Molecular Basis of the Pharmacological Difference between Rat and Human Bombesin Receptor Subtype-3 (BRS-3)

Jie Liu,‡ Zhege J. Lao, Jiaping Zhang, M. T. Schaeffer, Michael M. Jiang, Xiao-Ming Guan, Lex H. T. Van der Ploeg, and Tung M. Fong*

Department of Obesity and Metabolic Research, Merck Research Laboratories, Rahway, New Jersey 07065

Received April 12, 2002; Revised Manuscript Received May 22, 2002

ABSTRACT: We cloned the gene and cDNA for rat bombesin receptor subtype-3 (BRS-3) and characterized its mRNA expression pattern and pharmacological properties. Despite the high degree of sequence similarity (80% identical), rat and human BRS-3 differ markedly in their pharmacological properties. Although the natural ligand for BRS-3 is still unknown, a synthetic peptide, dY-Q-W-A-V-(β-A)-H-F-Nle-amide (dY-bombesin), activates human BRS-3 with an EC_{50} of 1.2 nM. In contrast, dY-bombesin had a very poor potency for rat BRS-3 ($EC_{50} = 2\ \mu M$). To understand the molecular basis of this pharmacological difference, we constructed chimeric receptors in which individual extracellular loops of rat BRS-3 were replaced with the corresponding human sequences. Switching the N-terminal region or the second extracellular loop did not significantly change receptor properties. However, switching the third extracellular loop (E3) in the rat BRS-3 resulted in a chimeric receptor (RB3-E3) that behaved almost identically to human BRS-3. RB3-E3 bound dY-bombesin with high affinity ($K_i = 1.2 \pm 0.7$ nM), and was activated by dY-bombesin with high potency ($EC_{50} = 1.8 \pm 0.5$ nM). Within the E3 loop, mutation of $Y^{298}E^{299}S^{300}$ to $S^{298}Q^{299}T^{300}$ (RB3-SQT) or of $D^{306}V^{307}P^{308}$ to $A^{306}M^{307}H^{308}$ (RB3-AMH) only partially mimicked the effect of switching the entire E3 loop, and mutation of $A^{302}E^{303}$ to $V^{302}D^{303}$ or of $V^{310}V^{311}$ to $I^{310}F^{311}$ had little effect on the dY-bombesin potency. These results indicate that the sequence variation in the E3 loop is responsible for the species difference between rat and human BRS-3, and multiple residues in the E3 loop are involved in interactions with the agonist dY-bombesin.

Bombesin, a tetradecapeptide isolated from frog skin, represents the first member of a family of regulatory peptides named bombesin-like peptides (*1*, *2*). Gastrin-releasing peptide (GRP)[1] and neuromedin B (NMB) are the two bombesin-like peptides found in mammals. Currently, three subtypes of mammalian bombesin receptors have been described including GRP receptor (also named BB2), NMB receptor (also named BB1) (*3*), and an orphan receptor named bombesin receptor subtype-3 (BRS-3, also named BB3) (*4*). BRS-3 was identified based on its high degree of homology to the two known mammalian bombesin receptors. The naturally occurring high-affinity ligand for BRS-3 has not been identified. Bombesin, NMB, and GRP activate BRS-3 only at micromolar concentrations. Recently, a synthetic peptide, [D-Tyr6, βAla11, Phe13, Nle14]-bombesin(6−14), was described to have high affinity for human BRS-3 (*5*). Bombesin receptors are members of the G protein-coupled receptor superfamily. Upon agonist stimulation, bombesin receptors activate Gq which in turn activates phospholipase C. These events generate second messengers inositol trisphosphate (IP3) and diacylglycerol, leading to intracellular calcium mobilization (*6*, *7*).

BRS-3 has been implicated in the regulation of neuroendocrine function and energy metabolism. Mice lacking functional BRS-3 were hyperphagic and hypometabolic and developed obesity, hypertension, and diabetes (*8*). The BRS-3 receptor has been cloned from human, mouse, and sheep, and tissue-specific expression patterns have been reported (*9*). To facilitate the understanding of the physiological function of BRS-3, we cloned rat BRS-3, and characterized its distribution pattern and pharmacological properties. We found a pharmacological difference between human and rodent BRS-3 and determined the structural basis of this difference.

MATERIALS AND METHODS

Molecular Cloning. Rat BRS-3 cDNA was cloned by polymerase chain reaction (PCR) from rat hypothalamus mRNA. PCR primers were designed based on the published genomic DNA sequence of mouse BRS-3 (*10*). The forward and reverse primers are located 100 bp upstream of the start codon and 60 bp downstream of the stop codon, respectively. *Bam*HI or *Hin*dIII restriction enzyme site was introduced at the 5′-end of the primers for the convenience of subsequent cloning of the PCR product into expression vector (forward, 5′-GGATCCTGACACCACCTC-ATTACTAGAC-3′; and reverse, 5′-AAGCTTAAAGCTGGATACACACAG-3′). The

* To whom correspondence should be addressed at Merck Research Laboratories, R80M-213, P.O. Box 2000, Rahway, NJ 07065. Tel.: 732-594-6711; fax: 732-594-3337; e-mail: tung_fong@merck.com.

‡ Current address: AstraZeneca Inc., CRDL C246, 1800 Concord Pike, Wilmington, DE 19850-5437.

