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Identification of key amino acids in the gastrin-releasing peptide receptor (GRPR) responsible for high affinity binding of gastrin-releasing peptide (GRP)

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Abstract

The bombesin (Bn) receptor family includes the gastrin-releasing peptide (GRPR) and neuromedin B (NMBR) receptors, Bn receptor subtype 3 (BRS-3) and Bn receptor subtype 4 (BB_4). They share 50% homology, yet their affinities for gastrin-releasing peptide (GRP) differ. The determinants of GRP high affinity for GRPR and BB_4, and low affinity for BRS-3 are largely unknown. To address this question we made an analysis of structural homologies in Bn receptor members correlated with their affinities for GRP to develop criteria to identify amino acids important for GRP selectivity. Fourteen differences were identified and each was mutated singly in GRPR to that found in hBRS-3. Eleven mutants had a loss of GRP affinity. Furthermore, three of four amino acids in the GRPR selected used a similar approach and previously reported to be important for high affinity Bn binding, were important for GRP affinity. Some GRPR mutants containing combinations of these mutations had greater decreases in GRP affinity than any single mutation. Particularly important for GRP selectivity were K101, Q121, A198, P199, S293, R288, T297 in GRPR. These results were confirmed by making the reverse mutations in BRS-3 to make GRP gain of affinity mutants. Modeling studies demonstrated a number of the important amino acids had side-chains oriented inward and within 6 Å of the binding pocket. These results demonstrated this approach could identify amino acids needed for GRP affinity and complemented results from chimera/mutagenesis studies by identifying which differences in the extracellular domains of Bn receptors were important for GRP affinity.
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1. Introduction

The bombesin (Bn) receptor family includes four subtypes of G protein-coupled receptors including the GRPR, NMBR, the amphibian BB_4, and the orphan receptor, BRS-3 [1–5]. Activation of these receptors mediates a wide spectrum of biological activities including important changes in the central nervous system including satiety [6], control of circadian rhythm [7], thermoregulation [8], and in peripheral tissues including stimulation of gastrointestinal hormone release, activation of macrophages, and effects on development [9–11]. Bn-related peptides also have potent growth effects causing proliferation of both normal cells and various tumor cell lines [9,10,12].

All of the bombesin receptor family shared a high degree of homology (~50% amino acid identity) [1,3–5,13]. However, they have a high degree of selectivity for various

Abbreviations: Bn, bombesin; BRS-3, bombesin receptor subtype 3; BB_4, bombesin receptor subtype 4; BSA, bovine serum albumin fraction V; DMEM, Dulbecco's minimum essential medium; DTT, dithiothreitol; EC, extracellular; FBS, fetal bovine serum; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor; NMB, neuromedin B; NMBR, neuromedin B receptor; PBS, phosphate-buffered saline; TM, transmembrane

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members of the Bn peptide family [5,9,14–20]. For example, the mammalian peptide, gastrin-releasing peptide (GRP), has a 50–310-fold higher affinity for the GRP than the NMB receptor and a 1000-fold higher affinity for the mammalian GRP receptor than the mammalian orphan receptor, BRS-3 [3,14,15,17,21]. In contrast to adrenergic and muscarinic cholinergic receptors, but similar to many other gastrointestinal (GI) hormones/neurotransmitters, little is known about the molecular basis for this selectivity. An understanding of this selectivity could be useful for not only developing more selective ligands for these receptors, both agonists and antagonists, but also in providing insights into the receptor domains that are responsible for high affinity interaction.

Numerous approaches have been used to attempt to define the important amino acids accounting for the specificity of various G protein-coupled receptors for a given ligand [21–24] and to a limited extent to the bombesin receptors, including formation of chimeric receptors from different receptor domains [21–23] and site-directed mutagenesis studies [11,21,22]. These studies have demonstrated the importance of the extracellular (EC) receptor domains and upper transmembrane (TM) regions of bombesin receptors for agonist and peptide antagonist selectivity and high affinity interaction, and have provided limited information on the specific receptor amino acid differences responsible for the ligand's selectivity [11,21,22,25]. Another methodology recently reported to be useful for identifying receptor key amino acids involved in determining ligand selectivity for closely related receptors involves analyzing amino similarities and differences from the comparative alignment of amino acids from hydropathy plots of different members of a receptor family and correlation with the ligand affinity for a given receptor, to develop criteria that allow the possible identification of specific amino acids responsible for a given ligand selectivity [26–28]. In the present study, we have used this approach coupled with the recently defined affinity of GRP for the different members of the bombesin receptor family [14–17,29,30] to examine the molecular basis for the selectivity and high affinity interaction of GRP with the GRP and BB_4 receptors compared with the BRS-3 receptor.

2. Materials and methods

2.1. Materials

The mammalian expression vector, pcDNA3 and pcDNA3.1, custom primers, restriction endonucleases (*Bam*HI, *Hin*dIII, *Xba*I, and *Eco*RI), penicillin–streptomycin, LipofectAMINE, PLUS Reagent, OPTI-MEM I Reduced-Serum Medium and GENETICIN selective antibiotic (G418 Sulfate) were from Invitrogen. QuikChange Site-Directed Mutagenesis Kit, ExSite PCR-Based Site-Directed Mutagenesis Kit, and QuikChange Multi Site-Directed Mutagenesis Kit were from Stratagene. Dulbecco's minimum essential medium (DMEM), phosphate-buffered saline (PBS), fetal bovine serum (FBS) and trypsin/versene solution were from Biosource International. Balb 3T3 cells (mouse embryo) were from American Type Culture Collection. CHOP cells (Polyoma large T antigen-expressing Chinese hamster ovary cells) were a gift from James W. Dennis (Samuel Lunenfeld Research Institute). Bombesin and gastrin-releasing peptide were from Bachem. $Na^{125}I$ (2200 Ci/mmol) was from Amersham Biosciences. 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (IODO-GEN), dithiothreitol (DTT), and Bond-Breaker TCEP solution were from Pierce Biotechnology Inc. Bovine serum albumin fraction V (BSA) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were from ICN Pharmaceutical Inc. Goat anti-rabbit IgG-horseradish peroxidase conjugate was from Santa Cruz Biotechnology, Inc. Soybean trypsin inhibitor type I-S, ANTI-FLAG M2 affinity gel, rabbit ANTI-FLAG polyclonal, affinity isolated antibody and bacitracin were from Sigma. Sodium dodecyl sulfate (SDS), 2-mercaptoethanol, protein assay dye reagent, Tris/glycine/SDS buffer (10-times concentrated) and Tris/glycine buffer (10-times concentrated) were from Bio-Rad. Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets was from Roche Diagnostics Corp. Nonidet P40 was from Fluka. Nitrocellulose membranes were from Schleicher & Schuell. All other chemicals were of the highest purity commercially available.

2.2. Methods

2.2.1. Strategy used to identify amino acids important for GRP affinity

To identify possible amino acids that might be important for high affinity for GRP a modification of the method described previously was used [26]. Briefly, the extracellular domains and transmembrane domains of all known members of the Bn family of receptors were identified using hydropathy plots [26]. Specifically, the rGRPR (GenBank accession no. P52500), hGRPR (GenBank accession no. P30550), mGRPR (GenBank accession no. P21729), rNMBR (GenBank accession no. P24053), hNMBR (GenBank accession no. P28336), mNMBR (GenBank accession no. O54799), hBRS-3 (GenBank accession no. P32247), mBRS-3 (GenBank accession no. O54798), and fBB_4 (GenBank accession no. P47751) were used. The receptor amino acids were aligned using CLUSTAL-W (URL: http://molbio.info.nih.gov/molbiolgcglite/clusta117.html). The default protein gap parameters used were: residue-specific penalties = on; hydrophilic penalties = on; hydrophobic residues = GPSNDQEKR; gap separation distance = 8; and end gap separation penalty = off. From the hydropathy plots, 14 potentially important amino acids were identified that might be important for high affinity of GRP interaction

using the following criteria: (1) the amino acid was predicted to lie either in the outer one-half of the TM helix or in an extracellular domain in the receptor area from TM2 to the carboxyl terminus of the GRP receptor. Because previous studies showed the N-terminus of the GRP is not essential for high affinity GRP binding [22], only the receptor area from TM2 to the carboxyl terminus of the GRP receptor was included in the analysis; (2) the amino acid at a given position in BRS-3 (which has a low affinity for GRP) [3,14] differed from that in a comparable position in GRP receptor or BB-4 (which have a high affinity for GRP) [1,5,16]. Using this approach, 30 amino acids were identified (3) to reduce the number of amino acids to a number that could be systematically examined alone or in combination, a third criteria was applied which was the differing amino acids did not resemble each other by having similar properties based on charge, aromatic groups or hydrophobicity. Using these criteria, 14 amino acids not previously examined in any study, were identified. In addition, four amino acids, Q121, P199, R288, and A308 reported to be important for high affinity interaction of bombesin with the GRP receptor [26], were examined. Each of these 18 amino acids was mutated singly in the GRP receptor to determine the effect on GRP affinity (i.e., loss of affinity GRP mutants). After determining each single amino acid-substituted GRP receptor mutant's affinity for GRP and the universal Bn receptor ligand, [D-Phe^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) [14], double mutants were constructed using combinations of single mutants with altered affinity. After identifying amino acid differences that were important for GRP affinity from GRP receptor loss of affinity mutants, four gains of GRP affinity mutants were made by inserting these amino acids into an hBRS-3 receptor with low affinity for GRP.

