Molecular Basis for Selectivity of High Affinity Peptide Antagonists for the Gastrin-releasing Peptide Receptor*

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Few gastrointestinal hormones/neurotransmitters have high affinity peptide receptor antagonists, and little is known about the molecular basis of their selectivity or affinity. The receptor mediating the action of the mammalian bombesin (Bn) peptide, gastrin-releasing peptide receptor (GRPR), is an exception, because numerous classes of peptide antagonists are described. To investigate the molecular basis for their high affinity for the GRPR, two classes of peptide antagonists, a statine analogue, JMV594 ([D-Phe6,Stat13]Bn(6–14)), and a pseudo-peptide analogue, JMV641 ([D-Phe-Gln-Trp-Ala-Val-Gly-His-Leu(CHOH-CH2)3(CHOH-CH2)3], were studied. Each had high affinity for the GRPR and >3,000-fold selectivity for GRPR over the closely related neuromedin B receptor (NMBR). To investigate the basis for this, we used a chimeric receptor approach to make both GRPR loss of affinity and NMBR gain of affinity chimeras and a site-directed mutagenesis approach. Chimeric or mutated receptors were transiently expressed in Balb/c 3T3. Only substitution of the fourth extracellular (EC) domain of the GRPR by the comparable NMBR domain markedly decreased the affinity for both antagonists. Substituting the fourth EC domain of NMBR into the GRPR resulted in a 300-fold gain in affinity for JMV594 and an 11-fold gain for JMV641. Each of the 11 amino acid differences between the GRPR and NMBR in this domain were exchanged. The substitution of Thr297 in GRPR by Pro from the comparable position in NMBR, Phe302 by Met, and Ser305 by Thr decreased the affinity of each antagonist. Simultaneous replacement of Thr297, Phe302, and Ser305 in GRPR by the three comparable NMBR amino acids caused a 500-fold decrease in affinity for both antagonists. Replacing the comparable three amino acids in NMBR by those from GRPR caused a gain in affinity for each antagonist. Receptor modeling showed that each of these three amino acids faced inward and was within 5 Å of the putative binding pocket. These results demonstrate that differences in the fourth EC domain of the mammalian Bn receptors are responsible for the selectivity of these two peptide antagonists. They demonstrate that Thr297, Phe302, and Ser305 of the fourth EC domain of GRPR are the critical residues for determining GRPR selectivity and suggest that both receptor-ligand cation-π interactions and hydrogen bonding are important for their high affinity interaction.

The gastrin-releasing peptide (GRP)1 receptor, which mediates the diverse actions of the mammalian bombesin (Bn)-related peptide (1, 2), GRP, has numerous high affinity peptide antagonists (3–5). This is in contrast to most other gastrointestinal (GI) hormone/neurotransmitter receptors for which no high affinity peptide antagonists exist (6). These GRP receptor antagonists are now widely used in both in vitro studies (5) and in vivo studies in animals (3, 7–11) and humans (12). Recent studies show that for many nonpeptide antagonists, differences in amino acids in the transmembrane domains between receptor subtypes are frequently particularly important for determining receptor subtype selectivity (13, 14). A recent study (15) shows a similar result with the peptoid antagonist PD168368 for the neuromedin B receptor. However, with peptide antagonists of non-GI hormone/neurotransmitter receptors, interactions with transmembrane regions (16, 17) or extracellular domains (18) are important for high affinity interaction or receptor subtype selectivity. Which if any of these results apply to the different classes of GRPR peptide antagonists, at present, is unclear.

GRP and neuromedin B (NMB), mammalian homologues of the amphibian tetradecapeptide bombesin, have structurally related carboxyl termini (19). These peptides mediate a spectrum of biological activities such as stimulating growth of both normal and neoplastic tissues (20–23), secretion (1), muscle contraction (24), central nervous system effects (including satiety (25), thermoregulation (26), and circadian rhythm (27)), changed developmental (28, 29) and immunologic effects (30). These effects are mediated by binding to two structurally and pharmacologically distinct receptors, the GRPR and NMB receptor (NMBR) (31–33). These two receptors are members of the bombesin receptor family within the G protein-coupled receptor (GPCR) superfamily and share 56% overall amino acid sequence identity (34). Both the GRPR and NMBR are widely distributed in the central nervous system and peripheral tissues including in the GI tract (1, 35). Which of the widespread effects of these peptides (1) are important physiologically or in pathologic processes is largely unknown at present. The availability of potent Bn receptor subtype-specific antagonists and a molecular understanding of their basis of action are important steps in addressing these questions.

In the present study, we have examined the molecular basis for the GRPR selectivity of two different classes of closely related high affinity Bn receptor peptide antagonists: a Bn statine analogue, JMV594 (36), and a Bn peptoid analogue, JMV641 (37). A receptor chimeric and site-directed mu-

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1 The abbreviations used are: GRP, gastrin-releasing peptide; GRPR, GRP receptor; Bn, bombesin; GI, gastrointestinal; NMB, neuromedin B; NMBR, NMB receptor; GPCR, G protein-coupled receptor; ET, endothelin.

