Phe^{303} in TMVI of the α_{1B}-Adrenergic Receptor Is a Key Residue Coupling TM Helical Movements to G-protein Activation†

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ABSTRACT: We showed previously that Phe^{303} in transmembrane segment (TM) VI of the α_{1B}-adrenergic receptor, a highly conserved residue in G-protein-coupled receptors (GPCRs), is critically involved in receptor-activation and G-protein-coupling [Chen, S. H., Lin, F., Xu, M., Hwa, J., and Graham, R. M. (2000) *EMBO J. 19*, 4265−4271]. Here, we show that saturation mutagenesis of Phe^{303} results in a series of mutants with different levels of constitutive activity for inositol phosphate (IP) signaling. Mutants F303G and F303N showed neither basal nor agonist-stimulated IP turnover, whereas F303A displayed increased basal activity but an attenuated maximal response to (−)-epinephrine-stimulation. F303L, on the other hand, showed all features of a typical constitutively active GPCR with markedly increased basal activity and increased potency and efficacy of agonist-stimulated IP signaling. All mutants displayed higher agonist-binding affinities than the wild-type receptor, and by thermal stability studies, those able to signal showed increased susceptibility to inactivation with an order of sensitivity (F303L > F303A > WT) directly related to their degree of constitutive activity. Using the substituted cysteine accessibility method (SCAM) and equilibrium binding studies, we further show that the F303A and F303L mutants result in TM helical movements that differ in accordance with their degree of constitutive activity. These findings, therefore, confirm and extend our previous data implicating Phe^{303} as a key residue coupling TM helical movements to G-protein-activation.

G-protein-coupled receptors (GPCRs) comprise a large family of cell-surface receptors that mediate diverse effects of extracellular signaling molecules. They all share a common structure characterized by seven transmembrane segments (TMs) with three intra- and extracellular loops, an extracellular N-terminus, and an intracellular C-terminus (*1*).

Based on amino acid homology, five families of GPCRs have been identified (*1*). Although their structures and functions vary markedly, it has been proposed that activation of GPCRs involves a common molecular mechanism, i.e., movement of TM helices. This was demonstrated by the finding that photoactivation of rhodopsin involves rigid-body movement of TMVI relative to TMIII (*2*). Inhibition of this movement, either by disulfide bond formation (*2*) or by construction of a His−Zn^{2+}−His bridge between TMVI and -III (*3*), abolished receptor-activation and G-protein-coupling. In agreement with these data, agonist-activation of the β_2-adrenergic receptor (β_2-AR) has also been shown to involve critical movement of TMVI and TMIII (*4*). Moreover, rearrangement of TMVI has been observed for various constitutively active β_2-ARs that result from point mutations of residues at several different receptor locations (*5, 6*). Finally, in agreement with the above studies, Sheikh et al. (*7*) recently showed that as with rhodopsin, inhibition of TMVI and TMIII movement by a His−Zn^{2+}−His bridge impaired ligand-mediated activation of G_s by both the β_2-hormone and parathyroid hormone receptors, members of two distinct GPCR subfamilies.

α_1-Adrenergic receptors belong to the biogenic amine subfamily of GPCRs. Activation of these receptors by catecholamines and subsequent coupling to the heterotrimeric G-proteins, $G_{q/11}$, or in some instances G_h, result in phospholipase C-activation and enhanced inositol phosphate (IP) generation (*8*). Like other GPCRs, activation of α_1-ARs has been hypothesized to involve TM helical movements. This was inferred from the finding that catcholamine binding involves important interactions with residues in TMIII, TMV, and TMVI that are also critical for receptor-activation (*8, 9*). In addition, mutations of α_{1B}-AR residues, some of which disrupt a salt-bridge linkage between TMIII and TMVII, result in constitutively active receptors (*8, 10, 11*). However, the nature of the conformational changes involved in α_{1B}-AR-activation, and how these conformational changes are transmitted to G-protein-activation, remains unknown.

Recently, we showed that Phe^{303} (Figure 1A,B) in TMVI of the α_{1B}-AR, a highly conserved residue in GPCRs, when mutated to either glycine or asparagine, completely abolished agonist-activated IP turnover, despite these mutants binding agonists with high affinity (*12*). More importantly, these

