The gastrin-releasing peptide receptor (GRP-R) is a G protein-coupled receptor that mediates a variety of cellular responses, including cell growth and modulation of neuronal activity by activation of heterotrimeric GTP-binding proteins in the Gq family. To understand the regulation of GRP-R signaling we have substituted alanine for each of 10 amino acid residues within the transmembrane (TM) helices of the GRP-R predicted to project into the binding pocket of the receptor and analyzed the importance of each of these residues for receptor function. Two mutations showed selective loss of either agonist (Y285A) or antagonist (F313A) affinity for the GRP-R. In addition, we identified two amino acid residues, Phe270 and Asn281, in the sixth TM segment, which are important for receptor-G protein interaction. In a competition-binding assay with an antagonist radioligand, bombesin showed a 20- to 100-fold decreased affinity for the N281A and F270A mutant GRP-R compared with wild-type GRP-R. The saturation-binding isotherms are best fit by a two-state model, indicating that the receptors are in either a low-affinity (K_D1) or a high-affinity (K_D2) state. The ratio of the two affinities (K_D2/K_D1) was significantly increased for both mutants compared with wild-type GRP-R, whereas the fraction of mutant receptors in the high-affinity state (R_1) was decreased. GDP/guanosine-5’-O-(3-thio)triphosphate exchange catalyzed by the N281A mutant was lower than that observed for the wild-type GRP-R. However, for both mutants, bombesin was still able to stimulate 1,4,5-inositol triphosphate in transfected cells albeit with reduced activity. We conclude that these two TM residues are important for receptor-G protein coupling, and postulate that each mutation may affect GRP-R conformational change to the high-affinity, G protein-coupled state.

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ABSTRACT

The gastrin-releasing peptide receptor (GRP-R) is a G protein-coupled receptor that mediates a variety of cellular responses, including cell growth and modulation of neuronal activity by activation of heterotrimeric GTP-binding proteins in the Gq family. To understand the regulation of GRP-R signaling we have substituted alanine for each of 10 amino acid residues within the transmembrane (TM) helices of the GRP-R predicted to project into the binding pocket of the receptor and analyzed the importance of each of these residues for receptor function. Two mutations showed selective loss of either agonist (Y285A) or antagonist (F313A) affinity for the GRP-R. In addition, we identified two amino acid residues, Phe270 and Asn281, in the sixth TM segment, which are important for receptor-G protein interaction. In a competition-binding assay with an antagonist radioligand, bombesin showed a 20- to 100-fold decreased affinity for the N281A and F270A mutant GRP-R compared with wild-type GRP-R. The saturation-binding isotherms are best fit by a two-state model, indicating that the receptors are in either a low-affinity (K_D1) or a high-affinity (K_D2) state. The ratio of the two affinities (K_D2/K_D1) was significantly increased for both mutants compared with wild-type GRP-R, whereas the fraction of mutant receptors in the high-affinity state (R_1) was decreased. GDP/guanosine-5’-O-(3-thio)triphosphate exchange catalyzed by the N281A mutant was lower than that observed for the wild-type GRP-R. However, for both mutants, bombesin was still able to stimulate 1,4,5-inositol triphosphate in transfected cells albeit with reduced activity. We conclude that these two TM residues are important for receptor-G protein coupling, and postulate that each mutation may affect GRP-R conformational change to the high-affinity, G protein-coupled state.

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ABBREVIATIONS: BN, bombesin; GRP, gastrin-releasing peptide; GRP-R, GRP receptor (bb2); NMB-R, neuromedin B-prefering peptide receptor (bb1); BRS-3, bombesin receptor subtype 3 (bb3); GPCR, G protein-coupled receptor; TM, transmembrane; DMEM, Dulbecco’s modified Eagle’s medium; [3H]-ME, [3H]-(o-Tyr6)BN(6-13)methyl ester; GTP-y-S, guanosine-5’-O-(3-thio)triphosphate; [125]I-BN, [125]I-[Tyr6]bombesin; 1,4,5-IP3, 1,4,5-inositol triphosphate; ic3, intracellular loop 3; EG, extracellular; 5HT2A, 5-hydroxytryptamine2A.
receptor interacts with a heterotrimeric GTP-binding protein (G protein), resulting in GTP binding and dissociation of the Go- from Gβγ-subunits, which in turn regulate downstream effector systems. All GPCR proteins share seven hydrophobic regions, which form a bundle of α-helical transmembrane (TM) domains. A ligand-regulated conformational change of the receptor molecule is thought to underlie activation of GPCRs (Gether et al., 1995; Bukusoglu and Jenness, 1996). Time-resolved spin-labeling studies on rhodopsin indicated that TMIII and TMVI are involved in receptor activation (Altenbach et al., 1996; Farrens et al., 1996). Thus, the TM domains can both contribute to the formation of the ligand-binding pocket, and convert agonist binding into receptor activation through receptor conformational change, leading to enhanced catalysis of nucleotide exchange on G proteins. Despite these elegant studies of rhodopsin, the specific residues required for this conformational change have not been determined for most GPCRs, including the GRP-R.