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tagenesis approach was used to identify critical domain and amino acid(s) responsible for these antagonists' selectivity and high affinity for GRPR. In this study we show that the selectivity of peptide antagonists JMVs94 and JMVs64 for the GRPR over the NMBR depends primarily on interactions with amino acids in the fourth extracellular region of the GRPR. Site-directed mutagenesis studies demonstrate that Thr397, Phe302, and Ser305 in this region of GRPR are the critical amino acids for high affinity binding and selectivity of these two antagonists. Computer modeling of this region of the GRPR demonstrates that these amino acids all face inward forward the proposed binding pocket and are all within 5 Å of it, suggesting that cation–π and hydrogen bonding interactions between these antagonists and the above three amino acids are essential for receptor subtype selectivity and high affinity interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—pCDNA3 was from Invitrogen (Carlsbad, CA). Oligonucleotides were from Midland Certified Reagent Company (Midland, TX) and Life Technologies, Inc. Sequenase™ Cloning Kit and QuikChange™ Site-Directed Mutagenesis Kit were from Stratagene (La Jolla, CA). Restriction endonucleases (HindIII, XbaI, and EcoRI), fetal bovine serum, penicillin-streptomycin, LipofectAMINE™ reagent, and tetradsDNA-EDA-4Na were from Life Technologies. Dulbecco's modified Eagle's medium and Dulbecco's phosphate-buffered saline were from Biofluids, Inc. (Rockville, MD). Balb/c 3T3 cells were from the American Type Culture Collection (Manassas, VA). A 100 × 20-mm tissue culture dish (Falcon® 3003) was from Becton Dickinson (Plymouth, United Kingdom). Bn and neuromedin B were from Peninsula Laboratories, Inc. (Belmont, CA). Na125I (2,200 Ci/mmol) was from Amersham Pharmacia Biotech. 1,3,4,6-Tetrachloro-3a,6a-di-phenylglycouril (IODO-GEN®) and diithiothreitol were from Pierce. Bovine serum albumin fraction V and HEPES were from ICN Pharmaceutical Inc. (Aurora, OH). Soybean trypsin inhibitor type I-S and bacitracin were from Sigma. Nysiol M20 oil (specific gravity 1.0337) was from Nyco 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 mouse GRPR and rat NMBR were identical to those described previously (31, 32). The cDNA of the wild-type mouse GRPR was cloned between the HindIII site and XbaI site of pCDNA3, and the wild-type rat NMBR was cloned into the EcoRI site of pcDNA3. The GRPR/NMBR chimeras were constructed using the Sequenase™ Cloning Kit (38) using hydropathy plots for the GRPR and for the NMBR as described previously (15). Mutant receptors were made by using the QuikChange™ Site-Directed Mutagenesis Kit, following the manufacturer's instructions. The spiking temperature was 60 °C and the DNA digestion was for 2 h. Nucleotide sequence analysis 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/c 3T3 cells were seeded in a 10-cm diameter tissue culture dish at a density of 105 cells/dis/100 mm plate overnight at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. The following morning, cells were transfected with 5 μg of plasmid DNA by the cationic lipid-mediated method (39) using 30 μl of LipofectAMINE™ reagent and 20 μl of LipofectAMINE™ Plus reagent in serum-free Dulbecco's modified Eagle's medium for 3 h at 37 °C. At the end of the incubation period, the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37 °C with a 5% CO2 atmosphere and were used 48 h later for binding assays.

**Preparation of 125I[Tyr]BrN.**—At a specific activity of 2,200 Ci/mmol was prepared by a modification of the method described previously (40, 41). 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 KH2PO4 (pH 7.4). To this vial, 20 μl of KH2PO4 (pH 7.4), 8 μg of peptide in 4 μl of water, and 2 mCi (20 μl) of Na125I 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 and 300 μl of 1.5 M diithiothreitol. The iodonotation mixture was incubated at 80 °C for 60 min. The reaction mixture was applied to a Sep-Pak column (Waters Associates, Milford, MA), and free 125I was eluted with 5 ml of water followed by 5 ml of 0.1% (v/v) trifluoroacetic acid. The radiolabeled peptides were eluted with 200 μl of sequential elutions (X 10) with 60% acetonitrile in 0.1% trifluoroacetic acid. The two or three fractions with the highest radioactivity were combined and purified on a reverse-phase, high performance liquid chromatography with a μBondpak column (0.46 × 25 cm). The column was eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (v/v) from 16 to 60% acetonitrile in 60 min. One-ml fractions were collected and checked for radioactivity and receptor binding. The pH values of the fractions were adjusted to 7 using 0.2 M Tris (pH 9.5), and radioligands were stored in aliquots with 0.5% bovine serum albumin at −20 °C.

**Whole Cell Radioligand Binding Assays—**Competitive binding assays were performed 48 h post-transfection. Disaggregated transiently transfected cells were incubated for 1 h at room temperature in 250 μl of binding buffer (pH 7.4) with the ligand 50 pm 125I[Tyr]BrN (2,200 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 KH2PO4, 1 mM MgCl2, 0.5 mM CaCl2, 0.2% (v/v) bovine serum albumin, 0.05% (w/v) bacitracin, and 0.01% (w/v) soybean trypsin inhibitor. The cell concentration was adjusted to 0.2–2 × 106 cells/ml to assure that no more than 20% of the total added radioactive ligand bound. Bound tracer was then separated from unbound tracer by layering 100 μl of the binding solution on top of 100 μl of 25% (w/v) sucrose. The reaction on top of the sucrose was centrifuged at 10,000 × g in a Microfuge E42 (Beckman Instruments) 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 Instrument Co.). A 100-μl aliquot of the incubation mixture was then used in duplicate to determine the total radioactivity. Binding was expressed as percentage of total radioactivity that remained in 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 IC50 values was performed with a curve-fitting program, KaleidaGraph graphing software (Synergy Software, Reading, PA). Affinity and receptor density were calculated using a least-squares curve-fitting program (LIGAND).