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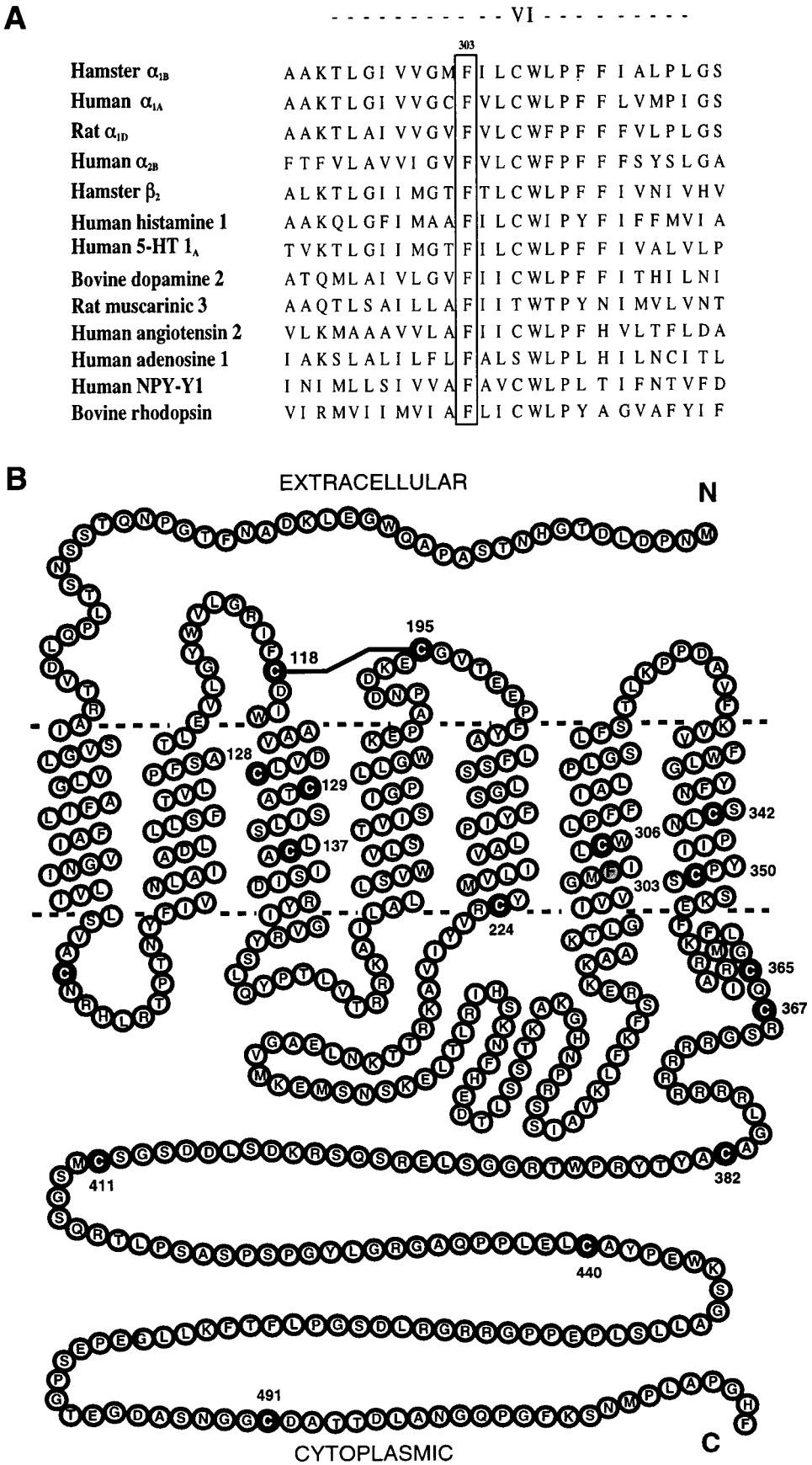

FIGURE 1: Sequence alignment of TMVI residues of the hamster α_{1B} and other G-protein-coupled receptors, and secondary structure model of the hamster α_{1B}-AR. (A) Sequences were aligned to maximize homology within this region using the GCG program "Pileup". Conserved phenylalanines corresponding to Phe[303], which is highly conserved among G-protein-coupled receptors, are boxed. The dashed line at the top delineates the transmembrane residues of helix VI. (B) Secondary structure model of the hamster α_{1B}-AR indicating the location of the native cysteine residues, including the solvent-inaccessible disulfide-linked extracellular pair Cys[118] and Cys[195] (*31*) and the Phe[303] residue evaluated in this study.

mutants induce a receptor conformation that allows tight binding but prevents activation of their cognate G-proteins. These mutants display dominant-negative activity, since the abortive receptor−G-protein complex, thus formed, inhibits PLC-signaling not only by the α_{1B}-AR but also by all $G_{q/11}$-coupled receptors.

Here, we show that substitution of Phe^{303} with various residues results in a series of mutants with differing degrees of constitutive IP-signaling activity. Thermal stability studies indicate that these mutants have a relaxed tertiary structure, as evidenced by increased susceptibility to denaturation, and that their thermal lability is directly related to their degree of constitutive activity. Using endogenous membrane-embedded cysteines as sensors, we further show that F303A and F303L induce conformational rearrangements of the TM helices. We propose, therefore, that Phe^{303} is a key residue coupling TMVI helical movements to G-protein-activation.

EXPERIMENTAL PROCEDURES

Materials. (−)-Epinephrine, cirazoline, oxymetazoline, phentolamine hydrochloride, lithium chloride, and *dl*-propanolol were purchased from Sigma. AG1-X8 (100−200 mesh, formate form) was from Bio-Rad, and fetal calf serum and culture media were from Gibco. [^{3}H]*myo*-Inositol (80 Ci/mmol) was from Amersham. [^{125}I]HEAT[1] (2200 Ci/mmol) was from Du Pont. Methanethiosulfonate ethylammonium (MTSEA) was from Toronto Research Chemicals Inc. Other chemicals were of the highest grade available commercially.

Total Synthesis of Hamster α_{1B}-AR. To allow extensive mutagenesis of the α_{1B}-AR, a synthetic gene was constructed by stepwise chemical/enzymatic synthesis, as detailed in Supporting Information. This material is available via the Internet at http://pubs.acs.org.

Mutagenesis. Construction of Phe^{303} single mutants (F303A, F303V, F303L, F303S, F303C, F303Y, and F303W) and single cysteine mutants (C128S, C129S, C136S, C224S, C306S, and C327S) was achieved by a "cassette mutagenesis" strategy based on the construction of a polymerase chain reaction (PCR)-generated mutagenic fragment, using the totally synthetic α_{1B}-AR gene as the template, as described previously (*13*). For construction of the double mutants F303G/C129S, F303N/C129S, F303A/C129S, and F303L/C129S, plasmid pMT2′ containing the C129S mutant was digested with *Xho*I and *Not*I to release a fragment encoding the Phe^{303} residue, and the plasmid backbone was isolated. The double mutants were then constructed by ligating the plasmid backbone with the fragments obtained from digesting each single Phe^{303} mutant with *Xho*I and *Not*I. The resulting constructs were confirmed by DNA sequencing.

Cell Culture and Transfection. COS-1 cells (American Type Culture Collection, Rockville, MD) were cultured and transiently transfected with the indicated constructs using the DEAE-dextran method, as described previously (*9*, *12*). Cells were harvested 72-h post-transfection.