Results from a previous study that compared the divergent residues of mouse GRP-R and BN receptor subtype BRS-3 showed that residues in TMIII and TMVI may constitute a major part of the binding pocket for GRP-R-selective agonists (Akeson et al., 1997). A three-dimensional model of the GRP-R was generated in an effort to define other TM residues likely to form the binding pocket (Baldwin, 1993). This model predicted that in addition to the previously identified residues in TMIII and TMVI several other amino acids would have side chains projecting into this binding pocket, suggesting that some of these residues may make contacts with ligands. Ten residues were chosen to be mutagenesis targets for alanine substitution to test their importance for ligand binding and receptor function. In this study we identify residues Tyr285 and Phe313 as essential amino acids for the binding of agonist (Tyr285) or antagonist (Phe313) peptides, respectively. In addition, alanine substitutions at Phe270 and Asn281 in TMVI of GRP-R seriously impaired BN binding when a radiolabeled antagonist, 125I-[d-Tyr4]BN(6-13)methyl ester (125I-ME), was used as the radioligand competitor.

Experimental Procedures

Materials. BN, NMB, and GRP were obtained from Peninsula Laboratories (Belmont, CA); Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and the aminoglycoside G418 (Life Technologies Inc., Gaithersburg, MD) in 24-well plates as recommended by the manufacturer. Approximately 48 h after transfection, cells were exposed to DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 800 μg/ml G418. Approximately 3 weeks after transfection, individual cell clones resistant to G418 were expanded and screened for receptor expression with both 125I-Tyr4]BN (125I-BN) and 125I-ME in whole cell ligand-banding assays (Manthey et al., 1993). Stable cell lines were maintained in DMEM containing 300 μg/ml G418.

Cell Membrane Preparation. GRP-R-containing cell membranes were obtained as a P2 fraction from the stably transfected cell lines. The cells were first washed twice at room temperature with PBS, then incubated with 5 ml/plate of lysis buffer (10 mM HEPES, pH 7.4, 1 mM EGTA) supplemented with 100 μM 4-2(aminomethyl)-benzene sulfonl fluoride hydrochloride at 4°C for 15 min. The swollen cells were harvested by scraping, homogenized in a Dounce homogenizer (15–20 strokes with a tight pestle), and the nuclei and cell debris removed by centrifugation at 750g for 10 min at 4°C. A P2 membrane fraction was collected from the supernatant by centrifugation at 75,000g for 30 min at 4°C.

Membrane Radioligand-Binding Assay. 125I-BN and 125I-ME were prepared as described (Manthey et al., 1993). Radioligand-binding assays were conducted as previously described (Hellmich et al., 1997). Briefly, membranes were diluted with membrane-binding solution (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.3% BSA, 3 mM MgSO4, and 1 mM EDTA) to a protein concentration of 0.005 to 1 mg/ml. In a typical experiment, each tube received a 200-μl aliquot with 20 to 30 pM 125I-BN or 125I-ME in the presence of varying concentrations of unlabeled peptide at room temperature (22°C). The incubation continued until equilibrium was achieved. After incubation, binding reactions were terminated by adding 4 ml of ice-cold TnMg solution (20 mM Tris-HCl, pH 8, 100 mM NaCl, 25 mM MgCl2 and filtering although GFP/glass filters. The binding tube and filter were washed with an additional 12 ml of TnMg solution. The filters were then transferred to counting vials, and bound radioactivity was determined with a gamma counter (COBRA II; Packard, Meriden, CT).