[1] Abbreviations: BRS-3, bombesin receptor subtype-3; BSA, bovine serum albumin; dY-bombesin, [D-Tyr6, βAla11, Phe13, Nle14]-bombesin(6−14) having the sequence dY-Q-W-A-V-(β-A)-H-F-Nle-amide; E3, third extracellular loop; FLIPR, fluorescence imaging plate reader; GRP, gastrin releasing peptide; NMB, neuromedin B; PCR, polymerase chain reaction.

10.1021/bi0202777 CCC: $22.00
Published on Web 06/19/2002

1.4 kb PCR product was cloned into pE3 expression vector (*11*) for sequence analysis and functional characterization. The GenBank accession number for the rat BRS-3 cDNA is AF510984.

The rat BRS-3 gene was cloned by screening a genomic library using rat BRS-3 cDNA as a probe. Approximately 1×10^6 plaques from Sprague−Dawley rat genomic library in Lambda FIX II vector (Stratagene, La Jolla, CA) were screened with ^{32}P-labeled probes. The filters were incubated overnight at 65 °C in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) containing 5×10^5 cpm/mL denatured probes. Filters were washed 3 times at 65 °C for 20 min in 2 × SSC, 0.1% SDS. Seven plaques were purified, and two of them encoded all three exons. These two clones were chosen for subsequent nucleotide sequence analysis.

Human BRS-3 cDNA was provided by Dr. James Battey at the National Institutes of Health. Mouse BRS-3 cDNA was cloned by PCR based on the published sequence (*4*).

Northern and in Situ Hybridization. Northern blotting and subsequent hybridizations were performed with MTN blot (Clontech, Palo Alto, CA) which contains mRNA from eight different rat tissues. The ^{32}P-labeled probes were prepared by random primer extension using rat BRS-3 cDNA as the template. Highly stringent conditions were used for hybridization and washing steps (*12*).

For in situ hybridization, 2−3 month old Sprague Dawley rats were euthanized by decapitation under CO_2 anesthesia. Brains were quickly removed and frozen in −40 °C isopentane. Coronal sections (14 μm thick) were cut with a cryostat at −17 °C and thaw-mounted on microslides. In situ hybridization was performed as detailed previously (*13*) with an equal mixture of three nonoverlapping ^{33}P-labeled oligonucleotide probes. The sequences of the probes are as follows: Oligo 451, 5′-GCACACAGTGCTTCTATTCCTGGAGAGTTGTCTCCGGTCCATCCT-3′; Oligo 452, 5′-AGGTAGTGGGTTGCATCCACTGGCACACAAGTCAGCAGGAGTAAC-3′; and Oligo 453, 5′-TGGCTTCACGACTGCTTTGTATCTGTCAGCGCTGAGAATTGTCAG-3′. Specificity of hybridization was determined by the blockade of hybridization signals in the presence of a 100-fold molar excess of nonlabeled probes.

Mutagenesis and Expression. Chimeric rat/human BRS-3 cDNA's and substitution mutants of rat BRS-3 were constructed by using PCR mutagenesis techniques (*14*). All the mutant and wild-type BRS-3 cDNA's were cloned into pE3 expression vector which contains CMV promoter, hygromycin-resistance gene, and OriP segment from Epstein-Bar virus. The authenticity of all mutant receptors was confirmed by determining the nucleotide sequence of the entire coding region.

The wild-type and mutant BRS-3 constructs were transfected by using lipofectamine reagents (Life Technologies, Rockville, MD) into 293EBNA cells (Invitrogen, Carlsbad, CA) which support episomal replication of plasmid containing Epstein Bar virus OriP segment. Transfected cells were selected with 250 μg/mL hygromycin. The cells are maintained as monolayers in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum and incubated at 37 °C in a humidified 5% CO_2 incubator.

The peptide agonist dY-bombesin [dY-Q-W-A-V-(β-A)-H-F-Nle-amide] was synthesized as described (*5*), and the [^{125}I]dY-bombesin was purchased from NEN Life Science (catalog no. NEX377, NEN, Boston, MA). The 293EBNA cells expressing wild-type or mutant BRS-3 grown in 150 mm^2 dishes were harvested in binding buffer (50 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 0.1% BSA) and disrupted with Dounce homogenization. After removal of cell nuclei by a low-speed centrifugation (1000*g*) step, the cell membrane proteins were collected by centrifugation at 35000*g* for 20 min. The membrane protein concentration was determined by the Bradford method using BSA as standard. For binding assays, 20 μg of membrane proteins was incubated with 0.25 nM ^{125}I-dY-bombesin and various concentrations of competitors in 200 μL of binding buffer. After a 2 h incubation at room temperature, the binding reaction was terminated by filtering through a GF/c filter and washing the filter with binding buffer using a Packard 96-well Harvester. The amount of radioligand bound to the receptor was determined by measurement of the radioactivities on the filter through liquid scintillation counting. The nonspecific binding was defined as the binding in the presence of 1 μM unlabeled dY-bombesin.

For IP1 assay, the transfected 293EBNA cells were plated onto poly-D-lysine-coated 12-well plates and incubated in the medium containing 2 μCi/mL [^{3}H]myo-inositol. After a 48 h labeling period, the cells were incubated for 30 min in Hank's balanced salt solution containing 10 mM HEPES, 0.1% BSA, and 10 mM LiCl. The cells were then stimulated, in the same buffer, with the peptide agonists for 1 h at 37 °C. The reaction was stopped by removal of medium and addition of 10 mM formic acid. The inositol monophosphate fraction was then isolated by anion exchange chromatography as described (*15*).