**Histology Modeling of Extracellular Loop Region**—The amino acid sequences of mouse GRPR and rat NMBR were retrieved from the G protein-coupled receptor data base (43). The three-dimensional crystal structure of bovine rhodopsin (1B88) was obtained from the Protein Data Bank (Research Collaboratory for Structural Bioinformatics, Rutgers University) (44). Examination of the hydrophathy profile of the fourth extracellular domain of bovine rhodopsin using SYBYL6.6 demonstrated that the hydrophobicity of the sequence is markedly less in the region of the extracellular loop as compared with the adjoining helical regions. This was confirmed by protein sequence analysis (45–47). Hydrophathy evaluation and protein sequence analysis were used to locate the fourth extracellular domain regions in both mouse GRPR and rat NMBR. An additional eight amino acids were included at each end of the putative regions, and the sequences were imported into Deep View, the Swiss-PdbViewer (48) and aligned to the structure of bovine rhodopsin. The threading energy (49) of the alignment was at a minimum and rose if the sequences were shifted in either direction. The orientation of the side chains was optimized to reduce clashes, and the three-dimensional structure of the extracellular loop region was exported to SYBYL as a pdb file.

**Binding Site Model**—The putative, solvent-accessible binding sites of the extracellular loop of the receptor were explored using the SiteID module in SYBYL6.6. Briefly, the loop region was solvated with water molecules, and clusters of solvent molecules adjacent to solvent-accessible residues were defined as a potential binding site. This demonstrated a possible binding site near the extracellular surface bordered by the extracellular loop and potentially extending into the interior of the helical bundle with a volume of 430 Å3. The surface of the solvent cluster adjacent to the extracellular loop was visualized using the SYBYL program Molcad.
increase the affinity for JMV594 by 300-fold (from NMBR the extracellular domain of the GRPR to attempt to
When the reverse study was performed by substituting in the pseudopeptide antagonist, JMV641 (Fig. 3, Table II).

/ H11006

affinity (from 1,500 / H11006

nM) for the GRPR (Table I), and neuromedin B (Fig. 1) had high

/H9274

affinity for the NMBR (IC 50 1.2 nM) (Table I). Bn had a 2-fold

/H11006

over the GRPR (Table I). Both the Bn statine antagonist, JMV594, and 1,200-fold (from 0.46

(1,200 nM) for the statine antagonist,

/JMV594

over NMBR for these two structurally different peptide

/JMV594

Bn-related natural occurring agonists Bn and GRP (Fig. 1) had high affinity (IC50 2.7

nm) for the GRPR (Table I), and neuromedin B (Fig. 1) had high affinity for the NMBR (IC50 1.2 nm) (Table I). Bn had a 2-fold and GRP a 12-fold selectivity for the GRPR over the NMBR, whereas neuromedin B had 200-fold selectivity for the NMBR over the GRPR (Table I). Both the Bn statine antagonist, JMV594 (Fig. 1), and the pseudopeptide antagonist, JMV641 (Fig. 1) had a high affinity (IC50 0.5–2.2 nm) for the wild-type GRPR as reported previously (36, 37) (Fig. 2, Table I). However, we found that each antagonist had a low affinity for the NMBR (IC50 1,500–10,000 nM) (Fig. 2, Table I). Therefore, JMV594 and JMV641 had >5,000 and 3,260 times higher selectivity, respectively, for the GRPR over the NMBR (Fig. 2, Table I). To explore the molecular basis for this GRPR selectivity of JMV594 and JMV641, we first made both loss of affinity GRP chimeric receptors (Fig. 3) and gain of affinity NMBR chimeric receptors (Fig. 4). Then to determine the exact amino acids involved we made GRPR loss of affinity point mutants (Figs. 5–8) and gain of affinity point mutants of NMBR (Figs. 9 and 10).

Extracellular Chimeric Receptors—Four loss of affinity GRPR chimeric receptors were made with the extracellular domains of NMBR substituted for the comparable domains in GRPR (Fig. 3), and four potential gain of affinity NMBR chimeras were made with the extracellular domains of GRPR substituted into NMBR (Fig. 4). Substitution of the first, second, and third extracellular domain in the GRPR by the comparable domain from the NMBR did not alter the affinity for JMV594 or JMV641, and each chimeric GRPR had similar affinities to wild-type GRPR (Fig. 3 and Table II). Substitution of the fourth extracellular domain in the GRPR by the comparable domain of the NMBR decreased the affinity 1,400-fold (from 2.2 ± 0.05 to 3,100 ± 120 nM) for the statine antagonist, JMV594, and 1,200-fold (from 0.46 ± 0.03 to 530 ± 22 nM) for the pseudopeptide antagonist, JMV641 (Fig. 3, Table II). When the reverse study was performed by substituting in the NMBR the extracellular domain of the GRPR to attempt to gain affinity, replacement of the fourth extracellular domain increased the affinity for JMV594 by 300-fold (from >10,000 to 29 ± 0.58 nM) and with JMV641 caused an 11-fold gain in affinity (from 1,500 ± 40 to 140 ± 4.2 nM) (Fig. 4, Table II). Replacement of the first, second, or third extracellular domain of the NMBR by the comparable domain of the GRPR had no effect on the affinity of JMV594. However, substitution of the third extracellular domain had a small effect on the affinity for JMV641, increasing affinity less than 1-fold (Fig. 4, Table II). These results from the study of chimeric receptors demonstrated that the fourth extracellular domain was principally involved in determining selectivity for GRPR over NMBR for these two structurally different peptide antagonists.

