45 $\pm$ 12 <sup>a</sup>	0.12 $\pm$ 0.01 <sup>a</sup>	ND
K269E	127 $\pm$ 39	32 $\pm$ 20	387 $\pm$ 132	6.4 $\pm$ 0.6	70 $\pm$ 8
K271E	70 $\pm$ 30	13 $\pm$ 18	344 $\pm$ 68 <sup>a</sup>	6.1 $\pm$ 0.5	22 $\pm$ 5
R276E	82 $\pm$ 28	10 $\pm$ 7	327 $\pm$ 52 <sup>a</sup>	7.2 $\pm$ 0.03	88 $\pm$ 9
K282E	182 $\pm$ 34	11 $\pm$ 8	314 $\pm$ 118	6.2 $\pm$ 0.06	25 $\pm$ 7
K285E	116 $\pm$ 32	19 $\pm$ 4	493 $\pm$ 141 <sup>a</sup>	3.3 $\pm$ 0.3	25 $\pm$ 5
R288A	130 $\pm$ 28	47 $\pm$ 12	280 $\pm$ 184	3.8 $\pm$ 0.4	23 $\pm$ 6
R288E	134 $\pm$ 30	55 $\pm$ 6	266 $\pm$ 54	4.4 $\pm$ 0.06	57 $\pm$ 4
K290E	113 $\pm$ 34	53 $\pm$ 12	565 $\pm$ 90 <sup>a</sup>	1.3 $\pm$ 0.2	34 $\pm$ 5
K291A	142 $\pm$ 29	14 $\pm$ 4	107 $\pm$ 17 <sup>a</sup>	8 $\pm$ 1.5	ND
K291E	177 $\pm$ 44	4 $\pm$ 2	77 $\pm$ 24 <sup>a</sup>	6.3 $\pm$ 0.7	ND
R288A/K291A	112 $\pm$ 31	26 $\pm$ 9	117 $\pm$ 7 <sup>a</sup>	3.1 $\pm$ 0.2	ND
R288E/K291E	161 $\pm$ 31	4 $\pm$ 1	81 $\pm$ 19 <sup>a</sup>	1.7 $\pm$ 0.2	ND
R288E/K291E/D142A	102 $\pm$ 41	20 $\pm$ 6	76 $\pm$ 22 <sup>a</sup>	0.22 $\pm$ 0.03 <sup>a</sup>	ND
R288E/K291E/A293E	137 $\pm$ 29	48 $\pm$ 10	299 $\pm$ 76	0.45 $\pm$ 0.1 <sup>a</sup>	ND
K294E	118 $\pm$ 38	9 $\pm$ 5	372 $\pm$ 84	5.3 $\pm$ 0.03	57 $\pm$ 15
Y144A	62 $\pm$ 5	79 $\pm$ 20 <sup>a</sup>	767 $\pm$ 149 <sup>a</sup>	3.5 $\pm$ 0.4	22 $\pm$ 8
V147A	94 $\pm$ 13	440 $\pm$ 160 <sup>a</sup>	744 $\pm$ 173 <sup>a</sup>	0.03 $\pm$ 0.01 <sup>a</sup>	86 $\pm$ 15
V147E	66 $\pm$ 7	7 $\pm$ 3	8 $\pm$ 6 <sup>a</sup>	0.02 $\pm$ 0.01 <sup>a</sup>	ND
V147E/A293E	84 $\pm$ 18	8 $\pm$ 3	46 $\pm$ 8 <sup>a</sup>	0.06 $\pm$ 0.01 <sup>a</sup>	ND
L151A	169 $\pm$ 33	28 $\pm$ 4	114 $\pm$ 7 <sup>a</sup>	5.3 $\pm$ 0.1	ND
L151D	208 $\pm$ 34	16 $\pm$ 6	50 $\pm$ 17 <sup>a</sup>	6.9 $\pm$ 0.8	ND
L151D/A293E	161 $\pm$ 10	48 $\pm$ 12	134 $\pm$ 12 <sup>a</sup>	0.04 $\pm$ 0.01 <sup>a</sup>	ND

<sup>a</sup>  $p < 0.05$  paired Student's  $t$  test.

<sup>b</sup> ND, not determined.

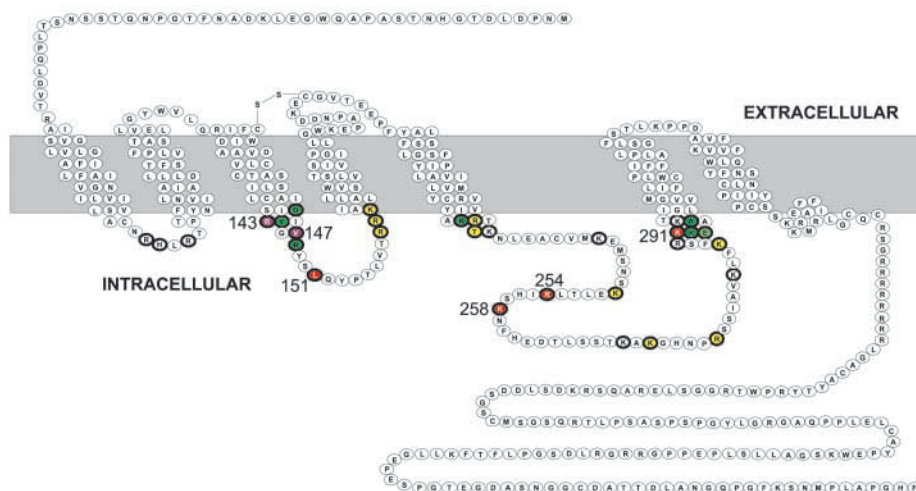
receptor mutants was similar to that for the wild type  $\alpha_{1b}$ -AR (results not shown). Receptor coupling to the G<sub>q</sub>/PLC pathway was assessed as the ability of the receptor mutants to mediate epinephrine-stimulated IP accumulation (Table I). Transfections using 3  $\mu$ g of DNA per  $1 \times 10^6$  cells resulted in the expression of all receptor mutants at levels ranging from 60 to over 250 fmol/well. In each experiment, the wild type  $\alpha_{1b}$ -AR was expressed using varying quantities of DNA (0.3, 1.3, and 3

$\mu$ g of DNA/ $1 \times 10^6$  cells) resulting in low (between 60 and 100 fmol/well), medium (between 100 and 200 fmol/well), and high (between 200 and 300 fmol/well) levels of expression. This allowed us to always be able to directly compare the properties of the mutated receptors with those of the wild type  $\alpha_{1b}$ -AR expressed at comparable levels within the same experiment.