2.2.2. Construction of mutant GRP receptors and BRS-3 receptors

Both GRP receptor loss of affinity and hBRS-3 gain of affinity mutants for GRP were constructed. To construct loss of GRP affinity mutants the cDNA of the mouse GRP receptor (mGRP receptor) was used, which was identical to that described previously [22,26]. The GRP mutant receptors were constructed by using the QuikChange Site-Directed Mutagenesis Kit or the QuikChange Multi Site-Directed Mutagenesis Kit, following the manufacturer's instructions with minor modifications. Each of 18 amino acids possibly important for GRP high affinity were mutated one at a time to the comparable amino acids in hBRS-3. If the GRP mutant receptor when expressed resulted in no saturable binding of the radioligand, a sequence encoding the FLAG epitope tag (DYKDDDDK) was inserted between the first (Met) and second (Ala) amino acid residue of the GRP mutant receptor using the ExSite PCR-Based Site-Directed Mutagenesis Kit, following the manufacturer's instruction. To make hBRS-3 gain of affinity mutants for GRP, the critical amino acids identified from the study of the loss of GRP receptor mutants were substituted in the hBRS-3 receptor. The cDNA of the hBRS-3 in pCD2 was a gift from Eduardo Sainz and James F. Battey (Laboratory of Molecular Biology, NIDCD, National Institutes of Health). The hBRS-3 cDNA was inserted into pcDNA3.1 (+) at the EcoR I site and hBRS-3 mutant receptors were constructed by using the QuikChange Site-Directed Mutagenesis Kit, following the manufacturer's instructions, with minor modifications. Nucleotide sequence analysis of the entire coding region was performed using an automated DNA sequencer (Applied Biosystems Inc.).

2.2.3. Growth and maintenance of cells

Balb 3T3 cells were grown in DMEM containing 10% (v/v) FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin. CHOP cells were grown in DMEM containing 10% (v/v) FBS, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 200 μg/ml of G418. All cells were maintained at 37 °C in a 5% CO_2 atmosphere. Cells were split every 3–4 days at confluence after detaching the cells with trypsin/versene solution.

2.2.4. Cell transfections

Balb 3T3 cells were seeded in a 10-cm tissue culture dish at a density of 10^6 cells/dish and grown overnight at 37 °C in growth medium. CHOP cells, which contain no native GRP receptors, were seeded in a 10-cm tissue culture dish at a density of 2.5×10^6 cells/dish and grown overnight at 37 °C in growth medium. On the following morning, 5 μg of plasmid DNA was transfected to Balb 3T3 cells, and 2.5 μg of plasmid DNA was transfected to CHOP cells by cationic lipid-mediated method using 30 μl of LipofectAMINE reagent and 20 μl of PLUS reagent in OPTI-MEM I Reduced-Serum Medium for 3 h at 37 °C. At the end of the incubation period, the medium was replaced with each growth medium. Cells were maintained at 37 °C in a 5% CO_2 atmosphere and were used 48 h later for binding assays or immunoprecipitation and Western blotting.

2.2.5. Preparation of ^{125}I-[Tyr^4]Bn and ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)

^{125}I-[Tyr^4]Bn and ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) at a specific activity of 2200 Ci/mmol were prepared by a modification of methods described previously [14]. Briefly, 0.8 μg of IODO-GEN (in 0.01 μg/ml chloroform) was transferred to a vial, dried under a stream of nitrogen, and washed with 100 μl of KH_2PO_4 (pH 7.4). To the reaction vial, 20 μl of 0.5 M KH_2PO_4 (pH 7.4), 8 μg of peptide in 4 μl of water, and 2 mCi (20 μl) Na^{125}I were added, mixed gently, and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 μl of distilled water. In the case of ^{125}I-[Tyr^4]Bn, 300 μl of 1.5 M dithiothreitol was also added and the iodination mixture was re-incubated at 80 °C for

60 min to reduce the oxidized methionines. Radiolabeled peptides were separated using a Sep-Pak (Waters Associates) and high-pressure liquid chromatography as described previously [11,14]. Radioligands were stored with 0.5% BSA at −20 °C.

2.2.6. Whole cell radioligand binding assays

Binding studies to expressed plasmid DNAs were performed as described previously [11,25,29]. For the wild type GRP and mutant GRP receptors, ^{125}I-[Tyr^4]Bn was used as the ligand to assess affinity with all single mutations that did not show a large decrease in affinity to bombesin. Because ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) is a universal, high-affinity ligand for all bombesin receptor subtypes [14,31], it could be used to assess affinity of mutants with a large decrease in affinity for bombesin such that they did not bind ^{125}I-[Tyr^4]Bn. In contrast, binding studies for the wild type and mutant hBRS-3 receptors were examined with ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) because these receptors have a low affinity for bombesin and do not bind ^{125}I-[Tyr^4]Bn [14,26]. Briefly, 48 h after transient transfection with LipofectAMINE disaggregated transfected cells were incubated for 1 h at 21 °C in 250 μl of binding buffer containing 24.5 mM HEPES (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM KH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.01% (w/v) soybean trypsin inhibitor, 0.2% (v/v) amino acid mixture, 0.2% (w/v) BSA, and 0.05% (w/v) bacitracin with 50 pM of ^{125}I-[Tyr^4]Bn (2200 Ci/mmol) or 50 pM of ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) (2200 Ci/mmol) in the presence of the indicated concentration of unlabeled peptides. Although GRP receptor expression varying as much as 160-fold [32] has not shown not to alter receptor affinity under the binding conditions used in this study, as an added precaution to correct for any differences in ligand bound by different mutant GRP receptors, binding results with each mutant GRP receptor were compared only to results with wild type GRP receptor-containing cells binding similar amounts of ligand. The cell concentration was adjusted between 0.05 and 4 × 10^6 cells/ml for each mutant receptor so that less than 20% of the total added radioactive ligand was bound during the incubation and the results compared to cells transfected with wild type GRP receptor adjusted in concentration to bind a similar amount of ligand. After the incubation, 100 μl aliquot were added to 400 μl microfuge tubes (PGC Scientific), which contained 100 μl of binding buffer to determine the total radioactivity. The bound tracer was separated from unbound tracer by pelleting the cells through the binding buffer by centrifugation at 10,000 × *g* in a Microfuge E (Beckman) for 3 min. The supernatant was aspirated and the pelleted cells were rinsed twice with a washing buffer which contained 1% (w/v) BSA in PBS. The amount of radioactivity bound to the cells was measured in a Cobra II Gamma counter (Packard Instruments). Binding was expressed as the percentage of total radioactivity that was associated with the cell pellet. All binding values represented saturable binding (i.e., total binding minus nonsaturable binding). Nonsaturable binding was defined as the amount of binding that occurred with 1 μM Bn or 1 μM [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) in the incubation solution. Nonsaturable binding was <15% of the total binding in all experiments. Each point was measured in duplicate, and each experiment was replicated at least four times. Calculation of affinity was performed by determining the IC_{50} (the GRP concentration causing half-maximum inhibition of binding) using the curve-fitting program KaleidaGraph (Synergy Software), and the Hill coefficient (n_H) was calculated from the displacement curve using GraphPad Prism. Statistical analysis was performed with Statview version 4.02 (BrainPower, Inc.). The Student's *t*-test was used to determine the statistical significance of differences in affinity of each GRP receptor mutant compared with its own wild type GRP receptor control, binding similar amounts of radioligand.