GRPR Fourth Extracellular Domain Mutants (Loss of Affinity Point Mutants)—To identify which amino acid(s) in the fourth extracellular domain of GRPR are responsible for the high affinity for JMV594 and JMV641, the amino acid differences and identities were compared between the mouse GRPR and rat NMBR in the fourth extracellular domain (Fig. 5). The rat, mouse, and human NMBR are identical in the fourth extracellular domain (33, 50). The human and mouse GRPR are identical also in this region and differ from the rat by one conservative substitution of isoleucine for valine in position 303 (33). In the fourth extracellular domain, 11 amino acid differences were present, occurring at positions 290–291, 293, 295, 297, 299–300, and 302–305 of GRPR, which are comparable with positions 291–292, 294, 296, 298, 300–301, and 303–306 of NMBR (Fig. 5). To study the 11 amino acid differences in the fourth extracellular domains these receptors, we first made six GRPR loss of affinity group point mutants (Fig. 5). Two of the six GRPR group point mutants caused a decrease in affinity for both antagonists (Fig. 6). The GRPR mutant with Thr297 of the GRPR replaced by Pro297 from the comparable position of NMBR (Thr297 → Pro297) and a second mutant with Phe-Val-Thr-Ser292–295 of the GRPR replaced by Met-Ile-Val-Thr302–305 of NMBR, decreased the affinities for JMV594 by 17- and 90-fold, respectively, and for JMV641 by 2- and 48-fold, respectively (Fig. 6 and Table III). The other four GRPR group point mutants, including replacement of Tyr292–293 of the GRPR by Phe, Asn290, 291–293 of the GRPR by Lys293, Val305 of the GRPR by Ile295, and Met-Leu309 of the GRPR by Leu-Gly from NMBR had no effect on the affinity for either antagonist (Fig. 6, Table III). However, neither of the two group changes alone (i.e., 2–100-fold decrease) caused a decrease in affinity equal to the >1,100-fold decrease in affinity seen for each antagonist when the entire fourth extracellular GRPR domain was replaced by that from the NMBR (Table III). Therefore, a series of point mutations and combinations were made to identify which other amino acids were important for determining the antagonists’ selectivity. To understand which amino acids were important in
NMBR amino acid replacements (Thr297 mutants with three combinations of fourth extracellular domain of NMBR (Fig. 5). GRPR mutants were replaced by the comparable different amino acids of the GRPR affinity point mutants in which combinations of amino acids in determining antagonist selectivity, we constructed and assessed the possible gain in affinity of four NMBR mutants by replacing the three most important amino acids identified from the study of GRPR point mutants either alone or in combination (i.e. Pro298 → Thr298, Met302 → Phe302 → Ser306, and Pro298, Met302, Thr306 → Thr298, Phe302, Ser306) (Fig. 9). These three amino acids (i.e. Pro298, Met302, and Thr306) in NMBR are in the position comparable with that of the amino acids in GRPR that were the key amino acids for high selectivity of GRPR for the antagonists identified in the loss of affinity studies (Fig. 9). For the statine antagonist JMV594, two single amino acid mutants (Pro298→Thr298 and
Thr\textsuperscript{306} → Ser\textsuperscript{306} caused a -3-fold gain in affinity, and the combination mutant (Pro\textsuperscript{298} Met\textsuperscript{303}, Thr\textsuperscript{306} → Thr\textsuperscript{298}, Phe\textsuperscript{303} Ser\textsuperscript{306}) demonstrated a >130-fold gain in affinity (Fig. 10, Table III). This increase in affinity was only 2.5-fold less than the gain of affinity seen when the entire fourth extracellular domain of the GRPR was substituted into the NMBR (Fig. 10 and Table III). With the pseudopeptide JMV641, the substitutions of these three amino acids singularly or together into NMBR showed a different result. The substitution of Thr\textsuperscript{298} for Pro\textsuperscript{298} alone was the most critical amino acid for the gain in affinity for JMV641, causing a 5-fold increase in affinity (Fig. 10 and Table III). In contrast to the statine analogue JMV594, with the pseudopeptide JMV641 the replacement of Thr\textsuperscript{306} in the NMBR by Ser\textsuperscript{306} from the comparable position in the GRPR had no effect. Furthermore, the combination replacement of all three of these key fourth extracellular domain amino acids of

**Fig. 4. Affinities of JMV594 and JMV641 for wild-type NMBR, extracellular chimeric NMBRs, and wild-type GRPR expressed in Balb/c 3T3 cells (gain of affinity chimeras).** Diagrams of the chimeric receptors formed are shown at the top. The chimeric NMBRs were formed by replacing each of the extracellular loops of NMBR by the comparable loop of the GRPR one at a time. The affinity was measured by competitive radioligand displacement of 50 pM \[\text{[Tyr}^4]\text{Bn}\] by JMV594 and JMV641 at the concentrations shown. Each point on a curve is the mean from three separate experiments, and in each experiment each point was measured in duplicate. All curves were best fit by a single binding site model (LIGAND) (42). e1-, e2-, e3- and e4-GRPR refer to substitution of this extracellular loop of the GRPR for the comparable extracellular loop of the NMBR. The arrows indicate large gains of affinity for the antagonists.