Rat and human BRS-3 were also tagged at the N-terminus with a nine amino acid epitope (YPYDVPDYA) derived from the influenza hemagglutinin protein (HA-tag). An indirect cellular ELISA approach was used to quantify the relative amount of tagged receptors on the cell surface (*16*). The 293EBNA cells transfected with tagged receptors were plated onto a 96-well plate. After overnight culture, the cells were fixed with 4% formaldehyde in PBS, and incubated with a monoclonal antibody directed against the HA-epitope tag (12CA5, Boehringer Mannheim). The plate was then washed and incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Amersham). The peroxidase activities present on the cell surface were measured by using TMB (Pierce, Rockford, IL) and H_2O_2 as substrate and chromogen.

The agonist-induced calcium mobilization response was studied using a fluorescence imaging plate reader (FLIPR made by Molecular Device). The transfected 293EBNA cells were plated onto poly-D-lysine-coated 96-well plates with black wall and clear bottom (Becton Dickinson, Franklin Lakes, NJ). After 16−48 h culture, the cells were loaded with 4 μM Fluo-3-AM fluorescent calcium-indicator dye (Molecular Probes, Eugene, OR) in assay buffer (Hank's balanced salts solution, 10 mM HEPES, pH 7.4, 0.1% bovine serum albumin, 2.5 mM probenecid). The compounds diluted in assay buffer were added to the cells, and the changes in intracellular calcium concentration as indicated by Fluo-3 fluorescence were immediately measured by FLIPR. Maximal changes in fluorescence over baseline were used to determine agonist response.

a)

```
TTCCGTTCTGTTCTGTTCTCCTGACTCCTCCTCATTGCTAGACGTAGGCACTAGACGTGA

CAGTCAACTACATCTGAACTGAGCAGAGAAGAGGTAGTGAAGGCAGAGTGTGCAGGAGAC

ATGTCTCAAAGGCAGCCTCAGTCACCTAATCAGACTTTAATTTCCATTACAAATGACACA
 M  S  Q  R  Q  P  Q  S  P  N  Q  T  L  I  S  I  T  N  D  T

GAAACATCAAGCTCTGCCGTCTCCAACGATACTACACCTAAAGGATGGACCGGAGACAAC
 E  T  S  S  S  A  V  S  N  D  T  T  P  K  G  W  T  G  D  N

TCTCCAGGAATAGAAGCACTGTGTGCCATCTATATCACTTATGCTGTGATCATTTCAGTG
 S  P  G  I  E  A  L  C  A  I  Y  I  T  Y  A  V  I  I  S  V

GGCATCCTCGGAAATGCTATCCTCATCAAAGTCTTTTTCAAGACTAAATCCATGCAAACA
 G  I  L  G  N  A  I  L  I  K  V  F  F  K  T  K  S  M  Q  T

GTTCCAAATATTTTCATCACCAGCCTGGCTTTTGGAGATCTGTTACTCCTGCTGACTTGT
 V  P  N  I  F  I  T  S  L  A  F  G  D  L  L  L  L  T  C

GTGCCAGTGGATGCAACCCACTACCTGGCAGAGGGATGGCTGTTtGGAAAGGTCGGTTGT
 V  P  V  D  A  T  H  Y  L  A  E  G  W  L  F  G  K  V  G  C

AAAGTGCTTTCCTTCATCCGGCTCACTTCTGTCGGTGTATCAGTGTTCACGCTGACAATT
 K  V  L  S  F  I  R  L  T  S  V  G  V  S  V  F  T  L  T  I

CTCAGCGCTGACAG gtgagtttcttttctctgttctatttgccgggatatgaaattagg
 L  S  A  D  R

-- (intron 1, ~2 kb) --  tcctatgtgtcactgatttcaactctgcctgtgat

tctgtgtttctag ATACAAAGCAGTCGTGAAGCCACTTGAACGACAGCCCTCCAATGCC
                Y  K  A  V  V  K  P  L  E  R  Q  P  S  N  A

ATTCTGAAGACCTGTGCCAAAGCTGGTGGCATCTGGATCATGGCTATGATATTTGCTCTG
 I  L  K  T  C  A  K  A  G  G  I  W  I  M  A  M  I  F  A  L

CCAGAGGCTATATTCTCAAATGTATACACTTTCCAAGATCCTAACAGAAACGTAACATTT
 P  E  A  I  F  S  N  V  Y  T  F  Q  D  P  N  R  N  V  T  F

GAATCCTGTAACTCCTACCCTATCTCTGAGAGGCTTTTGCAGGAAATACATTCTCTGTTG
 E  S  C  N  S  Y  P  I  S  E  R  L  L  Q  E  I  H  S  L  L

TGTTTCTTGGTGTTCTACATTATCCCGCTCTCGATTATCTCTGTCTATTATTCTTTGATT
 C  F  L  V  F  Y  I  I  P  L  S  I  I  S  V  Y  Y  S  L  I

GCCAGGACTCTTTACAAAAGCACCTTGAACATACCGACTGAGGAACAAAGCCATGCCCGA
 A  R  T  L  Y  K  S  T  L  N  I  P  T  E  E  Q  S  H  A  R

AAGCAG gtatgtataaattggtactcctgcaaatacagcctgaatttacgagtgagccc
 K  Q

-- (intron 2, ~ 2 kb) --  gtgtgtgtgtgtgtgtgtttatttttctcccc

atgtagATTGAATCCCGGAAGAGAATTGCCAAAACGGTACTGGTGCTGGTGGCTCTGTTC
        I  E  S  R  K  R  I  A  K  T  V  L  V  L  V  A  L  F

GCACTCTGCTGGTTGCCGAATCACCTCCTGTATCTCTATCACTCATTCACTTATGAAAGC
 A  L  C  W  L  P  N  H  L  L  Y  L  Y  H  S  F  T  Y  E  S

TACGCAGAGCCTTCTGATGTCCCTTTCGTTGTCACCATTTTCTCTCGGGTGCTGGCTTTC
 Y  A  E  P  S  D  V  P  F  V  V  T  I  F  S  R  V  L  A  F

AGTAATTCCTGCGTGAACCCCTTTGCTCTGTATTGGCTGAGCAAGACCTTCCAGAAGCAT
 S  N  S  C  V  N  P  F  A  L  Y  W  L  S  K  T  F  Q  K  H

TTTAAGGCTCAGCTCTGCTGCTTCAAGGCAGAGCAGCCTGAACCTCCTCTTGGTGACACC
 F  K  A  Q  L  C  C  F  K  A  E  Q  P  E  P  P  L  G  D  T

CCCCTTAACAACCTCACTGTGATGGGGCGGGTTCCAGCTACTGGGAGTGCACACGTCTCT
 P  L  N  N  L  T  V  M  G  R  V  P  A  T  G  S  A  H  V  S

GAAATTAGCGTGACCCTGTTTAGTGGCAGTACTGCCAAGAAAGGAGAGGACAAAGTTTAG
 E  I  S  V  T  L  F  S  G  S  T  A  K  K  G  E  D  K  V  *

ATGTTCCATTTAAAATGATTTTAAGTCTCACTGTGTGTATCCAGCTTTAAGCTGTGCATA
```

