Identification of Four Amino Acids in the Gastrin-releasing Peptide Receptor That Are Required for High Affinity Agonist Binding

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The bombesin family of G-protein-coupled receptors includes the gastrin-releasing peptide receptor (GRP-R), the neurenomed B receptor (NMB-R), bombesin receptor subtype 3 (BRS-3), and bombesin receptor subtype 4 (bb4). All species homologues of GRP-R, NMB-R, and bb4 bind bombesin with dissociation constants in the nanomolar range; by comparison, human BRS-3 binds bombesin at much lower affinity (Kd > 1 μM). We used this difference to help identify candidate residues that were potentially critical for forming the bombesin binding pocket. We reasoned that amino acids essential for bombesin binding would be conserved among all homologues of bb4, NMB-R, and GRP-R; conversely, at least one of these amino acids would not be conserved among homologues of BRS-3. Amino acid sequence alignment revealed nine residues that fit this model. We replaced each of these amino acids in mouse GRP-R with the homologous amino acid in human BRS-3. Four substitutions resulted in a significant decrease in bombesin affinity (R288H, Q121R, P199S, and A208S). The analogous mutations in BRS-3 (R127Q, H294R, S205P, and S315A) together resulted in a receptor with a 100-fold increase in bombesin and GRP affinities relative to wild-type BRS-3. From this, we propose a preliminary map of some of the amino acids comprising the agonist binding pocket.

Bombesin is a tetradecapeptide that was originally isolated from frog skin (1). Numerous polypeptides with structurally related carboxyl termini were subsequently isolated from amphibians and classified into three subfamilies (bombesin, ranatensin, and phyllolitorin) based upon the sequence of the last three residues in their amidated carboxy-terminal domains (2). To date, two bombesin-like peptides have been isolated from mammalian tissue: NMB1 in the ranatensin subfamily (3) and GRP in the bombesin subfamily (4). Bombesin, NMB, and GRP bind to a family of G-protein-coupled receptors known to include at least four receptor subtypes: the GRP-prefering receptor (GRP-R (or bb2)) (5, 6), the NMB-prefering receptor (NMB-R (or bb1)) (5, 7), bombesin receptor subtype 3 (BRS-3, (or bb3)) (8, 9), and bombesin receptor subtype 4 (bb4) (10). Although the four subtypes share about 50% identity when their primary amino acid sequences are aligned, the pharmacology of BRS-3 is significantly different from the pharmacology of the others.2 Notably, GRP-R, NMB-R, and bb4 all bind bombesin with affinities in the nanomolar range, whereas BRS-3 binds bombesin with much lower affinity (>10 μM).

We hypothesized that amino acids essential for high affinity bombesin binding would be conserved among all known GRP-R, NMB-R, and bb4 sequences and would diverge in the BRS-3 sequences. Nine amino acids in extracellular loops or in the outer half of transmembrane helices fulfilled these conditions. The importance of each of these residues for agonist binding to GRP-R was determined by using site-directed mutagenesis to exchange the residue found in GRP-R with its divergent counterpart in BRS-3. The affinities of BRS-3 or GRP-R mutants for bombesin and GRP were determined using quantitative ligand displacement analysis and, in instances where the affinity was low, were confirmed by dose-response analysis of ligand-activated inositol phosphate elaboration.

Reduced affinity in mouse GRP-R and increased affinity in human BRS-3 revealed that two residues (Gln-121 and Arg-288) were essential for bombesin and GRP binding to GRP-R; two other residues (Pro-199 and Ala-308) modulated binding. These four residues appear to form a pocket centered between TMIII, TMVI, and TMVII, near the membrane-solution interface of the receptor.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Site-directed Mutagenesis—cDNA inserts for the mouse GRP-R and for human BRS-3 were identical to those described previously (8, 11) except that a flag epitope tag was added to the amino terminus of human BRS-3. These were cloned into the EcoRI site of pcDNA3 (Invitrogen, San Diego, CA) or pCDII (12). To generate point mutations in these cDNAs, we initially used the Chameleon™ double-stranded site-directed mutagenesis kit as suggested by the manufacturer (Stratagene, La Jolla, CA). In our hands, the Chameleon procedure generated an unacceptable number of random mutations. Therefore, we introduced subsequent amino acid substitutions in GRP-R and BRS-3 with the QuickChange™ kit (Stratagene) following manufacturer instructions, except that the annealing step was at 60–62 °C and the DpnI digestion was incubated overnight. Nucleotide sequence analysis of the entire coding region was performed on an

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Automated DNA Sequencer (ABI 373, Applied Biosystems Inc., Foster City, CA).

