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Molecular Basis of the Selectivity of Gastrin-Releasing Peptide Receptor for Gastrin-Releasing Peptide

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ABSTRACT

The mammalian bombesin peptides [gastrin-releasing peptide (GRP) and neuromedin B (NMB)] are important in numerous biological and pathological processes. These effects are mediated by the heptahelical GRP receptor (GRPR) and NMB receptor (NMBR). GRP has high affinity for GRPR and lower affinity for NMBR. Almost nothing is known about the molecular basis for the selectivity of GRP. To address this question, we first studied four loss-of-affinity GRPR chimeric receptors formed by exchanging the four extracellular (EC) domains of GRPR with the corresponding NMBR EC domains. Receptors were transiently expressed, and affinities were determined by binding studies. Only substitution of the third EC domain (EC3) of GRPR markedly decreased GRP affinity. In the reverse study using gain-of-affinity NMBR chimeras, only replacement of EC3 of NMBR markedly increased GRP affinity. Replacing each of the 20 comparable EC3 amino acids that differed in the NMBR in GRPR showed that two separate NMBR substitutions in the GRPR, Ile for Phe^{185} or Ile for Ala^{198}, markedly decreased GRP affinity. Additional point mutants demonstrated that an amino acid with an aromatic ring in position 185 of GRPR and the size of the backbone substitution in position 198 of GRPR were important for GRP selectivity. These results demonstrate that selectivity of GRP for GRPR over NMBR is primarily determined by two amino acid differences in the EC3 domains of the receptor. Our results suggest that an interaction between the aromatic ring of Phe^{185} of the GRPR with GRP is the most important for GRP selectivity.

In contrast to adrenergic and muscarinic cholinergic receptors, with many receptors for gastrointestinal (GI) hormones/neurotransmitters, little is known about the molecular basis of their agonist selectivity. Some of the most important GI hormone/neurotransmitter receptors are those mediating the actions of the mammalian bombesin (Bn)-related peptides, gastrin-releasing peptide (GRP), and neuromedin B (NMB). These peptides mediate a wide spectrum of biological activities, including stimulating the growth of both normal and neoplastic tissues (Willey et al., 1984; Rozengurt, 1988; Tache et al., 1988), smooth-muscle contraction (Tache et al., 1988), secretion (Tache et al., 1988), widespread central nervous system (CNS) effects [including thermoregulation (Brown et al., 1988), regulation of circadian rhythm (Albers et al., 1991), and satiety (McCoy and Avery, 1990)], and have potent immunologic (DelaFuente et al., 1993) and developmental (Sunday et al., 1993) effects. These numerous effects are mediated by two closely related receptors, the GRP receptor (GRPR) and NMB receptor (NMBR) (Corjay et al., 1991; Kroog et al., 1995). These two receptors are both members of the G protein-coupled receptor (GPCR) superfamily and share 56% overall amino acid sequence identity (Kroog et al., 1995). Both the GRPR and NMBR are found in the CNS and peripheral tissues, especially the alimentary tract (Tache et al., 1988; Ladenheim et al., 1992; Kroog et al., 1995; Ohki-Hamazaki, 2000). Of these two receptors, the GRPR has been studied most extensively, because it mediates many of the important effects described for mammalian Bn-related peptides, such as potent growth effects and CNS effects (e.g., thermoregulation and satiety) (Brown et al., 1988; McCoy and Avery, 1990).

For the naturally occurring agonists GRP and NMB, the GRPR is reported to have a 50- to 310-fold higher affinity for GRP than NMB, and GRP is reported to have 11- to 820-fold higher affinity for GRPR than NMBR in different species (Benya et al., 1995; Tokita et al., 2001). No studies have yet investigated the molecular basis for the selectivity of the GRPR over the NMBR for GRP. Therefore, the molecular basis of the selectivity of this receptor is completely unknown. An understanding of this selectivity could provide not

ABBREVIATIONS: GI, gastrointestinal; Bn, bombesin; GRP, gastrin-releasing peptide; NMB, neuromedin B; CNS, central nervous system; GRPR, gastrin-releasing peptide receptor; NMBR, neuromedin B receptor; GPCR, G protein-coupled receptor; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's Medium; BSA, bovine serum albumin; TFA, trifluoroacetic acid; Nle, norleucine; EC, extracellular; ET, endothelin; L-365,260, (3*R*)-(+)-*N*-[2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-*N*-[3-methylphenyl] urea.

only insights that could be useful for developing more selective synthetic ligands for the GRPR but also insights into the important receptor domains that are required for high-affinity ligand interaction.

To address this question in the present study, we have examined the molecular basis for the selectivity and high-affinity interaction of GRP with the GRPR compared with the NMBR. To gain insight into the receptor domains responsible for this GRPR selectivity, we first made loss-of-affinity GRPR and gain-of-affinity NMBR chimeric receptors, which have proven useful in elucidating the structural basis of GPCR interaction with other receptors and ligands (Fathi et al., 1993; Tokita et al., 2001). A site-directed mutagenesis approach was then used to identify critical amino acid(s) within these domains. Here, we report that GRP selectivity for the GRPR over the NMBR depends primarily on differences in the amino acids in the third extracellular domains of these two receptors. Site-directed mutagenesis studies demonstrate that the presence of Phe^{185} in GRPR instead of Ile in NMBR and Ala^{198} in GRPR instead of Ile in NMBR in this domain are the critical differences responsible for high affinity and selectivity for GRP. Additional site-directed mutagenesis at these sites suggested that the presence or absence of π-π or cation-π interactions and steric factors introduced by the presence or absence of these two amino acids were important factors contributing to the receptor selectivity for GRP.

Experimental Procedures

Materials. pcDNA3 was from Invitrogen (Carlsbad, CA). Oligonucleotides were from Midland Certified Reagent Co. (Midland, TX) and Invitrogen. Seamless cloning kit and QuikChange site-directed mutagenesis kit were from Stratagene (La Jolla, CA). Restriction endonucleases (*Hin*dIII, *Xba*I, and *Eco*RI), fetal bovine serum (FBS), penicillin-streptomycin, LipofectAMINE and LipofectAMINE PLUS reagents, and trypsin-EDTA (0.05% trypsin and 0.53 mM EDTA-4Na) were from Invitrogen. Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate-buffered saline were from Biofluids, Inc. (Rockville, MD). BALB 3T3 cells were from American Type Culture Collection (Manassas, VA). Tissue culture dishes (100 × 20-mm; Falcon 3003) were from BD Biosciences (Plymouth, England). Bn, GRP, and NMB were from Peninsula Laboratories, Inc. (Belmont, CA). $Na^{125}I$ (2200 Ci/mmol) was from Amersham Biosciences (Piscataway, NJ). IODO-GEN (1,3,4,6-tetrachloro-3′,6′-diphenylglycouril) and dithiothreitol were from Pierce Chemical Co. (Rockford, IL). Bovine serum albumin (BSA) fraction V and HEPES were from ICN Pharmaceuticals Biochemicals Division (Aurora, OH). Soybean trypsin inhibitor type I-S and bacitracin were from Sigma-Aldrich (St. Louis, MO). Nyosil M20 oil was from Nye Lubricants Inc. (New Bedford, MA). All other chemicals were of the highest purity commercially available.