b)

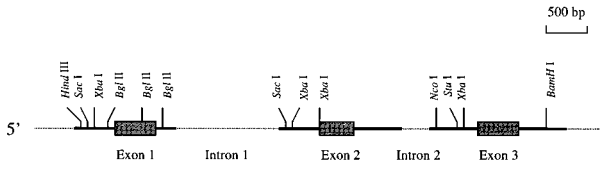

FIGURE 1: (a) Genomic nucleotide sequence and the predicted amino acid sequence of rat BRS-3. The exon/intron junctions were determined by comparing the cDNA and the genomic DNA sequences. The putative transmembrane regions are underlined. (b) Restriction map of the rat BRS-3 gene.

RESULTS

Cloning of Rat BRS-3. Rat BRS-3 cDNA was cloned from brain hypothalamus by homologous RT-PCR based on the published mouse sequence (*10*). To rule out PCR errors, a total of six independent PCR reactions were carried out, and the nucleotide sequence of two clones from each reaction was determined. In addition, the genomic DNA of rat BRS-3 was cloned through library screening. The genomic nucleotide sequence agreed with the cDNA sequence in their respective common regions.

Figure 1 shows the nucleotide and the predicted peptide sequences of rat BRS-3. The exon/intron junction sites were determined by comparing genomic and cDNA sequences. A single open reading frame of 1200 bp is encoded in three exons. The presumed peptide of 399 amino acids has seven predicted α-helical transmembrane domains containing highly conserved motifs, as observed in other members of the GPCR family.

At the amino acid level, rat BRS-3 is 92%, 80%, and 77% identical to mouse, human, and sheep homologues, respectively. The divergence in amino acid sequences is clustered in the N- and C-terminal regions, and the second and the third extracellular loops (Figure 2). Variations in the extracellular domains result in a species-dependent difference in ligand binding properties, as demonstrated below. The sequence variations in the C-terminal tail result in changes in potential phosphorylation sites which might affect receptor desensitization and regulation. Compared with other members of the bombesin receptor family, rat BRS-3 is 51% and 55% identical to rat BB1 (NMB receptor) and rat BB2 (GRP receptor), respectively.

Distribution of Rat BRS-3 mRNA. Tissue-specific expression of BRS-3 mRNA was studied by Northern blotting analysis using the rat BRS-3 cDNA coding region as a probe. Abundant expression of BRS-3 was detected in rat testis, and weak expression was detected in brain. In rat testis and brain, the BRS-3 message was expressed as an approximately 4 kb mRNA (Figure 3).

In the present study, the distribution pattern of BRS-3 mRNA in the rat brain was examined by in situ hybridization. Overall, the expression of BRS-3 mRNA is restricted, and is mainly detected in the medial habenula nucleus and various hypothalamic nuclei including the paraventricular nucleus (PVN), the dorsal hypothalamic area (DA), the posterior part of the anterior hypothalamic area (AHP), the dorsomedial hypothalamic nucleus (DMH), the ventromedial hypothalamic nucleus (VMH), and the lateroposterior arcuate nucleus (Figure 4). Weak to moderate hybridization was also observed in the vertical and horizontal limbs of the diagonal band, the bed nucleus of the stria terminalis, the medial amygdaloid nucleus, and the lateral parabrachial nucleus. Strong expression of BRS-3 was observed in the rat medial habenula nucleus, which was not reported in the mouse. The functional significance of BRS-3 in the medial habenula remains unknown at the present time.

Functional Expression of Rat BRS-3. The functional properties of rat BRS-3 were characterized in transfected 293EBNA cells. Since the natural agonist of BRS-3 has not been found, members of the bombesin-like peptide family, including GRP, NMB, bombesin, and the synthetic dY-bombesin, were used to stimulate rat BRS-3. As expected, each of the four peptides at 10 μM stimulated a significant increase in phosphatidylinositol (PI) hydrolysis, as indicated by inositol monophosphate production in the cell. The most efficacious peptide was dY-bombesin, which stimulated a 9-fold increase in PI hydrolysis, followed by NMB, bombesin, and GRP (Figure 5). As a control, nontransfected 293EBNA cells did not give any PI hydrolysis response to the four peptides tested (data not shown).

