An Aspartate Residue at the Extracellular Boundary of TMII and an Arginine Residue in TMVII of the Gastrin-Releasing Peptide Receptor Interact To Facilitate Heterotrimeric G Protein Coupling†

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ABSTRACT: The mammalian bombesin receptor subfamily of G protein-coupled receptors currently consists of the gastrin-releasing peptide receptor (GRP-R), neuromedin B receptor, and bombesin receptor subtype 3. All three receptors contain a conserved aspartate residue (D98) at the extracellular boundary of transmembrane domain II and a conserved arginine residue (R309) near the extracellular boundary of transmembrane domain VII. To evaluate the functional role of these residues, site-directed GRP-R mutants were expressed in fibroblasts and assayed for their ability to both bind agonist and catalyze exchange of guanine nucleotides. Alanine substitution at GRP-R position 98 or 309 reduced agonist binding affinity by 24- and 56-fold, respectively, compared to wild-type GRP-R. Single swap GRP-R mutations either resulted in no receptor expression in the membrane (D98R) or the protein was not able to bind agonist (R309D). In contrast, the double swap mutation (D98R/R309D) had high-affinity agonist binding, reduced from wild-type GRP-R by only 6-fold. In situ reconstitution of urea-extracted membranes expressing either wild-type or mutant (D98A or R309A) GRP-R with G_q indicated that alanine substitution greatly reduced G protein catalytic exchange compared to wild-type GRP-R. The D98R/R309D GRP-R had both a higher intrinsic basal activity and a higher overall catalytic exchange activity compared to wild-type; however, the wild-type GRP-R produced a larger agonist-stimulated response relative to the double swap mutant. Taken together, these data show that GRP-R residues D98 and R309 are critical for efficient coupling of GRP-R to G_q. Furthermore, our findings are consistent with a salt bridge interaction between these two polar and oppositely charged amino acids that maintains the proper receptor conformation necessary to interact with G proteins.

The G protein-coupled receptors (GPCRs)[1] are a family of proteins containing seven transmembrane (TM) domains (*1*). These receptors transduce extracellular signals into cells by activating heterotrimeric G proteins. Molecular cloning studies have resulted in classification of these 7TM receptors into three subfamilies: the rhodopsin-like (*2*), the metabotropic glutamate-like (*3*), and the VIP-like receptors (*4*). Residues conserved among the various subfamilies of GPCRs may play important structural and functional roles with regard to ligand binding, receptor activation, and coupling to heterotrimeric GTP-binding proteins. Receptors for bombesin-like peptides are members of the GPCR superfamily. To date, four structurally distinct receptors for bombesin-like peptides within the rhodopsin-like subfamily have been cloned and characterized: gastrin-releasing protein receptor (GRP-R) or bb2 (*5, 6*), neuromedin-B receptor (NMB-R) or bb1 (*7*), bombesin (Bn) receptor subtype 3 (BRS-3) or bb3 (*8, 9*), and Bn receptor subtype 4 (BB4) (*10*). Three of the four receptors (GRP-R, NMB-R, and BRS-3) are known to exist in mammals, while, at present, BB4 has only been identified in amphibians. The three mammalian subtypes share about 50% amino acid sequence identity. BRS-3 is selectively expressed in secondary spermatocytes (*9*), whereas GRP-R and NMB-R are widely expressed in the gastrointestinal tract (*11, 12*) and central nervous system (*7*). In contrast

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[1] Abbreviations: α_{1b}-AR, α_{1b}-adrenergic receptor; Bn, bombesin; BRS-3, Bn receptor subtype 3; BB4, Bn receptor subtype 4; G protein, guanine nucleotide binding protein; GPCR, G protein-coupled receptor; GRP, gastrin-releasing peptide; GRP-R, GRP receptor; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); 5-HT, 5-hydroxytryptamine; NMB-R, neuromedin-B receptor; TM, transmembrane; TMP, thymidine 5′-monophosphate.

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to NMB-R expression in the central nervous system, GRP-R and BRS-3 are expressed in several hypothalamic nuclei associated with glucose regulation, thermoregulation, and feeding behavior (*13*). Recently, analysis of a mouse with a targeted GRP-R allele unequivocally identified this receptor as having a role in satiety (*14*).

The three mammalian Bn-like receptors are pharmacologically distinct, binding the naturally occurring peptides Bn, GRP, and NMB with different affinities. The GRP-R binds Bn and GRP with highest affinity (nanomolar range) and NMB with lower affinity (hundreds of nanomolar range). NMB-R binds Bn and NMB with highest affinity (nanomolar range) and GRP with lower affinity (hundreds of nanomolar range). None of these naturally occurring peptides binds to BRS-3 with an affinity greater than the micromolar range (*15*). The biological responses elicited by Bn-like peptides binding to their cognate receptors result from the activation of specific signal transducing heterotrimeric G proteins within the $G\alpha_q$ family (*16*) with subsequent downstream effects on phospholipase C, calcium mobilization from intracellular stores, and protein kinase C (*17*−*20*).

A focus of our research has been to understand the structural motifs critical for various Bn receptor functions. We have recently identified four residues (Q121, P199, R288, and A308) in the mouse GRP-R required for high-affinity agonist binding (*21*). Akeson et al. (*21*) arrived at this conclusion based upon lower agonist affinity for the GRP-R when these residues are mutated and upon gain of Bn and GRP affinity when these residues are introduced into the corresponding position of BRS-3. These same four residues also are present in the same location within the NMB-R primary amino acid sequence (Q123, P200, R289, and A309) and are critical for high-affinity binding of NMB to its receptor (*22*). Two additional residues, D98 at the top of TMII and R309 in TMVII of the mouse GRP-R, are conserved in all three mammalian Bn receptor subtypes, and therefore we hypothesized that these loci may play a role in receptor-mediated signal transduction. To test this supposition, site-directed mutants of the GRP-R were constructed and expressed in murine fibroblasts, and the ability of these receptors both to bind agonist and to catalyze initial receptor-mediated signal transduction events was quantitated using in vitro assays.