Construction of Chimeric and Mutant Receptors. The cDNAs of the GRPR and NMBR were identical to those described previously (Tokita et al., 2001). The cDNA of the wild-type mouse GRPR was cloned between the *Hin*dIII site and *Xba*I site of pcDNA3, and the wild-type rat NMBR was cloned into the *Eco*RI site of pcDNA3. The GRPR/NMBR chimeras were constructed using the Seamless cloning kit as described previously (Tokita et al., 2001) using results from hydropathy plots of the GRPR and NMBR. Mutant receptors were made by using the QuikChange site-directed mutagenesis kit, following the instructions of the manufacturer except that the annealing temperature was 60°C and the *Dpn*I digestion was for 2 h. Nucleotide sequence analyses of the entire coding region was performed using an automated DNA sequencer (ABI Prism 377 DNA sequencer; Applied Biosystems Inc., Foster City, CA).

Cell Transfection. 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 DMEM supplemented with 10% (v/v) FBS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. The following morning, cells were transfected with 5 μg of plasmid DNA by a cationic lipid-mediated method (Felgner et al., 1987) using 30 μl of LipofectAMINE reagent and 20 μl of LipofectAMINE PLUS Reagent in serum-free DMEM for 3 h at 37°C. At the end of the incubation period, the medium was replaced with DMEM supplemented with 10% (v/v) FBS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. Cells were maintained at 37°C with a 5% CO_2 atmosphere and were used 48 h later for binding assays.

Preparation of ^{125}I-[Tyr^4]Bn. ^{125}I-[Tyr^4]Bn at a specific activity of 2200 Ci/mmol was prepared by a modification of the methods described previously (Mantey et al., 1993). Briefly, 0.8 μg of IODO-GEN in 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 this vial was added 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) of $Na^{125}I$, mixed gently, and incubated at room temperature for 6 min. The incubation was stopped by the addition of 100 μl of distilled water and 300 μl of 1.5 M dithiothreitol. The iodination mixture was incubated at 80°C for 60 min. The reaction mixture was applied to a Sep-Pak (Waters, Milford, MA), and free ^{125}I was eluted with 5 ml of water followed by 5 ml of 0.1% (v/v) trifluoroacetic acid (TFA). The radiolabeled peptides were eluted with 200 μl of sequential elutions (×10) with 60% acetonitrile in 0.1% TFA. The two or three fractions with the highest radioactivity were combined and purified on a reverse-phase, high-performance liquid chromatography with a μBondaPak column (0.46 × 25-cm; Waters). The column was eluted with a linear gradient of acetonitrile in 0.1% TFA (v/v) from 16 to 60% acetonitrile in 60 min. One-milliliter fractions were collected and checked for radioactivity and receptor binding. The pH of the fractions were adjusted to 7 using 0.2 M Tris, pH 9.5, and radioligands were stored in aliquots with 0.5% BSA at −20°C.

Whole-Cell Radioligand-Binding Assays. Competitive binding assays were performed 48 h after transfection. Disaggregated transiently transfected cells were incubated for 1 h at room temperature in 250 μl of binding buffer, pH 7.4, with 50 pM ^{125}I-[Tyr^4]Bn (2200 Ci/mmol) in the presence of the indicated concentration of unlabeled peptides. The binding buffer contained 98 mM NaCl, 6 mM KCl, 11.5 mM glucose, 5 mM fumarate, 5 mM glutamate, 5 mM pyruvate, 24.5 mM HEPES, 0.2% (v/v) essential amino acid solution, 2.5 mM KH_2PO_4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2% (w/v) BSA, 0.05% (w/v) bacitracin, and 0.01% (w/v) soybean trypsin inhibitor. The cell concentration was adjusted to 0.2 to 5.5 × 10^6 cells/ml to assure that no more than 20% of the total added radioactive ligand was bound. Bound tracer was then separated from unbound ligand by layering 100 μl of the binding reaction on top of an oil phase (100 μl of Nyosil M20) in a 0.4-ml microcentrifuge tube (PGC Scientific, Frederick, MD) and pelleting the cells through the oil by centrifugation at 10,000*g* in a Microfuge E (Beckman Coulter, Inc., Fullerton, CA) for 3 min. The supernatant was aspirated, and the pelleted cells were rinsed twice with distilled water. The amount of radioactivity bound to the cells was measured in a Cobra II γ counter (Packard BioScience, Meriden, CT). Aliquots (100-μl) of the incubation mixture were taken in duplicate to determine the total radioactivity. 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 <15% of the total binding in all experiments. Each point was measured in duplicate, and each experiment was replicated at least three times. Calculation of IC_{50} values was performed with a curve-fitting program, KaleidaGraph graphing software (Abelbeck/Synergy, Reading, PA). Changes in IC_{50} values were

compared using the Student's t test, and values that differed by $p < 0.05$ were considered significantly different.

Results

Wild-Type GRPR and NMBR. The Bn-related naturally occurring agonists Bn and GRP (Fig. 1; Table 1) had high affinity (IC_{50}, 2.7 nM) for the GRPR (Fig. 2; Table 1). Bn and GRP had 2- and 12-fold selectivity, respectively, for the GRPR over the NMBR (Fig. 2; Table 1). [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14), a synthetic Bn analog reported to have high affinity for all Bn receptors (Mantey et al., 1997; Pradhan et al., 1998), was found to have a high affinity for both the wild-type GRPR and the wild-type NMBR (IC_{50}, 0.42 and 1.6 nM, respectively) (Table 1).