2.2.7. Preparation of cell membrane fractions from transiently transfected CHOP cells

Forty-eight hours after CHOP cells were transiently transfected with GRP receptors using LipofectAMINE as described under "Cell Transfection", preparation of cell membrane fractions was carried out according to the procedure published previously [33] with minor modifications. The cells were scraped from dishes, washed twice in 1 ml of PBS at 4 °C in a 1.5 ml centrifuge tube and resuspended in 1 ml of chilled membrane buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% (w/v) NaN_3, 1 mM EGTA, 0.4 mM EDTA, 1 mM Bond-Breaker TCEP Solution, 3.5 mM DTT, Complete Mini, EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml of solution)]. The CHOP cells in the membrane buffer were homogenized with the use of a Polytron homogenizer (Brinkmann Instruments) for 20 s twice at power level of 8 at 4 °C. Homogenates were centrifuged first at 500 × *g* for 10 min at 4 °C to remove nuclei and debris and then for 30 min at 50,000 × *g* at 4 °C to obtain membrane and cytosol fractions. Precipitates were resuspended and washed twice with 1 ml of chilled membrane buffer, then centrifuged for 15 min at 15,000 × *g* at 4 °C. After aspirating the supernatant, precipitates were resuspended in 0.3 ml of lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 25 mM β-glycerophosphate, 1 mM sodium pyrophosphate, 1 mM EGTA, 0.4 mM EDTA, 1% (v/v) Nonidet P40, 0.5% (w/v) deoxycholic acid, 1 mM Bond-Breaker TCEP Solution, 3.5 mM DTT, and Complete Mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml of solution)] and sonicated for 5 s at 4 °C. Lysates were centrifuged at 15,000 × *g* for 15 min at 4 °C to remove insoluble substances. The supernatant was saved into the

solubilized membrane fraction. Protein concentration in the membrane fractions and cytosol fractions were measured using the Bio-Rad protein assay reagent.

2.2.8. GRP receptor immunoprecipitation and Western blotting

The volume of the membrane fraction or the cytosol fraction was adjusted to 250 μg of protein in 1 ml. Twenty-five microliters of ANTI-FLAG M2 Affinity Gel was added and incubated overnight at 4 °C. The immunoprecipitates were washed twice with PBS, 12.5 μl of 2× SDS sample buffer added, and then boiled for 5 min at 95 °C before analysis by SDS-PAGE and Western blotting. Western blotting was performed as described previously [33] with minor modifications. Briefly, immunoprecipitates were fractionated by SDS-PAGE using 4–20% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature using blotto [5% nonfat dried milk in a solution containing 50 mM Tris–HCl (pH 8.0), 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20] and were incubated for 90 min at room temperature with 1 μg/ml of polyclonal ANTI-FLAG antibody in 10 ml of blotto. After incubation with the primary antibody, membranes were washed twice for 4 min with blotto and were incubated for 45 min at room temperature with 0.4 μg/ml of anti-rabbit IgG-horseradish peroxidase conjugate in 15 ml of blotto. The membranes were finally washed twice for 4 min with blotto and twice for 4 min with washing buffer [50 mM Tris–HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl, 0.05% (v/v) Tween 20], then were incubated with enhanced chemiluminescence detection reagents for 5 min and were exposed to Kodak BioMax MR film (Eastman Kodak Co.).

2.2.9. Modeling of the GRP receptor

The primary sequence of the GRP receptor was input into Swiss-PDB Viewer, Deep View [34] and the suggested homology modeling template 1JFP [35], an NMR-derived solution structure of bovine Rhodopsin, downloaded from the PDB archives. This receptor model contained extracellular loops that were extended and solvated and thus not blocking access to the transmembrane interior region, unlike the crystallographic structure [36]. This solution structure was found to contain several D-amino acids, and many *cis*-peptide bonds with poor side chain geometries for some residues. The structure was annealed by brief, constrained, molecular dynamics and energy minimizations to relax the bad geometrical features. Helical phi/psi constraints were added for all the helical transmembrane regions, *trans* peptide bond constraints were added to each peptide bond, all D-amino acids were inverted to the correct configuration and the structure subjected to 1 ps of dynamics at 300 K using the Kollman all atom force field [37] in SYBYL 6.9 [38]. This was followed by energy minimization. The final structure was much improved: when viewed as a Ramachandran plot of the phi/psi angles, no amino acids were in forbidden regions and no *cis*-peptide bond or D-amino acids were present. This annealed structure was used as the basis for the homology modeling of the GRP receptor. The GRP receptor was read into Deep View and aligned to the sequence of bovine rhodopsin based on a CLUSTAL-W [39] alignment of the GRP receptor family to rhodopsin. Any gaps in the highly conserved transmembrane regions were relocated to the adjacent loop regions. The loop regions were built by Swiss-Model. The third extracellular domain (second extracellular loop) is thought to be constrained by a disulfide bridge between Cys197 and Cys114. This bond was formed by slowly moving the loop into contact with Cys114 through the use of increasing distance constraints and molecular dynamics at 300 K, in which the entire molecule was held as a rigid aggregate and only the loop region was allowed to relax. Once the disulfide bridge was formed the structure was optimized by energy minimization. The putative binding site was visualized by the SiteID module in SYBYL [38]. The model was solvated with water and solvent molecules capable of defining a continuous cluster within 6 Å of the model were visualized as a surface. Amino acid residues within this interaction distance of the solvent accessible region are labeled in Fig. 7.

3. Results

3.1. Comparison of GRP's affinity for single amino acid GRP receptor mutants and the wild type GRP receptor (single amino acid loss of affinity)

Fourteen amino acids were identified from the comparative alignment of the structure of members of the bombesin receptor family as having a possible important role in determining high affinity for GRP (Fig. 1). Each of the 14 amino acids fit the criteria of: (1) occurring in the extracellular domain or upper one-half of the transmembrane region from TM2 to the carboxyl terminus of the GRP receptor; (2) differing in comparable positions in BRS-3 from the GRP and the BB_4 receptor; and (3) having different physical properties. Each of the 14 amino acids was located in the extracellular domains of the GRP receptor with two in EC_2 (K101 and R106), eight in EC_3 (D181, P184, H186, V187, K188, D189, Q192, and A198), and four in EC_4 (S293, E294, T297, and T304) (Fig. 1). In addition, four amino acids in the GRP receptor identified in a previous study [26] as being important for high affinity for bombesin, a GRP-related peptide with high affinity for GRP receptors [9,19], which were located in the upper transmembrane regions (Q121, A308) or extracellular loops (P198, R288), were also investigated to compare with the 14 new amino acids investigated alone or in combination (Fig. 1). Each of these 18 amino acids were mutated singly in the GRP receptor to the comparable amino acid in BRS-3, which has a low affinity for GRP

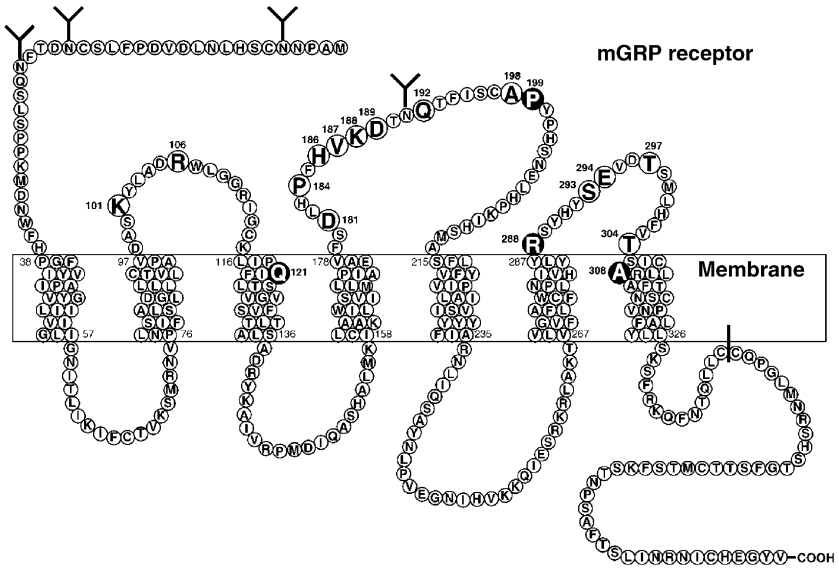

Fig. 1. Transmembrane plot of the GRP receptor showing amino acid residues examined by site-directed mutagenesis. The transmembrane areas of the mouse GRP receptor are those identified by hydropathy plots [26]. This diagram shows the extracellular side above, and the cytoplasmic side below, with the four glycosylation sites shown by the Y symbols [41]. The 14 amino acids identified as described in Section 2.2 from comparative alignment of bombesin receptors as possible having an important roles in determining high affinity for GRP and investigated in the present study are shown in the enlarged open circles. The four amino acids previously reported [26] to be important for GRP selectivity, which were also examined in the present study, are shown by the enlarged filled circles.

[3,14,20], to determine the effect on GRP affinity. Fourteen of the 18 mutant GRP receptors were found to have a significant loss of affinity for GRP (1.25- to >3000-fold) (Table 1). The relative decreases in affinity were: [Q121R], [R288H] >100-fold decrease in affinity resulting in no binding of ^{125}I-[Tyr^4]Bn > [K101H], [P199S] 11.3-fold decrease > [P184T], [H186R], [K188P], [D189N], [Q192M], [A198T], [S293Q], [E294Y], [T297P], [T304F] 1.25–2.5-fold decrease > [R106G], [V187D], [A308S] no decrease in affinity (Table 1; Fig. 2, top and bottom panels). Similar to results reported by others [26], the [Q121R] mutant GRP receptor was not able to bind either radioligand [^{125}I-[Tyr^4]Bn or ^{125}I-[D-Tyr^6,β-Ala^{11},-Phe^{13},Nle^{14}]Bn(6-14)] even though it has been shown to be expressed by Western blotting after transient transfection [26]. The [A308S] mutant GRP receptor, which is reported to have a marked decrease in affinity for bombesin [26] and this amino acid difference to therefore be important in determining the relative affinity of Bn for these receptors [26], in our study this amino acid difference was found to not be important for GRP's affinity for the GRP receptor. Furthermore, the [R288H] mutant GRP receptor was found to have a 13-fold decrease in affinity for GRP using ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14), which was less than the 100–1000-fold decrease in affinity for bombesin or GRP reported in a prior study [26]. Each of the mutant GRP receptors except the [Q121R] mutant retained high affinity for [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) with all showing either no change in affinity or less than a threefold decrease ([K101H], [P199S]), compared to wild type GRP receptor, demonstrating these mutations were selectively decreasing the affinity for GRP and not for all ligands.