Fig. 1 shows the localization of the amino acids mentioned in this study within a simplified topographical scheme of the



**FIG. 1. Topographical representation of the  $\alpha_{1b}$ -AR.** The sequence of the hamster  $\alpha_{1b}$ -AR is topographically arranged according to its alignment with bovine rhodopsin (see supplementary material). The amino acid residues mutated in this study are circled in boldface and some key amino acids are numbered. The background color for the mutated amino acids depicts the effect of the mutations at that residue with white representing no effect, green being constitutively activating, yellow increased efficacy for epinephrine, red impaired receptor-mediated signaling, and violet being either impaired signaling or constitutively activating depending upon the substituent amino acid.



$\alpha_{1b}$ -AR based on its sequence alignment with bovine rhodopsin. The mutated amino acids are colored according to the functional effects induced upon their mutation.

**Mutagenesis of Basic Residues in the *i1* Loop of the  $\alpha_{1b}$ -AR**—The first intracellular loop of the  $\alpha_{1b}$ -AR as with most GPCRs is short, being predicted to consist of just six amino acids (Fig. 1). Within this region there are three basic residues (Arg<sup>74</sup>, His<sup>75</sup>, and Arg<sup>77</sup>) forming a BBXXB/BBXB motif that has been described as important in the coupling of some receptors to G proteins. The individual mutations of Arg<sup>74</sup>, His<sup>75</sup>, and Arg<sup>77</sup> into Glu did not result in any significant change in the ligand binding properties of the receptors or in their ability to mediate epinephrine-induced IP accumulation (Table I).

To investigate whether the loss of more than a single basic residue had a greater effect than the single mutations, we generated the double mutants R74E/H75E and R74E/R77E. Both mutants displayed decreased levels of expression. However, their ability to mediate an agonist-induced IP response did not significantly differ from that of the wild type  $\alpha_{1b}$ -AR expressed at similar levels (Table I). It may therefore be concluded that the basic residues forming the BBXB motif in the *i1* loop of the  $\alpha_{1b}$ -AR do not play a significant role in receptor-G protein coupling. These findings are in agreement with those from other studies on various GPCRs indicating that amino acids in the *i1* loop are not important (22, 23) or only play a modest role (24, 25) in receptor-G protein coupling.

**Mutagenesis of Basic Residues in the *i2* Loop and Cytosolic Extension of Helix 4 of the  $\alpha_{1b}$ -AR**—The 13 amino acids that constitute the *i2* loop of the  $\alpha_{1b}$ -AR and the cytosolic extension of helix 4 contain four cationic amino acids, Arg<sup>148</sup>, Arg<sup>159</sup>, Arg<sup>160</sup>, and Lys<sup>161</sup>. Within this region is found the DRYXX(W/D)XXXL motif identified as a common feature in the rhodopsin family of GPCRs and an essential part of the receptor activation mechanism (5, 6). Mutations of the four positively charged residues, Arg<sup>148</sup>, Arg<sup>159</sup>, Arg<sup>160</sup>, and Lys<sup>161</sup>, did not result in any change in the ligand binding properties of the receptor mutants (Table I). Only the triple mutant R148E/R159E/R160E displayed a 9-fold increase in affinity for epinephrine (Table I).

Significantly, all the mutations resulted in an increased maximal epinephrine-stimulated activity of the receptor. However, the EC<sub>50</sub> values of epinephrine for all the receptor mutants were similar to that of the wild type  $\alpha_{1b}$ -AR (Table I). The R148E mutant also displayed a significant 6-fold increase in its constitutive activity (Table I). Interestingly, when the sequence of the  $\alpha_{1b}$ -AR is aligned with those of the muscarinic M1, M3, and M5 receptors a homologous arginine is similarly located. Mutation of this arginine in the M5 muscarinic receptor to

either Asp or Glu also produced constitutive activity (26).

The mutations of Arg<sup>148</sup> and Arg<sup>159</sup> to Ala resulted in a 2-fold increase in the efficacy of epinephrine, whereas the mutation of Arg<sup>160</sup> into Ala did not (results not shown). This suggests that the effect on agonist efficacy is linked to the loss of the positive charge at positions 148 and 159, rather than to the introduction of the anion. In contrast, at position 160 the introduction of the anion is responsible for the effects seen, rather than the loss of the positive charge.

To further explore the respective role of these residues, the mutations R148E, R159E, and R160E were combined. The triple mutant R148E/R159E/R160E, despite being expressed at a lower level as compared with the single mutants, displayed both a significantly increased constitutive and epinephrine-stimulated activity (Table I).

**Mutagenesis of Basic Residues in the *i3* Loop and Cytosolic Extensions of Helices 5 and 6 of the  $\alpha_{1b}$ -AR**—The region including the *i3* loop and the cytosolic extensions of helices 5 and 6 of the  $\alpha_{1b}$ -AR is rich in basic amino acids, containing 16 Arg and Lys residues (Fig. 1). Herein we have generated point mutations of each of these residues, and the mutations fall into three groups (Table I).