**Fig. 5. Alignment of amino acid sequences in the fourth extracellular domain of GRPR and NMBR.** The boxes indicate divergent amino acids between these two receptors in the fourth extracellular domain. Shown are the 21 GRPR mutants made to explore the importance of 11 amino acid differences for determining JMV594 and JMV641 selectivity. The arrow indicates that the top set of amino acids in the indicated GRPR position were replaced by the bottom set of amino acids from the comparable position in the NMBR.
the NMBR (Pro\textsuperscript{298}, Met\textsuperscript{303}, Phe\textsuperscript{306}) resulted in no greater gain in affinity than the effect of replacing the Pro\textsuperscript{298} in NMBR alone (Fig. 10 and Table III), whereas in the case of the statine antagonist JMV594 it caused a 40-fold gain in affinity over any single replacement (Fig. 10 and Table III), whereas in the case of the statine antagonist JMV594 it caused a 40-fold gain in affinity over any single replacement (Fig. 10 and Table III).

**Binding Site Model**—To attempt to gain additional insight into why certain amino acids within the fourth extracellular domain of GRPR are important for determining selectivity for the NMBR (Pro\textsuperscript{298}, Met\textsuperscript{303}, Phe\textsuperscript{306}) that were found to be the primary determinants for the specificity of these two antagonists for the GRPR all projected into the interior of the putative binding pocket, with most atoms within a 5-Å distance of the projected binding pocket (Fig. 11).

**DISCUSSION**

In general, the roles of GPCRs mediating the action of most GI hormones/neurotransmitters in physiological or in pathological processes are still unclear. This is in large part because, for many, specific receptor antagonists do not exist, and for others, only recently have high affinity antagonists been developed (51). The antagonists for these receptors generally fall into one of three types: nonpeptide antagonists (the largest group); peptide antagonists; or, in a few cases, peptoid antagonists, which have features of both peptides and nonpeptides (52, 53). There are a number of studies of the molecular basis of action of nonpeptide antagonists for various GI hormone receptors (14, 54–57); however, there are only a few studies for peptide an-
Basis of GRPR Selectivity of Peptide Antagonists

| TABLE III | Affinities of GRP, JMV594, and JMV641 for wild-type GRPR, wild-type NMBR, and fourth extracellular domain amino acid mutants of GRPR and NMBR |

|                      | Wild-type receptors | Fourth extracellular domain group point mutants (loss of affinity) | Fourth extracellular domain point mutants (loss of affinity) | Fourth extracellular domain combination point mutants (loss of affinity) | Fourth extracellular domain point mutants (gain of affinity) |
|----------------------|---------------------|---------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------------------------|-------------------------------------------------------------|
|                      | GRPR | NMBR | [e4-NMBR]GRPR | [Met302]GRPR | [Ile303]GRPR | [Lys293]GRPR | [Pro297,Met302]GRPR | [Pro297,Thr305]GRPR | [Ile295,Pro297,Met-Ile-Val-Thr302]GRPR | [Phe-Asn290,291,Ile295,Pro297,Met-Ile-Val-Thr302]GRPR | [Phe-Asn290,291,Leu-Gly299,300,Met-Ile-Val-Thr302]GRPR | [Thr298,Phe303,Ser306]NMBR |
|                      | GRPR | NMBR | [e4-NMBR]GRPR | [Met302]GRPR | [Ile303]GRPR | [Lys293]GRPR | [Pro297,Met302]GRPR | [Pro297,Thr305]GRPR | [Ile295,Pro297,Met-Ile-Val-Thr302]GRPR | [Phe-Asn290,291,Ile295,Pro297,Met-Ile-Val-Thr302]GRPR | [Phe-Asn290,291,Leu-Gly299,300,Met-Ile-Val-Thr302]GRPR | [Thr298,Phe303,Ser306]NMBR |
|                      | 13.5 | 12.7 | 9.0 | 15.6 | 12.9 | 14.1 | 11.2 | 12.5 | 11.0 | 12.1 | 14.6 | 7.5 |
|                      | 0.4  | 0.8  | 0.5 | 0.7  | 0.4  | 0.5  | 1.0  | 1.2  | 1.5  | 0.4  | 0.6  | 0.7  |
|                      | 0.5  | 2.6  | 1.0 | 1.2  | 1.3  | 1.0  | 2.3  | 1.2  | 1.1  | 1.3  | 1.1  | 0.7  |
|                      | 2.2±0.05 | >10,000 | 3,100±120 | 72±4.2 | 180±5.1 | 1,100±25 | 310±14 | 72±4.2 | 180±5.1 | 1,100±25 | 310±14 | 29±0.58 |
|                      | 0.46±0.03 | 1,500±40 | 530±22 | 16±1 | 24±1 | 230±14 | 13±1 | 16±1 | 24±1 | 230±14 | 14±1 | 140±4.2 |

Tortagonists (14, 16, 17, 55). This has occurred in large part, because potent peptide agonists have been described for only a few GI hormone/neurotransmitter receptors (51). One of these exceptions is GRPR, which mediates the actions of the mammalian bombesin-related peptide, GRP, in the central nervous system and peripheral tissues (1–3, 58). Six different classes of peptide receptor antagonists are described for the GRPR (3, 4, 36, 37, 59–62), some with sufficient potency and stability to be used recently for in vivo studies to determine GRP’s action in humans (12). At present, for each of these peptide GRPR antagonists, their molecular basis of action (the molecular determinants of their receptor selectivity or for their high affinity receptor interaction) is unknown. In this study, we examined the molecular basis of action of two of the most potent GRPR peptide antagonists, the statine analogue, JMV594, and the pseudopeptide analogue, JMV641 (36, 37).