3.2. Comparison of the affinities for GRP for double amino acid mutant GRP receptors (double amino acid loss of affinity)

To investigate the effects of combinations of mutations, 21 double amino acid mutant GRP receptors were constructed from the single amino acid mutant GRP receptors, which demonstrated a significant loss of affinity for GRP compared to wild type GRP receptor (Fig. 2, Table 1). The double mutants in general demonstrate Hill coefficients not significantly different from unity, similar to the wild type receptor (Table 2). Fourteen of the 21 double mutant GRP receptors were found to have a significant loss of affinity for GRP compared to the wild type GRP receptor (Table 2). The double GRP receptor mutations with the greatest effect (>10-fold decrease in affinity) were: [P199S, R288H] (>361-fold decrease), [Q192M, R288H] (42.0-fold decrease) (Table 2; Figs. 3 and 4), [P184T, P199S] (13.4-fold decrease), [Q192M, P199S] (10.9-fold decrease) (Table 2; Figs. 3 and 4), and [K101H, T297P] (10-fold decrease) (Table 2, Fig. 3). In contrast, six combinations of mutations had no effect on GRP affinity [K101H, K188P], [D184T, E294Y], [P184T, A308S], [Q192M, E294Y], [P199S, E294Y] and [T304F, A308S] (Table 2). The remaining seven GRP receptor combination mutations had an intermediate effect on decreasing GRP affinity (i.e., 1.3–5.5-fold) (Table 2). Seven of the 19 double GRP receptor mutants had a significantly lower affinity for GRP than occurred with either single mutation alone (Fig. 3). Compared to the single GRP receptor mutant with the lowest affinity, the additional significant decrease caused by a double mutation was [P199S, R288H]

Table 1
Comparison of the affinities of GRP and [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) for wild type GRP receptors and single amino acid mutant GRP receptors

Mutant	IC_{50} (nM)	
	GRP	[D-Phe^6,β-Ala^{11}, Phe^{13},Nle^{14}]Bn(6-14)
GRP receptor	2.8 ± 0.2	0.15 ± 0.17
[K101H]	31.2 ± 8.8[c]	0.47 ± 0.01[b]
[R106G]	1.4 ± 0.1	0.12 ± 0.03
[D181N]	1.5 ± 0.2	0.13 ± 0.03
[P184T]	4.2 ± 0.5[b]	0.11 ± 0.01
[H186R]	4.1 ± 0.4[a]	0.19 ± 0.02[a]
[V187D]	1.1 ± 0.1	0.15 ± 0.00
[K188P]	3.8 ± 0.4[b]	0.14 ± 0.01
[D189N]	3.7 ± 0.3[a]	0.19 ± 0.05[a]
[Q192M]	4.8 ± 0.9[b]	0.21 ± 0.05
[A198T]	5.5 ± 1.1[a]	0.12 ± 0.02
[P199S]	31.8 ± 5.5[c]	0.38 ± 0.01[a]
[S293Q]	7.1 ± 1.5[a]	0.31 ± 0.09
[E294Y]	3.9 ± 1.4[a]	0.24 ± 0.10
[T297P]	6.3 ± 0.4[a]	0.23 ± 0.01
[T304F]	3.5 ± 0.8[b]	0.26 ± 0.10
[A308S]	2.2 ± 0.1	0.12 ± 0.01

The affinities (IC_{50}) were measured by competitive displacement of 50 pM ^{125}I-[Tyr^4]Bn by GRP or [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) in whole cell radioligand binding assays as described under Section 2. Each single GRPR mutant's affinity for GRP or [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) was compared to that of cells expressing wild type GRPR, which bound similar amounts of radioligand as described in Section 2.2. Values are means ± S.E. from at least four experiments and in each experiment each point was measured in duplicate. [Q121R] was not able to bind either 50 pM ^{125}I-[Tyr^4]Bn or ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) even though it has been shown to be expressed by Western blotting [26]. [R288H] was not able to bind 50 pM ^{125}I-[Tyr^4]Bn. However, [R288H] was able to bind 50 pM ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) and its affinity for GRP was 75.2 ± 15.9 nM (Fig. 4). Abbreviations: Nle, norleucine; Bn, bombesin; GRP, gastrin-releasing peptide; β-Ala^{11}, beta-alanine in position 11.

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$ lower affinity than the wild type GRP receptor.

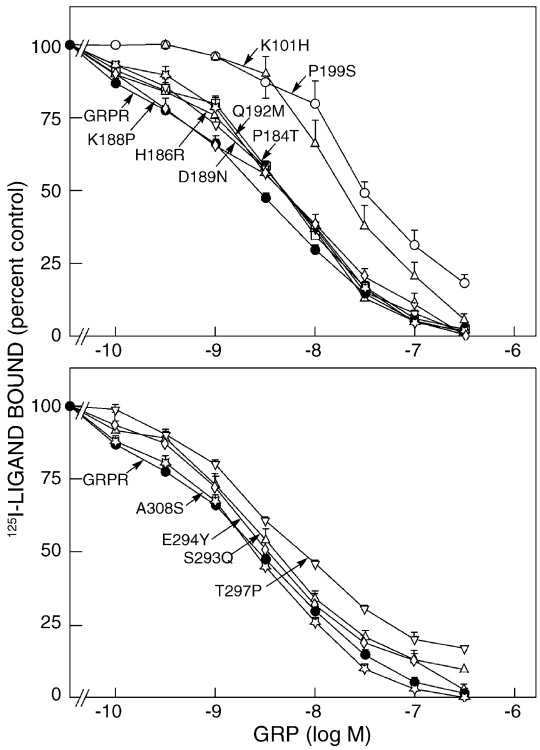

Fig. 2. Affinities of GRP for wild type mGRP receptor and selected single amino acid mutant GRP receptors. The dose-inhibition curves for GRP for the wild type GRP receptor and the 11 single amino acid mutant receptors with decreased affinity for GRP are shown. [K101H] refers to the replacement of lysine in position 101 of the GRPR (Fig. 1) by histidine from the comparable position in hBRS-3. Results with [Q121R] and [R288H] are not included because these GRP receptor mutants did not bind ^{125}I-[Tyr^4]Bn. Results with GRP mutant receptors with alterations in amino acids 101–199 are shown in the top panel, and alterations in amino acids 293–308 in the bottom panel. Each point on the dose-inhibition curve is the means ± S.E. from at least four separate experiments, and each point in the each experiment was determined in duplicate. Data are expressed as the percent of saturable binding when no unlabeled peptide was present. Data from single mutants showing a significant change from the wild type using ^{125}I-[Tyr^4]Bn (Table 1) and with the [A308S] mutant are shown because its result differ from that reported in a previous study [26].

(>34-fold decrease) > [Q192M, R288H], [K101H, T297P], [P184T, H186R], [Q192M, P199S] (2–3.2-fold decrease) > [P184T, D189N], [P184T, Q192M] (1.3-fold decrease) (Table 2, Fig. 3). One double amino acid mutant GRP receptor, [P199S, R288H], was unable to bind radioligand [^{125}I-[Tyr^4]Bn. However, it demonstrated low levels of binding with ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)] (bound only 1.2% of added counts saturably) and it had an affinity >3000 nM for [Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) and >3000 nM for GRP (Table 2, Fig. 3). To confirm that this double mutant was being expressed on the cell membrane, [P199S, R288H] expression was compared to that of the wild type GRP receptor using Western blotting to a FLAG-epitope (DYKDDDDK), which was inserted between the first (Met) and second (Ala) amino acid residue of the amino terminus mutant receptor as well as the wild type GRP receptor, as described in Section 2.2 (Fig. 5). In cytosolic fractions of cells transiently transfected with vector only, neither wild type GRP receptor nor the [P199S, R288H] mutant were detected. In contrast, in the membrane fraction three components of molecular mass, about 250, 98 and 50 kDa, were seen after transfection with the FLAG epitope-tagged wild type GRP receptor or the [P199S, R288H] mutant, but not when the vector only was used in the transfection (Fig. 5). Equal expression of the wild type and [P199S, R288H] double mutant in the membrane fraction was seen (Fig. 5). The mouse GRP receptor has been shown to have four extracellular sites glycosylated [40,41], accounting for 40 kDa of molecular weight and therefore the two major bands of 50 and 98 kDa for the wild type GRP receptor and [P199S, R288H] GRP receptor mutant are consistent with receptor expression of the fully glycosylated and unglycosylated form of equal receptor (Fig. 5).