The functional activity of rat BRS-3 was further evaluated in the calcium mobilization assay using a FLIPR. A transient increase in intracellular calcium concentration, as indicated by Fluo-3 fluorescence, was observed when the receptor was stimulated with 10 μM GRP, NMB, bombesin, and dY-

```
                1                                                   50
Rat-BRS3        MSQRQPQSPN QTLISITNDT ETSSSAVSND TTPKGWTGDN SPGIEALCAI
MouseBRS3       MSQRQSQSPN QTLISITNDT ETSSSVAPND TTHKGWTGDN SPGIEALCAI
Hum-BRS3        MAQRQPHSPN QTLISITNDT ESSSSVVSND NTNKGWSGDN SPGIEALCAI

               51                                                  100
Rat-BRS3        YITYAVIISV GILGNAILIK VFFKTKSMQT VPNIFITSLA FGDLLLLLTC
MouseBRS3       YITYAGIISV GILGNAILIK VFFKTKSMQT VPNIFITSLA FGDLLLLLTC
Hum-BRS3        YITYAVIISV GILGNAILIK VFFKTKSMQT VPNIFITSLA FGDLLLLLTC
                     TM1                                TM2
               101                                                 150
Rat-BRS3        VPVDATHYLA EGWLFGKVGC KVLSFIRLTS VGVSVFTLTI LSADRYKAVV
MouseBRS3       VPVDATHYLA EGWLFGKVGC KVLSFIRLTS VGVSVFTLTI LSADRYKAVV
Hum-BRS3        VPVDATHYLA EGWLFGRIGC KVLSFIRLTS VGVSVFTLTI LSADRYKAVV
                                          TM3
               151                                                 200
Rat-BRS3        KPLERQPSNA ILKTCAKAGG IWIMAMIFAL PEAIFSNVYT FQDPNRNVTF
MouseBRS3       KPLERQPPNA ILKTCAKAGG IWIVSMIFAL PEAIFSNVYT FQDPNRNVTF
Hum-BRS3        KPLERQPSNA ILKTCVKAGC VWIVSMIFAL PEAIFSNVYT FRDPNKNMTF
                                          TM4
               201                                                 250
Rat-BRS3        ESCNSYPISE RLLQEIHSLL CFLVFYIIPL SIISVYYSLI ARTLYKSTLN
MouseBRS3       ESCNSYPISE RLLQEIHSLL CFLVFYIIPL SIISVYYSLI ARTLYKSTLN
Hum-BRS3        ESCTSYPVSK KLLQEIHSLL CFLVFYIIPL SIISVYYSLI ARTLYKSTLN
                                          TM5
               251                                                 300
Rat-BRS3        IPTEEQSHAR KQIESRKRIA KTVLVLVALF ALCWLPNHLL YLYHSFTYES
MouseBRS3       IPTEEQSHAR KQIESRKRIA KTVLVLVALF ALCWLPNHLL YLYHSFTYES
Hum-BRS3        IPTEEQSHAR KQIESRKRIA RTVLVLVALF ALCWLPNHLL YLYHSFTSQT
                                          TM6
               301                                                 350
Rat-BRS3        YAEPSDVPFV VTIFSRVLAF SNSCVNPFAL YWLSKTFQKH FKAQLCCFKA
MouseBRS3       YANHSDVPFV LIIFSRVLAF SNSCVNPFAL YWLSKTFQQH FKAQLCCLKA
Hum-BRS3        YVDPSAMHFI FTIFSRVLAF SNSCVNPFAL YWLSKSFQKH FKAQLFCCKA
                                    TM7
               351                                                 400
Rat-BRS3        EQPEPPLGDT PLNNLTVMGR VPATGSAHVS EISVTLFSGS TAKKGEDKV.
MouseBRS3       EQPEPPLGDI PLNNLTVMGR VPATGSAHVS EISVTLFSGS SAKKGEDKV.
Hum-BRS3        ERPEPPVADT SLTTLAVMGT VPGTGSIQMS EISVTSFTGC SVKQAEDRF.
```

FIGURE 2: Alignment of rat, mouse, and human BRS-3 peptide sequences. The putative transmembrane regions are underlined. The putative N-terminal region, the second extracellular loop, and the third extracellular loop are outlined by a box. Bold residues in the third extracellular loop indicate positions of those residues mutated by site-directed mutagenesis as shown in Table 1.

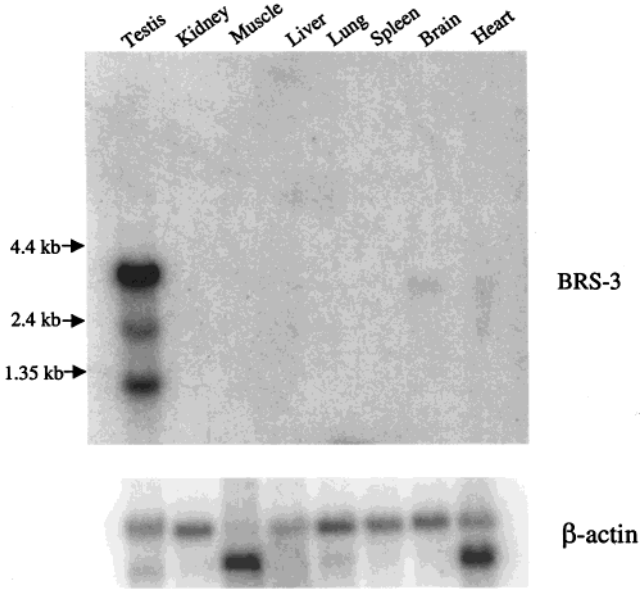

FIGURE 3: Northern blotting analysis of BRS-3 mRNA expression in different rat tissues with the full-length random-primed probe.