Cell Transfection—Balb 3T3 (13) and CHO cells (14) were transfectected with 10 μg of plasmid DNA by calcium phosphate precipitation as described previously (15). Transiently expressing CHO cells (16) were used for binding assays. Stably transfected Balb 3T3 cells were selected using 800 μg/ml G418 in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), penicillin, and streptomycin. Clones were then screened for GRP-R or BRS-3 mRNA expression by Northern blot analysis of total RNA, performed essentially as described in Davis et al. (15). Stable cell lines were maintained in DMEM supplemented with 10% (v/v) FBS, penicillin, streptomycin, and 300 μg/ml G418. These cell lines were used for binding or inositol phosphate assays. All reagents used in cell transfection are from Life Technologies, Inc.

Whole Cell Radioligand Binding Assays—For the initial screen, 10-cm diameter plates of transiently transfected CHO cells were trypsinized approximately 24 h post-transfection and seeded into 24-well plates at 1 × 10⁵ cells/well. Cells were allowed to incubate an additional 24 h in 1 ml of DMEM containing 10% FBS, penicillin/streptomycin, and 300 μg/ml G418. These assays were performed in binding buffer containing 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 50 mM HEPES/NaOH (pH 7.4), 0.1% (v/v) bovine serum albumin, and 0.1% bacitracin (Sigma). The cells were rinsed twice with 1 ml of binding buffer at room temperature and then incubated for 2 h at room temperature in 0.2 ml of binding buffer containing 100,000 cpm/ml of [125I-Tyr⁴]bombesin (2200 Ci/mmol) together with varying concentrations of unlabeled bombesin or GRP. The tracer was stable, and the reaction was at equilibrium as judged by stable binding maxima between 1.5 and 3 h. Therefore, the reaction was terminated at 2 h by rinsing twice in buffer and incubating the cells at 57 °C in 0.2 ml of 0.65% (v/v) trypsin, 0.53 mM EDTA (Life Technologies) for 15 min. This suspension and an equal volume rinse were transferred to a glass tube and assayed on a gamma counter. Each point was in duplicate, and each experiment was replicated three times. Kᵢ values represent means ± S.E. for the three replicates.

Competition binding experiments to measure gain of function in human BRS-3 or to measure low affinity binding to mouse GRP-R mutants were performed with a novel synthetic radioligand similar in structure to the carboxy-terminal end of bombesin. In contrast to bombesin, however, this radioligand ([125I-Tyr⁴], β Ala¹¹, β-Phe¹³, Nle¹⁴]bombesin 6–14, which we call 125I-BRS-3) binds BRS-3 with high affinity (Kᵢ = 5–10 nM). Competition binding studies were performed as described earlier (16), except that the protease inhibitor 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride (ICN Biomedicals Inc., Aurora, OH) was added to a final concentration of 0.1 mM. Briefly, disaggregated transfected Balb 3T3 cells (1–3 × 10⁶ cells/ml) were incubated for 1 h at room temperature in 100,000–600,000 cpm/ml of [125I-BRS-3] (2200 Ci/mmol) in the presence of unlabeled bombesin or GRP up to 30 μM. At 1 h, the binding reaction was at equilibrium as indicated by a plateau in radioligand binding as a function of time (data not shown), and the tracer was not measurably degraded as determined by high performance liquid chromatography of the radioligand from the binding reaction. Each point was measured in triplicate, and each experiment was replicated three times. Kᵢ values represent means ± S.E. for the three replicates.

Analysis of Ligand-activated Inositol Phosphate Generation—Stably transfected Balb 3T3 cells were assayed for GRP or bombesin-induced inositol phosphate hydrolysis as described previously (16). Briefly, cells in 24-well tissue culture dishes were incubated in the presence of 100 μM/ml of myo-[2-³²P]inositol (NEN Life Science Products) in DMEM containing 0.25% FBS for 24 h. The cells were washed and incubated in phosphate-buffered saline containing calcium and magnesium (Life Technologies) supplemented with 20 mM LiCl for 15 min. After this incubation, the wash solution was removed, and cells were incubated for 1 h with varying concentrations of GRP or bombesin in a buffer solution containing 135 mM NaCl, 20 mM HEPES, 2 mM CaCl₂, 1.2 mM MgSO₄, 10 mM LiCl, 11.1 mM glucose, and 0.5% (w/v) bovine serum albumin adjusted to pH 7.45. The reaction was stopped by the addition of ice-cold 1% (v/v) HCl in methanol, and total [³²P]inositol phosphate was recovered by anion exchange chromatography (17). Error estimates are standard errors of the mean.