Table 2
Comparison of the affinities of GRP and [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) for wild type (Wt) GRP receptors and double amino acid mutant GRP receptors

Mutant	GRP		[D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)
	IC_{50} (nM)	n_H	IC_{50} (nM)
^{125}I-[Tyr^4]Bn binding			
Wt GRP receptor	2.8 ± 0.2	0.94 ± 0.04	0.15 ± 0.17
[P184T, H186R]	10.2 ± 1.9[a]	0.95 ± 0.02	0.61 ± 0.20
[P184T, D189N]	5.5 ± 1.2[c]	0.95 ± 0.02	0.43 ± 0.12[b]
[P184T, Q192M]	6.5 ± 1.3[c]	1.2 ± 0.04	0.44 ± 0.15[b]
[P184T, P199S]	36.9 ± 7.8[b]	0.87 ± 0.08	0.46 ± 0.19
[P184T, E294Y]	3.5 ± 0.6	0.86 ± 0.05	0.18 ± 0.03
[P184T, A308S]	4.0 ± 0.7	0.93 ± 0.04	0.20 ± 0.03
[Q192M, E294Y]	2.5 ± 0.3	0.75 ± 0.7	0.32 ± 0.13
[E294Y, T304F]	3.7 ± 0.1[a]	0.75 ± 0.07	0.33 ± 0.09[a]
[T304F, A308S]	2.9 ± 0.6	1.03 ± 0.05	0.22 ± 0.03[b]
^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)			
Wt GRP receptor	8.3 ± 0.6	0.93 ± 0.04	0.29 ± 0.02
[K101H, P184T]	26.5 ± 1.3[d]	1.25 ± 0.08	0.21 ± 0.02
[P101H, H186R]	37.2 ± 6.3[b]	0.96 ± 0.06	0.18 ± 0.02
[K101H, K188P]	23.9 ± 5.3	0.98 ± 0.06	0.01 ± 0.00
[K101H, D189N]	33.6 ± 1.5[d]	0.13 ± 0.18	0.09 ± 0.01
[K101H, Q192M]	45.4 ± 1.8[d]	1.08 ± 0.05	0.21 ± 0.04
[K101H, E294Y]	28.9 ± 4.4[b]	0.77 ± 0.07	0.11 ± 0.01
[K101H, T297P]	82.2 ± 16.3[b]	1.17 ± 0.05	0.62 ± 0.03[d]
[K101H, T304F]	33.6 ± 2.6[c]	0.75 ± 0.03	0.14 ± 0.01
[Q192M, P199S]	90.2 ± 21.4[a]	0.90 ± 0.04	0.38 ± 0.22
[Q192M, R288H]	348.9 ± 21.6[d]	0.75 ± 0.04	0.47 ± 0.01[d]
[P199S, R288H]	>3000[d]	–	>3000[d]
[P199S, E294Y]	28.2 ± 6.7	1.08 ± 0.09	0.32 ± 0.09

Each double mutant's affinity for GRP or [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) was compared with wild type GRPR-containing cells that bound a similar amount of radioligand as described in Section 2.2. These affinities (IC_{50}) and the Hill coefficient (n_H) were measured by competitive displacement of 50 pM radioligand by GRP or [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) as described in Section 2. Values are means ± S.E. from at least four experiments and in each experiment each value was determined in duplicate. The upper half of the table shows results examined with ^{125}I-[Tyr^4]Bn. The lower half shows results with ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) because those double mutant receptors had low levels of binding or no binding with ^{125}I-[Tyr^4]Bn because of loss of affinity for bombesin. The [P199S, R288H] GRPR mutant demonstrated no binding with [^{125}I-[Tyr^4]Bn and very low levels of binding with ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14)] due to its low affinity for GRP and [Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) (i.e., >3000 nM). The abbreviations used are Nle, norleucine; Bn, bombesin; GRP, gastrin-releasing peptide.

[a] $P < 0.05$.
[b] $P < 0.02$.
[c] $P < 0.01$.
[d] $P < 0.001$ lower affinity than the wild type GRP receptor.

3.3. Comparison of the affinities for GRP of hBRS-3 mutant receptors (GRP gain of affinity hBRS-3 mutants)

To confirm the importance of the amino acids in determining GRP affinity identified in the study of loss of affinity GRP receptor mutants, the reverse study was performed by constructing hBRS-3 mutant receptors with substitution of the identified GRP receptor amino acids to attempt to obtain gain receptor affinity for GRP. To assess gain in affinity for GRP by the identified amino acids the effect of their substitution on the triple mutant, [R127Q, S205P, H294R]hBRS-3 (Δ3-hBRS-3), was determined (Fig. 6). The effect on Δ-hBRS-3 was assessed because hBRS-3 has very low affinity for GRP [3,14,26] (IC_{50} > 10 μM, Fig. 6) and the substitution of these three amino acids is reported to moderately increase the affinity for GRP to allow binding studies to be better performed [26]. We found that the triple mutant hBRS-3 receptor, Δ3-hBRS-3, had a fivefold greater affinity for GRP rather than wild type hBRS-3 (2.4 ± 0.4 μM versus 12 μM) (Fig. 6). The substitution of [H107K], [T204A], [Q299S] or [P304T] in Δ3-hBRS-3, which were the comparable substitutions to the loss of affinity GRP mutants, [K101H], [A198T], [S293Q] or [T297P], respectively, were made one at a time and their affinity for GRP assessed (Fig. 6). Each of the four quadruple mutant hBRS-3 receptors, [H107K] + Δ3-hBRS-3, [T204A] + Δ3-hBRS-3, [Q299S] + Δ3-hBRS-3, and [P304T]+ Δ3-hBRS-3, demonstrated an increase in affinity for GRP compared to the Δ3-hBRS-3 alone (Fig. 6). Specifically, [H107K] + Δ3-hBRS-3 had an eightfold increase in affinity for GRP (294 ± 23 nM) over Δ3-hBRS-3 alone (2.4 μM), [T204A] + Δ3-hBRS-3 had a 17-fold increase in affinity (138 ± 29 nM), [Q299S] + Δ3-hBRS-3 had threefold increase affinity (831 ± 110 nM), and [P304T] + Δ3-hBRS-3 had a fivefold increase in affinity (495 ± 61 nM) comparing with the Δ3-hBRS-3 (Fig. 6). In contrast, all four quadruple mutant

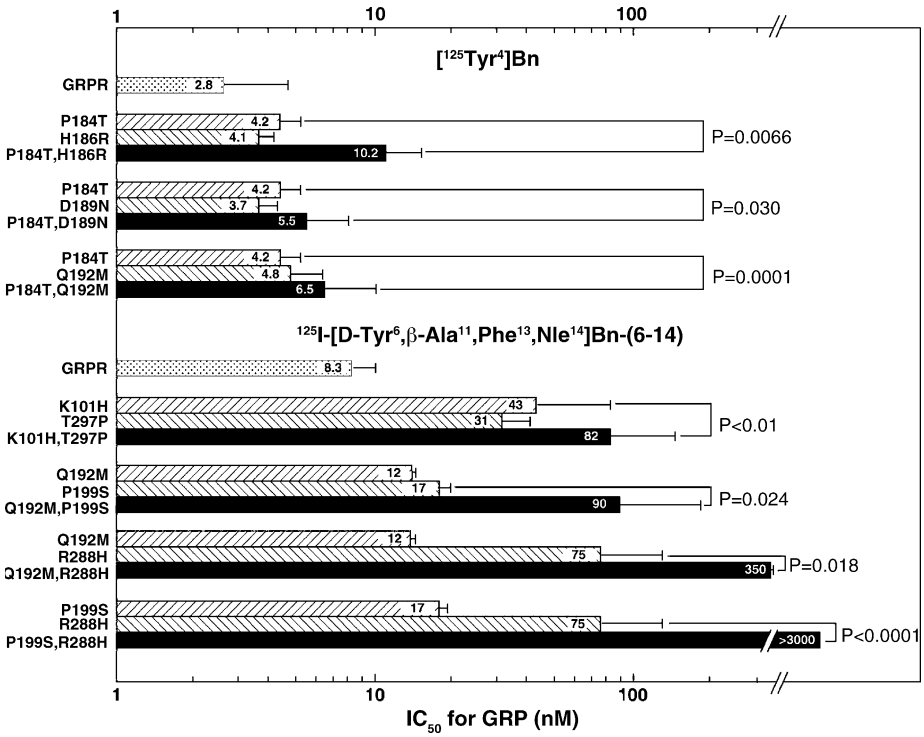

Fig. 3. Comparison of the affinities for GRP of selected double amino acid mutant GRP Receptors. Results from double mutants with a significantly greater decrease in affinity for GRP than either single mutation alone are shown. Shown are the IC_{50}s for GRP for each single and double GRP receptor mutant. Each double mutant's affinity was compared to that of the single mutant with the lowest affinity. The upper panel shows comparisons in which mutant receptors were able to be examined with ^{125}I-[Tyr^4]Bn, and the lower panel shows comparisons where ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) was used as the ligand because of low or no binding with ^{125}I-[Tyr^4]Bn due to a loss of affinity for bombesin. Significance was determined by the unpaired *t*-test. IC_{50} values are means ± S.E. from at least four separate experiments, and each number in each experiment was determined in duplicate.