The first group contains the 6 basic residues, Lys<sup>235</sup>, Lys<sup>243</sup>, Lys<sup>269</sup>, Lys<sup>282</sup>, Arg<sup>288</sup>, and Lys<sup>294</sup> that, when mutated into Glu, did not significantly change the ligand binding or G protein-coupling properties of the receptor (Table I). We therefore did not perform other mutations at these positions. The second group concerns the basic amino acids Lys<sup>231</sup>, Arg<sup>232</sup>, Lys<sup>249</sup>, Lys<sup>271</sup>, Arg<sup>276</sup>, Lys<sup>285</sup>, and Lys<sup>290</sup>. Mutation of all these residues into Glu significantly increased the efficacy of epinephrine without any change in its binding affinity (Table I). Interestingly, the mutation of Lys<sup>231</sup> also resulted in a 3.5-fold increase in constitutive activity.

The third group of mutations concern the three residues, Arg<sup>254</sup>, Lys<sup>258</sup>, and Lys<sup>291</sup>, whose mutations resulted in a significant impairment of the receptor-mediated IP response without changing the ligand binding properties of the receptor (Table I). Mutations of Arg<sup>254</sup> and Lys<sup>258</sup> into Glu resulted in a 65% and a 45% decrease in epinephrine-stimulated IP response, respectively (Fig. 2A). When these two mutations were combined to make the R254E/K258E mutant, the receptor-mediated IP response was almost completely abolished, suggesting an important role for these two residues in receptor G protein coupling (Table I and Fig. 2A).

To determine whether the effects observed were due to either the loss of the positive charge or to the introduction of the anions, Arg<sup>254</sup> and Lys<sup>258</sup> were also mutated into alanine, both individually and in combination. Although the maximal ago-

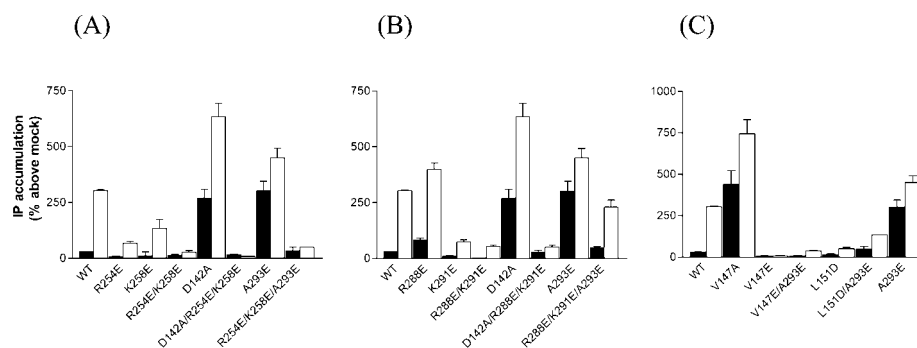


FIG. 2. **Inositol phosphate response of wild type and mutated  $\alpha_{1b}$ -AR.** Cos-7 cells were transiently transfected with DNAs encoding the wild type  $\alpha_{1b}$ -AR (WT) and its mutants. Inositol phosphates (IP) were measured in the absence (*black*) or presence (*white*) of 100  $\mu$ M epinephrine for 45 min. The IP levels are expressed as the percentage above that of cells transfected with the vector, pRK5 only (mock). Receptor expression measured in membrane preparations was in the range of 80–160 fmol/well for all the receptors. The results are the mean  $\pm$  S.E. for three independent experiments.

nist-stimulated IP response of the R254A mutant remained impaired, that of the K258A mutant was similar to the wild type  $\alpha_{1b}$ -AR (Table I). However, when the two mutations were combined to make the R254A/K258A mutant, the maximal epinephrine-stimulated activity was profoundly impaired (75% lower than the WT average), suggesting that the integrity of both Arg<sup>254</sup> and Lys<sup>258</sup> is important for receptor-mediated signaling (Table I). It is noteworthy that combining the mutations of Arg<sup>254</sup> and Lys<sup>258</sup> (whether replaced by Ala or Glu) had a greater effect than either of the two individual mutations. These findings suggest that, due to their relative locations, Arg<sup>254</sup> and Lys<sup>258</sup> may partially substitute for each other.

We made the hypothesis that, if the integrity of both Arg<sup>254</sup> and Lys<sup>258</sup> was essential for receptor function, then their mutation should abolish both the constitutive and agonist-induced activity of the constitutively active mutants (CAMs) D142A and A293E previously described (5, 27). Thus, the double mutation of Arg<sup>254</sup> and Lys<sup>258</sup> into Glu was combined with the constitutively active D142A and A293E mutants to generate the triple mutants R254E/K258E/D142A and R254E/K258E/A293E, respectively. In agreement with our hypothesis, the mutation of Arg<sup>254</sup> and Lys<sup>258</sup> in the context of the CAMs abolished both the constitutive and the epinephrine-stimulated activities of the receptor (Fig. 2A and Table I). The binding affinity of epinephrine for the triple mutants was increased by about 30-fold as for the constitutively active receptors D142A and A293E (5, 27). However, although the triple mutants maintained the ligand binding properties of the CAMs, they had lost their signaling ability.

The third basic residue found to be important for receptor-mediated signaling was Lys<sup>291</sup> found at the cytosolic extension of helix 6 (Fig. 1). The mutation of Lys<sup>291</sup> into Ala and Glu resulted in 65 and 75% decrease in epinephrine-stimulated IP production, respectively, without any significant effect on the ligand binding properties of the receptor (Table I). The EC<sub>50</sub> value for epinephrine was not measured for the K291E mutant due to its low activity.

Because Arg<sup>288</sup> and Lys<sup>291</sup> are predicted in our receptor models to be one helix turn apart on the cytosolic extension of helix 6 (Fig. 1), we combined the mutations of these two residues so as to assess the effect of a greater loss of cationic charge at this location. The R288A/K291A and R288E/K291E mutants each displayed properties similar to those of the single mutants K291A and K291E, respectively (Fig. 2B and Table I).