EXPERIMENTAL PROCEDURES

Construction of Plasmids, Site-Directed Mutagenesis, and cDNA Sequence Analysis. The mouse GRP-R cDNA was identical to that previously described (*5*) and cloned into the *Eco*RI site of pcDNA3 (Invitrogen). Point mutations were generated using Stratagene's QuikChange Site-Directed Mutagenesis Kit. Plasmid DNA was purified using a Wizard Miniprep Kit (Promega Corp.), and both strands of mutated GRP-R cDNAs were sequenced by the dideoxynucleotide chain termination method using an automated Applied Biosystems Model 373A DNA sequencer or manually using an *fmol* DNA Sequencing System (Promega). The nucleic acid sequence was compared to wild-type GRP-R sequence using IBI AssemblyLign software.

Generation of G418-Selected Stable Clones. A clonal mouse fibroblast cell line (BALB/B1) was obtained by recloning the BALB 3T3 cell line (American Type Culture Collection) and grown at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% (v/v) qualified fetal bovine serum (Life Technologies) and a 1:200 dilution of a penicillin−streptomycin solution (Life Technologies). The cells were expanded by detachment using trypsin−EDTA (Life Technologies) treatment and subcultured at a split ratio of 1:20 every 3−4 days. BALB/B1 cells were transfected with 3 μg of either wild-type or mutant GRP-R plasmid DNA with 18 μg of Lipofectamine (Life Technologies) in Opti-MEM (Life Technologies) for 1 h at 37 °C. Twenty-four hours post-transfection, the cells were released from 10 cm^2 culture dishes by treatment with trypsin−EDTA, and seeded at varying densities ranging from 100 to 10 000 cells/10 cm^2 dish. Forty-eight hours post-transfection, the cells were placed in medium containing 900 μg/mL G418, and selection was continued for 10−14 days. Clones were screened for wild-type and mutant GRP-R expression by ligand binding and Western blot assays as described below.

Western Blot Analysis. The expression of wild-type and mutant GRP-R in P2 membrane fractions was determined using an anti-GRP-R antiserum (*23*). Forty micrograms of each sample was added per lane and electrophoresed in an SDS−polyacrylamide gel at 120 V. The protein was transferred onto a 0.2 μM nitrocellulose membrane at 25 V overnight in transfer buffer at 4 °C. Immunoblotting was performed at room temperature. Briefly, nitrocellulose membranes were blocked for 1 h in Blotto [50 mM Tris, pH 8, 2 mM $CaCl_2$, 80 mM NaCl, 5% (w/v) nonfat milk, and 0.2% (v/v) Nonidet P-40] containing 10% bovine serum and then incubated for 1 h in GRP-R antiserum (*23*) diluted 1:200 in Blotto. After two washes in Blotto for 10 min each, the immunoblots were incubated with anti-rabbit Ig (Amersham) diluted 1:5000 in Blotto for 1 h. Membranes were then washed twice in Blotto for 10 min each and then twice for 10 min each with 50 mM Tris, pH 8, 2 mM $CaCl_2$, and 80 mM NaCl. Detection was by ultra-chemiluminescence (Pierce) as recommended by the manufacturer.

Whole Cell Radioligand Binding Assays. Competitive binding assays using the tracer ^{125}I-[$_D$Tyr^6,βAla^{11},Phe^{13},Nle^{14}]-Bn-(6−14) (^{125}I-[$_D$Tyr^6]-697) (*24*) were performed as described previously (*18*). Briefly, disaggregated transfected BALB/B1 fibroblasts [(1×10^5)−(3×10^6) cells/mL] were incubated for 45 min at room temperature in a modified HEPES buffer containing 0.16 mg/mL soybean trypsin inhibitor (Sigma), 0.14% (w/v) bovine serum albumin, 0.1% (w/v) bacitracin (Sigma), 10 μM 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride (AEBSF; ICN), and 250 000 cpm/mL tracer (2200 Ci/mmol) without or with unlabeled [$_D$Phe^6,βAla^{11},Phe^{13},Nle^{14}]Bn-(6−14) peptide ([$_D$Phe^6]-697; gift from J. Taylor, Biomeasure). In other binding experiments, the ability of Bn (Bachem), GRP (Bachem), NMB (Bachem), and [$_D$Phe^6]Bn(6−13) methyl ester (ME; gift from D. Coy, Tulane University) to displace ^{125}I-[$_D$Tyr^6]-697 was assayed. The cell number was adjusted to ensure that no more than 10% of the total radioligand added to the assay was bound. Bound ligand is separated from unbound by pelleting the cells through an oil (Nyosil-50, William Nye, Inc.) phase. Radioactivity measurements were determined using a Cobra II gamma counter (Packard). Binding data were analyzed using the nonlinear curve-fitting program LIGAND.