RESULTS

Analysis of Bombesin Binding to Transiently Expressed Mouse GRP-R Receptors with Single Point Mutations—Amino acid residues potentially critical for high affinity bombesin binding were revealed by an alignment of bombesin receptor sequences, which maximizes identity (Fig. 1). Nine positions are highlighted based on three criteria. (i) The amino acid at that position is conserved among all receptors that bind bombesin with high affinity. (ii) The amino acid at that position in BRS-3 is not the same as the conserved residue in the previous criterion. (iii) The amino acid at that position is predicted to lie outside the cell, and five are believed to be in transmembrane helices (when the boundaries of the transmembrane domains are determined by Kyte-Doolittle hydropathy analysis).

We changed single amino acid residues in the mouse GRP-R, converting each of the nine highlighted residues to the analog-
duplicates within an experiment; each curve represents the average of three experiments shown in a whole cell binding assay as described under “Experimental Procedures.” ○, wild-type GRP-R; ●, R288H GRP-R. Each point on the graphs represents the average of duplicates within an experiment; each curve shown is representative of three experiments.

Analysis of R288H and Q121R Mutants of Mouse GRP-R

To examine the R288H and Q121R mutant GRP-R receptors in more detail, we created stably transfected Balb 3T3 clonal cell lines expressing high levels of GRP-R proteins in membranes, as demonstrated by Western blot analysis using an anti-GRP-R antiserum (18) (data not shown). Agonist affinity was measured by displacement of $^{125}I$-BRS-3 tracer (Fig. 2, filled circles in top and bottom panels), and displacement by both GRP and bombesin indicated $K_d$ values in the $\mu M$ range. This represents a 1000-fold reduction in affinity relative to wild-type mouse GRP-R (Fig. 2, open circles in top and bottom panels). Low affinity of R288H for bombesin was confirmed by determining saturation curves for bombesin-stimulated inositol phosphate hydrolysis, where it is predicted that higher doses of bombesin will be needed to elicit a response comparable to that observed in the wild-type GRP-R. A saturation analysis of bombesin-stimulated inositol phosphate hydrolysis indicated a nearly 100-fold-reduced bombesin potency in the R288H GRP-R mutant (Fig. 3, filled circles) relative to the wild-type mouse GRP-R (Fig. 3, open circles). These data indicate a striking decrease in binding affinity for GRP and bombesin in an otherwise functional R288H GRP-R mutant.

GRP and Bombesin Affinities for Human BRS-3 Are Increased by the Combined Point Mutations R127Q, H294R, S205P, and S315A—The loss of function analysis identified four amino acids that might contribute to the agonist binding pocket of mouse GRP-R. If this interpretation is correct, then changing the same four amino acids in human BRS-3 (R127Q, H294R, S205P, and S315A) to their corresponding residues in the GRP-R would be expected to increase the affinity of bombesin and GRP for that BRS-3 mutant. To test this hypothesis, we constructed two human BRS-3 mutants: 2ABRS-3 contained the R127Q and H294R substitutions, and 4ABRS-3 contained all four key substitutions (R127Q, H294R, S205P, and S315A).
The affinity of GRP and bombesin for these mutant receptors and for wild-type human BRS-3 (Fig. 4, open circles, top and bottom panels) was measured by displacement of $^{125}$I-BRS-3 tracer by bombesin or GRP at the concentrations shown using a whole cell binding assay as described under "Experimental Procedures." Each point on the graphs represents the average of duplicates within an experiment; each curve shown is representative of three experiments. The $K_i$ values for the wild type and 2$\Delta$BRS-3 were too low to estimate. The $K_i$ values for the 4$\Delta$BRS-3 receptor were 370 ± 70 nM (GRP) and 1.5 ± 0.3 μM (bombesin).

To substantiate this result, we compared bombesin and GRP-stimulated inositol phosphate hydrolysis by wild-type BRS-3 and by 4$\Delta$BRS-3 expressed in Balb 3T3 cells. If the binding for bombesin is shifted to higher affinity in the two BRS-3 mutant receptors, it is predicted that lower doses of bombesin or GRP should be needed to elicit comparable inositol phosphate hydrolysis when BRS-3 mutants are compared with wild-type BRS-3. In agreement with the competitive binding assay, the agonist saturation curves for the 4$\Delta$BRS-3 (Fig. 5, filled circles) were shifted to higher potency relative to wild-type human BRS-3 (Fig. 5, open circles).

DISCUSSION

Gln-121, Arg-288, Ala-308, and Pro-199 are at least some of the residues necessary for high affinity bombesin and GRP binding to the mouse GRP-R. This conclusion is based upon lower agonist affinity for the GRP-R when these residues are altered and upon gain of bombesin and GRP affinity when these residues are introduced into human BRS-3 (Figs. 4 and 5). A conceptually similar analysis was recently used to identify amino acids that influence agonist binding among opioid receptors (19). In both the study presented here and in the opioid receptor study, the gain of function mutations present the most compelling evidence that key amino acids play a direct role in determining agonist affinity.