To further assess the importance of Lys<sup>291</sup> in receptor function, the double mutation R288E/K291E was combined with the constitutively active D142A and A293E mutants to generate the triple mutants R288E/K291E/D142A and R288E/K291E/A293E, respectively. Interestingly, the triple mutants

did not display a significantly altered constitutive activity as compared with the wild type  $\alpha_{1b}$ -AR. However, the R288E/K291E/D142A receptor displayed some epinephrine-stimulated activity whereas the R288E/K291E/A293E mutant was as good as the wild type receptor in its response to epinephrine (Fig. 1B and Table I). Both triple mutants displayed a 30-fold increase in binding affinity for epinephrine as found for the constitutively active receptors D142A and A293E (5, 27). The double mutants K291E/D142A and K291E/A293E displayed ligand binding and G protein-coupling properties similar to those of the triple mutants R288E/K291E/D142A and R288E/K291E/A293E, respectively (results not shown).

In conclusion, these results suggest that Lys<sup>291</sup> plays an important role in receptor function. However, the finding that its mutation does not completely abolish the agonist-induced activity of the CAMs suggests that its integrity is not essential.

**Mutagenesis of Conserved Hydrophobic Residues of the  $\alpha_{1b}$ -AR**—We have previously investigated the role of Asp<sup>142</sup> and Arg<sup>143</sup> belonging to the DRYXX(V/I)XXXL motif that has been identified as an essential part of the activation mechanism in GPCRs. Herein we have mutated the other conserved residues of this motif, Tyr<sup>144</sup>, Val<sup>147</sup>, and Leu<sup>151</sup>.

The Y144A mutant displayed ligand binding properties similar to those of the wild type  $\alpha_{1b}$ -AR. However, it was characterized by a small but significant increase in its constitutive activity and a 5-fold increase in epinephrine-induced IP response (Table I).

The replacement of Val<sup>147</sup> by alanine resulted in a marked increase in the constitutive activity as well as in the epinephrine-induced IP response of the receptor. In contrast, the mutation V147E resulted in a complete loss of receptor-mediated signaling. Interestingly, the affinity of both the V147A and V147E mutants for epinephrine was increased by more than 100-fold. The introduction of the V147E mutation into the constitutively active receptor A293E abolished both its constitutive and epinephrine-induced activity (Fig. 2C and Table I). Altogether the features of the V147E mutant are similar to those of the previously described R143E mutant that displayed high affinity for epinephrine, despite being completely impaired in its signaling properties (6). Mutations of the homologous valine in other GPCRs has also resulted in a profound impairment of receptor-G protein coupling (26, 28), whereas increased constitutive activity induced by its mutation has not been reported to date.

Mutation of Leu<sup>151</sup> in the  $\alpha_{1b}$ -AR into both Ala and Asp resulted in 62 and 83% impairment of epinephrine-induced IP response, respectively, without any significant change in the ligand binding properties of the receptor (Fig. 2C and Table I).

**FIG. 3. Location of mutated amino acids within the *ab initio* and homology models of the  $\alpha_{1b}$ -AR.** Each figure shows three different views of a “super average” structure for the *ab initio* (A) and homology (B) models of the wild type  $\alpha_{1b}$ -AR. The models represent an average structure derived from mutant receptors displaying the functional properties of the wild type receptor. In the *right hand* and *lower left* views, the receptor is seen from a direction parallel to the membrane surface, whereas in the *upper left* view the helical bundle is seen from the intracellular side in a direction perpendicular to the membrane surface. In the *upper left* panel, the intracellular loops are omitted so as to permit the location of amino acids studied on the helical extensions to be clearly visible. The amino acids mutated in this study are represented by *spheres centered on the  $\beta$ -carbon of the side chain of the amino acid*. The effect of the mutations at each residue is depicted by the *color of the sphere*, with *white* representing no effect, *green* being constitutively activating, *yellow* increased efficacy for epinephrine, *red* impaired receptor-mediated signaling, and *violet* being either impairing or constitutively activating depending upon the substituent amino acid.