In the present study, we not only found that each of these peptide antagonists had an affinity as high as GRP or bombesin for the GRPR (as reported previously (36, 37), but also that they were highly selective for the GRPR over the other mammalian Bn receptor, the NMBR. Each of the antagonists had >3,000-fold higher affinity for GRPR than NMBR, despite the fact that these receptors share an ~50% overall amino acid identity (2, 33). The analyses of both the loss of affinity GRPR chimera and gain of affinity NMBR chimera support the conclusion that differences in the fourth extracellular domains of these two receptors play the major role in determining selectivity of these two different classes of peptide agonists.

This result has both similarities with and differences from studies on the interaction of peptide agonists and agonists with other GPCRs. Studies of several GPCRs demonstrate that the receptor extracellular domains can be an important receptor region for determining high affinity ligand binding (63). However, only a few studies have explored whether the determinants of high affinity interaction or selectivity for a peptide antagonist are due to interactions with the receptor extracellular domains. Such an interaction is not important for determining high affinity interaction of the peptide antagonist D-Arg-[Hyp3,D-Phe7]bradykinin (NPC567) with the B2 bradykinin receptor (16) or BQ-123 with the endothelin A receptor (14). However, it is important for the high affinity interaction of the peptide antagonists JMV179 with the human CCK-A receptor (55) and [Sar1,Ile8]angiotensin II with the AT 1 receptor (18).
FIG. 7. Affinities of JMV594 and JMV641 for wild-type GRPR, fourth extracellular domain point mutants of GRPR, and wild-type NMBR expressed in Balb/c 3T3 cells (loss of affinity point mutants). The point mutants of GRPR were formed by replacing the amino acids in positions 302–305 of GRPR by the comparable amino acids of the NMBR as shown in Fig. 5. The affinity was measured by competitive radioligand displacement of 50 pM [125I-Tyr4]Bn by JMV594 and JMV641 at the concentrations shown. Each point on a curve is the mean from three separate experiments, and in each experiment each point was measured in duplicate. All curves were best fit by a single binding site model (LIGAND) (42). [Met302]GRPR refers to replacement of the amino acid residue in position 302 of the GRPR by methionine, which exists in the comparable position in NMBR. The arrows indicate large changes in affinity of the GRPR by the mutations.

FIG. 8. Affinities of JMV594 and JMV641 for wild-type GRPR, fourth extracellular domain combination amino acid(s) mutants of GRPR, and wild-type NMBR expressed in Balb/c 3T3 cells (loss of affinity point mutant combinations). The mutants of GRPR were formed by replacing the amino acid(s) of the fourth extracellular domain of GRPR by the comparable amino acid(s) of the NMBR as shown in Fig. 5. The affinity was measured by competitive radioligand displacement of 50 pM [125I-Tyr4]Bn by JMV594 and JMV641 at the concentrations shown. Each point on a curve is the mean from three separate experiments, and in each experiment each point was measured in duplicate. All curves were best fit by a single binding site model (LIGAND) (42). [Pro297,Met302]GRPR refers to replacement of the amino acid residues in positions 297 and 302 of the GRPR by proline and methionine, which exist in the comparable positions in NMBR. The arrows indicate large changes in affinity of the GRPR by the mutations.

FIG. 9. Alignment of amino acid sequences in the fourth extracellular domain of NMBR compared with GRPR. Boxes indicate divergent amino acids between these two receptors in the fourth extracellular domain. Shown are the four NMBR gain of affinity point mutants made to explore the importance of amino acid differences for determining JMV594 and JMV641 selectivity.
NMB with the NMBR (67), endothelin (ET) with the ET<sub>A</sub> receptor (57), and neuropeptide Y with neuropeptide Y<sub>1</sub> receptor (68), the high affinity interaction or selectivity is primarily determined by amino acids in the transmembrane regions. Similarly, with many nonpeptide antagonists such as the interaction of L365,260 with the CCK-B receptor (54), losartan with the AT<sub>1</sub>B angiotensin II receptor (69), bosentan with the ET<sub>A</sub> receptor (57), and L161,664 with the neurokinin-1 receptor (70), the extracellular domains do not contain determinants for high affinity interactions or receptor subtype selectivity.

To determine which amino acids in the fourth extracellular domain of the GRPR account for the high affinity of these two antagonists for the GRPR and its selectivity for this receptor over the NMBR, we performed a comparative alignment of the amino acids in this region between the GRPR and the NMBR. Our results support the conclusion that the threonine residue in position 297 of GRPR instead of a proline in the NMBR, a phenylalanine in position 302 of GRPR instead of a methionine in NMBR, and a serine in position 305 of GRPR rather than a threonine in NMBR are the key amino acid differences responsible for the high affinity of the two peptides for the GRPR and their high selectivity for the GRPR over the NMBR. Whereas the individual replacement of these three amino acids caused only a 10-fold change in affinity for each antagonist, a combined substitution of these three residues by the comparable NMBR amino acids led to a marked decrease in affinity (~500-fold) for each of the antagonists. This result demonstrates that amino acid differences at the both the amino and carboxyl terminus of the fourth extracellular domain are involved in determining the high affinity and selectivity for the GRPR of both antagonists.