Membrane Preparation. Membranes prepared from transfected COS-1 cells, as described previously (*9*, *12*), were resuspended in TEM buffer (10 mM Tris-HCl, pH 7.5, 1.5 mM EGTA, 12.5 mM $MgCl_2$) containing 10% (v/v) glycerol, and stored at −70 °C. Protein concentration was determined by the Bradford method (*14*).

Ligand Binding. The ligand-binding characteristics of the membrane-expressed receptors were determined, as described previously (*9*, *12*), using [^{125}I]HEAT, an α_1-specific antagonist, as the radioligand. Binding data were analyzed using the iterative, nonlinear, curve-fitting program Prism.

Phosphatidylinositol Hydrolysis in Intact Cells. Phosphatidylinositol (PI) hydrolysis in intact, transfected COS-1 cells was determined exactly as described (*9*, *12*).

Thermal Stability. COS-1 cell membranes expressing the mutant and wild-type α_{1B}-ARs were resuspended at 1 mg/mL in Tris binding buffer (10 mM Tris-HCl, pH 7.5, 12.5 mM $MgCl_2$, 1.5 mM EGTA) containing protease inhibitors (20 μg/mL each of aprotinin, leupeptin, bacitracin, benzamidine, and phenylmethylsulfonyl fluoride), and 50 μL aliquots were incubated at 37 °C for up to 30 h. The amount of functional receptor was determined by equilibrium binding using a saturating concentration of [^{125}I]HEAT (700 pM) (*15*). To evaluate the ability of ligand to protect the receptor from denaturation, membranes were incubated in the presence or absence of phentolamine (0.1 mM) for 6 h. After being washed 8 times, each with 1 mL of buffer, using a vacuum filtration system (Millipore), the amount of functional receptor was determined, as above.

Reaction with MTSEA and Binding Assays in Intact Cells. Transfected COS-1 cells were harvested by trypsinization and resuspended in HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1 mM EDTA, 0.006% bovine serum albumin, 25 mM HEPES, pH 7.4) as described previously (*9*). Aliquots (80 μL) of the cell suspension were incubated with 20 μL of freshly prepared MTSEA at the stated concentrations at room temperature. Cell suspensions were then diluted with HEPES buffer, and 100 μL aliquots were used to assay for [^{125}I]-HEAT binding with either a single concentration (600−800 pM) or a series of concentrations of [^{125}I]HEAT, as indicated, in the presence or absence of 0.1 mM phentolamine in a total volume of 250 μL in triplicate. The result was analyzed as described above.

Residue Numbering. The residue numbers are those for the hamster α_{1B}-AR. For comparison with the Ballesteros−Weinstein numbering scheme (*16*), residues Cys^{129} and Phe^{303} are Cys$^{3.36}$ and Phe$^{6.44}$, respectively.

Data Analysis. Results are expressed as the mean ± 1 SE (shown as error bars in the figures). Student's *t*-test was used to determine significant differences ($p < 0.05$).

RESULTS

Using the synthetic gene as a template, Phe^{303} in TMVI of the α_{1B}-AR was mutated to Tyr, Trp, Ala, Val, Leu, Ser, Cys, and Glu. The antagonist- and agonist-binding affinities of these mutants, plus two previously characterized mutants, F303G and F303N, were evaluated following expression in COS-1 cells at densities comparable to the wild-type α_{1B}-AR. All mutants showed no change or only small decreases (1.7−4.8-fold) in affinity for the antagonist radioligand [^{125}I]-HEAT (Table 1). However, their affinity for the agonists

[1] Abbreviations: [^{125}I]HEAT, 2-[β-(4-hydroxyl-3-[^{125}I]iodophenyl)-ethylaminomethyl]tetralone; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; EC_{50}, concentration of agonist required to produce 50% of the maximal response; IC_{50}, concentration of compound required to produce a 50% decrease in maximal specific radioligand binding; $t_{1/2}$, time required to produce a 50% decrease in maximal specific radioligand binding.

Table 1: Ligand-Binding Profiles of Wild-Type and Phe[303] Mutant α_{1B}-ARs[a]

ligand	WT	F303G	F303N	F303A	F303S	F303C
(−)-epinephrine (nM)	539 ± 60	11 ± 1[b] (49)	6.8 ± 2.6[b] (79)	11 ± 8[b] (49)	31 ± 9[b] (17)	22 ± 3[b] (25)
cirazoline (nM)	1107 ± 220	110 ± 22[b] (10)	56 ± 7[b] (20)	128 ± 40[b] (8.6)	150 ± 69[b] (7.3)	654 ± 77 (1.7)
HEAT (pM)	67 ± 12	95 ± 24	324 ± 26[b]	121 ± 32	246 ± 29[b]	63 ± 8

ligand	F303E	F303V	F303L	F303Y	F303W
(−)-epinephrine (nM)	254 ± 82[b] (2.1)	21 ± 3[b] (26)	4.7 ± 1.8[b] (115)	5662 ± 1717[b] (0.1)	21 ± 3[b] (26)
cirazoline (nM)	926 ± 227 (1.2)	544 ± 117 (2)	50 ± 12[b] (22)	3966 ± 1376 (0.28)	212 ± 21[b] (5.2)
HEAT (pM)	205 ± 25[b]	86 ± 5	116 ± 36	296 ± 25[b]	258 ± 62[b]

[a] Ligand-binding affinity (K_i) was determined from [^{125}I]HEAT competition binding studies using membranes prepared from transfected COS-1 cells except the affinity of [^{125}I]HEAT (K_D), which was determined by saturation binding as described under Experimental Procedures. Data are presented as the mean ± 1 SE of at least three independent experiments, each performed in duplicate. Values in parentheses are the ratio of K_i values of the wild-type to the mutant α_{1B}-ARs. [b] < 0.05 indicates significant difference versus the wild-type α_{1B}-AR.