Binding Data Analysis. Competition-binding data were analyzed with the computer program PRISM version 2.0 (GraphPad, San Diego, CA). Data can be simultaneously fit to one- and two-site models and the two fits compared. In this analysis, the KD of radioligand for the two sites, KD1 and KD2, were assigned to constant values. These values were obtained by measuring displacement of 125I-ME by unlabeled ME for wild-type GRP-R and mutants. This program provided an estimate of the affinity (Kd) for each radioligand competitor at one or two sites (Kd1, Kd2) along with the capacity associated with each affinity site. The statistical significance of the fit to either a one-site or two-site model was determined by a calculated fitting of weighted residual variance. A two-site fit was assigned only if the fit was significantly better than a one-site fit with an F test (P < .05) (Draper and Smith, 1966).

Receptor-Catalyzed GDP/GTP-S Exchange Assay. In vitro reconstitution of wild-type and mutant GRP-R with Gq was performed by the procedure of Hartman and Northup (1996) with modifications (Hellmich et al., 1997; Jian et al., 1999). Briefly, the endogenous background GTP-binding activity of P2 membranes was reduced by chaotropic extraction with 7 M urea. After extraction, the number of receptor-binding sites was determined by Scatchard analysis of 125I-ME binding. Urea-extracted membranes containing wild-type and N281A GRP-R (0.5 mM) were reconstituted with G protein subunits (Goq, 100 mM; Gβγ, 250 mM) and incubated for 5 min at 30°C in a final volume of 50 μl of reaction solution (final concentration 50 mM 3-(N-morpholino)propanesulfonic acid, pH 7.5, 100 mM
Fig. 1. Schematic representations of the mouse GRP-R. A, proposed topological model for the GRP-R. Residues targeted for mutagenesis in this study are shown as bold in enlarged circles [near TMIV Ser^{180} (S) and Asp^{181} (D); TMV Ser^{212} (S), Ser^{215} (S), and Phe^{216} (F); TMVI Phe^{270} (F), Trp^{278} (W), Asn^{281} (N), and Tyr^{285} (Y); TMVII Phe^{313} (F)]. Residues previously identified to be involved in the binding of BN and other agonists (Akeson et al., 1997) [Gln (Q) in TMIII, Pro (P) in ECHIII, Arg (R) in TMVI, Ala (A) in TMVII] are shown as bold in enlarged squares. Y denotes sites of N-linked glycosylation. B, helical wheel diagram presents an EC view of GRP-R model based on the projection map of bovine rhodopsin (Baldwin, 1993). The Baldwin number for each TM residue mutated in this study is enclosed in circles; the Baldwin number for those residues previously identified to be involved in the binding of BN are shown in squares.
either an anti-G

Nonidet P-40] for2ha troom temperature. After this blocking step,

pH 8, 2 mM CaCl2, and 80 mM NaCl) for 15 min each. Specific
twice for 15 min with Blotto and twice with solution A (50 mM Tris,
temperature. After the 1-h incubation, the membrane was washed
twice for 15 min with Blotto, and then incubated with a horseradish
peroxidase-conjugated anti-rabbit secondary antiserum (Calbio-
described.

Western Blot of G Protein Expression in Wild-Type and
Mutant GRP-R Membranes. F2 membrane protein concentration
from cell lines expressing mutant or wild-type GRPR was deter-
mined with amido black protein assay (Schaffner and Weissmann,
1973) with BSA as a standard. Membrane samples were separated
by SDS-polyacrylamide gel electrophoresis (12% polyacrylamide)
electrophoretically transferred to a nitrocellulose membrane.
The nitrocellulose membrane was incubated in Blotto [50 mM Tris,
P2 membrane protein concentration in Blotto and incubated for 2 h at
and 0.3% BSA) with 0.2 to 0.4 Ci [35S]GTP-S. Reactions were
terminated by the addition of 4 ml of ice-cold TNMg solution, fol-
lowed by filtration over a nitrocellulose membrane (Whatman,
Tewksbury, MA). Filters were washed four times, with 2 ml of
ice-cold TNMg solution. The filters were dried and bound radioac-
tivity was determined by liquid scintillation with a Wallac 1219 beta
counter. Squid retinal Gq (Hartman and Northup, 1996) and bovine
brain Gβγ (Sternweis and Robishaw, 1984) were prepared as
described.