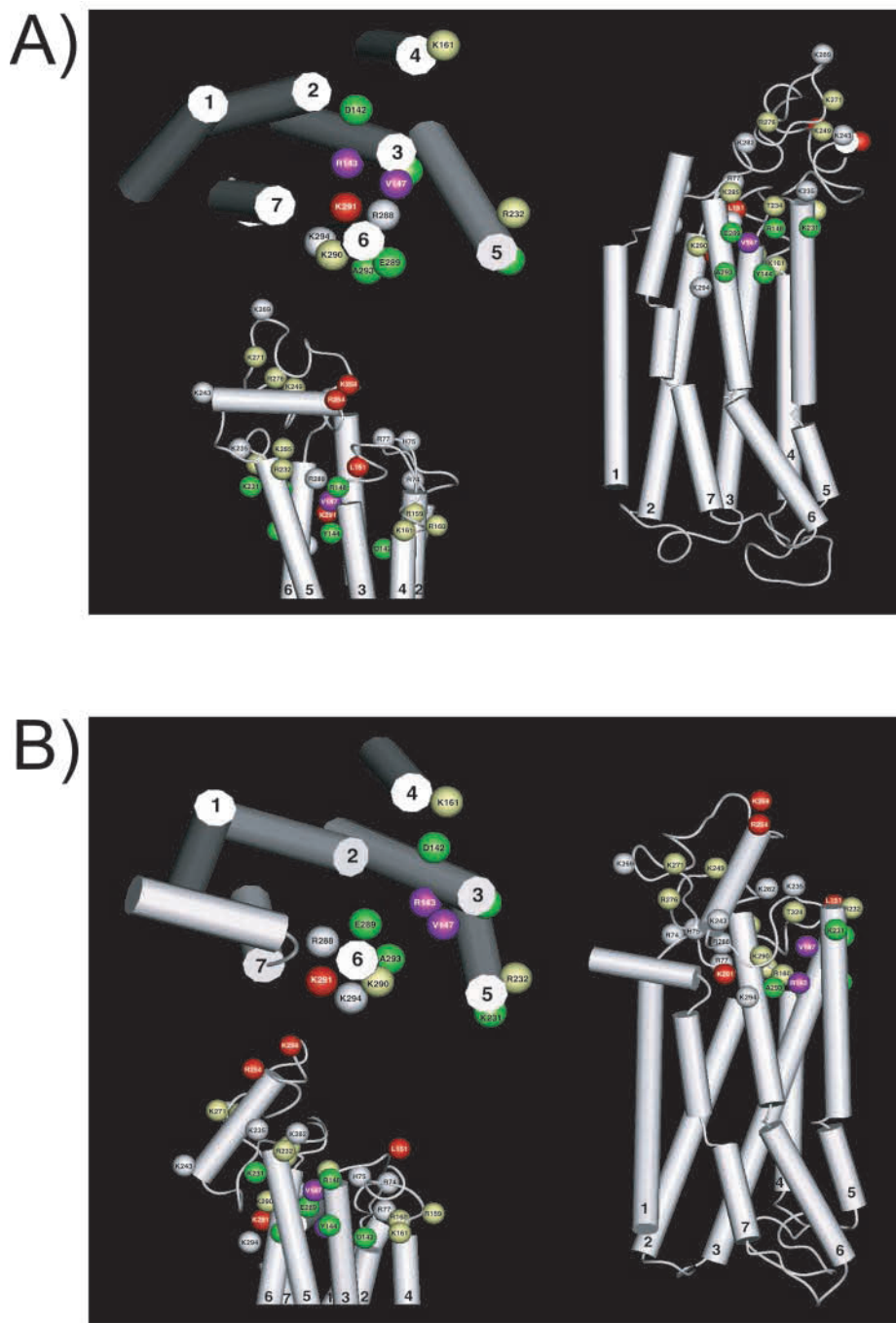




Fig. 3 shows three different views of the models representing the inactive state of the  $\alpha_{1b}$ -AR obtained following either the *ab initio* (Fig. 3A) or homology (Fig. 3B) modeling approaches. Each model was obtained by averaging the structures of the wild type  $\alpha_{1b}$ -AR and those of all mutants with functional properties equivalent to the wild type receptor. We consider this “super average” structure to be more representative of the inactive state of the receptor than that of the wild type  $\alpha_{1b}$ -AR alone.

The *ab initio* and homology models each displayed a high degree of similarity in the arrangement of the transmembrane helices. In addition, the i3 loop in both models contains an  $\alpha$ -helical segment (ranging from Met<sup>242</sup> to Arg<sup>254</sup>) within its N-terminal half and a less structured C-terminal portion. Most importantly, the i2 and i3 loops in both models contribute to the formation of a cytosolic crevice that potentially represents a site with electrostatic and shape complementarity with G proteins as previously described (8).

However, there are also some important differences between the two models that result in different mechanistic hypotheses for receptor function. In the homology model, helices 2 and 3, respectively, begin three and five amino acids earlier than in the *ab initio* model. Furthermore, helix 3 in the homology model displays a greater degree of tilt such that its cytosolic extension is closer to helix 5 than in the *ab initio* model. In addition, the orientation of the cytosolic extension of helix 6 is slightly different in the two models (Fig. 3). As a result, important differences in the amino acids contributing to the helix 3/helix 6 interface as well as in the environment of Arg<sup>143</sup> of the DRY motif are found (Fig. 3).

In particular, Arg<sup>143</sup> in the *ab initio* model (Fig. 3A) is directed toward helix 2, and we have previously predicted that its interaction with Asp<sup>91</sup> was an important constraint in maintaining the receptor in its inactive state (30). It is worth noting that in the “super average” structure shown in Fig. 3A, the interaction between Arg<sup>143</sup> and Asp<sup>91</sup> is not as apparent as in the original model of the wild type receptor described in our previous studies (17). In fact the Asp<sup>91</sup> to Arg<sup>143</sup> distance in the super average structure is 7.86 Å in comparison to 4.82 Å in the previously described wild type model. This suggests that the Arg<sup>143</sup> to Asp<sup>91</sup> interaction in the *ab initio* model is not a feature common to all the inactive conformations of the receptor as would have been expected when looking at the model of the wild type  $\alpha_{1b}$ -AR alone.

In the homology model, Asp<sup>91</sup> in helix 2 is not in the proximity of Arg<sup>143</sup>. Instead, Arg<sup>143</sup> makes a salt bridge with both the adjacent Asp<sup>142</sup> and Glu<sup>289</sup> on helix 6 (Fig. 3B). The latter interaction introduces a link between helices 3 and 6 that potentially represents an important constraint keeping the  $\alpha_{1b}$ -AR in the inactive state. This hypothesis seems to be supported by our preliminary findings, which show that mutations of Glu<sup>289</sup> in helix 6 can increase the constitutive activity of the receptor (results not shown).

We cannot say at present which receptor model is more representative of the actual structure of the  $\alpha_{1b}$ -AR. Although the arrangement of the seven helices in the homology model may be more reliable, the lack of sequence and length similarity in the extra- and intracellular portions of  $\alpha_{1b}$ -AR and rhodopsin does not favor the homology model in preference to the *ab initio* model. In this study we have therefore used both models of the  $\alpha_{1b}$ -AR to interpret the results of the mutagenesis experiments. The modeling analysis was mainly used to locate the mutated residues in the inactive state of the  $\alpha_{1b}$ -AR and to highlight potential relationships between the effect of the mutations on receptor function and their position in the receptor structure.

Molecular dynamics analysis of the basic amino acids in the three intracellular loops revealed a number of structural features consistent with the experimental findings. In both models the majority of the basic amino acids of the i1 (Arg<sup>74</sup> and Arg<sup>77</sup>) and i2 loops (Arg<sup>159</sup> and Arg<sup>160</sup>) as well as of the cytosolic extension of helix 4 (Lys<sup>161</sup>), lie at the putative lipid/water interface (Fig. 1). Modeling the mutations of these residues does not predict dramatic functional changes for the receptor in agreement with the experimental effects observed for these mutations.