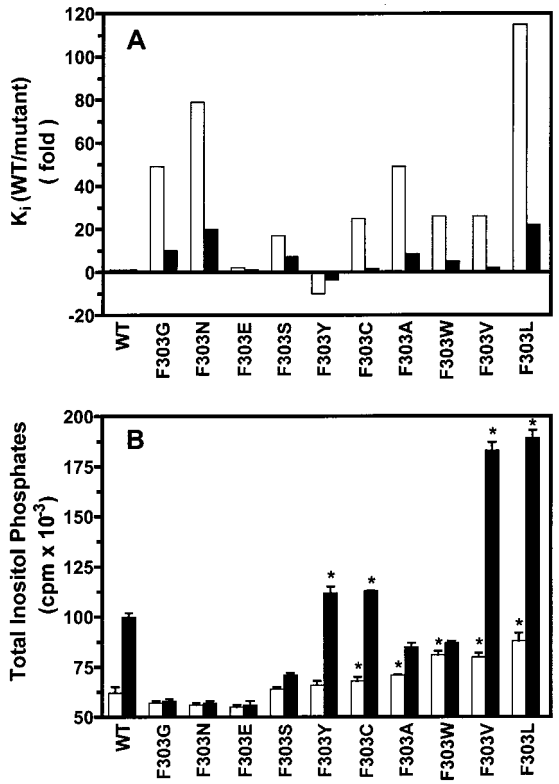

FIGURE 2: Agonist-binding and PI hydrolysis by wild-type and mutant α_{1B}-ARs. (A) The binding affinity for agonists, (−)-epinephrine (open bars) and cirazoline (closed bars), was determined in competition studies using transfected COS-1 cell membrane, as described under Experimental Procedures. The fold change in K_i values for the various mutants, as compared with those determined for the wild-type α_{1B}-AR, is shown. (B) PI hydrolysis was determined in intact COS-1 cells expressing wild-type and mutant α_{1B}-ARs in the absence (open bars) or presence (closed bars) of epinephrine (0.1 mM), as described under Experimental Procedures. Receptor densities were approximately 0.6−1.1 pmol/mg of protein, as determined by radioligand binding assay using a single saturating concentration of radioligand. *$p < 0.05$ indicates significant difference versus PI hydrolysis in cells expressing the wild-type α_{1B}-AR.

epinephrine and cirazoline differed. As seen in Figure 2A, none of the mutants maintained wild-type binding affinity. While mutant F303Y showed a decrease in binding affinity (5−10-fold) for epinephrine and cirazoline, the rest of the mutants displayed 2−115-fold increases in their affinity for agonists (Figure 2A). In general, these changes were larger for the full agonist, epinephrine, than for the partial agonist, cirazoline.

The influence of Phe[303] mutations on inositol phosphate (IP) production was determined in COS-1 cells expressing the wild-type or Phe[303] mutant α_{1B}-ARs. All receptors, except F303W, were expressed at levels between 0.6 and 1.1 pmol/mg of proteins, and showed appropriate maturational glycosylation, as evidenced by an M_r of ~80 000 by PAGE (data not shown). F303W was expressed at 0.1 pmol/mg of protein, a level that could not be enhanced by transfection of larger amounts of plasmid. As shown in Figure 2B, consistent with their higher agonist-binding affinity, mutants F303A, F303W, F303C, F303V, and F303L generated agonist-independent IP turnover, to levels significantly higher than the wild-type receptor, indicating that these mutations induced constitutive receptor activity. However, the degree of this basal activity varied. Whereas F303A and F303C produced only a 20% increase in basal IP production, that of F303W, F303V, and F303L was increased by 50−90%. Additionally, these mutants showed different profiles of response to agonist stimulation. Maximal epinephrine-mediated IP production was partially impaired with the F303A and F303W mutants, but was significantly increased with the F303C, F303V, and F303L mutants (Figure 2B). For mutants F303A and F303L, a similar profile of IP responses was observed with the agonists cirazoline and oxymetazoline (data not shown). In addition, F303L showed a significant increase in potency (EC_{50} = 0.93 ± 0.14 nM) for epinephrine-mediated PI hydrolysis as compared to the wild-type receptor (EC_{50} = 55 ± 16 nM), whereas the potency of F303A was not altered (EC_{50} = 39 ± 4 nM) (Figure 3).

In contrast to the constitutively active mutants, F303E, like the previously characterized mutants, F303G and F303N, was unable to activate IP-signaling, either in the absence of agonist or with epinephrine-stimulation (Figures 2B and 3). The other mutants, F303S and F303Y, showed no change in basal activity but displayed either impaired or enhanced IP-responses to epinephrine stimulation, respectively (Figure 2B). Interestingly, compared to the wild-type receptor, the binding affinity for epinephrine was increased more than 20-fold for F303S, but was 10-fold lower for F303Y (Figure 2A).

The diverging effects of the Phe[303] mutations on the functional properties of the receptor support the pivotal role of this residue in α_{1B}-AR activation. However, the structural basis for the altered phenotypes displayed by the various substitutions—increased (F303C/A/W/V/L) or decreased (F303G/N/E) basal signaling and increased (F303Y/C/V/L) or decreased (F303G/N/E) agonist-stimulated signaling—

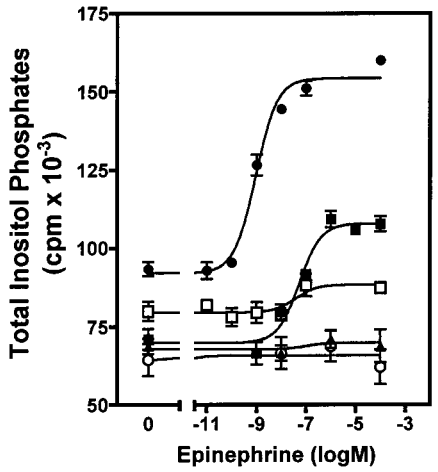

FIGURE 3: (−)-Epinephrine-stimulated PI hydrolysis mediated by wild-type and mutant α_{1B}-ARs. PI hydrolysis stimulated by the indicated concentration of (−)-epinephrine was determined in cells expressing the wild-type α_{1B}-AR (■), F303G (▲), F303N (○), F303A (□), and F303L (●). The concentration of (−)-epinephrine required to produce a half-maximal response (EC_{50}) was 55 ± 2, 39 ± 4, and 0.93 ± 1.4 nM for the wild-type α_{1B}-AR, F303A, and F303L, respectively.

remains unclear. To further explore the structural and functional effects of the Phe[303] substitutions, the inactive mutants, F303G and F303N, and the constitutively active mutants, F303A and F303L, were selected for additional study.