Results

Construction of GRP-R Point Mutants Based on a
Molecular Model. In an effort to identify residues involved
in GRP-R ligand binding, we built a molecular model of
GRP-R with rhodopsin as a template for the folding of a
generic G protein-coupled receptor (Baldwin, 1993). Ten
amino acids with side chains predicted to project into the
binding pocket of GRP-R were then targeted for alanine
substitution with site-directed mutagenesis. The position of
these amino acids in the mouse GRP-R is shown in a topo-
projection model showing the seven TM segments (Fig. 1A).
We initially examined the cell-surface ligand-binding prop-
ties of the 10 mutant receptors with both a radiolabeled agonist and a radiolabeled antagonist in intact cell-binding
experiments. As shown in Table 1, mutation of Tyr285 sub-
stantially decreases agonist binding, and mutation of Phe313
decreases affinity for antagonist ME by 10- to 20-fold. Alane-
nine substitutions at residues Ser180, Asp181, Ser212, Ser215,
Phe216, and Trp278 had no demonstrable impact on the bind-
ing of either ligand. Mutations of residues Phe270 and Asn281
in TMVI resulted in a more complex and interesting pheno-
type affecting agonist binding only when a radiolabeled an-
tagonist was used as the radioligand. We therefore selected
the F270A and N281A mutants for additional analysis.

F270A and N281A Mutations Do Not Disrupt Ligand
Binding to GRP-R. When agonist peptide [125]I-BN was used as
the radioligand, BN displaced [125]I-BN with only a slightly
reduced affinity for N281A (2.3 ± 0.4 nM), and a slightly
enhanced affinity for F270A (0.5 ± 0.2 nM) compared with
wild-type GRP-R (1.6 ± 0.2 nM) (Table 1). Both mutants also
bound GRP-R-specific antagonist ME with high affinity.
However, for both mutants, the agonist BN did not compete
well for binding of the radiolabeled antagonist (125)I-ME
(N281A, KI = 56.8 ± 3.6 nM; F270A, KI = 126 ± 34 nM;
Table 1). For both mutants, the apparent Bmax value is lower
(N281A, 0.34 ± 0.14 pmol/mg of protein; F270A, 0.03 ± 0.01
pmol/mg of protein) for the agonist radioligand than for
the antagonist radioligand (N281A, 1.0 ± 0.1 pmol/mg of protein;
F270A, 0.08 ± 0.01 pmol/mg of protein) (Table 2). To deter-
mine whether the failure of BN to compete with ME was due
to altered rates of attaining equilibrium in the mutants, we
measured the time courses of association of [125]I-BN to wild-
type and mutant receptors at room temperature. As shown in
Fig. 2, A and B, binding of [125]I-BN to either wild-type or
mutant N281A reaches equilibrium in less than 40 min. For
the mutant F270A, binding of [125]I-BN reaches equilibrium
after approximately 60 min (Fig. 2C). Hence, for the compe-
tition experiments that were performed at 60 and 80 min for
mutants N281A and F270A, respectively, failure to reach
equilibrium cannot explain the differences between wild-type
and mutant GRP-Rs. Both mutations affect the apparent
affinity of the agonist peptide BN, but not the antagonist ME,

---

TABLE 1

Effects of alanine substitution on agonist and antagonist binding for the wild-type and mutant GRP-R