hBRS-3 receptors had less than a twofold increase in affinity for the universal Bn receptor ligand, [D-Phe^6,β-Ala^{11},-Phe^{13},Nle^{14}]Bn(6-14) (data not shown). Furthermore, three of the four quadruple mutant hBRS-3 receptors ([H107K] + Δ3-hBRS-3, [Q299S] + Δ3-hBRS-3, and [P304T] + Δ3-hBRS-3) were found to be able to bind ^{125}I-[Tyr^4]Bn, which has high affinity for GRP receptors, whereas the Δ3-hBRS-3 and hBRS-3 had such low affinities for ^{125}I-[Tyr^4]Bn that no saturable binding of ^{125}I-[Tyr^4]Bn was seen (data not shown). The conclusion that the loss of affinity for GRP in the GRPR mutants and gain of affinity for GRP in the hBRS-3 mutants reflected the importance of the altered amino acid for GRP selectivity and not due to a global confirmation change in the receptor resulting in a decrease in affinity for all ligands is supported by the results of studies of the change in affinity for [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14) of the various mutant receptors. This ligand has high affinity for all members of the bombesin receptor family except for rat and mouse BRS-3 [3,14,21,42,43] and therefore has different structural determinants of binding affinity than GRP, bombesin, or NMB. We found that all GRP and hBRS-3 receptor mutants in our study retained high affinity for this ligand, demonstrating the specific mutations made were not globally altering the affinity of the mutated receptors for all ligands (Tables 1 and 2).

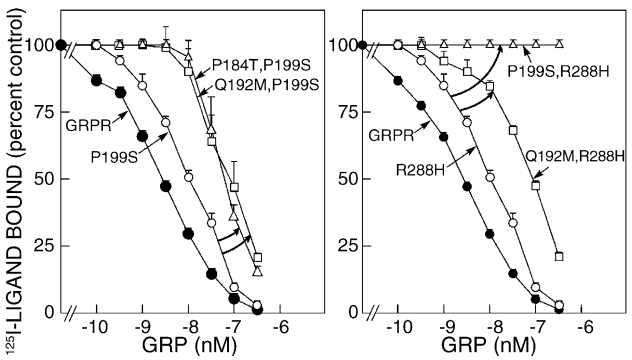

Fig. 4. Effect of mutations alone or combination at positions Q192, P199 or R288 on GRP receptor affinity. The dose-inhibition curve for GRP for each of the mutant GRP receptors was determined as described in Methods using ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14). Each point on the dose-inhibition curve is the means ± S.E. from at least four separate experiments, and each point in the each experiment was determined in duplicate. Data are expressed as the percent of saturable binding when no unlabeled peptide was present. *Arrows* indicate changes in affinity for the double mutant compared to the single mutant.

3.4. Molecular modeling of GRP receptor

To attempt to gain additional insight into why these different amino acids in the GRP receptor extracellular

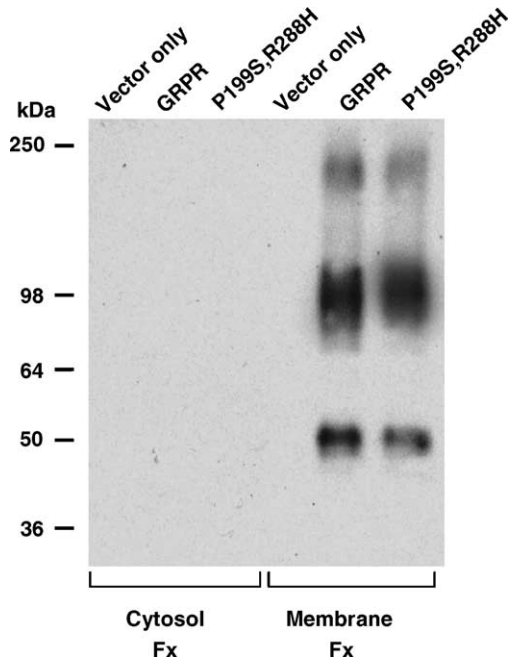

Fig. 5. Cell surface expression of the [P199S, R288H] GRP receptor compared to wild type GRP receptor. [P199S, R288H] GRP receptor expression on the cell membrane was assessed using Western blotting to a FLAG-epitope on the GRP receptor because this mutant receptor did not bind either radioligand [^{125}I-[Tyr^4]Bn or ^{125}I-[D-Tyr^6,β-Ala^{11},Phe^{13},N-le^{14}]Bn(6-14)]. The *left half* of this figure shows results with the cytosolic fractions from cells transfected with empty vector, wild type GRPR or the double mutant GRPR, which were negative in all cases. The *right half* of the figure shows results with the membrane fractions from the same cells, demonstrating that both the wild type GRP receptor and the [P199S, R288H] GRP receptor are expressed. The 98 kDa band is the fully glycosylated receptor and the 50 kDa band the unglycosylated receptor. For both the wild type and double mutant receptors, equal numbers of transfected cells (50×10^6 ml^{-1}) were used to make cell fractions.

domains identified by the criteria used in this study are important for selectivity for the GRP, three-dimensional modeling of the GRP receptor was performed (Fig. 7). This was accomplished by starting from a three-dimensional solution structure of bovine rhodopsin from NMR studies (1JFP) [35]. This receptor configuration was chosen as the basis for the three-dimensional model of the GRP receptor as it had the flexible extracellular domains exposed and was more accessible to solvation by the extracellular medium than the structures of bovine rhodopsin from X-ray crystallography in which the major loop between helix 4 and 5 is folded down into the interior of the transmembrane cavity, and interacts with the ligated rhodopsin moiety [36]. Thus, the interior of the transmembrane domain and extracellular loop regions are more accessible for interaction with a large peptide ligand such as GRP. The three-dimensional structure was generated after alignment of the GRP receptor to the three-dimensional scaffold of the conserved transmembrane regions of rhodopsin, generation of the extracellular loops and optimization by molecular dynamics and energy minimization. The three-dimensional structure was then solvated to determine the solvent-accessible regions of the receptor, which may be involved in potential interactions with a ligand (Fig. 7). The model revealed the presence of a large solvent-accessible cavity within the transmembrane domain/extracellular domain interface, with potential interactions between a ligand and the interior-located transmembrane moieties and the flexible extracellular domains (orange area, Fig. 7). In our model seven of the amino acids studied had their backbone residues inverted toward the binding pocket, and 8 of the 14 amino acids examined in our study (H186, V187, K188, D189, A198, S293, E294, and T304) (yellow, green, amino acids in Fig. 7) were found to be within 6 Å of the proposed binding pocket as well as two amino acids (Q121 and R288) identified in a previous study [26] as being important for GRP selectivity using a similar approach to that used in our study. Alteration of four (Q121, A198, S293, and R288) (yellow, amino acids, Fig. 7) of the 10 amino acids studied within 6 Å of the binding pocket caused the most significant decrease in affinity when present alone (3–3000-fold), and two additional members of this group (H186 and D189) caused a greater than additive decrease in affinity when added to another mutation. In contrast, six amino acids examined in the present study (K101, R106, D181, P184, Q192, and T297) (orange and blue amino acids in Fig. 7), and one reported in an earlier study (P199) [26] to be important for GRP affinity which were identified by a similar experimental approach, were not within 6 Å of the binding pocket. However, alterations in three of these (K101, P199, and T297) resulted in a >2-fold decrease in affinity for the GRP receptor. At present our model does not account for the change in affinity caused by altering these three amino acids because they are beyond 6 Å of the binding pocket and would not be assumed to easily interact with GRP. In a previous study [26], it was proposed that alteration of P199 in the GRPR might decrease GRP affinity because of its proximity to a critical Cys^{114}–Cys^{197} disulfide bridge between the EC_2 and EC_3 domains (Fig. 7). Residues occurring within the conserved transmembrane region can reasonably be expected to have little conformational flexibility since the helical nature of the region is fixed and so only rotations of the side-chains are possible. For residues occurring in the middle of the loop regions the range of conformational space available to each is considerable as the loop regions are flexible and dynamic. These residues have the most flexibility in terms of possible interactions with a ligand. The residues that occur at the interface between the loops and the helical domains and near to the disulfide bridge have an intermediate level of conformational flexibility. Therefore, it is possible that in some formations, alterations in the three amino acids (K101, P199, T297) that were beyond 6 Å of the binding pocket in our model could cause a decrease in GRP affinity, because in another conformation they were within a critical distance to interact with the ligand.