This result has both similarities and differences from studies of binding of other peptide ligands to G1 hormone/transmitter GPCRs. It is similar to results with interaction of the peptide antagonist [Sar<sup>1</sup>,Ile<sup>8</sup>]angiotensin II (18) with the AT<sub>1</sub> receptor, in which a cooperative interaction between His<sup>24</sup>, Tyr<sup>26</sup>, and Ile<sup>37</sup> in the first extracellular domain is required for high affinity ligand interaction or for the peptide antagonist D-Arg-[Hyp<sup>3</sup>,Phe<sup>7</sup>]bradykinin's interaction with the B<sub>2</sub> bradykinin receptor, in which the ligand's interaction with multiple amino acids in the sixth transmembrane domain is required for high affinity interaction and selectivity. Similarly, with almost all studies on peptide agonists, such as bradykinin's interaction with the bradykinin 1 or 2 receptor (65); CCK-8 interaction with the CCK-B receptor (64); and substance P, neurokinin A, or neurokinin B interaction with the neurokinin-1 receptor (66), cooperative interactions with a number of residues in the same extracellular domain are required for high affinity interaction and selectivity for the one receptor subtype. In contrast to these cooperative interactions, with some peptide antagonists such as the interaction of the pentapeptide BQ123 with the ET<sub>1</sub>B receptor (14) or JMV179 with the CCK-A receptor (55) and with many nonpeptide antagonists such as L365,260 interaction with the CCK-B receptor (54), BMS-182874 with the ET<sub>A</sub> receptor (14) or SB209670, and Ro 46–2005 with the ET<sub>B</sub>
receptor (56), a single amino acid difference between receptor subtypes accounts primarily for the high affinity or selectivity of the antagonist.

The threonine, serine, and phenylalanine residues found to be important in determining GRPR selectivity for both classes of antagonists in the present study have been reported in several studies in other GPCRs to play a critical role in determining high affinity interaction and selectivity of the ligand for a GPCR subtype (16, 17, 57, 64, 69). A threonine in the sixth transmembrane domain of the bradykinin 2 receptor is required for high affinity interaction with the peptide antagonist des-Arg10-Leu9[BK (NPC567) (16), two threonine residues in the fifth transmembrane domain of the m3 muscarinic receptor are critical amino acids for high affinity interaction with agonists (acetylcholine, carbachol) (71), and a serine in the third transmembrane domain of the AT1A receptor is one of the critical amino acids required for high affinity interaction with the peptide antagonist [Sar1, Ile8]AII (72). In the former two studies (16, 71), it was proposed that the mechanism of the enhanced affinity due to the threonine or serine was by enhancing hydrogen bonding with the ligand. The presence of a phenylalanine residue in either a transmembrane domain or extracellular domain in other GPCRs also has been shown to play an important role in high affinity ligand-receptor interaction and ligand GPCR selectivity (16, 17, 57, 64, 69). The presence of a phenylalanine in the sixth transmembrane domain of the bradykinin 2 receptor (Phe301) is necessary for high affinity and selectivity of the peptide antagonist NPC567, and this effect was proposed to be mediated by an amino-aromatic or aromatic-aromatic interaction (16). A phenylalanine residue in the second transmembrane domain of the CCK-A receptor (Phe107) or two phenylalanine residues in the second extracellular domain of the CCK-B receptor are critical amino acids for high affinity interaction with peptide agonist CCK-8 (64). Similarly, with substance P interaction with the neurokinin-1 receptor (66), endothelin with the ET_A receptor (57), and the peptide antagonist [des-Arg10-Leu9]kallidin with the bradykinin 1 receptor (17), phenylalanines in either extracellular domains or transmembrane regions are essential for high affinity interaction and selectivity. Phenylalanine, similar to other aromatic amino acids, characteristically interacts with ligands by functioning as a strong locus of cation-π binding (73). The cation-π binding occurs through the side chains from the aromatic amino acids such as phenylalanine, tyrosine, or tryptophan (74). In our study, the substitution of methionine from the comparable position of NMBR for phenylalanine in the GRPR resulted in a marked decrease in affinity for JMV594 and JMV641, supporting the conclusion that cation-π interactions are important for the high affinity and selectivity of both of the peptide antagonists.