<table>
<thead>
<tr>
<th></th>
<th>125I-BN</th>
<th></th>
<th>125I-ME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BN</td>
<td>ME</td>
<td>BN</td>
</tr>
<tr>
<td>wt GRP-R</td>
<td>1.6 ± 0.2 (6)</td>
<td>0.9 ± 0.2 (6)</td>
<td>4.4 ± 0.6 (6)</td>
</tr>
<tr>
<td>S180A</td>
<td>2.1 ± 0.3 (3)</td>
<td>1.1 ± 0.3 (3)</td>
<td>4.6 ± 0.4 (3)</td>
</tr>
<tr>
<td>D181A</td>
<td>2.3 ± 0.2 (3)</td>
<td>1.2 ± 0.2 (3)</td>
<td>4.6 ± 0.4 (3)</td>
</tr>
<tr>
<td>S212A</td>
<td>1.8 ± 0.2 (3)</td>
<td>0.9 ± 0.1 (3)</td>
<td>4.3 ± 0.2 (3)</td>
</tr>
<tr>
<td>S215A</td>
<td>2.0 ± 0.2 (3)</td>
<td>1.1 ± 0.2 (3)</td>
<td>4.6 ± 0.3 (3)</td>
</tr>
<tr>
<td>F216A</td>
<td>2.1 ± 0.3 (3)</td>
<td>1.0 ± 0.2 (3)</td>
<td>4.3 ± 0.3 (3)</td>
</tr>
<tr>
<td>F270A</td>
<td>0.5 ± 0.2 (3)</td>
<td>0.8 ± 0.1 (3)</td>
<td>126 ± 34 (3)</td>
</tr>
<tr>
<td>W278A</td>
<td>1.8 ± 0.2 (3)</td>
<td>1.0 ± 0.2 (3)</td>
<td>4.6 ± 0.5 (3)</td>
</tr>
<tr>
<td>N281A</td>
<td>2.3 ± 0.4 (3)</td>
<td>1.1 ± 0.3 (3)</td>
<td>56.8 ± 3.6 (3)</td>
</tr>
<tr>
<td>Y285A</td>
<td>&gt;1000 (3)</td>
<td>ND</td>
<td>&gt;1000 (3)</td>
</tr>
<tr>
<td>F313A</td>
<td>1.8 ± 0.2 (3)</td>
<td>15 ± 3.5 (3)</td>
<td>4.2 ± 0.5 (3)</td>
</tr>
</tbody>
</table>

ND, not determined; wt, wild-type.
suggesting that an effect on G protein coupling may underlie the phenotypes.

**Analysis of BN Binding with Antagonist Radioligand.** Allosteric models of ligand–receptor activation assume that the receptor exists in two freely interconvertible states, a basal state and an active state. Pure antagonists are thought to bind to both states indiscriminately, whereas agonists preferentially bind to receptors in the active state. For a GPCR agonist ligands will display a higher affinity for the G protein-coupled conformation of the receptor. To examine the conformational interconversion of our GRP-R mutants we used $^{125}$I-ME, a high-affinity radiolabeled antagonist that specifically binds GRP-R (Mantey et al., 1993), as the radioligand to analyze agonist BN binding. We analyzed the equilibrium-binding data for conformity to single- or two-state binding models. Analysis of BN/$^{125}$I-ME competition curves indicated that BN binding to wild-type GRP-R was better fit by a two-state binding model with the high affinity, $K_{D1} = 2.54 \pm 0.3$ nM, and the low affinity, $K_{D2} = 31.2 \pm 0.5$ nM (Table 3; Fig. 3A). The estimated fraction of GRP-R showing the high-affinity state ($R_1$) is 0.89 $\pm$ 0.04 of total receptor population. In contrast, the competition curves for BN/$^{125}$I-ME in both F270A and N281A membranes were shallower than for the wild-type GRP-R. These data also were better fit by a two-state model (Table 3; Fig. 3, B and C) than a one-state model. In N281A, $K_{D1} = 8.05 \pm 0.5$ nM, $K_{D2} = 188.5 \pm 4.3$ nM, and the fraction of receptor showing a high-affinity state ($R_1$) is 0.35 $\pm$ 0.1 of the total receptor population. In F270A, $K_{D1} = 0.25 \pm 0.05$ nM, $K_{D2} = 312.1 \pm 20.3$ nM, and $R_1 = 0.36 \pm 0.02$. Both mutations increased the ratio of two affinities ($K_{D2}/K_{D1}$, wild-type GRP-R, 12.5 $\pm$ 1.6; N281A, 22.8 $\pm$ 1.3; F270A, 1317.5 $\pm$ 344.5), and the fraction of receptor in the high-affinity state was significantly decreased in both GRP-R mutants.

One factor other than receptor itself that might inhibit F270A and N281A from assuming the high-affinity agonist-binding state would be a lack of sufficient quantities of Gq, the GRP-R-coupling partner. To examine whether there was any difference in the amount of G protein subunits present in the F270A and N281A membranes, we used Western blot analysis to compare the abundance of Goq and Gqβ in membranes from the wild-type- and mutant GRP-R-expressing cell lines. We found that cell lines expressing F270A and N281A have similar amounts of G protein as the cell lines expressing the wild-type receptor (Fig. 4), ruling out differ-