Membrane Preparation. Wild-type and mutant GRP-R transfected fibroblasts, grown in 20−150 (mm) × 25 (mm) dishes to near-confluence, were washed twice with PBS. Cells were placed into ice-cold homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EGTA, 100 μM AEBSF) for 15 min at 4 °C, released by scraping, and subsequently homogenized using a 40 mL Dounce homogenizer. The cellular homogenate was centrifuged for 15 min at 540*g* at 4 °C and the resulting supernatant centrifuged at 75600*g* for 30 min at 4 °C (P2 membrane). The P2 pellet was resuspended in 150 mL of homogenization buffer containing 7 M urea with the aid of the Dounce homogenizer and then incubated on ice for an additional 30 min. At the end of the incubation period, an equal volume of homogenization buffer (without urea) was added to the membrane suspension, and then the membrane preparation was centrifuged at 75000*g* for 30 min at 4 °C. After being washed with homogenization buffer to remove residual urea, the pellet was resuspended in 4−6 mL of homogenization buffer containing 12% sucrose with the aid of a Dounce homogenizer. Aliquots of the resuspended P2 membranes were snap-frozen on dry ice and stored at −70 °C until use.

Receptor-Catalyzed GDP/GTPγS Exchange Assay. The capacity of wild-type and mutant GRP-R to activate $G\alpha_q$ was determined by in situ reconstitution of urea-washed P2 membranes with $G\alpha_q$ and $G\beta\gamma$. Receptor concentration was determined by binding of ^{125}I-[DTyr^6]-697 to membrane preparations. G protein purification was carried out as described (*25*, *26*). The assay protocol has been modified from that previously published (*16*) and is briefly summarized here. The reaction components were assembled in a PCR tube (0.2 mL) on ice with final concentrations of receptor, bovine brain $G\beta\gamma$, squid photoreceptor $G\alpha_q$, TMP, and agonist, when required, at concentrations of 0.5 nM, 300 nM, 100 nM, 1 mM, and 1 μM, respectively. Due to the presence of 5′-nucleotidase in some preparations, TMP was added to ensure stability of GDP and GTP during the time course assays (G. Kroog, unpublished observation). Once preequilibrated to 30 °C, the 150 μL reaction was initiated by the addition of 33 μL of a 4.5 times concentrated reaction solution (also preequilibrated to 30 °C), resulting in a final concentration of 5 mM MOPS, pH 7.5, 100 mM NaCl, 3 mM $MgSO_4$, 1 mM DTT, 1 mM EDTA, 0.3% BSA, 2 μM GDP, and [^{35}S]GTPγS (3×10^6 cpm). Reactions were incubated at 30 °C, and at the indicated times, a 10 μL aliquot was removed and terminated by dispensing into a 12 × 75 mm siliconized borosilicate glass test tube containing 2 mL of ice-cold stopping solution (20 mM, Tris pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$). Samples were processed by filtration using nitrocellulose membranes (Millipore HAWP02500) and then washed 4 times with 4 mL each of ice-cold stop solution. Membranes were dried using a heat lamp then placed into 5 mL of Betamax scintillation fluid (ICN), and radioactivity was determined using a Packard beta counter.

RESULTS

GRP-R Residues Critical for High-Affinity Ligand Binding. The predicted positions of residues D98 and R309 in the TM helices of the mouse GRP-R are shown in Figure 1A. The model used for helix and residue orientation is derived from Baldwin (*27*). The aspartate at the extracellular boundry of TMII and the arginine in TMVII are conserved residues

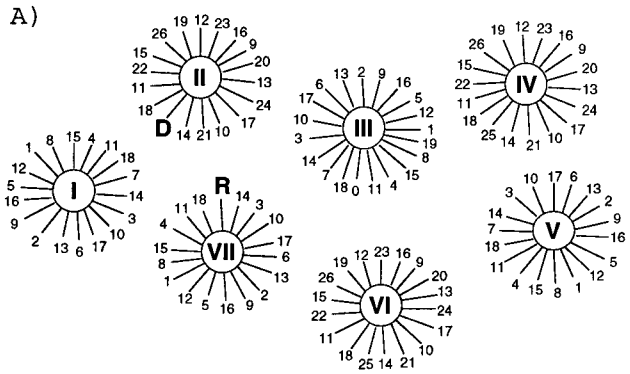

FIGURE 1: Structural model of the GRP-R TM domains and sequence conservation of two polar amino acid residues in juxtaposed helices. (A) The location and orientation of the helices are from a model for the GPCR superfamily proposed by Baldwin (*27*). The relative positions of the D98 (D) and R309 (R) residues are depicted. (B) Selective sequence alignment of the extracellular portions of TM helices II and VII in all cloned mammalian Bn receptors. D98 and R309 in the mouse GRP-R and homologous residues in other Bn receptors are in boldface.

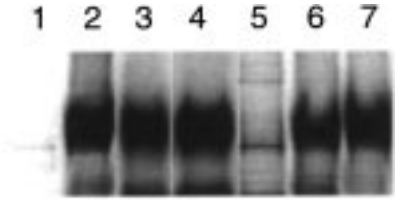

FIGURE 2: Western blot analysis of wild-type and mutant GRP-R P2 membrane fractions using an anti-GRP-R antiserum. Nitrocellulose transfers were probed for immunoreactivity with anti-GRP-R antibody and developed as described under Experimental Procedures. Untransfected control (lane 1); wild-type GRP-R (lane 2); D98A GRP-R (lane 3); R309A GRP-R (lane 4); D98R GRP-R (lane 5); R309D GRP-R (lane 6); D98R/R309D GRP-R (lane 7).