Like other members of the G-protein-coupled receptor superfamily, the primary sequence of the GRP-R suggests seven helical transmembrane domains that form a tightly packed bundle. Recently, two structural models of these transmembrane helices have been independently proposed, based upon electron diffraction analysis of bovine rhodopsin (20) or a sum-
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The location, orientation, and tilt of the helices are from Baldwin (21), with shaded circles representing the helices at three depths. The scale of the amino acid side chains between helices is derived from a NMR-based model of the retinal ligand within the binding domain of rhodopsin (23). Gln-121 (Q121), Arg-288 (R288), and Ala-308 (A308) are amino acids determined to affect agonist binding to the mouse GRP-R in this study. Ser-216 (S216) was previously shown to prevent high affinity NMB binding to this receptor (13). Pro-199, which also modulates agonist binding to the GRP-R, is in extracellular loop 3 near a cysteine-cysteine disulfide bridge at the top of TMIII above Gln-121.

The natural ligand binding pocket is formed between TMIII and TMVII (24). The cysteine at position 114 is believed to lie at the top of TMIII above Gln-121. It is noteworthy that a highly conserved aspartate occupies the same position in TMIII of the biogenic amine receptors appears to form a disulfide bridge and Pro-199 is in extracellular loop 3 near a cysteine-cysteine disulfide bridge at the top of TMIII above Gln-121.

The model we used for helix and residue orientation is from Baldwin (21, 22), and the approximate size of the side chains within the cavity circumscribed by the helices is derived from an NMR study of retinal-associated rhodopsin (23). All three side chains are predicted to point into a pocket formed between TMIII, TMIV, and TMVII. Pro-199 is in extracellular domain 3 (Fig. 1) two positions from a cysteine (Cys-197), which is presumed to form a disulfide bridge with Cys-114 of the GRP-R. The cysteine at position 114 is believed to lie at the top of TMIII; therefore, the disulfide bridge and Pro-199 are predicted to be in close proximity to the pocket between Gln-121, Ala-308, and Arg-288.

This model for receptor structure predicts that these four residues lie in a plane at or near the membrane solution interface, in agreement with earlier work in our laboratory showing that serine or isoleucine at position 216 atop TMV (shown in Fig. 6) strongly influences NMB affinity within the bombesin family (13). This model also predicts that the agonist binding pocket for the GRP-R occupies space between alpha helices similar to the biogenic amine receptors and rhodopsin. In the former case, agonists bind to residues in TMIII, TMV, and TMVI (24). It is noteworthy that a highly conserved aspartate in TMIII of the biogenic amine receptors appears to form an ionic bond to the protonated amine that is shared by all agonists in that class (24). This aspartate occupies the same position in TMIII as does Gln-121 of the GRP-R. In rhodopsin, Lys-296 at the top of TMVII is the site of Schiff base linkage to the retinal chromophore, and Gln-113 at the top of TMIII is the Schiff base counterion (25). These residues in rhodopsin are positioned about one helical turn above the positions of Ala-308 and Gln-121 in the GRP-R.

We are presently testing two hypotheses about receptor-ligand interactions in the bombesin family that follow from this study. First, we hypothesize that there is a shared domain among mutational analyses performed on receptors throughout the superfamily (21, 22). The two models agree in most respects and present a useful structural framework to analyze ligand and heterotrimeric G-protein binding to this class of receptors. In Fig. 6, we show the predicted position of the three residues (Gln-121, Arg-288, Ala-308) in the transmembrane helices of the GRP-R. The model we used for helix and residue orientation is from Baldwin (21, 22), and the approximate size of the side chains within the cavity circumscribed by the helices is derived from an NMR study of retinal-associated rhodopsin (23). All three side chains are predicted to point into a pocket formed between TMIII, TMIV, and TMVII. Pro-199 is in extracellular domain 3 (Fig. 1) two positions from a cysteine (Cys-197), which is presumed to form a disulfide bridge with Cys-114 of the GRP-R. The cysteine at position 114 is believed to lie at the top of TMIII; therefore, the disulfide bridge and Pro-199 are predicted to be in close proximity to the pocket between Gln-121, Ala-308, and Arg-288.

Second, we hypothesize that if the pocket defined by Gln-121, Pro-199, Arg-288, and Ala-308 is important for agonist binding within this family, then other conserved residues facing this pocket would be expected to significantly affect agonist binding. We are in the process of examining several candidate amino acids to test this hypothesis.

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