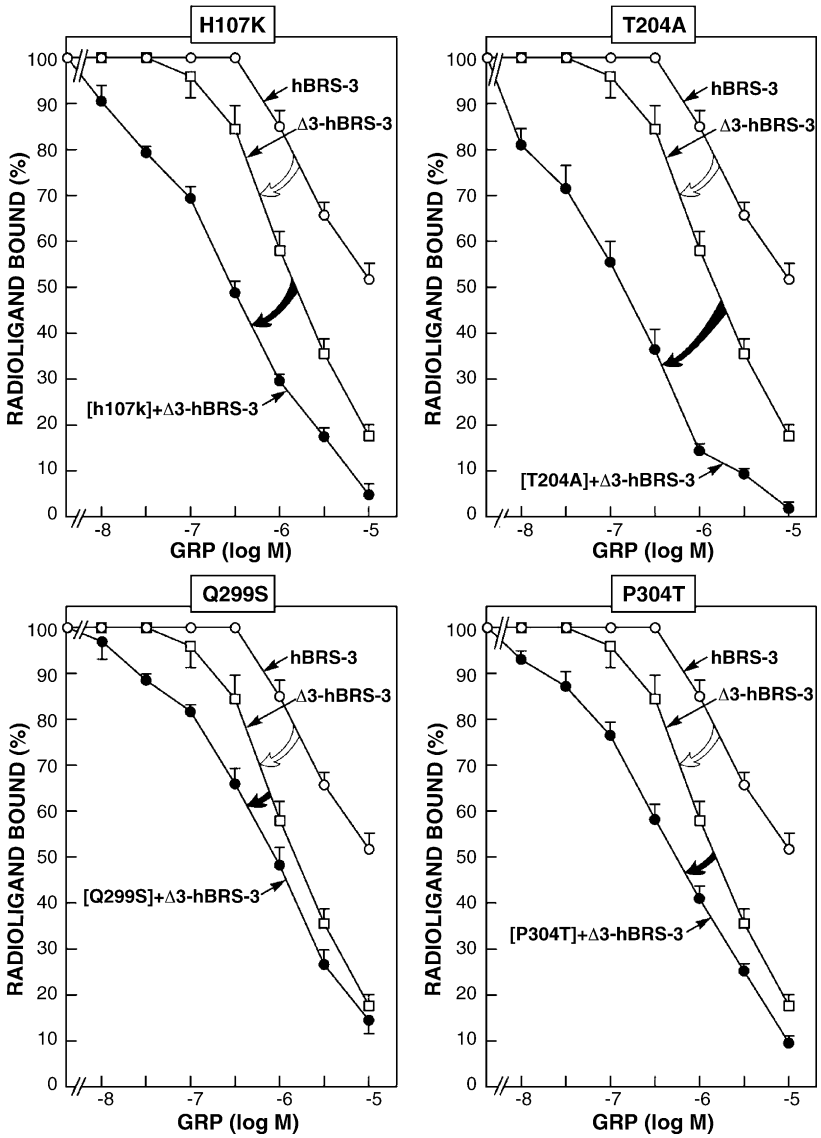

Fig. 6. Gain of affinity of GRP for BRS-3 mutant receptors. The dose-inhibition curve for GRP for each of the mutant BRS-3 receptors was determined as described in Methods using ^{125}I-[D-Tyr^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6-14). Δ3-hBRS-3 refers to [R127Q, S205P, H294R]hBRS-3, which contains three amino acids substituted from the GRP receptor into hBRS-3, which were reported previously to increase affinity for GRP to the micromolar range [26]. [H107K], [T204A], [Q299S], and [P304T] refer to the amino acid and their position in hBRS-3 that were substituted by the corresponding amino acid from the GRP receptor, that had been identified by the loss of affinity GRP mutants as being important for GRP affinity and correspond to the GRP receptor substitutions, [K101H], [A198T], [S293Q], and [T297P] respectively, shown in Fig. 2. In each panel is shown the dose-inhibition curve for GRP of wild type hBRS-3, Δ3-hBRS-3, and each of the above amino acid substitutions. Each point on the dose-inhibition curve is the means ± S.E. from at least four separate experiments, and each point in the each experiment was determined in duplicate. Data are expressed as the percent of saturable binding when no unlabeled peptide was present. *White arrows* indicate the changes in affinity for the Δ3-hBRS-3 compared to the wild type hBRS-3 and the *black arrows* indicate the additional gain in affinity for GRP of each of the quadruple mutants, compared to the Δ3-hBRS-3.

4. Discussion

The GRPR interacts preferentially with one member (i.e., GRP) of a family of closely related, natural-occurring peptide ligands [14,16,21,26]. The molecular basis for this selectivity is largely unknown. Studies of the molecular basis of ligand selectivity in various G protein-coupled receptors, including a few studies on Bn receptors, have generally used a chimeric receptor approach or site-directed mutagenesis studies [11,21–24]. A recent receptor chimeric/mutagenesis study of Bn receptors has provided some limited insights [21], suggesting the importance of Phe^{185} and Ala^{198} in the GRPR for GRP selectivity. Another approach recently reported to be useful for identifying receptor amino acid differences between closely related receptors important for ligand selectivity [26–28] is the analysis of amino acid similarities/differences revealed by alignment of members of a given receptor family compared to the affinity of each receptor for a given ligand to identify residues that might determine ligand selectivity.

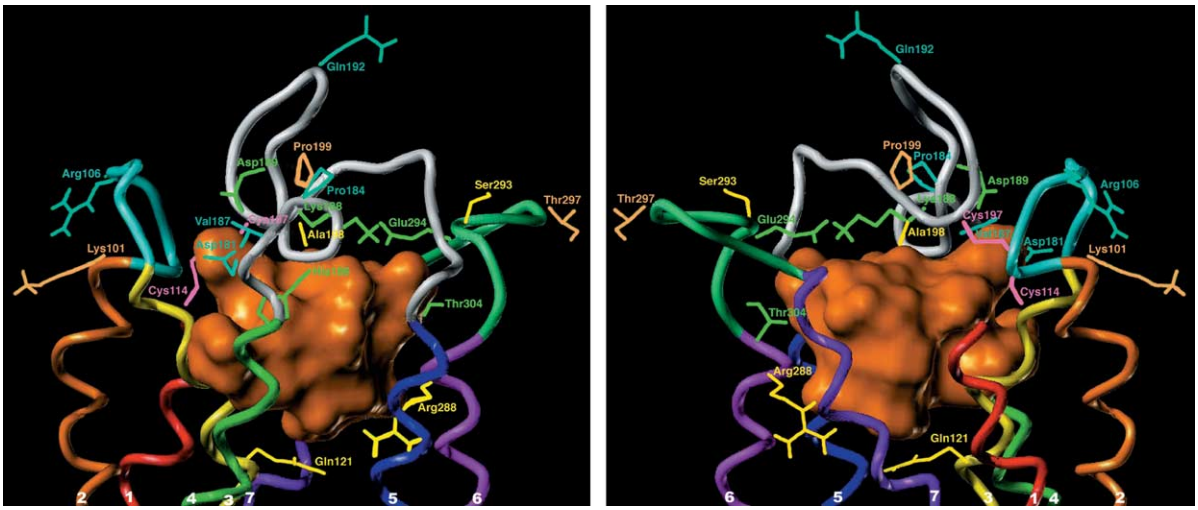

Fig. 7. Three-dimensional model of the putative binding site of GRP receptor. Two different side-views with different rotations of the three-dimensional model of the GRP receptor are shown in which the helical domains are indicated as backbone helices that are colored *red* for helix 1, *orange* for helix 2, *yellow* for helix 3, *green* for helix 4, *blue* for helix 5, *indigo* for helix 6, and *violet* for helix 7. The second extracellular domain is colored *cyan* (helix 2–3), the third extracellular domain is colored *white* (helix 4–5), and the fourth extracellular domain is colored *seagreen* (helix 6–7). The model and binding pocket were visualized by the SiteID module in SYBYL, as described in Section 2.2. The putative binding pocket is depicted as an *orange space-filled structure*. The location and identity of each amino acid included in the present study are shown. The amino acids within 6 Å of the binding pocket are shown in *yellow* and *green* and those >6 Å from the binding pocket in *orange* and *blue*. *Pink* indicates the disulfide bridge. Alteration of the four amino acids in *yellow* (Gln121, Ala198, Arg288, and Ser293) and three in *orange* (Pro199, Lys101, and Thr297) resulted in a >2-fold decrease in GRP receptor affinity, whereas alteration of the others caused either no change or a <2-fold decrease in affinity.