Extracellular Chimeric Receptors. To investigate the selectivity of GRPR for the GRPR over the NMBR, four chimeric receptors with the extracellular domains of NMBR substituted for the comparable domains in GRPR (GRPR loss-of-affinity chimeras) (Fig. 3), and four chimeras with the extracellular domains of GRPR substituted into NMBR (NMBR gain-of-affinity chimeras) (Fig. 4) were made. The affinities of Bn for all of the GRPR and NMBR extracellular chimeras were equal to or greater than the wild-type GRPR and NMBR (Table 1). The affinities of [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) were either equal to or only minimally decreased (< 2.4-fold) for each chimeric receptor compared with the wild-type GRPR and NMBR (Table 1). The affinities of GRP for the GRPR chimeras in which the first and second extracellular domains in the GRPR were substituted with the comparable domain of NMBR showed no change compared with wild-type GRPR (Fig. 3, Table 1). However, substitution of the third and fourth extracellular domains in the GRPR with the comparable domain of the NMBR decreased the affinity for GRP by 15-fold (from 2.7 ± 0.16 to 39.7 ± 1.04 nM, $p < 0.001$) and 2.2-fold (from 2.7 ± 0.16 to 5.8 ± 0.36 nM, $p < 0.01$), respectively (Fig. 3). When the reverse study was performed by substituting in the NMBR the extracellular domain of the GRPR to attempt to increase affinity, replacement of the second and third extracellular domains increased affinity for GRP by 2.4-fold (from 30.8 ± 1.2 to 13 ± 0.65 nM, $p < 0.001$) and 8.7-fold (from 30.8 ± 1.2 to 3.5 ± 0.26 nM, $p < 0.001$), respectively (Fig. 4; Table 1). In contrast, substitution of the first or fourth extracellular domains of the NMBR by the comparable domains of the GRPR had no effect (Fig. 4; Table 1). These results demonstrated that the amino acid differences in the third extracellular domains were principally involved in determining the selectivity of GRP for GRPR over NMBR.

GRPR Third Extracellular Domain Mutants (GRPR Loss-of-Affinity Point or Group Point Mutants). To identify which amino acid(s) in the third extracellular domain of GRPR were responsible for the selectivity of GRP for the GRPR over the NMBR, the amino acid differences and identities were compared between the GRPR and NMBR in this domain (Fig. 5). In the third extracellular domain, 20 amino acid differences were present occurring at positions 181 to 190, 192, 193, 195, 196, 198, 202 to 204, 213, and 214 of GRPR, which are comparable with positions 183 to 191, 193, 194, 196, 197, 199, 203 to 205, 214, and 215 of NMBR (Fig. 5). A histidine residue is in position 186 of the GRPR, but there is no comparable position in the NMBR (Fig. 5). We divided the differences into 12 groups and made 12 GRPR point or group point mutants (Fig. 5; Table 2). Of the 12 point GRPR mutants, 10 demonstrated a decrease in affinity for GRPR (Fig. 6; Table 2) ($p < 0.05$). The decrease in affinity for GRP with eight of the point mutants [D181E, L182V, H183A, P184R, H185(−), IS195,196TA, HSN202-204QTD, and MA213,214VL] was relatively small (< 2.5-fold) (Table 2). For two mutants, however, the GRPR point mutant with Phe^{185} of the GRPR replaced by Ile^{185} from the comparable

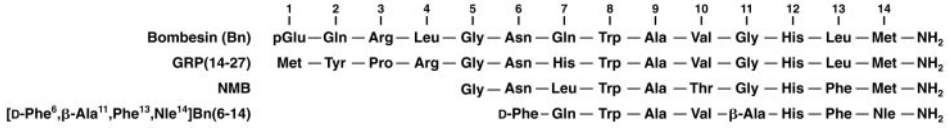

Fig. 1. Structure of the agonists, Bn, GRP(14–27), NMB, and [D-Phe^6,β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14).

TABLE 1

Affinities of three agonists for wild-type GRPR and NMBR and EC chimeric GRPRs and NMBRs.

Balb 3T3 cells were transfected with either wild-type, chimeric GRPRs, or chimeric NMBRs and incubated with 50 pM ^{125}I-[Tyr^4]Bn alone or with increasing concentrations of the indicated unlabeled three agonists. The concentration causing half-maximal inhibition of binding (IC_{50}) was determined by using the curve-fitting program KaleidaGraph. Values are mean ± S.E.M. from three different experiments and in each experiment each value was determined in duplicate. [e1-NMBR]GRPR refers to replacement of the first extracellular domain of the GRPR by that from NMBR, and so forth.

	IC_{50}		
	Bombesin	GRP	[D-Phe^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn(6–14)
	nM		
Wild-Type Receptors			
GRPR	2.7 ± 0.2	2.7 ± 0.2	0.42 ± 0.04
NMBR	5.3 ± 0.4	30.8 ± 1.2	1.6 ± 0.1
GRPR EC Chimeras (Loss of Affinity Chimeras)			
[e1-NMBR]GRPR	1.5 ± 0.04	1.5 ± 0.07	0.43 ± 0.03
[e2-NMBR]GRPR	1.6 ± 0.06	1.1 ± 0.08	1.0 ± 0.04
[e3-NMBR]GRPR	1.4 ± 0.08	39.7 ± 1.0	0.14 ± 0.004
[e4-NMBR]GRPR	1.6 ± 0.04	5.8 ± 0.4	0.28 ± 0.01
NMBR EC Chimeras (Gain of Affinity Chimeras)			
[e1-GRPR]NMBR	3.2 ± 0.08	57.5 ± 3.3	2.1 ± 0.05
[e2-GRPR]NMBR	3.0 ± 0.2	13.0 ± 0.7	3.2 ± 0.09
[e3-GRPR]NMBR	2.1 ± 0.2	3.5 ± 0.3	2.9 ± 0.1
[e4-GRPR]NMBR	2.7 ± 0.09	31.8 ± 1.3	0.75 ± 0.04

position of NMBR (Phe^{185} → Ile^{185}) and the point mutant with Ala^{198} of the GRPR replaced by Ile^{198} the decrease in affinity for GRP was as great as that seen with native NMBR or replacement of the entire third extracellular domain (Fig. 6; Table 2). These latter two mutants also had decreased affinity for Bn ($p < 0.001$); however, they retained high affinity for [D-Phe^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14) (Table 2), demonstrating that the insertion of these mutations was unlikely to be causing a global structural change in the mutant receptor. These results demonstrated that the Phe^{185} and Ala^{198} in GRPR are the key amino acid differences from NMBR responsible for determining the selectivity of GRP for GRPR over the NMBR.