As structural instability has been shown to be associated with constitutive activity of GPCR mutants (*15*), we first studied the thermal stability of these mutants. As shown in Figure 4A, mutation of Phe[303] resulted in mutants with different degrees of thermal lability. Assuming that denaturation proceeds at an exponential rate, the $t_{1/2}$ of receptor inactivation was 367 h for the wild-type α_{1B}-AR, and was decreased to 30, 30, 19, and 4 h for F303G, F303N, F303A, and F303L, respectively. Preincubation of these mutants with the α_1-antagonist phentolamine prevented thermal inactivation (Figure 4B). Since degradation of the receptor-protein was not evident by Western blot analysis despite incubation at room temperature for 24 h (data not shown), thermal instability is likely due to protein denaturation resulting from the relaxed tertiary structure of the mutants.

To further elucidate the TM helical movements associated with the Phe[303] mutants, a SCAM approach was used. We showed previously that modification of intact COS-1 cells expressing the wild-type α_{1B}-AR with the charged, sulfhydryl-specific agent MTSEA irreversibly inhibits binding of the radioligand [^{125}I]HEAT (*9*). Combined mutagenesis of the three native cysteines (Cys[128], Cys[129], and Cys[137]) (Figure 1B) in TMIII of the α_{1B}-AR significantly reduced both the sensitivity and the reactivity of the receptor to MTSEA (*9*). To further identify which cysteine is responsible for MTSEA-modification, we now substituted each cysteine (Figure 1B) in TMIII (Cys[128], Cys[129], Cys[137]), TMIV (Cys[224]), TMVI (Cys[306]), and TMVII (Cys[342]) to serine, one at a time, and then assessed their sensitivity to MTSEA. As shown in Figure 5, substitution of Cys[129] in TMIII completely inhibited inactivation by MTSEA. In contrast, other serine substitution mutants remained sensitive to MTSEA modification. This indicates that Cys[129] in TMIII is solely responsible for the sensitivity of the wild-type α_{1B}-AR to MTSEA. Importantly, this mutant displayed similar binding affinities for various α_1-AR antagonists and agonists, and similar activity in stimulating PI hydrolysis, as the wild-type receptor (Figure 6A,B). Thus, this mutant was used as a template for SCAM studies of Phe[303] mutants.

Combining the Phe[303] mutants with C129S to produce the double mutants F303G/C129S, F303N/C129S, F303A/C129S, and F303L/C129S did not alter their radioligand-binding affinity (data not shown), their higher affinity for epinephrine (Figure 6A), or their ability to stimulate PI hydrolysis (Figure 6B). Like the wild-type receptor, the Phe[303] single mutants displayed similar reactivity for MTSEA-mediated inactivation. However, like C129S, the double mutants F303G/C129S and F303N/C129S were resistant to MTSEA-inactivation (Figure 7A). The time course of MTSEA (10 mM)-inactivation of radioligand binding by these mutants was also evaluated as shown in Figure 7B. Although the rapid inactivation kinetics precluded accurate determination of the rates of inactivation, it is evident that whereas C129S and F303G/C129S were insensitive to MTSEA, the extent of MTSEA-inactivation of the F303L/C129S mutant (~80%) was significantly greater ($p < 0.01$) than that for the F303A/C129S mutant (~30%). Further analysis showed

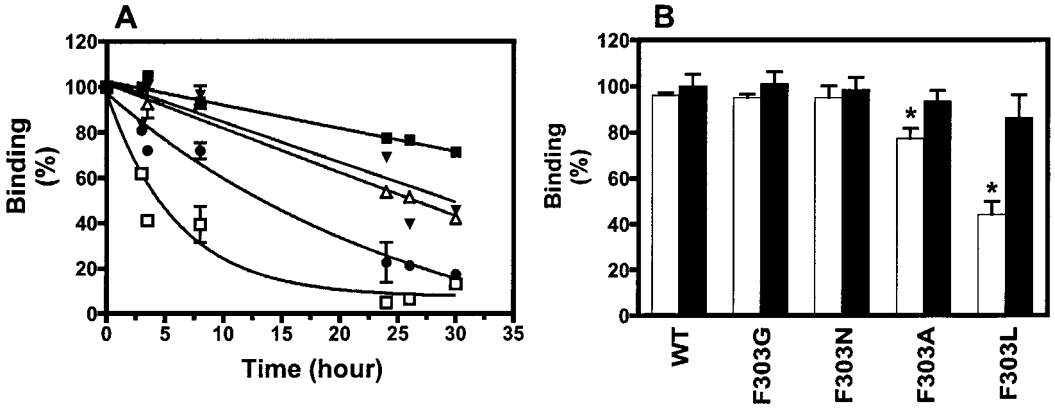

FIGURE 4: Thermal stability of wild-type and mutant α_{1B}-ARs. COS-1 cell membranes expressing the wild-type α_{1B}-AR (■), F303G (△), F303N (▼), F303A (●), and F303L (□) were incubated at 37 °C in the presence of protease inhibitors for the indicated time (A) or incubated at 37 °C for 6 h in the absence (open bars) or presence (closed bars) of phentolamine (0.1 mM) (B). Residual [^{125}I]HEAT binding was determined as described under Experimental Procedures using a saturating concentration of the radioligand, and is expressed as a percentage of control binding at $t = 0$. *$p < 0.05$, indicates significant differences versus [^{125}I]HEAT binding in the presence of phentolamine treatment.