However, the mutations of Arg<sup>159</sup>, Arg<sup>160</sup>, and Lys<sup>161</sup> resulted in a significant increase in the efficacy for epinephrine. This effect may be in part due to the influence of these mutations on the pK<sub>a</sub> of Asp<sup>142</sup> of the (E/D)RY motif that is in their proximity. Mutation of Arg<sup>159</sup>, Arg<sup>160</sup>, or Lys<sup>161</sup> to Glu could potentially increase the pK<sub>a</sub> of Asp<sup>142</sup>, thereby favoring its protonation. Computer simulations (5), as well as experimental studies on rhodopsin (31), have suggested that protonation of this residue is one of the key events in the activation process of these receptors.

The majority of the basic amino acids in the i3 loop are not directed toward the solvent-accessible crevice formed between the i2 and i3 loops but instead are involved in intra-loop interactions. In particular, most of the cationic amino acids of the i3 loop that when mutated did not alter receptor function (Lys<sup>235</sup>, Lys<sup>243</sup>, Lys<sup>269</sup>, Lys<sup>282</sup>) or increased the efficacy of the agonist (Lys<sup>249</sup> and Lys<sup>271</sup>), were found to form salt bridges with anionic amino acids within the loop. Thus, both the *ab initio* and homology models suggest that the main role of the majority of the cationic amino acids in the i3 loop is to stabilize its structure.

Interestingly, the few basic amino acids in the i3 loop that are directed toward the cytosolic crevice of the receptor include Arg<sup>254</sup> and Lys<sup>258</sup>, both of which are fully exposed to the solvent in both models (Fig. 3). The potential key position of Arg<sup>254</sup> and Lys<sup>258</sup> has been previously highlighted by the results of our study on docking between the  $\alpha_{1b}$ -AR and G<sub>q</sub> (8). Docking solutions between active forms of the  $\alpha_{1b}$ -AR and a G<sub>q</sub> heterotrimer identified a number of cationic residues (Arg<sup>148</sup>, Arg<sup>160</sup>, Arg<sup>232</sup>, Arg<sup>243</sup>, Arg<sup>254</sup>, Lys<sup>258</sup>, Lys<sup>282</sup>, and Arg<sup>288</sup>) on the cytosolic surface of the receptor as being available to make contact with anionic amino acids in the  $\alpha_q$  subunit. Interestingly, Arg<sup>254</sup> and Lys<sup>258</sup> were among the few residues shared by all the docking solutions proposed.

The position of residues whose mutations resulted in important functional effects, either constitutively activating the receptor (Tyr<sup>144</sup>, Arg<sup>148</sup>), impairing receptor-G protein coupling (Lys<sup>291</sup>) or both (Val<sup>147</sup>), have given insight into their structural functional role. In particular, both models demonstrate that the receptor sites susceptible to activating mutations (Asp<sup>142</sup>, Tyr<sup>144</sup>, Val<sup>147</sup>, Arg<sup>148</sup>, Glu<sup>289</sup>, and Arg<sup>293</sup>) reported in this and previous studies (5, 27) belong to, or are close by, the helix 3/helix 6 interface. As a result, all are in close proximity of the highly conserved arginine, Arg<sup>143</sup>, of the DRY motif (Fig. 3). In both models, these constitutively activating mutations all perturb the intramolecular interactions involving Arg<sup>143</sup>, which represents an important constraint that stabilizes the inactive state (R) of the  $\alpha_{1b}$ -AR (results not shown). These findings strongly support a rearrangement between helix 3 and helix 6 as a fundamental step in the activation process of GPCRs (32, 33).

The amino acid Lys<sup>291</sup> at the cytosolic extension of helix 6 belongs to the helix 3/helix 6 interface in the *ab initio* model, whereas it is directed toward the outer face of helix 7 in the homology model (Fig. 3). Despite these topographical differences, the lack of solvent accessibility of Lys<sup>291</sup> in the *ab initio*

model and its orientation in the homology model appear to exclude that this residue is directly involved in G protein interaction and/or activation. Mutations of Lys<sup>291</sup> therefore probably indirectly impair receptor-G protein coupling by inducing a structural perturbation in the helix 6/helix 3 or helix 6/helix 7 packing. The experimental results support the hypothesis that the integrity of Lys<sup>291</sup> is important for productive receptor-mediated signaling, but it is not an essential mediator of receptor-G protein coupling. In fact, mutations of Lys<sup>291</sup> impaired the agonist-induced response of the wild type  $\alpha_{1b}$ -AR but not that of the constitutively active D142A and A293E mutants (Fig. 2B).

Mutations of Val<sup>147</sup> at the cytosolic extension of helix 3 and of Leu<sup>151</sup> in the i2 loop resulted in marked effects on receptor function. Both models are consistent with mutations of Val<sup>147</sup> introducing structural perturbations in the helix 3/helix 6 packing. According to the *ab initio* model, the V147A mutation that results in an increased constitutive activity of the receptor changes the interaction pattern of Arg<sup>143</sup> and increases the solvent accessibility of the cytosolic crevice between the i2 and i3 loops, which is a feature of the active receptor state (R\*). In contrast, the mutation V147E introduces a link between the replacing glutamate and Lys<sup>291</sup> on helix 6. Consistent with this additional constraint between helices 3 and 6, this mutation almost completely abolished the receptor-mediated signaling response. The homology model suggests that the activating mutation of Val<sup>147</sup> into Ala destabilizes the interaction found in the inactive state of the wild type receptor between Arg<sup>143</sup> and Glu<sup>289</sup>. In contrast, the V147E mutation reinforces the link between the two helices, by introducing a new inter-helical interaction between the replacing glutamate and Lys-290 on helix 6. In addition, the replacing glutamate at position 147 can also interact with the neighboring Arg<sup>143</sup>. Thus, both models highlight Val<sup>147</sup> as a crucial residue by its close proximity to Arg<sup>143</sup>, and its inactivating mutation V147E introduces additional constraints into the receptor. We therefore propose that the integrity of Val<sup>147</sup> is important because it supports the mechanistic role of Arg<sup>143</sup> in the activation process of the  $\alpha_{1b}$ -AR.