GRPR Point Mutants for Phe^{185} and Ala^{198}. The importance of Phe^{185} and Ala^{198} in GRPR for high-affinity GRP interaction suggests that either an interaction between the

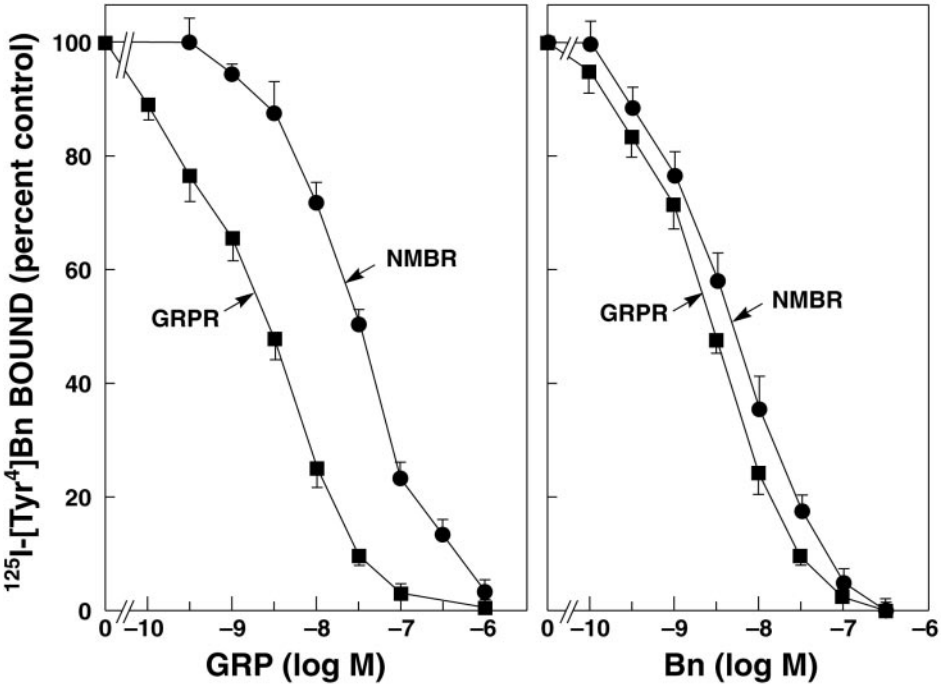

Fig. 2. Affinities of GRP and Bn for wild-type GRPR and wild-type NMBR expressed in BALB 3T3 cells. BALB 3T3 cells were transfected with either wild-type receptor as outlined under *Experimental Procedures* and were incubated with 50 pM ^{125}I-[Tyr^{4}]Bn and the indicated concentrations of peptides. Each point is the mean of three separate experiments; in each experiment, each point was determined in duplicate.

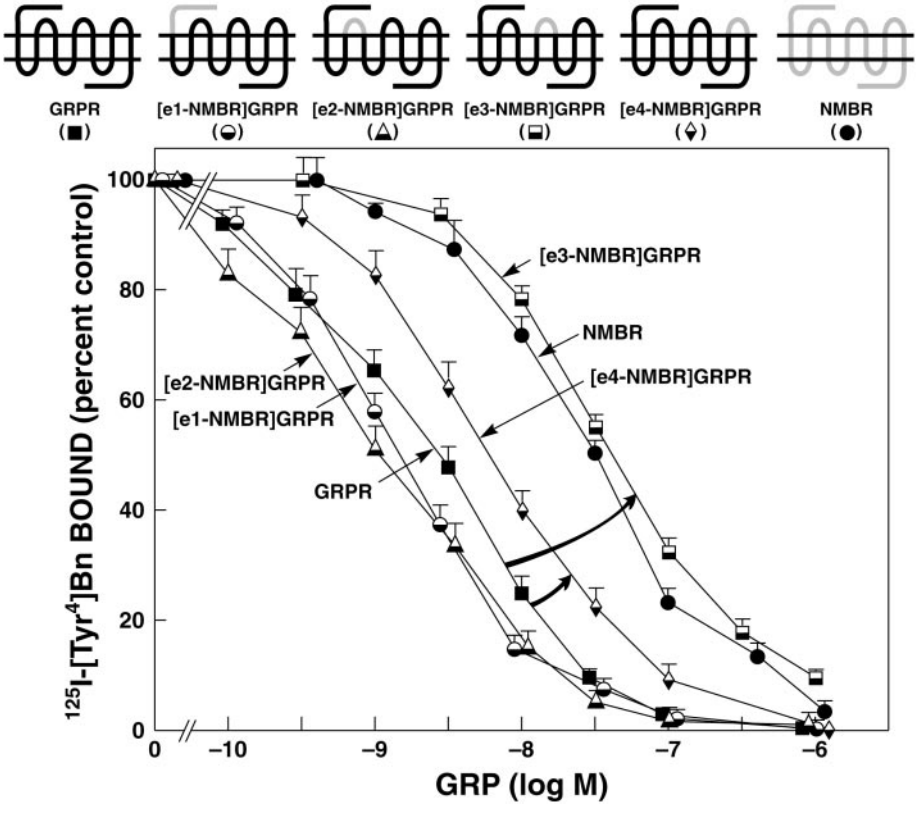

Fig. 3. Affinities of GRP for wild-type GRPR, extracellular chimeric GRPRs, and wild-type NMBR expressed in BALB 3T3 cells (GRPR loss-of-affinity chimeras). The diagrams of the chimeric receptors formed are shown at the top of the figure. The chimeric GRPRs were formed by replacing each of its extracellular loops one at a time with the comparable NMBR extracellular loop. The affinity was measured by competitive radioligand displacement of 50 pM ^{125}I-[Tyr^{4}]Bn by GRP at the concentrations shown. Each point on a curve is the mean from three separate experiments; in each experiment, each point was measured in duplicate. e1-, e2-, e3-, and e4-NMBR refer to the substitution of this extracellular loop of the NMBR for the comparable extracellular loop in GRPR. Arrows indicate large changes in affinity compared with native GRPR for the GRP in the indicated chimeric receptor.

aromatic ring of Phe^{185} with GRP is probably important or steric differences introduced by the substitution of isoleucine for Phe^{185} could be important factors mediating the differences in GRP affinity with these two amino acids. The decrease in affinity for GRP in the position 198 mutant of GRPR caused by replacing alanine with isoleucine in this position suggests that either changes in steric factors or hydrophobicity could be important determinants at this site. To explore these possibilities, additional GRPR point mutants were made by replacing Phe^{185} of GRPR with two different amino acids with aromatic rings (Tyr^{185} and Trp^{185}) or with an aliphatic backbone substitution (Ala^{185}). To provide additional insight into the basis of differences in affinities in position 198 with different replacements, we made a point mutant GRPR with Gly^{198} instead of Ala^{198} in the native GRPR. The substitutions of Tyr or Trp for Phe^{185} caused only a small decrease in affinity for GRP (<2.7-fold) ($p < 0.001$) (Fig. 7; Table 3). However, an Ala substitution for Phe^{185} decreased affinity for GRP 8.4-fold (2.7 to 22.4 nM, $p < 0.001$) (Fig. 7; Table 3). On the other hand, substitution of Gly for Ala^{198} caused no change in affinity for GRP (Fig. 8; Table 3) compared with the 16-fold decrease seen with Ile^{198} substitution.