in all three mammalian Bn receptor subtypes (Figure 1B). These conserved residues were hypothesized to play an important role in ligand binding and/or receptor activation. To investigate these possibilities, we constructed GRP-R mutants at these two loci (D98, R309) and expressed these constructs in BALB/B1 fibroblasts. Western blot analysis of transfected fibroblast P2 membrane fractions using an anti-GRP-R antiserum (*23*) identified clones that expressed similar levels of wild-type or mutant GRP-R protein (Figure 2), and hence these isolates were further studied. The D98R GRP-R was not detected in the postnuclear membrane fraction, probably due to misfolding. Wild-type and mutant GRP-R binding of the agonist radioligand ^{125}I-[DTyr^6]-697 was then assayed using quantitative ligand displacement analysis. Recently, this peptide, [DPhe^6]-697, was identified as an agonist with greater affinity for GRP-R than either GRP or Bn (*28*); hence, this ligand was most appropriate to detect specific binding of mutated receptors with decreased

Table 1: Binding Parameters of ^{125}I-[DTyr^6]-697 to Transfected BALB/B1 Cells[a]

		K_i				
receptor	B_{max}	[DPhe^6]-697	GRP	Bn	NMB	ME
wild-type	9030	0.21 ± 0.06	6.2 ± 1.9	1.8 ± 0.4	166 ± 64	2.3 ± 0.4
D98A	28900	5.1 ± 1.4	310 ± 38	277 ± 43	9076 ± 1377	766 ± 164
R309A	12040	12.3 ± 1.9	208 ± 20	321 ± 29	2306 ± 838	4 ± 1.5
D98R[b]						
R309D[c]						
D98R/R309D	59440	1.3 ± 0.1	411 ± 75	270 ± 17	5160 ± 466	350 ± 29

[a] Binding constant values are expressed in nanomolar and represent the mean ± SE obtained from at least three separate experiments in which each data point is performed in triplicate within an experiment. Representative receptor density values (B_{max}) were determined from saturation binding experiments and are expressed as sites/cell. [b] No receptor expression. The P2 fractions of 15 stable transfectants were assayed by Western blot analyses (see Figure 2). [c] No detectable specific binding.

binding affinity. D98A and R309A GRP-R substitutions decreased the apparent affinity for the synthetic agonist by a factor of 24.3-fold and 58.6-fold, respectively (Table 1).

We further hypothesized that these two oppositely charged, highly conserved residues in this subset of GPCRs might contribute to receptor structure and function by interacting with one another. If this interpretation is correct, then swapping the two loci within the same receptor (D98R/R309D) might be expected to increase ligand affinity for this double mutant receptor. Although neither mutation alone produced a receptor capable of binding agonist, reciprocal mutations within the same receptor did restore high-affinity [DTyr^6]-697 agonist binding that differed from wild-type by only 6.2-fold (Table 1). The ability of naturally occurring agonists (Bn, GRP, and NMB) and the synthetic antagonist ME to displace ^{125}I-[DTyr^6]-697 also was examined. We found that the agonists' binding affinities for the mutant receptors were significantly reduced in all cases compared to wild-type. Interestingly, ME affinity for either the D98A or the D98R/R309D GRP-R was diminshed; however, ME affinity for the R309A GRP-R was virtually identical to wild-type receptor. These data show that the structural determinants required for GRP-R binding to the naturally occurring agonists versus the synthetic agonist are different. In addition to altering the ligand binding pocket, this finding suggests that the different binding properties of the three agonists and the antagonist to R309A GRP-R may be due to the coupling status of the receptor.

Substitution at D98 or R309 Alters GRP-R Function. The decreased affinity of the D98A or R309A GRP-R for agonist could be due in part to altered G protein coupling; GPCRs must be coupled to heterotrimeric G proteins to attain the high-affinity agonist state. Therefore, we employed an in situ reconstitution assay, which uses urea-stripped membranes expressing recombinant receptor to catalyze the agonist- and $\beta\gamma$-dependent exchange of GTP for GDP on the Gα subunit (*16*, *26*). Functional coupling of the GRP-R with the Gα_q subunit but not other Gα subunits has previously been reported (*16*). Due to the requirement for high-level receptor expression input in this assay, additional wild-type, D98A, and R309A GRP-R transfectants were selected. Initially, the K_i for ^{125}I-[DTyr^6]-697 binding to extracted P2 membranes was determined to confirm that no loss of specific receptor agonist binding sites took place during the urea-stripping procedure (data not shown). The ability of [DPhe^6]-697 to stimulate receptor-mediated guanine nucleotide exchange in reconstituted P2 membranes expressing either wild-type or mutant GRP-R is shown in Figure 3. Extracted membranes expressing wild-type or D98R/R309D GRP-R exhibited a basal receptor catalytic activity similar to that reported for the reconstitution of $5HT_{2c}$ (*26*). Membranes expressing D98A or R309A GRP-R show a noncatalytically driven increase in guanine nucleotide exchange, attributable to the higher urea-washed membrane levels in these reactions. Only the R309A mutation failed to generate an agonist-dependent GTP exchange. Both the intrinsic basal activity and the overall catalytic activity of D98R/R309D GRP-R are greater than those generated by wild-type GRP-R; however, the wild-type GRP-R produced a larger agonist-stimulated response when compared to the double mutant. Taken together, these data show that GRP-R residues D98 and R309 functionally contribute to the first biochemical process after ligand binding: receptor-catalyzed exchange of GDP for GTP on Gα_q.