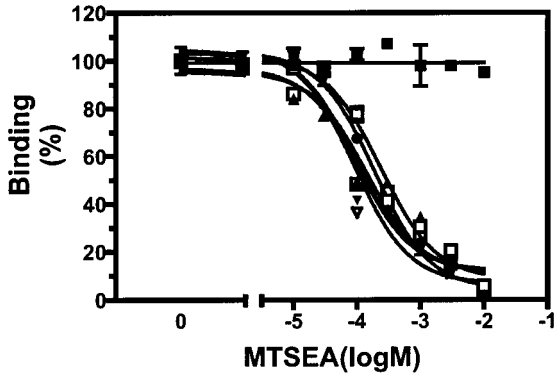

FIGURE 5: Effect of MTSEA on [^{125}I]HEAT binding to wild-type and mutant α_{1B}-ARs. COS-1 cells transfected with plasmids encoding the wild-type (□) or mutants C128S (▲), C129S (■), C137S (●), C224S (▼), C306S (△), and C342S (▽) were incubated with the indicated concentrations of MTSEA for 2 min, and the specific binding of [^{125}I]HEAT to intact cells was then determined as detailed under Experimental Procedures using a saturating concentration of the radioligand. The IC_{50} for MTSEA-induced inhibition of [^{125}I]HEAT binding was 0.14 ± 0.07, 0.12 ± 0.02, 0.19 ± 0.02, 0.10 ± 0.02, 0.24 ± 0.03, and 0.09 ± 0.02 mM for the wild-type α_{1B}-AR and mutants C128S, C137S, C224S, C306S, and C342S, respectively.

that after repeated treatment with 20 mM MTSEA, radioligand binding by F303L/C129S, but not by F303A/C129S, could be completely inhibited (Figure 8A,B). Equilibrium binding studies showed that MTSEA-treatment of F303A/C129S caused a small (3-fold) reduction in radioligand-binding affinity that failed to achieve statistical significance (pK_D = 9.32 ± 0.11 vs 8.91 ± 0.21, before and after MTSEA treatment, respectively, $p > 0.05$), without loss of binding-site density (B_{max}) (Figure 8A,B). In contrast, neither B_{max} nor pK_D was altered by MTSEA-treatment of C129S or F303G/C129S (Figure 8A,B).

DISCUSSION

In the present study, we show that a wide range of substitutions at the 303-position result in mutants with altered signaling-activity. Based on detailed analyses of these mutants, several lines of evidence provide further support for, and extend our notion that Phe[303] is a key residue in coupling TM helical movements to G-protein-activation. First, the Phe[303] mutants that were capable of signaling displayed an increased susceptibility to thermal inactivation with an order of sensitivity (F303L > F303A > WT) directly related to their degree of constitutive activity. That this is likely due to the mutations inducing a relaxed tertiary structure that renders the receptor susceptible to thermal denaturation, is the finding that it could be prevented by stabilization of the binding pocket and, hence, the maintenance of a compact tertiary structure, by the binding of the antagonist, phentolamine. Second, the altered signaling activity of the Phe[303] mutants is unlikely to be due merely to global perturbations in receptor structure, since all mutants (except F303W) showed membrane expression and correct maturational processing, as well as radioligand-binding activity that was largely unaltered from that observed with the native receptor. Third, our SCAM studies provide evidence that the Phe[303] mutants do indeed induce TM helical movements. Thus, the constitutively active mutants, F303A and F303L, when combined with C129S, restored sensitivity of the C129S mutant to MTSEA. This restoration of MTSEA-sensitivity in the double mutants indicates that the addition of the F303A and F303L mutations has altered the conformation of the transmembrane helical bundle forming the ligand-binding pocket, such that one or more native cysteines has now become water-accessible.

Given that both double mutants (F303A/C129S and F303L/C129S) are constitutively active, and that the magnitude of this activity correlates with their degree of MTSEA-sensitivity, we speculated that both the Ala and Leu substitutions induced similar conformational changes that differed only in magnitude. Surprisingly, however, both time-resolved SCAM studies and equilibrium binding studies suggest, rather, that the Ala and Leu substitutions induce qualitatively distinct conformational perturbations. Thus, both the extent of MTSEA-inactivation and its effects on radioligand binding by the two mutants differed significantly. With respect to the latter, whereas derivatization of F303A with MTSEA did not alter binding-site density and caused only a 3-fold decrease in binding affinity, modification of F303L completely abolished radioligand binding. Importantly, this loss of binding was not due to the enhanced thermal lability of this mutant, since MTSEA derivatization was performed