Discussion

The GRPR interacts preferentially with one member (i.e., GRP) of a family of closely related naturally occurring peptide ligands. Despite the importance of this receptor in mediating the effects of GRP in many physiological and pathological processes (Tache et al., 1988; Bunnett, 1994), almost nothing is known about the molecular basis for the selectivity of GRP for the GRPR. One study (Akeson et al., 1997) investigated the ability of the GRPR and NMBR, but not the closely related orphan receptor BRS-3, to both interact with high affinity with the amphibian peptide Bn, which has a carboxyl terminal heptapeptide identical to that of the biologically active end of GRP (Erspamer, 1988; Tache et al., 1988; Bunnett, 1994). In that study (Akeson et al., 1997), four amino acids (Gln^{121}, Pro^{199}, Arg^{288}, and Ala^{308}) in the GRPR were required for high-affinity Bn interaction. However, these four amino acids are not determinants of the selectivity of the GRPR for GRP over the NMBR, because they are all conserved in the comparable positions in the NMBR (Akeson et al., 1997). In the present study, we examined the molecular basis of selectivity of GRPR for GRP over NMBR by using

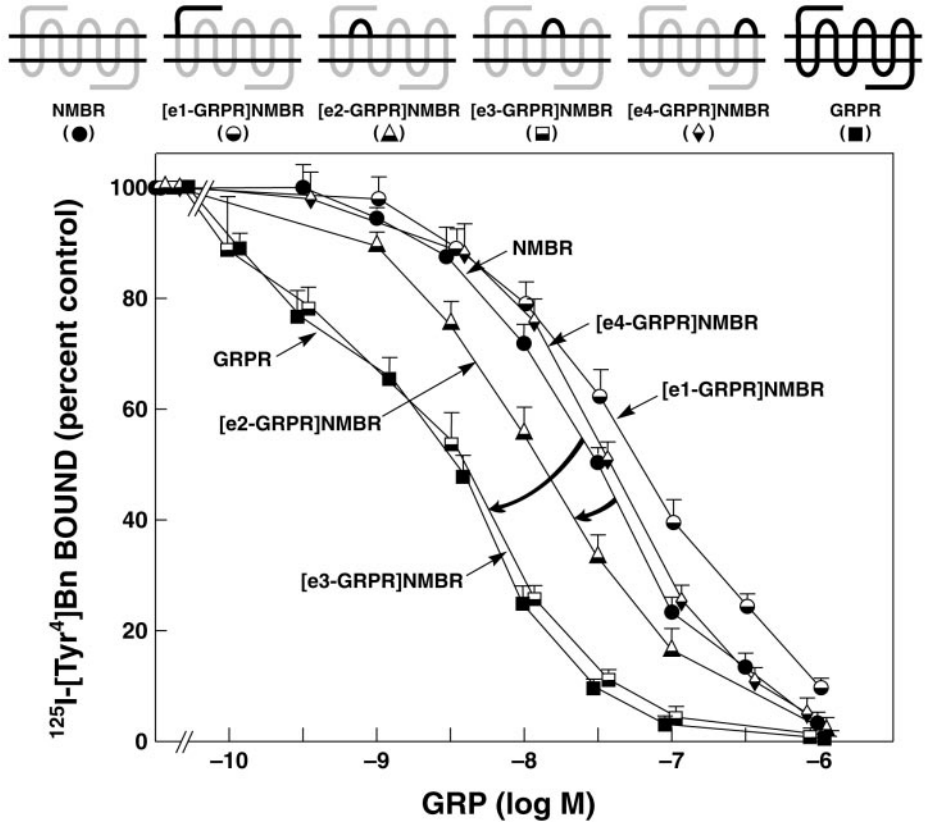

Fig. 4. Affinities of GRP for wild-type NMBR, extracellular chimeric NMBRs, and wild-type GRPR expressed in BALB 3T3 cells (NMBR gain-of-affinity chimeras). The diagrams of the chimeric receptors formed are shown at the top of the figure. The chimeric NMBRs were formed by replacing each of its extracellular loops with the comparable loop of the GRPR one at a time. The affinity was measured by competitive radioligand displacement of 50 pM ^{125}I-[Tyr^4]Bn by GRP at the concentrations shown. Each point on a curve is the mean from three separate experiments; in each experiment, each point was measured in duplicate. e1-, e2-, e3-, and e4-GRPR refer to substitution of this extracellular loop of the GRPR for the comparable extracellular loop in the NMBR. Arrows indicate large changes in affinity compared with native NMBR for GRP in the indicated chimeric receptor.

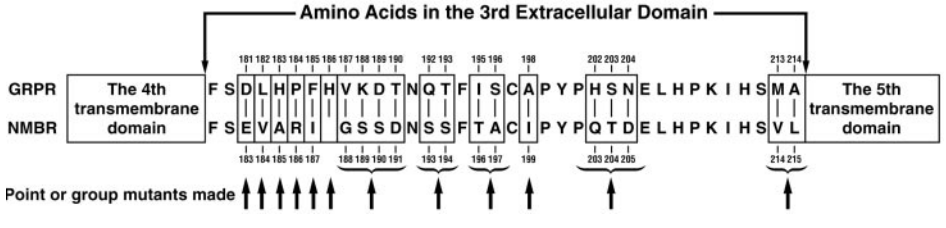

Fig. 5. Alignment of amino acid sequences in the third extracellular domain of GRPR and NMBR. Boxes indicate divergent amino acids between these two receptors in the third extracellular domain. Arrows indicate 12 point mutations or group point mutations made in GRPR by replacing them with the comparable amino acids from NMBR.

TABLE 2
Affinities of three agonists for wild-type GRPR and NMBR and third extracellular GRPR point or group mutants.