Figure 4 presents the saturation of the GTPγS exchange reaction with Gα_q in the presence of both agonist and saturating G$\beta\gamma$ to determine the affinities and catalytic activities of the GRP-R mutants for G_q. In other time-course experiments conducted in the absence of TMP, we have observed that the initial velocity for the wild-type and double mutant GRP-R reactions was linear up to 10 min while the single point mutation receptor reaction was linear up to 30 min (data not shown). These data conformed to a single-site model with K_m's for Gα_q of 87, 68, 97, and 103 nM for wild-type, D98A, R309A, and D98R/R309D GRP-Rs, respectively. Wild-type and double mutant GRP-Rs produce typical hyperbolic response curves approaching saturation levels at higher Gα_q concentrations. Notably the D98R/R309D GRP-R attains a higher V_{max} compared to wild-type, and this is consistent with the results shown in Figure 3. Receptors bearing single point mutations show little or no response at the assayed receptor concentration of 0.2 nM. At a higher receptor concentration (0.5 nM), the D98A and R309A GRP-Rs displayed the expected saturation kinetics in a 20 min reaction (Figure 4, inset). The D98A and R309A GRP-R's have a reduced turnover number relative to both wild-type and double mutant receptor.

DISCUSSION

It is estimated that more than 1000 members of the GPCR superfamily exist and these receptors represent more than 1% of the ~100 000 human gene products (*29*, *30*). Members of the GPCR superfamily share in common a topological motif of seven TM α-helical segments, with extracellular amino-terminal and intracellular carboxy-terminal sequences. The transduction of signaling by heterotrimeric G proteins

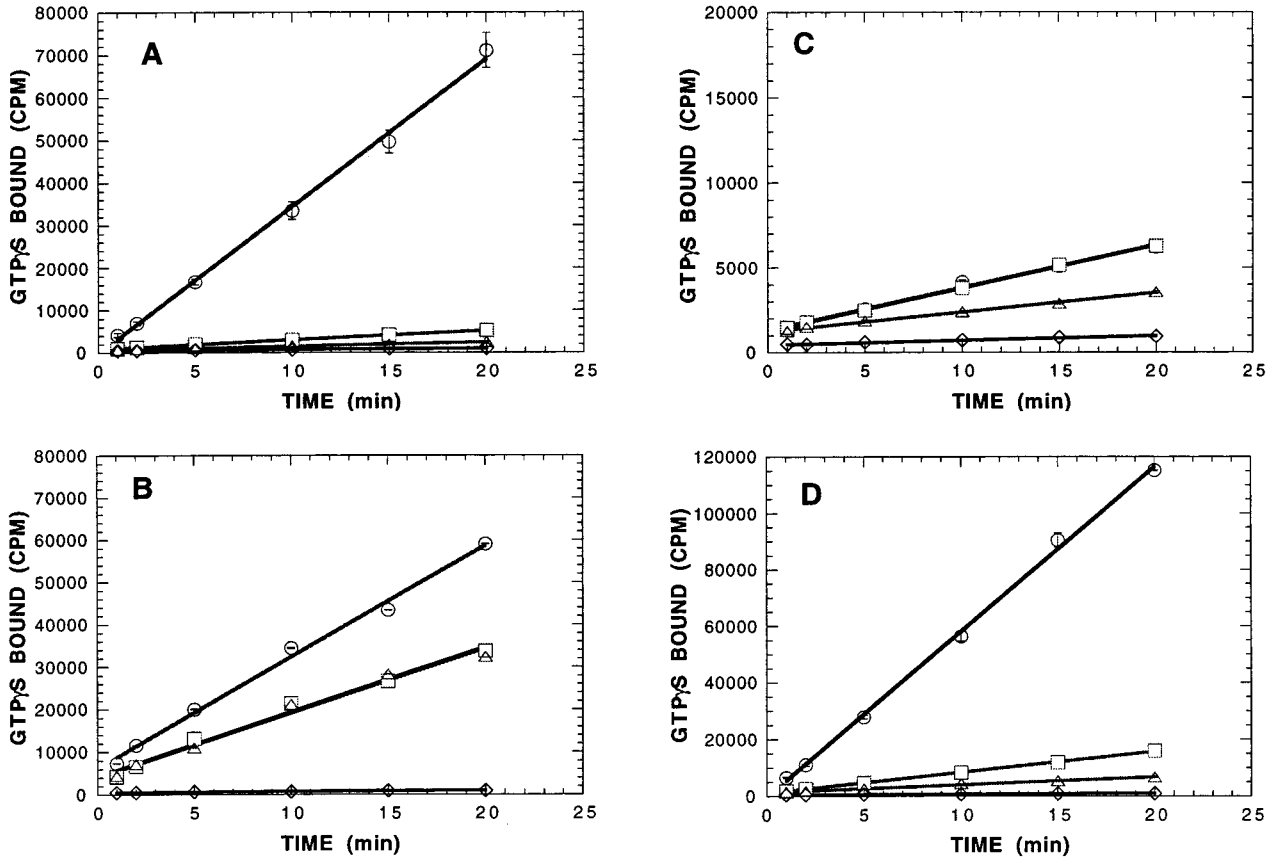

FIGURE 3: Time course of agonist-stimulated wild-type or mutant GRP-R-catalyzed GDP/GTPγS exchange. The catalyzed exchange of GTPγS by 0.5 nM wild-type (panel A), D98A (panel B), R309A (panel C), or D98R/R309D (panel D) GRP-R was determined with 100 nM α_q, 300 nM $\beta\gamma$ in the presence (○) or absence (□) of 1 μM agonist. Membrane control (△) contains no added G proteins. G protein subunit control (◇) contains no added membrane. The amount of urea-washed membrane protein added to the D98A, R309A, and D98R/R309D GRP-R reactions was 56.7-, 10.8-, and 2.1-fold higher, respectively, compared to the wild-type reaction. GTPγS binding reactions were performed as described under Experimental Procedures.

also implies a similarity of protein contacts between these receptors and G protein subunits. Since the overall structure of G proteins is highly conserved (*31*), it seems reasonable to infer that the molecular mechanism(s) for receptor signaling must also be conserved among the GPCR superfamily. These might include, in addition to the protein−protein contacts between receptor and G protein, the conformational transitions involved in the activation mechanism. To address these issues experimentally, we have developed techniques for the in situ reconstitution of recombinant receptors expressed in their native membrane environment (*16*, *26*). These procedures provide a means both to dissect the contribution of specific G proteins and receptors to the regulation of cellular responsiveness and to examine the effect of mutation on the interaction between receptor and G protein by measuring G protein activation, the proximal biochemical event in GPCR signaling.