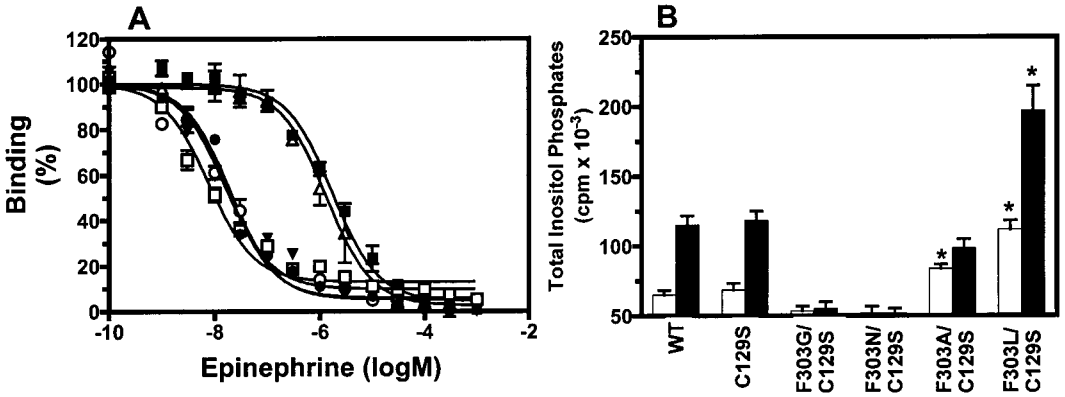

FIGURE 6: (−)-Epinephrine-binding and stimulated PI hydrolysis by wild-type and mutant α_{1B}-ARs. (A) (−)-Epinephrine binding affinity was determined in competition binding studies using membranes prepared from COS-1 cells transfected with plasmid DNA encoding the wild-type α_{1B}-AR (■) and mutants C129S (△), F303G/C129S (○), F303N/C129S (▼), F303A/C129S (●), and F303L/C129S (□). Respective K_i values were 781 ± 52, 598 ± 63, 9.83 ± 0.87, 12.8 ± 0.9, 11.9 ± 0.6, and 4.53 ± 0.76 nM. (B) Total inositol phosphates generated in the absence (open bars) or presence (closed bars) of (−)-epinephrine (0.1 mM) were determined in transfected cells as detailed in Figure 2B. *$p < 0.05$ indicates significant difference versus the response mediated by the wild-type α_{1B}-AR.

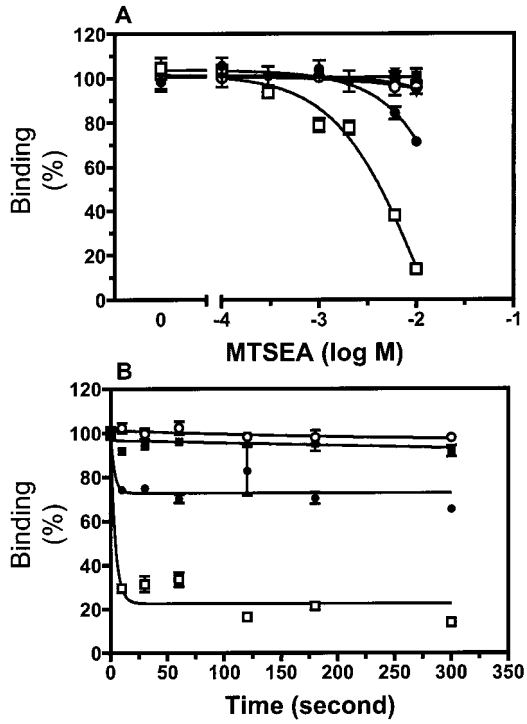

FIGURE 7: Effect of MTSEA on [^{125}I]HEAT binding to mutant α_{1B}-ARs. COS-1 cells expressing the α_{1B}-AR mutant C129S (■) or the double mutants F303G/C129S (○), F303N/C129S (▼), F303A/C129S (●), or F303L/C129S (□) were treated with the indicated concentrations of MTSEA for 2 min (A) or incubated with 10 mM MTSEA for the times indicated (B). Specific [^{125}I]HEAT binding to intact cells was then determined as detailed in Figure 5.

at room temperature for a total time of only 2 min. Under these conditions, the extent of thermal inactivation would be minimal. Although we cannot exclude the possibility that the differences in MTSEA sensitivity are merely due to differing degrees of movement of a single cysteine residue into the water-accessible binding pocket, for the reasons detailed below we believe it is much more likely that they are due to the mutations inducing qualitatively distinct conformational changes.

Theoretically, it should be possible to determine which native cysteine has moved into the water-accessible binding pocket by mutating each native cysteine, one at a time, on the background of the F303A/C129S and F303L/C129S mutants, and then evaluating each resulting triple mutant for loss of MTSEA sensitivity. Unfortunately, when such triple mutants were constructed, radioligand binding was markedly perturbed due, presumably, to a global change in receptor structure; further analysis of such mutants was, thus, not possible. We, thus, turned to delineation of such native cysteine(s) movements in the β_2-AR. This receptor does not require initial substitution of the Cys^{129} homologue as the wild-type receptor lacks a Cys at the 129-position and is resistant to MTSEA. Indeed, with this receptor, substitution of the corresponding Phe^{282} resulted in mutants that, like the equivalent Phe^{303} α_{1B}-AR mutants, displayed not only altered agonist-binding and signaling activity but also TM helical movements that were clearly qualitatively distinct (*17*).

Interestingly, combining the C129S mutation with the inactive F303G and F303N mutants did not restore their sensitivity to MTSEA. This suggests that these mutants may not induce significant TM helical movement. Alternatively, the TM movements induced by the double mutants (F303G/C129S and F303N/C129S) may not be detectable by the use of native cysteines as sensors, since they may not cause such cysteines to become water-accessible. The latter is more likely, since both double mutants (F303G/C129S and F303N/C129S) displayed enhanced susceptibility to denaturation and increased agonist-binding affinities, indicating that they had, indeed, induced significant conformational changes. Nonetheless, as the TM helical movements induced by these two mutants are undetectable, they are distinct from those for the F303A and F303L mutants. Thus, these findings provide further evidence for the notion that mutations at Phe^{303} induce multiple receptor conformers with differing functional activities.

The demonstration that mutations at a single residue can induce multiple receptor conformers may have important implications for our understanding of the receptor-activation process. An allosteric ternary complex model is currently widely implicated to explain the mechanism of receptor-activation (*18*, *19*). Essentially this model predicts that receptor isomerizes between two states, an inactive R-state and an active R*-state. Further, in the absence of agonist, only a small proportion of receptor exists in the R*-state.