	IC_{50} ()		
	Bombesin	GRP	[D-PHE^6, β-Ala^{11}, Phe^{13}, Nle^{14}]Bn(6–14)
		nM	
GRPR	2.7 ± 0.2	2.7 ± 0.2	0.42 ± 0.04
[D181E]GRPR	2.7 ± 0.09	6.1 ± 0.2	0.50 ± 0.02
[L182V]GRPR	1.8 ± 0.09	5.7 ± 0.2	0.25 ± 0.02
[H183A]GRPR	1.6 ± 0.03	6.9 ± 0.4	0.60 ± 0.02
[P184R]GRPR	1.9 ± 0.07	5.9 ± 0.3	0.56 ± 0.04
[F185I]GRPR	71.8 ± 8.3	65.8 ± 7.6	0.75 ± 0.1
[H186(–)]GRPR	1.3 ± 0.02	6.4 ± 0.2	0.23 ± 0.02
[VKDT187–190GSSD]GRPR		2.8 ± 0.1	
[QT193,194SS]GRPR		1.9 ± 0.06	
[IS195,196TA]GRPR	1.0 ± 0.05	4.7 ± 0.3	0.68 ± 0.02
[A198I]GRPR	4.7 ± 0.4	42.8 ± 1.5	0.39 ± 0.02
[HSN202–204QTD]GRPR	1.6 ± 0.05	5.1 ± 0.1	0.43 ± 0.03
[MA213,214VL]GRPR	1.6 ± 0.1	6.3 ± 0.2	0.31 ± 0.04
NMBR	5.3 ± 0.4	30.8 ± 1.2	1.6 ± 0.1

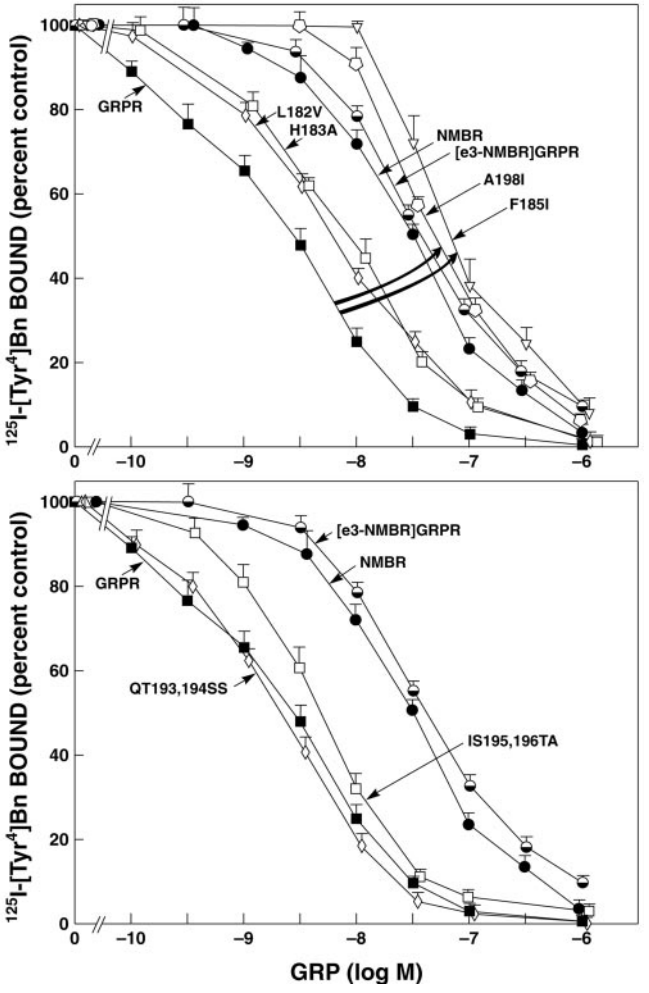

Fig. 6. Affinities of GRP for wild-type GRPR, third extracellular domain group or point mutants of GRPR, and wild-type NMBR expressed in BALB 3T3 cells (GRPR loss-of-affinity group or point mutants). The amino acid mutants of GRPR were formed by replacing the amino acid(s) of the 3rd extracellular domain of GRPR in the positions indicated with the comparable amino acid(s) of the NMBR as shown in Fig. 5. The affinity was measured by competitive radioligand displacement of 50 pM ^{125}I-[Tyr^4]Bn by GRP at the concentrations shown. Each point on a curve is the mean from three separate experiments; in each experiment, each point was measured in duplicate. 181E refers to replacement of aspartic acid in position 181 of the GRPR by glutamic acid, which exists in the comparable position in NMBR. Arrows indicate large changes in affinity compared with native GRPR for GRP in the indicated mutant receptor.

a combined chimeric approach and site-directed mutagenesis.

Our results support the conclusion that differences in the third extracellular domains of these two receptors are the most important in determining the selectivity of GRP for GRPR. When the extracellular domains of both receptors were exchanged to make either potential loss- or gain-of-affinity chimeric receptors, an analysis of both sets of chimeras supported this conclusion. Specifically, when the extracellular domains of the NMBR were substituted into the GRPR, only the substitution of the third extracellular domain decreased the affinity to the same level seen with native NMBR. When the reverse study was performed, only substitution of the third extracellular domain of GRPR in the NMBR resulted in an NMBR chimeric receptor with almost the same affinity for GRP as the wild-type GRPR.

This result has both similarities and differences from studies on the interaction of peptide agonists with other GPCRs. With several gastrointestinal hormone/neurotransmitter GPCRs, the extracellular domains of the receptor are the important receptor regions for determining selective agonist binding. Such an interaction is important for determining selectivity of gastrin for the cholecystokinin-B receptor (Silvente-Poirot and Wank, 1996), secretin for the secretin receptor (Holtmann et al., 1995), and substance P for the neurokinin 1 receptor (Fong et al., 1992b). However, for other GI peptide agonists such as endothelin-3 with the ET_B receptor (Krystek et al., 1994), ET-1 with the ET_A receptor (Breu et al., 1995), and bradykinin with the B2 bradykinin receptor (Nardone and Hogan, 1994), high-affinity interaction and selectivity are primarily determined by amino acids in the transmembrane regions of the receptor. Furthermore, with the NMBR, which shares 56% homology with the GRPR (Corjay et al., 1991; Kroog et al., 1995), the presence in the upper fifth transmembrane region of an isoleucine (Ile^{216}) (Fathi et al., 1993) rather than a serine in GRPR is critical for the high affinity and selectivity of the NMBR over the GRPR for the naturally occurring ligand, NMB. These latter results demonstrated that even with very closely related receptors, such as the GRPR and NMBR, the receptor domains responsible for selectivity for their respective naturally occurring agonist ligands can be very different.