<table>
<thead>
<tr>
<th>B$_{max}$ values for wild-type and mutant GRP-R using either agonist $^{125}$I-BN or antagonist $^{125}$I-ME</th>
<th>$^{125}$I-BN</th>
<th>$^{125}$I-ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRP-R-Hi</td>
<td>8.9 ± 1.2</td>
<td>10.2 ± 1.4</td>
</tr>
<tr>
<td>GRP-R-Med</td>
<td>0.76 ± 0.05</td>
<td>0.87 ± 0.03</td>
</tr>
<tr>
<td>N281A</td>
<td>0.34 ± 0.14</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>F270A</td>
<td>0.03 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Fig. 2. Association kinetics for BN binding to GRP-R. The progress curves for the association of $^{125}$I-BN with wild-type (A), N281A (B), and F270A (C) GRP-R are presented. P2 membranes from each cell line (wild type, 53 μg; N281A, 388 μg; F270A 2.5 mg) were incubated with 50 pM $^{125}$I-BN in a final volume of 2 ml. At the indicated time point, a 100-μl aliquot was removed and the bound radioactivity was determined as described under Experimental Procedures. The curves shown are the best-fit to a simple exponential approach to equilibrium with GraphPad PRISM.
Quantitative Assessment of Gq Coupling to GRP-R.

We have measured BN-induced transient increases of 1,4,5-inositol triphosphate (1,4,5-IP$_3$) in wild-type GRP-R, F270A, and N281A. Both mutants are able to activate phospholipase C; however, the magnitude of the IP$_3$ response was much lower when either mutant was compared with wild-type GRP-R (data not shown). This difference in phospholipase C activation may be explained by the fact that the expression levels of F270A and N281A are less than that of the wild-type GRP-R (Table 2). To examine the coupling properties of wild-type and mutant GRP-R under conditions where receptor number is defined and equalized, we used an in situ reconstitution method to measure receptor-catalyzed exchange of GTP$^\gamma$S for GDP bound to Gq. This method allowed a direct, quantitative comparison of the ability of wild-type and mutant receptors to assume the agonist-activated state and catalyze nucleotide exchange. As shown in Fig. 5A, N281A-catalyzed exchange of GTP$^\gamma$S for GDP is less than one-third of that for the wild-type GRP-R. To exclude the possibility that a difference in receptor density might explain the activity difference between wild-type and N281A receptors, we compared the GRP-R activities of two cell lines, GRP-R-Hi and GRP-R-Med, expressing wild-type receptor with differing abundance. GRP-R-Hi expresses more receptors than the N281A, whereas GRP-R-Med expresses fewer receptors than the N281A (Table 2). This experiment examines the influence of receptor density and membrane concentration on the reconstitution of wild-type GRP-R activation of Gq. As shown in Fig. 5B, GRP-R-Hi-2 and GRP-R-Med, we found no difference in the BN-stimulated activation of Gq when equal concentrations of receptors were added either from the wild-type high- or low-expressing cells. Furthermore, the only influence of the addition of membranes from the untransfected BALB 3T3 cells to equalize total membrane added for the GRP-R-Hi sample was to increase the blank value for the GRP-R-Hi membranes to be identical with that for the GRP-R-Med membranes. Thus, we conclude that the lower N281A-catalyzed exchange of GTP$^\gamma$S for GDP is the result of less efficient catalytic function rather than a difference in receptor expression level. We note that the apparent activity differences seen between the experiments presented in Fig. 5, A and B, are due to differences in the concentrations of GTP$^\gamma$S$^\text{35S}$ used in the two assays. The activity measured by in vitro reconstitution of F270A was below the sensitivity threshold of the nucleotide exchange assay, preventing a quantitative analysis of this GRP-R mutant.