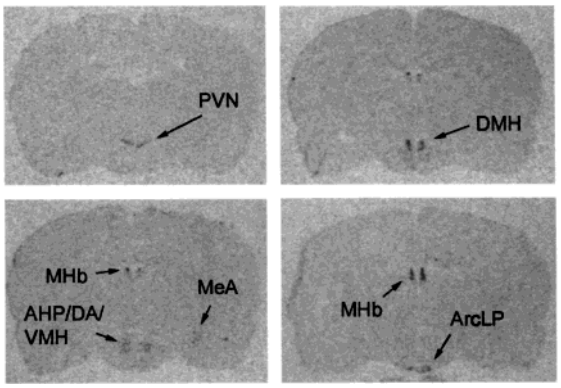

FIGURE 4: In situ hybridization pattern of BRS-3 mRNA in the rat brain. Signals marked by the arrowheads are specific since they can be completely blocked by the addition of a 100-fold molar excess of unlabeled probes. AHP, posterior part of the anterior hypothalamic area; ArcLP, lateroposterior arcuate nucleus; DA, the dorsal hypothalamic area; DMH, the dorsomedial hypothalamic nucleus; MeA, medial amygdaloid nucleus; MHb, medial habenula nucleus; PVN, paraventricular hypothalamic nucleus; VMH, the ventromedial hypothalamic nucleus.

bombesin. This calcium response is believed to be downstream of inositol triphosphate (IP3) generation and results from release of calcium from intracellular storages. These peptides showed low potency at rat BRS-3. At concentrations below 1 μM, none of the four peptides could stimulate a significant calcium response.

Pharmacological Differences between Rat and Human BRS-3. The synthetic peptide dY-bombesin was reported to have a nanomolar high affinity and high potency for human

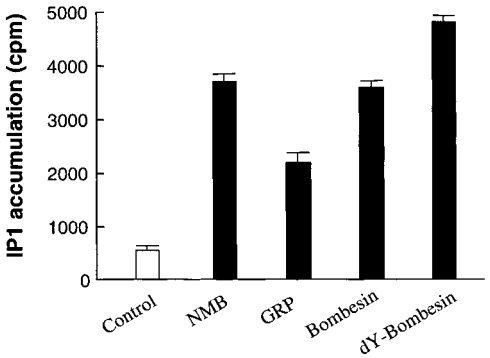

FIGURE 5: Functional activity of rat BRS-3 in transfected 293EBNA cells. Stimulation of phosphatidylinositol hydrolysis by peptide agonists, GRP, NMB, bombesin, and dY-bombesin at 10 μM. The values are mean ± SE of triplicates.

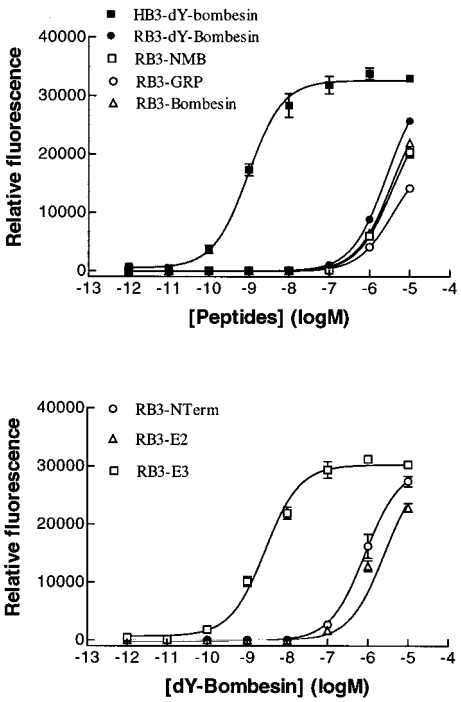

FIGURE 6: Stimulation of calcium mobilization through activation of rat, human, and chimeric BRS-3 receptors by dY-bombesin. Changes in Fluo-3 fluorescence, an indicator of intracellular calcium concentration, were measured by a FLIPR. The data shown are representative of 3−5 experiments. The results of multiple experiments and receptor abbreviations are summarized in Table 1. Top panel: Comparison of rat BRS-3 (open symbols) and human BRS-3 (closed symbols). Lower panel: Functional activities of chimeric rat BRS-3. RB3-Nterm represents the chimeric rat BRS-3 in which the N-terminal sequence has been replaced with the equivalent sequence from the human BRS-3. RB3-E2 and BR3-E3 are as defined in Table 1.