At present, it is not possible to compare the receptor structural determinants of high affinity interaction and GRPR selectivity of these two different classes of peptide agonists with that for either the agonist bombesin or GRP, peptide agonists of which both antagonists are close structural analogues. There are no studies on the molecular determinants of either bombesin or GRP’s high affinity interaction with the GRPR or of the selectivity of GRP for GRPR over the NMBR. However, our study does demonstrate that these two different antagonists have both similarities and differences in their molecular determinants of high affinity GRPR interaction and selectivity. They are similar in that with both the statine antagonist JMV594 and the pseudopeptide antagonist JMV641, the amino acid differences in the fourth extracellular domains of NMBR and GRPR are primarily responsible for their high affinity and selectivity for GRPR. They are also similar in that of the 11 amino acid differences in this domain between GRPR and NMBR, the three most important for each antagonist for high affinity interaction are Thr297, Phe302, and Ser305 in the GRPR. Furthermore, with each antagonist, these three amino acids had a potentiating effect on enhancing affinity, with the combination having a much greater effect than any of the three substitutions alone. These results suggest that for both antagonists cation-π interactions and hydrogen bonding through serine and threonine receptor residues are of primary importance in determining high affinity interaction. However, some results suggest that these two different classes of antagonists have some important differences in the molecular determinants of their GRPR selectivity. First, for the pseudopeptide antagonist, JMV641, the GRPR loss of affinity and NMBR gain of affinity chimeric studies showed that differences in second and third extracellular domains were of some importance in determining its GRPR selectivity, whereas for the statine analogue, JMV594, they were not important. Second, within the fourth extracellular domain, which was the most important domain for GRPR selectivity for both antagonists, the presence of Thr297, Phe302, and Ser305 in the GRPR was relatively more important for the statine antagonist JMV594’s selectivity than for the pseudopeptide antagonist, JMV641. With both NMBR gain of affinity chimeric receptors made by substituting the extracellular domain of NMBR with that from GRPR and with the gain of affinity combination point mutant, [Thr298, Phe303, Ser306]NMBR, the gain in affinity was much greater for JMV594 than JMV641. Specifically, with the replacement of the fourth extracellular domain of NMBR by that of GRPR ([4-GRPR]NMBR) there was a 30-fold greater gain in affinity for JMV594 than JMV641 (i.e. 345-versus 11-fold increase, respectively). Similarly, with insertion of Thr298, Phe303, and Ser306 in NMBR, there was a >20-fold greater increase in affinity for JMV594 than JMV641 (>133-versus 6.5-fold increase, respectively). These results demonstrate that while the molecular determinants of receptor subtype selectivity for these two closely related classes of GRPR antagonists is generally similar, there are also some important differences.

To attempt to gain additional insight into why these three amino acids of the fourth extracellular domain are important in determining the high GRPR selectivity of these two antagonists, three-dimensional modeling of this region of the GRPR was undertaken. Modeling of the GRPR was performed based on the results with bovine rhodopsin, which has recently been crystallized and characterized by x-ray diffraction (75). In rhodopsin, the fourth extracellular domain is well defined and solvent-accessible (75). The hydropathy profile of the sequence is markedly more hydrophilic in the loop region than in the adjoining transmembrane helical regions. This profiling was used to determine the ends of the loop region in GRPR, and the sequence of the extracellular loop was threaded onto the three-dimensional structure of rhodopsin. When the sequences were aligned, the loop regions coincided, and any shift in the alignment resulted in a higher threading energy, indicating a less favorable environment for the sequence. In this model, the critical Thr297, Phe302, and Ser305 residues in the fourth extracellular domain of the GRPR were found to face the interior of the 5 Å of the putative binding site and could interact with the antagonists (Fig. 11). However, Arg296, Ser289, Tyr292, Glu294, Asp296, and His301 are in a comparable position in the NMBR and therefore are un-
likely to be important in the selectivity of the JMV594 and JMV641 for the GRPR. Of the other six amino acids (Tyr^{290}, Ser^{293}, Val^{295}, Thr^{297}, Phe^{302}, and Ser^{305}), our mutagenesis studies show that only Thr^{297}, Phe^{302}, and Ser^{305} are important in determining the selectivity of JMV594 and JMV641 for the GRPR over the NMBR. Substitution of the other three amino acids (Tyr^{290}, Ser^{293}, and Val^{295}) by the comparable amino acids from the NMBR (Phe^{291}, Lys^{294}, and Ile^{296}) caused no change in the affinities of JMV594 and JMV641, suggesting that either the backbone substitutions of these amino acids were not involved in ligand interaction or the comparable amino acid from NMBR was sufficiently similar to effectively replace the GRPR amino acid. Therefore, we conclude that Thr^{297}, Phe^{302}, and Ser^{305}, which are the critical amino acids in determining the selectivity of JMV594 and JMV641, each are facing and within 5 Å of the proposal binding pocket.

In conclusion, our receptor chimeric gain and loss of affinity studies showed that the fourth extracellular domain of the GRPR was the principal receptor region responsible for the high affinity and selectivity of the statin antagonist JMV594 and the pseudopeptide antagonist JMV641 for the GRPR over the NMBR. Our mutagenesis studies show that Thr^{297}, Phe^{302}, and Ser^{305} in the fourth extracellular domain of the GRPR were likely to be important in the selectivity of the JMV594 and JMV641 for the GRPR. Of the other six amino acids (Tyr^{290}, Ser^{293}, and Val^{295}), our mutagenesis studies show that only Thr^{297}, Phe^{302}, and Ser^{305} are important in determining the selectivity of JMV594 and JMV641 for the GRPR. Of the other six amino acids (Tyr^{290}, Ser^{293}, and Val^{295}), our mutagenesis studies show that only Thr^{297}, Phe^{302}, and Ser^{305} are important in determining the selectivity of JMV594 and JMV641 for the GRPR. Of the other six amino acids (Tyr^{290}, Ser^{293}, and Val^{295}), our mutagenesis studies show that only Thr^{297}, Phe^{302}, and Ser^{305} are important in determining the selectivity of JMV594 and JMV641 for the GRPR.
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Molecular Basis for Selectivity of High Affinity Peptide Antagonists for the Gastrin-releasing Peptide Receptor
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