**Discussion**

In this study we have examined the ligand-binding and signaling properties of GRP-R mutants for which alanine was substituted for an amino acid in TM domains postulated to participate in ligand binding. Of the 10 mutations constructed, 6 showed no significant impact on the binding properties of either antagonist or agonist ligands, indicating that the Ser$^{180}$, Asp$^{181}$, Ser$^{212}$, Ser$^{215}$, Phe$^{216}$, and Trp$^{278}$ residues are not essential for binding of BN peptides. For the remaining four mutations, the Tyr$^{285}$ residue in TMVI was found to be critical for agonist interaction because alanine mutation abrogated BN binding, whereas the affinity of antagonist (ME) remained similar to wild-type receptor. Conversely, the F313A substitution in TMVII selectively diminished the binding of the antagonist peptide ME, and the affinity for BN remained similar to wild-type receptor. These data, then, define two additional residues in the GRP-R critical for binding peptide ligands. Previous investigations showed that residues Gln$^{121}$ of TMIII and Arg$^{288}$ of TMVI are essential for agonist recognition (Akeson et al., 1997). Furthermore, these residues along with the EC residues Pro$^{199}$ and Ala$^{308}$ clearly confer agonist selectivity for the BN receptor family because altering these four amino acids in BRS-3 to their counterparts in GRP-R increases the affinity of BRS-3 for BN approximately 100-fold (Akeson et al., 1997). Similarly, substitution of these same four residues to their counterparts in the NMB-R increases the affinity for NMB by more than 200-fold (Sainz et al., 1998). These published studies, together with the data presented in this report, underscore the importance of residues in TMIII and TMVI of the GRP-R for ligand binding. Moreover, our data also identify the Phe$^{313}$ residue as essential for binding the synthetic peptide antagonist ME, although not essential for agonist binding. This finding is consistent with data obtained from chimeric receptors that defined regions of ECIV and TMVII near ECVI as important.
for selective antagonist recognition by GRP-R (Katsuno et al., 1997).

The Phe270 and Asn281 residues of TMVI provided the most interesting and complex alteration in the properties of the GRP-R, with alanine substitutions diminishing the capacities of the mutant receptors to activate Gq as well as altering agonist binding. Previously, Ala263, located near the intracellular junction of TMVI was found to be crucial for GRP-R activation of Gq (Benya et al., 1994). In this study we found that only in a heterologous competition-binding assay with antagonist ME as the radioligand, BN affinity was significantly decreased for both F270A and N281A mutants. Mutations producing similar phenotypes to F270A and N281A of mouse GRP-R also have been reported in the neurokinin NK1 receptor system (Rosenkilde et al., 1994) and k-opioid receptor systems (Hjorth et al., 1996). Those residues located in TMII face inwards in the receptor toward TMIII and TMVII. For the TMII mutants in the NK1 receptor system, the apparent affinity for the agonist substance P is dramatically reduced as determined in a heterologous binding assay with radiolabeled antagonist. Hjorth et al. (1996) proposed that TMII mutations could hinder the receptor interconversion from low- to high-affinity conformations because the agonist is not able to compete for binding with the antagonist. Our detailed analysis of the two mutations of GRP-R (F270A and N281A) was similarly facilitated by the use of a radiolabeled antagonist ligand 125I-ME (Mantey et al., 1993), which enabled us to observe the alteration in agonist-ligand affinities resulting from the mutations. We have previously shown that uncoupling the GRP-R from G protein by urea extraction of fibroblast membranes decreases affinity for BN, GRP, or NMB, but leaves the binding of the antagonist peptide ME unchanged (Hellmich et al., 1997). Because ME retains identical affinity for both coupled and uncoupled GRP-R, radioligand binding with this peptide allows measurement of the affinity of uncoupled GRP-R for agonist peptides in a competition ligand displacement assay. For wild-type GRP-R, F270A, and N281A, BN competition for ME binding displays a biphasic saturation profile. However, the ratio of the low-

**Fig. 3.** BN competition for 125I-ME binding to GRP-R. The saturation of BN binding to wild-type (A), N281A (B), and F270A (C) GRP-Rs was determined by competition for 125I-ME. P2 membranes from each cell line (wild type, 8 µg; N281A, 100 µg; F270A, 1.8 mg) was diluted with binding solution and incubated with the indicated concentration of BN and 20 pM 125I-ME in a final volume of 6 ml as described under Experimental Procedures. Non-specific binding was determined by measuring 125I-ME binding in the presence of 10-6 M BN. The binding data were analyzed with PRISM program by comparing one-site (dotted line) and two-site (solid line) fits. Results are summarized in Table 3. For wild-type GRP-R, the maximal bound 125I-ME was 1400 ± 13 cpm, and the non-specific binding was 30 ± 5 cpm. For N281A GRP-R, the maximal bound 125I-ME was 1200 ± 24 cpm, and the non-specific binding was 100 ± 9 cpm. For F270A GRP-R, the maximal bound 125I-ME was 2400 ± 12 cpm, and the non-specific binding was 400 ± 12 cpm. The incubation time for wild-type and mutant N281A was 60 min. For F270A, the incubation was extended to 80 min to ensure that equilibrium was attained.
and high-affinity binding constants \((K_{D1}/K_{D2})\) is significantly increased in the two mutants compared with wild-type GRP-R. Because the antagonist ME binds to a single class of sites, independent of G protein coupling and unaltered by these two mutations, we conclude that ME must bind without preference to both high- and low-affinity conformational states of the GRP-R \((K_{D1} = K_{D2})\). We have ruled out a difference in the cellular content of G protein subunits as the basis for the differences between the two mutant clones and the wild-type GRP-R. Therefore, we propose that these two residues are critical for receptor conformational transition to the high-affinity, G protein-coupled state.