Within the third extracellular domain there were 20 amino acid residues that differed between GRPR and NMBR. Our

results show that a phenylalanine residue in position 185 of GRPR instead of an isoleucine in NMBR and the alanine in position 198 of GRPR instead of an isoleucine in NMBR are the key differences responsible for the high selectivity of GRP for the GRPR. The decrease in affinity for GRP with the replacement of phenylalanine in position 185 of GRPR by isoleucine or alanine in position 198 by isoleucine was not caused by a global change in structure, because the receptors with these mutations retained high affinity for the synthetic GRPR agonist, [D-Phe^{6},β-Ala^{11},Phe^{13},Nle^{14}]Bn(6–14).

Phenylalanine in other GPCRs plays a critical role in determining high-affinity interaction and selectivity of peptide agonists (Fong et al., 1992a; Nardone and Hogan, 1994; Breu et al., 1995; Leeb et al., 1997; Silvente-Poirot et al., 1998). Similar to our results, a phenylalanine in the first extracellular domain of the neurokinin-1 receptor is necessary for high-affinity interaction with substance P, and a phenylalanine residue in the fourth extracellular domain of the galanin receptor is necessary for high-affinity interaction with galanin (Berthold et al., 1997). In contrast, a phenylalanine in the sixth transmembrane domain of the bradykinin 2 receptor is required for bradykinin high affinity and selectivity (Leeb et al., 1997). Phenylalanine also plays an important role for high-affinity interaction of peptide and nonpeptide antagonists with some GPCRs (Ji et al., 1994; Nardone and Hogan, 1994; Bastian et al., 2000). A phenylalanine in the seventh transmembrane domain of the bradykinin 1 receptor is required for high affinity for the peptide antagonist desArg^{10}-Leu^{9}-kallidin (Bastian et al., 2000), and a phenylalanine in the sixth extracellular domain of the bradykinin 2 receptor is critical for high-affinity interaction with the peptide antagonist NPC567 (Nardone and Hogan, 1994). With the nonpeptide AT1 angiotensin receptor antagonist losartan, a phenylalanine in the seventh transmembrane domain is required for high-affinity interaction (Ji et al., 1994). Aromatic amino acids, including phenylalanine, may interact with ligands through cation-π binding or π-π binding (Burley and Petsko, 1985; Serrano et al., 1991; Dougherty, 1996; Mecozzi et al., 1996; Gallivan and Dougherty, 1999). The cation-π or π-π binding usually occurs through the side chains of phenylalanine, tyrosine, or tryptophan (Dougherty, 1996). Our results suggest that such an interaction might be contributing to the selectivity of GRP for GRPR. In our study, the substitution of tyrosine or tryptophan for phenylalanine in position 185 in the GRPR resulted in only a small decrease in affinity for GRP compared with an alanine substitution. This result suggests that the presence of an aromatic ring in the amino acid in this position contributes to the selectivity of GRP for the GRPR. However, whether the mechanism of this effect is by a cation-π or π-π ligand-receptor interaction or some other type of interaction is unknown.

In the present study, the alanine residue at position 198 in the GRPR was also important in determining GRPR high affinity and selectivity for GRP. With other GPCRs, we were

TABLE 3

Affinities of GRP for wild-type GRPR, NMBR and GRPR position 185 or 198 point mutants.

Affinities were calculated by competitive displacement of 50 pM ^{125}I-[Tyr^{4}]Bn by GRP. Values represent the mean ± S.E.M. from at least three independent experiments. The numbers refer to the position in the GRPR of the amino acid substitutions as shown in Fig. 5.

	GRP IC50
	nM
GRPR	2.7 ± 0.2
[F185I]GRPR	65.8 ± 7.6
[F185Y]GRPR	7.3 ± 0.2
[F185W]GRPR	6.4 ± 0.2
[F185A]GRPR	22.4 ± 0.3
[A198I]GRPR	42.8 ± 1.5
[A198G]GRPR	3.0 ± 0.3
NMBR	30.8 ± 1.2

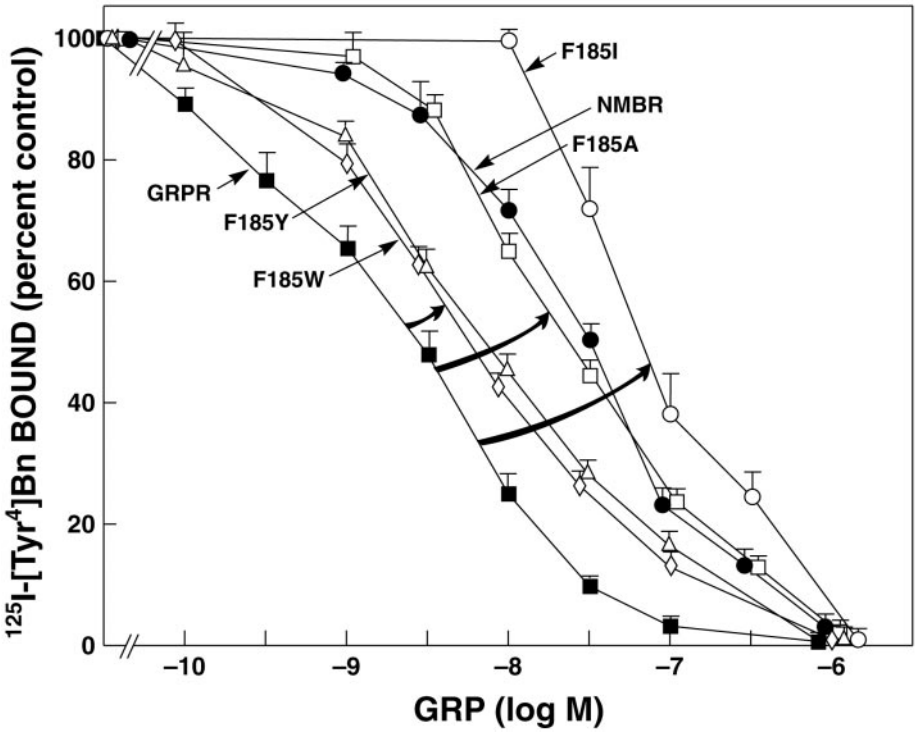

Fig. 7. Importance of the aromatic ring in position 185 of GRPR for high-affinity GRP interaction. The amino acid point mutants of GRPR were formed by replacing phenylalanine in position 185 of GRPR with isoleucine, tyrosine, tryptophan, or alanine. The affinity was measured by competitive radioligand displacement of 50 pM ^{125}I-[Tyr^{4}]Bn by GRP at the concentrations shown. Each point on a curve is the mean from three separate experiments; in each experiment, each point was measured in duplicate. F185I refers to replacement of phenylalanine in position 185 of the GRPR by isoleucine. Arrows indicate large changes in affinity compared with native GRPR for GRP in the indicated point mutant receptor.