BRS-3 (*5*, *7*). However, we could not detect specific binding of the [^{125}I]dY-bombesin to the rat BRS-3 or potent activation of rat BRS-3 by dY-bombesin, while we confirmed the high affinity of this peptide for human BRS-3. Species-dependent variation in the response to these peptide ligands might account for this discrepancy. We therefore compared rat BRS-3 with its human and mouse homologue in the same expression system and found a ~1000-fold difference in dY-bombesin potency between human and rodent BRS-3. As shown in Figure 6, dY-bombesin potently activated human BRS-3 with an EC_{50} value of 1.2 ± 0.4 nM, whereas it poorly activated rat BRS-3 with an EC_{50} value of approximately

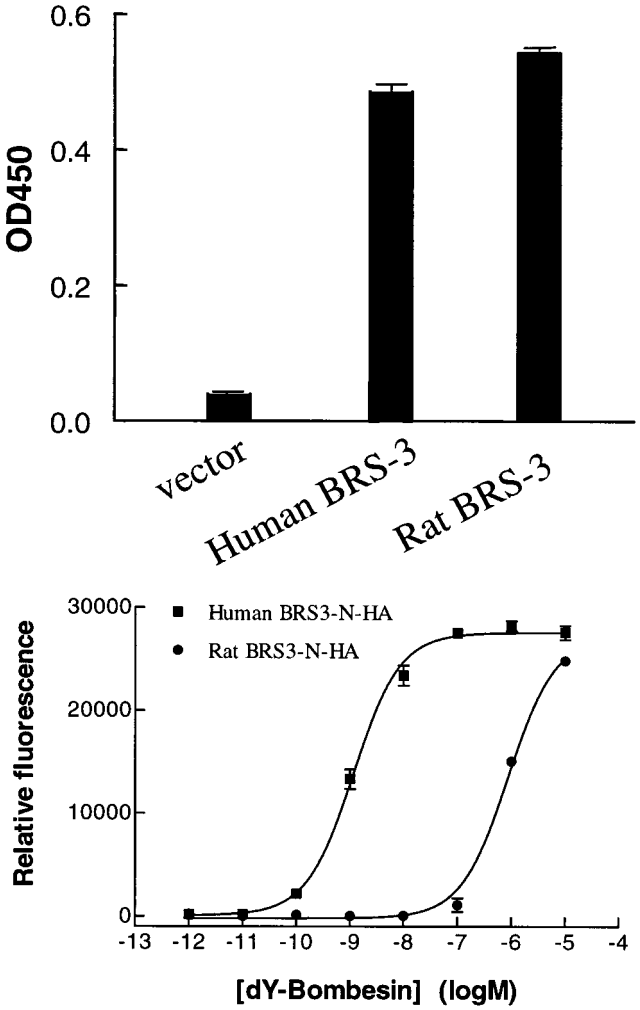

FIGURE 7: Top panel: The expression levels of HA-epitope-tagged rat and human BRS-3 on the surface of transfected 293EBNA cells measured by ELISA using a monoclonal antibody against HA-epitope. Lower panel: Stimulation of calcium mobilization of the HA-tagged rat or human BRS-3.

2 μM. Similar to rat BRS-3, dY-bombesin was also a poor agonist for mouse BRS-3 (EC_{50} in the micromolar range, data not shown).

One other possible explanation for the different potency of dY-bombesin for human BRS-3 and rat BRS-3 is that the observed difference in dY-bombesin potency might simply be due to a much lower level of expression of rat BRS-3 as compared to that of the human homologue. To test this hypothesis, we added a HA-epitope tag to the N-terminus of both rat and human receptors and quantitated the receptor expressed on the cell surface using a monoclonal antibody against the HA-epitope. The result of the ELISA experiments is shown in Figure 7. Rat BRS-3 was expressed at a high level in transfected 293EBNA cells, and the expression level was similar to that of human BRS-3. We next confirmed that the difference in dY-bombesin potency was also observed in tagged receptors. In contrast, the natural peptides GRP, NMB, and bombesin all had poor activation EC_{50} values (>1 μM) for both human and rat BRS-3. Both the immunochemical data and the lack of species difference for the three natural peptides argue against the possibility that different receptor densities might be responsible for the observed dY-bombesin potency difference between species.

Table 1: Functional Activities of dY-Bombesin at Wild-Type and Mutant BRS-3 Receptors[a]

receptors	abbreviation	mutations	EC_{50} (nM)
rat BRS-3	RB3	/	2200 ± 550
human BRS-3	HB3	/	1.2 ± 0.4
rat BRS-3/E2	RB3-E2	switch E2 loop	990 ± 140
rat BRS-3/E3	RB3-E3	switch E3 loop	2.8 ± 0.6
rat BRS-3/SQT	RB3-SQT	$Y^{298}E^{299}S^{300} \rightarrow S^{298}Q^{299}T^{300}$	390 ± 80
rat BRS-3/VD	RB3-VD	$A^{302}E^{303} \rightarrow V^{302}D^{303}$	930 ± 150
rat BRS-3/AMH	RB3-AMH	$D^{306}V^{307}P^{308} \rightarrow A^{306}M^{307}H^{308}$	370 ± 70
rat BRS-3/IF	RB3-IF	$V^{310}V^{311} \rightarrow I^{310}F^{311}$	1300 ± 230

[a] The EC_{50} values were mean ± SE calculated from dose−response curves of 3−5 repeated experiments using FLIPR assays.

The Roles of Extracellular Loops. It has been demonstrated extensively that the extracellular and the outer portion of transmembrane domains of G protein-coupled receptors are directly involved in ligand binding and induction of receptor conformational changes that trigger downstream signaling cascades (*17*). Alignment of rat, mouse, and human BRS-3 peptide sequences shows that variation in amino acid sequences is mostly located in N- and C-terminal regions, E2 and E3 loops (Figure 2). The C-terminal tail is unlikely to participate in ligand binding. The species-dependent pharmacological difference of BRS-3 may therefore be dictated by the amino acid differences in the extracellular domains.