In this study, we have examined the contribution of two polar amino acid residues, D98 and R309, in TM domains II and VII to the signaling function of the GRP-R. Our experiments were designed to test whether these two residues provided intramolecular contacts within the GRP-R by introducing complementing mutations singly or in tandem. Substitution of alanine at either D98 or R309 greatly reduced agonist binding. This loss of affinity could be attributable to either a mutation disrupting the binding pocket or the inability of the receptor to properly couple to G proteins. To clarify this issue, an in situ reconstitution assay was used to determine the ability of wild-type or mutant GRP-R to catalyze binding of GTPγS to purified G α protein subunits. The data obtained from the reconstitution assay clearly indicate that the conserved D98 and R309 residues play a critical functional role in GRP-R coupling to Gα_q. It is proposed that replacement of D98 or R309 by alanine precludes formation of hydrogen bonds between the two loci; the disrupted interaction prevents receptor activation. Restoration of a functional GRP-R by the introduction of complementing mutations (D98R/R309D) implies that these residues share a salt bridge within the same microenvironment (*32*, *33*); the swapping of these TM domain residues within the same receptor accommodated both polarity and side chain size. Hence, our data support the notion that the structural backbone of the receptor is formed, in part, by the interaction of interhelical amino acid residue side chains and this network is essential for the activation capacity of the protein.

Although the catalytic activity of the D98R/R309D double mutant receptor exceeds that of wild-type, it is significantly attenuated in its ability to bind the naturally occurring agonists GRP, Bn, and NMB. This decreased binding affinity in comparison to wild-type receptor implies that structural determinants for binding natural agonists differ from those essential for efficient catalysis of guanine nucleotide exchange on G protein. D98A GRP-R similarly has diminished affinity for all Bn peptides tested. However, D98A GRP-R has diminished catalytic activity compared to the high

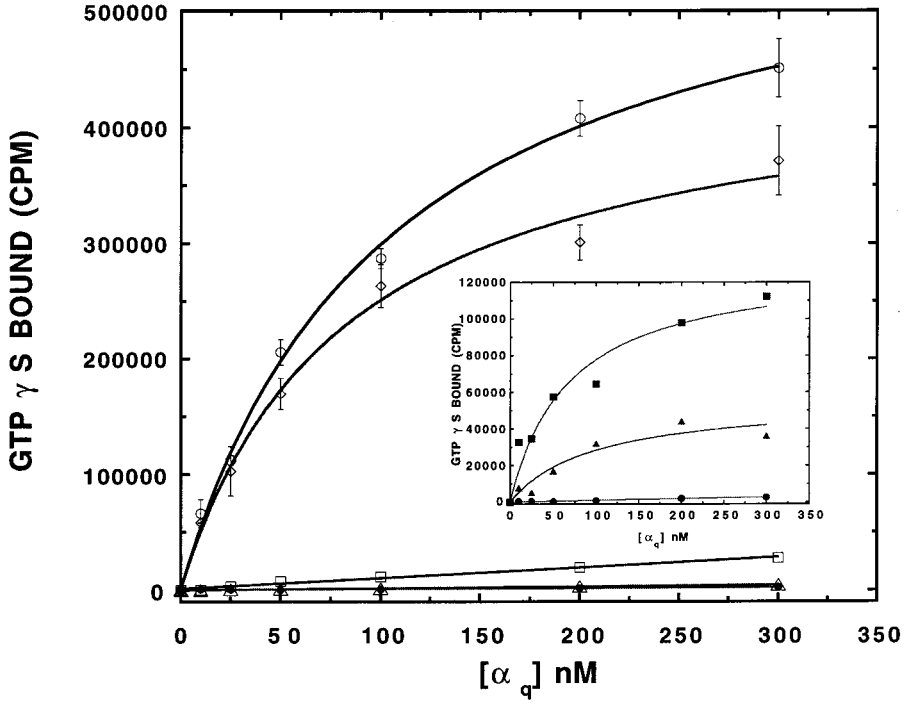

FIGURE 4: Saturation of the rate of agonist-stimulated GRP-R-catalyzed GDP/GTPγS exchange with Gα_q. Agonist-dependent catalyzed exchange of GTPγS by 0.2 nM wild-type (◇), D98A (□), R309A (△), or D98R/R309D (○) GRP-R was determined with 500 nM $\beta\gamma$ and the indicated concentrations of α_q. Reactions were terminated after 5 min at 30 °C in a final volume of 50 μL, and the binding of GTPγS was determined as described under Experimental Procedures. The G$\beta\gamma$ concentration of 500 nM is greater than the estimated K_m value of 150 nM (data not shown). The inset shows the ability of 0.5 nM D98A (■) or R309A (▲) GRP-R to catalyze GTP exchange in a 20 min reaction.

catalytic activity of the double mutant receptor. Intriguingly, the R309A mutant, while abrogating agonist peptide binding, retains nearly wild-type affinity for the antagonist peptide ME. These data indicate that the R309 residue may provide a contact site for agonist ligands in addition to forming a salt bridge with D98. The swapped polar residue side chains may distort the binding pocket or repel the ligand peptide side chains. We propose that the internal β-alanine of the synthetic agonist (peptide [DPhe^6]-697) is better able to accommodate a modified receptor binding pocket when compared with the naturally occurring agonists and that the antagonist ME does not require the same binding contacts.