According to the *ab initio* model, Leu<sup>151</sup> in the i2 loop is directed toward helix 6 and buried with respect to the cytosol. Mutation of Leu<sup>151</sup> to Asp is predicted to trigger the formation of a salt bridge across the core of the helical bundle between the replacing aspartate and Arg<sup>288</sup> on helix 6. This new constraint may be expected to impair receptor function. In contrast, Leu<sup>151</sup> in the homology model is accessible to the cytosol. Its mutation to Asp may introduce an intra-loop salt bridge with Arg<sup>148</sup>, thereby changing the conformation and solvent accessibility of the loop. Thus, the homology model suggests that Leu<sup>151</sup> may either be important for maintaining the conformation and orientation of the i2 loop and/or play a direct role in receptor-G protein coupling.

#### DISCUSSION

In this study we have applied a systematic mutagenesis approach upon the  $\alpha_{1b}$ -AR to investigate the role in receptor-G protein coupling of all the basic amino acids as well as of some conserved hydrophobic residues located in the cytosolic portion of the receptor. Interpreting the effects of the mutations in conjunction with results from molecular modeling analysis has provided some insight on the structure function role of several amino acids.

The  $\alpha_{1b}$ -AR-mediated IP response in cells can be mediated by different members of the G<sub>q</sub> family (34). However, the results of this study have only been interpreted in the context of the  $\alpha_{1b}$ -AR coupling to G<sub>q</sub>, because our previous modeling study investigated the docking of a  $\alpha_{1b}$ -AR model with a modeled G<sub>q</sub>

heterotrimer (8).

*The Majority of the Cationic Amino Acids in the Intracellular Loops Are Not Directly Involved in Receptor-G Protein Coupling*—An important finding of this study is that mutating the majority of basic residues in the cytosolic loops and extensions of helices 4, 5, and 6 of the  $\alpha_{1b}$ -AR did not impair the receptor-mediated IP response. This result is in good agreement with the predictions made in our previous modeling work docking the  $\alpha_{1b}$ -AR with G<sub>q</sub> (8). In that study we suggested that, although the majority of cationic residues on the cytosolic surface of the  $\alpha_{1b}$ -AR contribute to reciprocal electrostatic properties between the receptor and the  $\alpha_q$  subunit, only a selected number of cationic residues could be contact sites on the receptor for the G protein.

It is noteworthy that several mutations in the i3 loop of the  $\alpha_{1b}$ -AR (colored yellow in Figs. 1 and 3) increased the agonist-induced response of the receptor. This in conjunction with our molecular modeling studies suggests that the i3 loop is highly constrained by a number of intramolecular interactions, thereby limiting its propensity to interact with and/or activate the G protein. Mutations of these residues would reduce these constraints leading to increased receptor-G protein coupling.

*Arg<sup>254</sup> and Lys<sup>258</sup> in the i3 Loop and Leu<sup>151</sup> in the i2 Loop Are Directly Involved in Receptor-G Protein Coupling*—We have found that, among all the basic residues of the cytosolic surface of the receptor, only the combined mutations of Arg<sup>254</sup> and Lys<sup>258</sup> in the i3 loop almost totally impaired the IP response of the receptor as well as of the constitutively active mutants D142A and A293E.

In both the *ab initio* and homology models of the  $\alpha_{1b}$ -AR, Arg<sup>254</sup> and Lys<sup>258</sup> are the only cationic amino acids in the i3 loop that are solvent-accessible and directed toward the cytosolic crevice of the receptor. Thus, we hypothesize that Arg<sup>254</sup> and Lys<sup>258</sup> are among the contact sites on the receptor for the  $\alpha_q$  subunit and may therefore play a direct role in receptor-G protein coupling. This is also supported by the results of a previous study showing that Arg<sup>254</sup> and Lys<sup>258</sup> belonged to the only stretch of residues identified in the  $\alpha_{1b}$ -AR that could confer to  $\beta_2$ -AR the ability to activate the G<sub>q</sub>/PLC pathway (19). It will be interesting to assess whether mutations of the amino acids predicted to be the partners to Arg<sup>254</sup> and Lys<sup>258</sup> in the  $\alpha_q$  subunit also impair receptor G protein coupling.

Our experiments, however, do not directly assess the functional role played by Arg<sup>254</sup> and Lys<sup>258</sup> at a mechanistic level. The impairment of the  $\alpha_{1b}$ -AR-mediated IP response induced by mutations of these residues could result from their effect on any of the steps leading to a receptor-mediated response, *i.e.* receptor activation, receptor-G protein interaction or receptor-induced G protein activation. Arg<sup>254</sup> and Lys<sup>258</sup> are located far from the transmembrane helical bundle and from the membrane/cytosol boundary where constitutively activating mutations have principally been found in GPCRs (3). This location appears to exclude Arg<sup>254</sup> and Lys<sup>258</sup> from being involved in the process of receptor activation, *i.e.* the transition of the receptor from its inactive (R) to active (R\*) state.