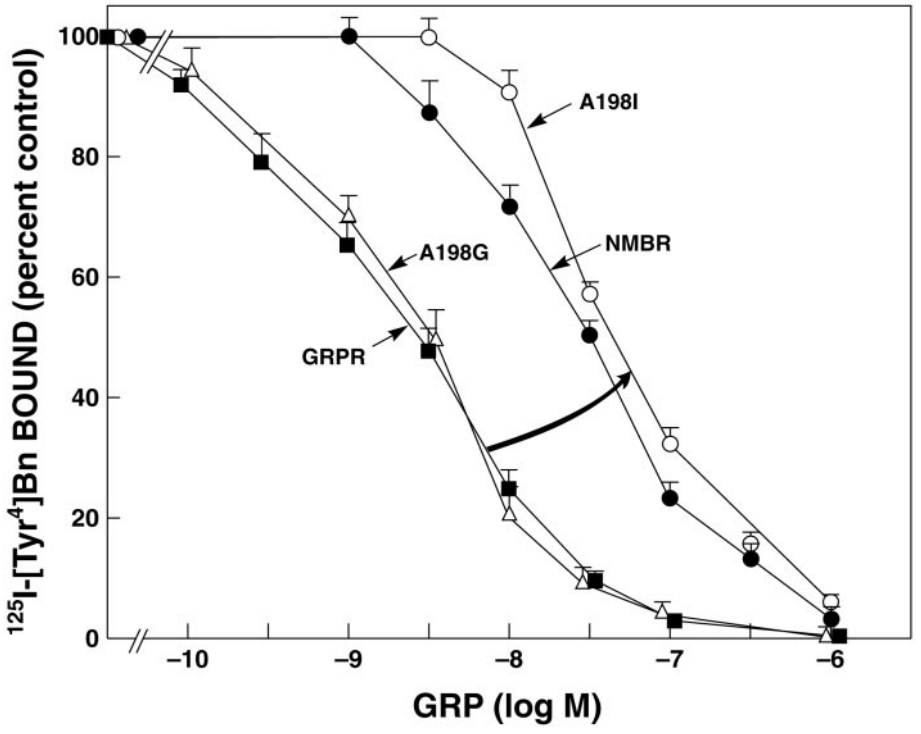

Fig. 8. Affinities of GRP for wild-type GRPR, point mutants of GRPR in position 198, and wild-type NMBR. The amino acid point mutants of GRPR were formed by replacing alanine in position 198 of GRPR by isoleucine or glycine. The affinity was measured by competitive radioligand displacement of 50 pM ^{125}I-[Tyr^4]Bn by GRP at the concentrations shown. Each point on a curve is the mean from three separate experiments; in each experiment, each point was measured in duplicate. A198I refers to replacement of alanine in position 198 of the GRPR by isoleucine. Arrows indicate large changes in affinity compared with native GRPR for the GRP in the indicated point mutant.

unable to find any other reports where an alanine residue was critical for peptide agonist high affinity or selectivity. However, the presence of an alanine in the fourth transmembrane domain of the AT1 angiotensin receptor (Ala^{163}) is necessary for high-affinity interaction with the nonpeptide antagonist losartan (Ji et al., 1994), and an alanine residue in the third transmembrane domain of the cholecystokinin-B receptor (Ala^{129}) is a critical amino acid for high-affinity interaction with nonpeptide antagonist L-365,260 (Kopin et al., 1995). The principal difference introduced by the substitution of alanine by isoleucine is in the size of the backbone substitution, possibly contributing to steric effects or differences in hydrophobicity. Our results provide some support that one of the important effects of this substitution may be a steric effect because the substitution of glycine for alanine caused no change in affinity of the GRPR for GRP compared with a decrease in affinity seen with the isoleucine substitution. However, whether a change in steric factors actually occurs was not investigated in this study and remains unproven.

To attempt to develop a model for our results, the three-dimensional structure of the GRPR receptor was modeled from the structure of bovine rhodopsin (Palczewski et al., 2000). The sequence for mouse GRPR was threaded onto the three-dimensional template of bovine rhodopsin using Swiss Pdb Viewer Deep View (http://www.expasy.ch/spdbv/) and the SYBYL modeling program (Tripos, St. Louis, MO). The model was refined by energy minimization. The backbones of the transmembrane and intracellular domains were then constrained, the extracellular domains optimized by molecular dynamics, and the disulfide bond was formed and the structure optimized further. The resulting structures were minimized to give models of the loop regions, which gave structures that were very stable during the dynamic simulation. With either implicit or explicit solvation of the extracellular domain, the domain did not undergo a large change in backbone conformation. In the implicit solvation model, the long third extracellular loop remained largely within the body of the transmembrane region. The critical residues identified by mutation studies were located within ~6 Å, with the Phe^{185} residue exposed to the extracellular environment, oriented above the Ala^{198} residue, which was oriented toward the interior of the receptor. With explicit solvation of the polar residues, the loop relaxed further into the extracellular environment, with the Phe and Ala residues further separated, but no solvent accessible binding site involving both of these critical residues could be defined in either model. The failure of the bovine rhodopsin third extracellular loop template to be a useful model for the GRPR is probably related to the fact that it is folded into the interior in rhodopsin and is in contact with the covalently bound 11-*cis*-retinal, whereas with the larger peptide ligands, GRP or NMB, it is unlikely this occurs in GRPR or NMBR. Without the three-dimensional structures of the extracellular loop and the bound peptide ligand, there is too great a degree of uncertainty in the structures of the loop derived purely from homology modeling to be of value in explaining these data.

In conclusion, our receptor chimeric gain- and loss-of-affinity studies show that the third extracellular domain of the GRPR and NMBR is the principal receptor region responsible for the selectivity of the naturally occurring peptide agonist GRP for the GRPR over the NMBR. Our mutagenesis studies show that Phe^{185} and Ala^{198} in the third extracellular domain of the GRPR, rather than isoleucine in these positions in NMBR, are the key amino acid differences in determining the GRPR selectivity of GRP. Our additional mutagenesis studies show the presence or absence of an aromatic ring on the amino acid at position 185, and possibly the size of the backbone substitution on the amino acid in position 198, were important factors caused by the amino acid changes that probably contributed to the different GRP affinities in the two receptors. The availability of this data will for the

first time allow comparisons of the molecular basis of high-affinity interaction of the numerous selective GRPR peptide antagonists (Jensen and Coy, 1991; de Castiglione and Gozzini, 1996) and the naturally occurring agonist GRP, as well as a comparison of the basis for their high selectivity for the GRPR.

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