Experimental evidence from several GPCR studies supports the idea that interhelical interactions are mediated by polar amino acid residues. The activation of the visual pigment rhodopsin involves the breaking of a salt bridge between E113 in TMIII and K296 in TMVII (*34*). Also, similar to what we have proposed for R309 and agonist peptide binding to GRP-R, K296 of rhodopsin makes the essential Schiff's base linkage with the aldehyde of 11-*cis*-retinal (*35*). A network of hydrogen bonds between N63, D91, N344, and Y348 has been postulated to form a ligand binding pocket in the α_{1B}-adrenergic receptor (*36*). Recently, Perlman and colleagues (*37*) have proposed that a conserved aspartate in TMII interacts with asparagine residues in both TMI and TMVII of the thyrotropin-releasing hormone receptor.

Mutational analyses of residues that form interhelical interactions required to maintain the appropriate receptor activation state have been examined in several GPCRs. For example, substitution of alanine for D120 (TMII) or N376 (TMVII) in the serotonin 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptor resulted in a receptor having near-wild-type receptor affinity values for agonist, while neither mutated receptor produced an agonist-dependent accumulation of inositol phosphates (*33*). Either single swap (D120N or N376D) or double swap (D120N/N376D) 5-HT$_{2A}$ mutations resulted in receptors having near-wild-type receptor affinity values for agonist. Furthermore, the D120N 5-HT$_{2A}$ receptor did not generate an agonist-stimulated second messenger response whereas the D120N/N376D produced a response ~50% of wild-type. Mutation of N87 to aspartate (TMII) eliminated detectable agonist or antagonist binding in the gonadotropin-releasing hormone receptor (GnRHR) (*32*). Introduction of a second mutation, generating the D87/N318 GnRHR, restored high-affinity agonist and antagonist binding, but this receptor was unable to generate inositol phosphates. In the α_{1b}-adrenergic receptor (α_{1b}-AR), substitution of alanine for D125 (TMIII) produces a mutant receptor having a K_i agonist affinity value 3-fold less than wild-type receptor, whereas the K331A (TMVII) binding affinity for agonist improved 5.6-fold relative to wild-type (*38*). The alanine substitutions at positions 125 or 331 of the α_{1b}-AR produced receptors that had elevated basal inositol phosphate levels. Many aspects of these previously reported findings for the 5-HT$_{2A}$ receptor, the GnRHR, and α_{1b}-AR both parallel and contrast the results presented here. First, in contrast to our results, neither the 5-HT$_{2A}$ receptor nor the α_{1b}-AR is adversely affected by alanine substitutions with regard to agonist binding. Second, the single swap 5-HT$_{2A}$ mutations are well tolerated in terms of agonist binding and receptor expression in contrast to the GRP-R presented here. Third, the D87/N316 GnRHR was unable to generate an agonist-stimulated second messenger response in contrast to the restored

function of the D98R/R309D GRP-R. Last, in contrast to our results, alanine substitutions of the α_{1b}-AR produced constitutively active receptors. In our experiments, only the D98R/R309D double mutation increased the constitutive activity of the GRP-R, while the individual alanine substitutions abrogated measurable signaling function.

In summary, we have investigated the functional role of two conserved polar amino acid residues, D98 and R309, of the GRP-R. Mutations of D98 or R309 to alanine result in a receptor incapable of catalyzing the exchange of guanine nucleotides and unable to bind agonists with high affinity. The signaling function of the GRP-R is restored by the complementing double mutation of these two residues. These data imply that a salt bridge exists between these two residues that maintains the receptor conformation necessary to properly interact with G proteins and which is essential in the transition to the activated conformation. Taken together with the data from other GPCRs, these findings argue that the salt bridge residues that underlie receptor activation play unique roles in terms of their impact on the maintenance of the ligand binding pocket as well as the receptor's ability to interact with heterotrimeric G proteins.