For most GPCRs, receptor-G protein interaction cannot be conclusively distinguished from receptor-mediated G protein activation at the experimental level. The guanine nucleotide-sensitive high affinity binding of agonists to the  $\beta_2$ -AR has been interpreted as a measure of its physical interaction with G<sub>s</sub> and some of its mutants (35). Unfortunately, this experimental tool cannot be applied to the  $\alpha_{1b}$ -AR which, like other GPCRs coupled to the G<sub>q</sub>/PLC pathway, displays monophasic binding isotherms for agonists that are insensitive to GTP analogues. We have also explored the possibility of assaying the ability of different receptor mutants to co-immunoprecipitate the  $\alpha_q$  sub-



unit as a tool to potentially measure the receptor-G protein physical interaction in COS-7 cells. However, the inability to detect a significant agonist-dependent regulation of the  $\alpha_{1b}$ -AR- $\alpha_q$  subunit association (results not shown), combined with the difficulty in finding a convincing theoretical interpretation of the co-immunoprecipitation experiments, discouraged us from further pursuit of this assay.

In conclusion, despite the lack of direct evidence, both the functional analysis of the receptor mutants and the predictions of molecular modeling support the hypothesis that Arg<sup>254</sup> and Lys<sup>258</sup> are directly involved in the receptor-mediated activation of the G protein.

The experimental results indicate that Leu<sup>151</sup> also plays an important role in receptor-mediated signaling. Despite the fact that the *ab initio* and homology models of the  $\alpha_{1b}$ -AR do not provide a consistent interpretation of the structural functional role of Leu<sup>151</sup>, the fact that its mutation into Asp almost completely abolished the IP response mediated by the wild type  $\alpha_{1b}$ -AR as well as by the constitutively active mutant A293E suggests that this residue may be part of the receptor-G protein interface. The high degree of conservation of this residue, in conjunction with the similar effects found upon its mutation in other receptors, suggests that its role in receptor-G protein coupling is conserved among different GPCRs.

*The Cytosolic Extensions of Helices 3 and 6 Play a Crucial Role in Receptor Activation*—Both the *ab initio* and homology models of the  $\alpha_{1b}$ -AR predict that the majority of amino acids susceptible to activating mutations identified in this study (Tyr<sup>144</sup>, Val<sup>147</sup>, Arg<sup>148</sup>, and Glu<sup>289</sup>) or in our previous work (Asp<sup>142</sup> (5) and Arg<sup>293</sup> (27)) belong to or are close to the interface between the cytosolic extensions of helices 3 and 6 (Fig. 3). Altogether, our results suggest that the residues located in the environment of the interface between the cytosolic extensions of helices 3 and 6 are mainly involved in the activation process of the  $\alpha_{1b}$ -AR, *i.e.* its transition between the inactive (R) and active (R\*) states. This is in agreement with the conclusions of other studies on rhodopsin (36, 37) and  $\beta_2$ -AR (38), suggesting that a rearrangement in the relative positioning of helices 3 and 6 is a fundamental step in receptor activation.

The findings of this study exclude the basic amino acids in the cytosolic extension of helix 6 of the  $\alpha_{1b}$ -AR from playing a direct role in receptor-G protein coupling. In fact, mutating most of these residues (Arg<sup>288</sup>, Lys<sup>290</sup>, and Lys<sup>294</sup>) had no significant effect on receptor function. Only mutations of Lys<sup>291</sup> profoundly impaired the  $\alpha_{1b}$ -AR-mediated IP response. The functional as well as modeling analysis of these mutations suggest that Lys<sup>291</sup> is not a contact site on the receptor for the G protein, but rather it plays a structural role in helix 3/helix 6 or helix 6/helix 7 packing, thereby allowing productive receptor-G protein coupling.

Previous studies on muscarinic cholinergic receptors have provided evidence that it is mainly hydrophobic residues in the cytosolic extension of helix 6 that dictate receptor-G protein coupling selectivity (reviewed in Ref. 2). Future mutagenesis studies of the  $\alpha_{1b}$ -AR targeting other amino acids that have not been considered in this study will further investigate the functional role of this portion of the receptor.

*Conclusions*—The findings of this study significantly improve our knowledge of the molecular determinants of the  $\alpha_{1b}$ -AR/ $G_q$  protein interface. The role of cationic as well as hydrophobic residues in receptor-G protein coupling has been suggested for several GPCRs (2). However, a systematic mutational analysis of these residues has been lacking. It is therefore difficult to build a complete map of the different amino acids involved in receptor-G protein coupling for various receptors and to compare the positions of the basic residues found

functionally important among different receptors. Our findings do not support the hypothesis that simple motifs like the BBXXB or BBXB sequences found in different cytosolic loops of GPCRs can predict receptor-G protein coupling. In fact, among the three BBXXB or BBXB motifs found in the  $\alpha_{1b}$ -AR, in the i1 loop, the N-terminal portion of the i3 loop, and in the cytosolic extension of helix 6, only mutations of a single residue (Lys<sup>291</sup>) in the later motif (<sup>290</sup>KKAAK<sup>294</sup>) impaired the receptor-mediated response. This suggests that the effect of this mutation is linked to an important structural functional role of Lys<sup>291</sup> rather than to the disruption of the motif.

The results of the mutagenic analysis of the  $\alpha_{1b}$ -AR are in agreement with the conclusions of docking simulations between the  $\alpha_{1b}$ -AR and  $G_q$  models (8). It is noteworthy that the complementary areas of charge and shape driving the docking between the  $\alpha_{1b}$ -AR and  $G_q$  display similarities with that recently described between the rhodopsin structure and transducin (39). The involvement of the i2 and i3 loops of rhodopsin in G protein interaction was recently demonstrated by elegant studies, in which different sites in these loops were cross-linked to transducin (40, 41).

Despite the large number of experimental studies on GPCRs, our knowledge on how agonist binding to receptors results in G protein activation still remains unclear. The crystal structure of rhodopsin in its ground state has represented a significant breakthrough in GPCR research (18). However, a better understanding of how the active conformation of rhodopsin interacts with and activates transducin awaits the resolution of the active structure of the receptor. New structural approaches like those recently described by the group of Khorana (40–42) will be extremely useful in elucidating the architecture of the receptor-G protein interface. However, the results from systematic mutational analysis of different receptors, such as those presented herein, represent an important step in determining the role of individual amino acids in GPCR function.

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