Using this approach [26], four amino acids (Gln^{121}, Pro^{199}, Arg^{288}, and Ala^{308}) in GRPR were reported to be important for the selectivity of bombesin for GRP, NMB and BB_4 receptors over BRS-3. A subsequent study [16] revealed that the amphibian BB_4 receptor also had a moderately high affinity for GRP compared to the BRS-3 receptor [16]. In the present study, we used this finding, combined with an analysis of amino acid similarities and differences revealed by comparative alignment of receptor structures, to develop criteria for identifying additional residues that may contribute to the selectivity of GRP for the GRPR/BB_4 receptors.

Using this approach, 14 amino acids were identified in the GRP/BB_4 receptors that might contribute to high GRP affinity. A number of our results support the conclusion that this approach and the criteria developed identified important amino acids involved in GRP selectivity. First, 11 of the mutated GRPRs demonstrated a decrease in GRP affinity. Second, a number of the amino acids, whether mutated singly or in combination, resulted in a >10-fold decrease in GRP affinity. Third, these results were confirmed by showing gain of affinity for GRP by making the reverse changes in hBRS-3. Fourth, double mutations in the GRPR formed by altering combinations of these amino acids had, in many cases, a greater effect than either one. Fifth, we confirmed that three of four amino acid differences between the GRPR and hBRS-3 receptors reported in a previous study [26] important for Bn affinity, are also important for high GRP affinity. Sixth, the approach used in the present study identified one of the key amino acids (Ala198) determined by using a chimeric/mutagenesis strategy [21] to be important for the GRPR's selectivity for GRP. Furthermore, this previous study [21] suggested amino acid differences in both the second and third extracellular domains of Bn receptors were important for selectivity/higher affinity for GRP, and the present approach identified specific amino acids in both domains important for GRP selectivity. Seventh, the conclusion that the loss of affinity for GRP in the GRP receptor mutants and gain of affinity for GRP in the hBRS-3 mutants reflected the importance of these amino acids for GRP selectivity and not done to a global conformational change in the receptor, resulting in altered affinity for all ligands is supported by the results of assessing changes in affinity of these mutants for the agonist, [D-Phe^{6},β-Ala^{11},Phe^{13},N-le^{14}]Bn(6-14). This ligand has high affinity for all members of the bombesin receptor family except for rat and mouse BRS-3 [3,14,15,17,21,42,43] and therefore, has different structural determinants of binding affinity than GRP, bombesin or NMB. We found that all of the new, single GRP and hBRS-3 receptor mutants examined in our study retained high affinity for this ligand, demonstrating the receptor mutations were not causing global changes in the receptor conformation, altering the affinity of the mutant receptor for all ligands, and therefore the ligand affinity changes represented a specific change for the binding of the ligand investigated.

Our results have both similarities and differences from studies of the molecular basis of affinity/selectivity of various gastrointestinal peptide hormones/neurotransmitters for other G protein-coupled receptors. Each of the 11 amino acids identified that was important for GRP

selectivity was in GRPR extracellular domains, as were two (P199 and R288) of three amino acids reported in a previous study using a similar approach [26] and confirmed in our study. One (Q121) of the two amino acids occurred in the upper TM domains (Q121 and A308), reported in a previous study [26] to be important for the GRPR selectivity for GRP was confirmed in the present study to also be important for GRP selectivity. These results are consistent with other studies that demonstrate both amino acids in the extracellular domains and in the TM region can be important in determining agonist peptide ligand selectivity/affinity. Extracellular receptor domains are important for determining the selectivity of secretin for the secretin receptor [44], substance P for the NK1 receptor [45] and gastrin for the CCK_B receptor [46]. However, high affinity/selectivity for endothelin by the endothelin type A receptor [47], bradykinin by the B_2 bradykinin receptor [48], and ET-3 by the endothelin type B receptor [49], are primarily determined by the TM regions. These results are also consistent with recent studies of the selectivity of the NMBR for NMB, which demonstrate receptor differences both in the upper TM5 [22] and in amino acids in extracellular domains [27], are the most important for NMB selectivity. These latter results show that even with closely related receptors such as the GRPR and NMBR, which share 56% homology [4], the receptor domains responsible for selectivity for their preferred agonist ligand can be very different.

We found four amino acid differences in the GRP/BB_4 receptor and BRS-3 receptors (K101, A198, S293, and T294) had the greatest effect on GRP selectivity. Of these four differences, the lysine101 in the second extracellular domain (EC_2) of GRPR instead of histidine in BRS-3, had the greatest effect. This result is similar to the importance of lysine173 in the EC_2 domain for secretin's affinity for the secretin receptor [50], lysine174 in the EC_3 of the bradykinin B_2 receptor for bradykinin affinity [51], and lysine195 in the EC_2 domain of the VPAC-1 receptor in VIP affinity [52]. Moreover, some studies show the importance of lysine in the upper TM for determining gastrointestinal peptide/neurotransmitter agonist affinity for its receptor [i.e., GLP-1 receptor [53], histamine H1 receptor [54], and angiotensin receptor [55]]. In the secretin receptor [50], an interaction of lysine173 of the receptor with asparatic acid of secretin was proposed, whereas in the VPAC-1 [56] the lysine195 in the EC_2 domain was proposed to participate in the formation of an electrophilic pocket that recognizes the negatively-charged Asp^3 side-chain of VIP with which it interacts by strong hydrogen bonding (e.g., dipole–dipole or ion–dipole interactions) [56]. In GRP, there are no asparatic/glutamic acids and it is unclear which amino acids in GRP interact with the lysine in GRPR. In our study, when the lysine101 of the GRPR was replaced by histidine, which is in the comparable position in BRS-3, a loss of affinity for GRP was seen, suggesting that the presence of a basic group in the 101 position of the GRPR may be less important than the ability of the amino acid in this position to have strong hydrogen bonding to the peptide. A second important difference we found that contributed to the high affinity of the GRPR/BB_4 receptors for GRP was the presence of an alanine in the EC_3 domain of the GRP/BB_4 receptors rather than a threonine in the comparable position in hBRS-3 or isoleucine in the NMB receptor. In a recent study [21], using a chimeric/site-directed mutagenesis approach, the mutation of alanine198 in GRPR to isoleucine, which occurs in this position in the NMBR [21], caused a decrease in affinity for GRP, whereas an A198G mutation in the GRPR caused no change. These results suggested that the size of the amino acid backbone substitution in position 198 was important for GRP affinity, possibly due to steric effects or differences in hydrophobicity [21]. A similar result may explain the decrease in affinity for GRP by the GRPR with the substitution of alanine by threonine in the present study. Lastly, the presence of a serine or threonine at positions 293 and 297, respectively, in the EC_4 domain of the GRPR instead of a glutamine or proline in hBRS-3, had a moderate effect on GRP affinity. The presence of a serine in the CCK_B receptor rather than the threonine in the CCK_A receptor in the upper TM3 (position S131) has been shown to be important for gastrin affinity [57] and the substitution of an alanine for serine in the B_2 bradykinin receptor to reduce affinity for bradykinin [58]. A threonine in TM6 of the bradykinin B_2 receptor is required for high affinity interaction with the peptide antagonist, D-Arg-[Hys^3,D-Phe^7] BK[NP (567)] [48] and two threonines residues in the TM5 of the M3 muscarinic receptor are critical for high affinity interaction with agonists (acetylcholine, carbachol) [59]. In these latter two studies [48,59] it was proposed that the mechanism of the enhanced affinity due to the threonine was by enhancing hydrogen bonding to the ligand. A recent study [25] has demonstrated that alteration of [T297] in the fourth extracellular domain of GRPR to a proline, which exists in this position in the hBRS-3 receptor, results in loss of affinity for the selective statine peptide antagonist, JMV594, but not for the selective pseudopeptide antagonist, JMV641, demonstrating that JMV594, but not JMV641, share the importance of this amino acid difference between the GRPR and the hBRS-3 for their selectivity. At present, whether steric factors, hydrophobicity or hydrogen bonding account for the effect of differences in position 293 and 297 of GRP receptor from the hBRS-3 receptor in their difference in affinity for GRP, is unclear.

Our modeling results support the conclusion that the differences in a number of the amino acids identified could be determinants of GRP affinity because they demonstrate that a number of the important amino acids (Q121, A198, S293, R288) for high affinity for GRP in the GRPR, are within 6 Å of the binding pocket and could therefore interact with GRP. Furthermore, the side-chains of a number of these important amino acids are oriented inward

toward the proposed binding pocket, which increases the probability they can interact with the ligand. The results from this study demonstrate that the use of analysis of amino acid homologies between Bn receptor family members in comparison to the different affinities for a selective ligand, GRP, can identify important amino acids needed for determining GRP affinity. Our results complement those from receptor chimera/mutagenesis studies by identifying which amino acid differences in the extracellular domains established by that approach are important for GRP affinity.

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