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REFERENCES

1. Strader, C. D., Fong, T. M., Graziano, M. P., and Tota, M. R. (1995) *FASEB J. 9*, 745−754.
2. Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., and Dixon, R. A. (1994) *Annu. Rev. Biochem. 63*, 101−132.
3. Conn, P. J., and Pin, J. P. (1997) *Annu. Rev. Pharmacol. Toxicol. 37*, 205−237.
4. Laburthe, M., Couvineau, A., Gaudin, P., Maoret, J. J., Rouyer-Fessard, C., and Nicole, P. (1996) *Ann. N.Y. Acad. Sci. 805*, 94−109; discussion 110−111.
5. Battey, J. F., Way, J. M., Corjay, M. H., Shapira, H., Kusano, K., Harkins, R., Wu, J. M., Slattery, T., Mann, E., and Feldman, R. I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 395−399.
6. Spindel, E. R., Giladi, E., Brehm, P., Goodman, R. H., and Segerson, T. P. (1990) *Mol. Endocrinol. 4*, 1956−1963.
7. Wada, E., Way, J., Shapira, H., Kusano, K., Lebacq-Verheyden, A. M., Coy, D., Jensen, R., and Battery, J. (1991) *Neuron 6*, 421−430.
8. Gorbulev, V., Akhundova, A., Buchner, H., and Fahrenholz, F. (1992) *Eur. J. Biochem. 208*, 405−410.
9. Fathi, Z., Corjay, M. H., Shapira, H., Wada, E., Benya, R., Jensen, R., Viallet, J., Sausville, E. A., and Battey, J. F. (1993) *J. Biol. Chem. 268*, 5979−5984.
10. Nagalla, S. R., Barry, B. J., Creswick, K. C., Eden, P., Taylor, J. T., and Spindel, E. R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 6205−6209.
11. Moran, T. H., Moody, T. W., Hostetler, A. M., Robinson, P. H., Goldrich, M., and McHugh, P. R. (1988) *Peptides 9*, 643−649.
12. von Schrenck, T., Heinz-Erian, P., Moran, T., Mantey, S. A., Gardner, J. D., and Jensen, R. T. (1989) *Am. J. Physiol. 256*, G747−G758.
13. Wada, E., Wray, S., Key, S., and Battey, J. (1992) *Mol. Cell. Neurosci. 3*, 446−460.
14. Hampton, L. L., Ladenheim, E. E., Akeson, M., Way, J. M., Weber, H. C., Sutliff, V. E., Jensen, R. T., Wine, L. J., Arnheiter, H., and Battey, J. F. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3188−3192.
15. Mantey, S. A., Weber, H. C., Sainz, E., Akeson, M., Ryan, R. R., Pradhan, T. K., Searles, R. P., Spindel, E. R., Battey, J. F., Coy, D. C., and Jensen, R. T. (1997) *J. Biol. Chem. 272*, 26062−26071.
16. Hellmich, M. R., Battey, J. F., and Northup, J. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 751−756.
17. Brown, K. D., Blay, J., Irvine, R. F., Heslop, J. P., and Berridge, M. J. (1984) *Biochem. Biophys. Res. Commun. 123*, 377−384.
18. Benya, R. V., Wada, E., Battey, J. F., Fathi, Z., Wang, L. H., Mantey, S. A., Coy, D. H., and Jensen, R. T. (1992) *Mol. Pharmacol. 42*, 1058−1068.
19. Benya, R. V., Fathi, Z., Kusui, T., Pradhan, T., Battey, J. F., and Jensen, R. T. (1994) *Mol. Pharmacol. 46*, 235−245.
20. Benya, R. V., Kusui, T., Pradhan, T. K., Battey, J. F., and Jensen, R. T. (1995) *Mol. Pharmacol. 47*, 10−20.
21. Akeson, M., Sainz, E., Mantey, S. A., Jensen, R. T., and Battey, J. F. (1997) *J. Biol. Chem. 272*, 17405−17409.
22. Sainz, E., Akeson, M., Mantey, S. A., Jensen, R. T., and Battey, J. F. (1998) *J. Biol. Chem. 273*, 15927−15932.
23. Kroog, G. S., Sainz, E., Worland, P. J., Akeson, M. A., Benya, R. V., Jensen, R. T., and Battey, J. F. (1995) *J. Biol. Chem. 270*, 8217−8224.
24. Pradhan, T. K., Katsuno, T., Taylor, J. E., Kim, S. H., Mantey, S. A., Donohue, P. J., Weber, H. C., Sainz, E., Battey, J. F., Coy, D. H., and Jensen, R. T. (1998) *Eur. J. Pharmacol. 343*, 275−287.
25. Sternweis, P. C., and Robishaw, J. D. (1984) *J. Biol. Chem. 259*, 13806−13813.
26. Hartman, J. I., and Northup, J. K. (1996) *J. Biol. Chem. 271*, 22591−11597.
27. Baldwin, J. M. (1994) *Curr. Opin. Cell Biol. 6*, 180−190.
28. Pradhan, T. K., Katsuno, T., Taylor, J. E., Kim, S. H., Ryan, R. R., Mantey, S. A., Donohue, P. J., Weber, H. C., Sainz, E., Battey, J. F., Coy, D. H., and Jensen, R. T. (1998) *Eur. J. Pharmacol. 343*, 275−287.
29. Ji, T. H., Grossmann, M., and Ji, I. H. (1998) *J. Biol. Chem. 273*, 17299−17302.
30. Gutkind, J. S. (1998) *Oncogene 17*, 1331−1342.
31. Sprang, S. R. (1997) *Annu. Rev. Biochem. 66*, 639−678.
32. Zhou, W., Flanagan, C., Ballesteros, J. A., Konvicka, K., Davidson, J. S., Weinstein, H., Millar, R. P., and Sealfon, S. C. (1994) *Mol. Pharmacol. 45*, 165−170.
33. Sealfon, S. C., Chi, L., Ebersole, B. J., Rodic, V., Zhang, D., Ballesteros, J. A., and Weinstein, H. (1995) *J. Biol. Chem. 270*, 16683−16688.
34. Cohen, G. B., Oprian, D. D., and Robinson, P. R. (1992) *Biochemistry 31*, 12592−12601.
35. Findlay, J. B., and Pappin, D. J. (1986) *Biochem. J. 238*, 625−642.
36. Scheer, A., Fanelli, F., Costa, T., De Benedetti, P. G., and Cotecchia, S. (1996) *EMBO J. 15*, 3566−3578.
37. Perlman, J. H., Colson, A. O., Wang, W., Bence, K., Osman, R., and Gershengorn, M. C. (1997) *J. Biol. Chem. 272*, 11937−11942.
38. Porter, J. E., Hwa, J., and Perez, D. M. (1996) *J. Biol. Chem. 271*, 28318−28323.

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