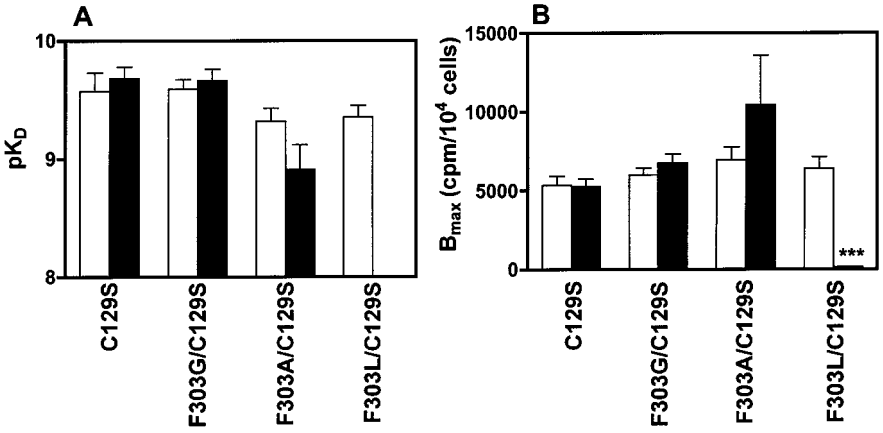

FIGURE 8: Effect of MTSEA modification on [^{125}I]HEAT binding affinity (pK_D) and B_{max} for mutant α_{1B}-ARs. The binding affinity (pK_D) (A) and B_{max} (B) of [^{125}I]HEAT to intact COS-1 cells expressing α_{1B}-AR mutants, C129S, F303G, F303A, and F303L, were determined from equilibrium binding assays performed as described in Table 1, with (closed bar) or without (open bar) MTSEA (20 mM) pretreatment, which was added twice, each for 1 min, as described in Figure 5. Asterisks indicate significant differences (***, $p < 0.001$) versus the response without MTSEA pretreatment.

Agonists bind with high affinity to, and thus stabilize, the R*-state, resulting in receptor-activation. It has also been proposed that mutations that cause constitutive activation of the receptor do so by lowering the activation energy required for the transition from the R- to the R*-state, leading to a higher proportion of receptor in the active, R*-state (*19*). However, whether GPCRs exist in only two active states, or whether they can adopt multiple, active conformations that are the result of intrinsic structural changes in the receptor, rather than merely to secondary transitions resulting from auxiliary protein−protein interactions (*20*, *21*), remains unclear. In addition, it is not clear if constitutively activating mutations represent forms of the receptor that can more readily isomerize to the active conformation, due merely to an altered *J* constant, or rather represent distinct transitional intermediates between R and R*. The present findings suggest that the latter is more likely, since the Phe^{303} mutants, even in the absence of agonist, displayed distinct conformational states that are directly related to their constitutive activity. Moreover, as is evident from Figure 2B, the relationship between substituting-residue character and phenotype is complex, since no single side chain property (e.g., volume, aromaticity, polarity, hydrophobicity) readily explains the observed alterations in either basal or agonist-activated signaling by the Phe^{303} mutants. Based on these considerations, those Phe^{303} substitutions that enhance or impair basal signaling can be interpreted as stabilizing or destabilizing a partially active conformation, R′, that is intermediate between R and R*, whereas those that alter agonist-stimulated signaling promote or inhibit isomerization from R′ to R*. In keeping with this notion, by examining the fluorescence lifetimes of purified β_2-AR labeled with a fluorescence probe, Ghanouni et al. (*22*) recently provided evidence that agonists with different intrinsic activities each induce distinct receptor conformations. It is evident, therefore, that the current two-state model of receptor-activation is clearly an oversimplification that will require modification to accommodate multiple, active receptor conformations.

Recently, mutations at Arg^{143} in TMIII of the α_{1B}-AR, a residue of the highly conserved E/DRY motif of GPCRs, have been shown to result in mutants which, like those of Phe^{303} studied here, demonstrate different degrees of constitutive activity and differential responses to agonist-stimulation (*23*). Although not tested experimentally, based on computational simulations, these mutants were shown to produce a variety of structural changes that mimic either active or inactive receptor conformations. These results are consistent with the present findings and further emphasize the close relationship between constitutive activity and specific conformational states of the receptor.

The location of Phe^{303} is of great interest, since it is highly conserved in GPCRs and is located several residues below those identified in many GPCRs to be important for ligand interaction and receptor activation; e.g., in rhodopsin, it is one turn below the important contact reside, Tyr^{265}, which forms a critical contact with the β-ionine ring of 11-*cis*-retinal (*24*, *25*). Similarly, in the biogenic amine class of GPCRs, it is about two turns below a phenylalanine (Phe^{310} in the α_{1B}-AR) that not only is critical for catechol ring bonding but also is a switch residue for receptor activation (*9*). Studies of rhodopsin suggest that the residue equivalent to Phe^{303} in the α_{1B}-AR, i.e., Phe^{261}, interacts functionally with Gly^{121} in TMIII, and that this interaction is involved in rhodopsin-activation (*26*). In biogenic amine receptors, based on computer modeling (*27*), Phe^{303} is also suggested to line the face of TMVI that orients toward TMIII and, thus, is likely involved in forming an interhelical interaction between these two helices. TM helical movements induced either by agonist-stimulation or by the Phe^{303} mutants may, therefore, perturb this interaction and constitute an important step in the activation process. Interestingly, in a recent report by Baranski et al. (*28*), using random saturation mutagenesis of the C5a receptor, its Phe^{303}-equivalent was identified as one of the residues that comprise the core switch-domain for G-protein activation. Moreover, a wide range of substitutions of this residue in the muscarinic (M_5) receptor resulted in mutants that, like the α_{1B}-AR Phe^{303} mutants, had altered signaling activity (*29*). Mutation of the Phe^{303}-equivalent to alanine has also been shown to result in inactivation of the cholecystokinin B receptor (*30*). These findings, therefore, further underscore the importance of this TMVI Phe in the GPCR activation process.

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SUPPORTING INFORMATION AVAILABLE

Experimental Procedures and Results involved in the construction of the entire synthetic gene for hamster α_{1B}-AR. This material is available free of charge via the Internet at http://pubs.acs.org.

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