Our interpretation of these binding data is that alanine substitutions at either Phe\(^{270}\) or Asn\(^{281}\) result in receptors with an increased free energy difference between the low- and high-affinity conformations. Compared with the wild-type GRP-R, which has a 10-fold separation between the two affinities (calculated \(K_{D1} = 2.54\) nM and \(K_{D2} = 31.2\) nM), the N281A mutant has about a 20-fold separation \((K_{D1} = 8.05\) nM and \(K_{D2} = 188.5\) nM), whereas the F270A mutant has a more than 1200-fold separation \((K_{D1} = 0.25\) nM and \(K_{D2} = 312.1\) nM). Although this alteration predicts a higher fraction of the two mutant receptors would accumulate in the high-affinity state at equilibrium compared with the wild-type receptor, what we observed for both mutants, is a decreased ratio. Thus, we proposed that these mutations also must increase the energy barrier for the interconversion between basal and active receptor conformations, thereby disrupting the free equilibrium between the two states and decreasing the capacity of these mutants to activate G protein efficiently. This proposition was supported by the in situ reconstitution assay, which showed that N281A mutant receptor does not catalyze nucleotide exchange on Gq as well as wild-type GRP-R.

Residues of the TMVI have been identified as essential for ligand binding and activation for a variety of GPCR structures (Oliveira et al., 1994). Several aromatic residues in TMVI have been shown to be critical for agonist binding to the serotonin receptor subtype 5-hydroxytryptamine\(_2\)A (5HT\(_2\)A) (Roth et al., 1997). Mutations of these residues, as found for Phe\(^{270}\) and Asn\(^{281}\) of the GRP-R, selectively disrupted agonist binding measured by displacement of antagonist radioligand. Furthermore, the F340L mutation in TMVI of the rat 5HT\(_2\)A receptor diminished agonist efficacy similar to the F270A and N281A mutations of GRP-R. Although the Phe\(^{329}\) of rat 5HT\(_2\)A receptor is best aligned with Asn\(^{281}\) of mouse GRP-R (Fig. 6), the F340L mutant, rather than P339L mutant, is most similar to N281A. However, these latter mutations of the 5HT\(_2\)A receptor are thought to involve aromatic residues interacting with the indole ring of serotonin. It seems unlikely that either the F270A or N281A mutation directly alters the ligand recognition site of the GRP-R because when in the high-affinity state both mutants retained affinity for BN that is similar to or greater than that observed for wild-type GRP-R. We suggest, rather, that Asn\(^{281}\) and Phe\(^{270}\) might serve as sites for helix-helix interactions.
actions of TMVI that are essential for the activation transition of the GRP-R.

The investigation of a number of different GPCRs has provided a model for the relationship between the agonist high-affinity conformation of a receptor and its interaction with the appropriate G protein (Wreggett and De Lean, 1984). This relationship was elegantly demonstrated by the alteration in the binding properties of the β-adrenergic receptor in Gsα mutants of the S49 lymphoma cell (Ross et al., 1977; Bourne et al., 1982). Also, the chromatographic separation of the muscarinic acetylcholine receptor from G protein abrogated all high-affinity binding of the agonists, whereas reconstitution with purified G protein restored high-affinity binding (Florio and Sternweis, 1989). Although more complex models of G protein-receptor coupling with multiple intermediate conformations have been proposed (Weiss et al., 1996; Leff et al., 1996; Waelbroeck, 1999), the data in this report are consistent with the simpler two-state models. In a complex models of G protein-receptor coupling with multiple affinity binding (Florio and Sternweis, 1989). Although more competitive of the muscarinic acetylcholine receptor from G protein and mRNA of GRP-R is a major determinant of the agonist-regulated conformational transition required for nucleotide exchange on G protein.

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References


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