To determine the structural basis of the marked difference in dY-bombesin potencies between rat and human BRS-3, we constructed three chimeric receptor mutants in which the N-terminal region, E2 loop, or E3 loop of rat BRS-3 was replaced with the corresponding human BRS-3 sequence. These chimeric receptors, RB3-Nterm, RB3-E2, and RB3-E3, along with wild-type rat and human BRS-3 were transfected into 293EBNA cells and functionally characterized by calcium mobilization assays using FLIPR. Switching the N-terminal region or the second extracellular loop did not significantly change the receptor property (Figure 6, lower panel). However, switching the third extracellular loop of rat BRS-3 resulted in a chimeric receptor, RB3-E3, that behaved almost identically to human BRS-3. RB3-E3 could be activated by dY-bombesin with a high potency ($EC_{50} = 2.8 \pm 0.6$ nM) which was close to that of human BRS-3 ($EC_{50} = 1.2 \pm 0.4$ nM) (see Table 1).

The binding activities of the chimeric BRS-3 receptors were also evaluated using [^{125}I]dY-bombesin. Similar to wild-type rat BRS-3, RBB3-Nterm and RBB3-E2 did not exhibit a detectable specific binding signal with 0.1−2 nM [^{125}I]-dY-bombesin in either membrane or whole cell binding assays. In contrast, the chimeric receptor RBB3-E3 showed a significant binding of [^{125}I]dY-bombesin that could be competitively inhibited by unlabeled dY-bombesin with a high affinity ($K_i = 1.2 \pm 0.7$ nM). Such a high affinity was almost identical to human BRS-3 ($K_i = 1.8 \pm 0.6$ nM). Similar to human BRS-3, RB3-E3 bound GRP, NMB, bombesin, and phyllolitorin with low micromolar affinities (Figure 8).

Among the 17 amino acids in the E3 loop, 10 are conserved in rodents but different in human BRS-3 (see Figure 2). To determine which amino acids in the E3 loop of human BRS-3 conferred dY-bombesin high-affinity binding, several substitution BRS-3 mutants were made in which

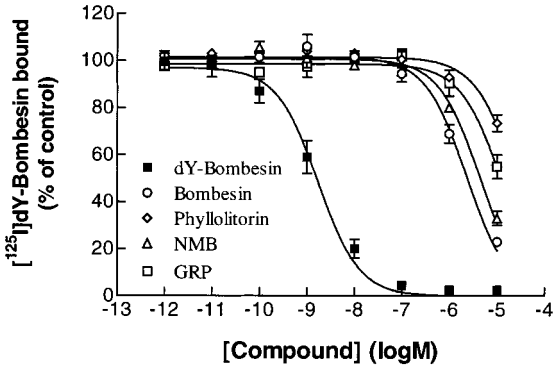

FIGURE 8: Binding activity of the chimeric receptor RBB3-E3 using 0.25 nM [^{125}I]dY-bombesin. Similar results were obtained from two repeated experiments.

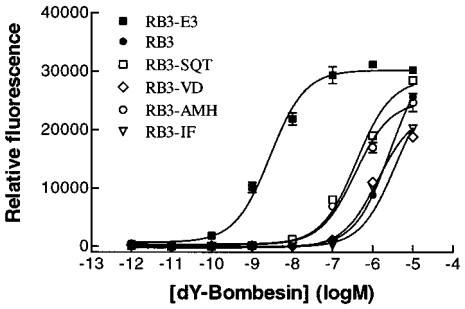

FIGURE 9: Functional activity of dY-bombesin on rat BRS-3 substitution mutants. Changes in Fluo-3 fluorescence were measured by a FLIPR. The data shown are representative of three experiments. The results of multiple experiments and receptor abbreviations are summarized in Table 1.

the amino acids in the E3 loop of rat BRS-3 were substituted with equivalent residues from human BRS-3. As shown in Figure 9 and Table 1, mutation of $Y^{298}E^{299}S^{300}$ to $S^{298}Q^{299}T^{300}$ (RB3-SQT) or $D^{306}V^{307}P^{308}$ to $A^{306}M^{307}H^{308}$ (RB3-AMH) partially mimicked the effect of switching the entire E3 loop. The potency of dY-bombesin in stimulation of the receptor-mediated calcium mobilization was improved by 5- and 6-fold, respectively. On the other hand, mutations of $A^{302}E^{303}$ to $V^{302}D^{303}$ (RB3-VD) or $V^{310}V^{311}$ to $I^{310}F^{311}$ (RB3-IF) had little effect on the dY-bombesin potency. These results indicate that substitution of the entire E3 loop is sufficient to dictate the binding affinity of dY-bombesin, and substitution of only three residues in the E3 loop is not sufficient to confer high-affinity dY-bombesin binding to the rat BRS-3.

DISCUSSION

The present study characterized the rat BRS-3 anatomically and pharmacologically. While BRS-3 has been found to be present in the brain in all species reported so far, the distribution of BRS-3 mRNA in peripheral tissues appears to be species-dependent. High levels of expression were detected in rat testis, but were barely detected in human and mouse testis and not found in sheep testis (*4*, *9*, *18*). BRS-3 expression was detected in guinea pig and human uterus, but not in mouse and sheep uterus (*19*, *20*).

The distribution pattern of BRS-3 mRNA in different human brain regions has not been well studied. Here, we found that the pattern of rat hypothalamic localization of BRS-3 mRNA resembles that described in the mouse (*18*), and is in support of its role in the regulation of feeding and energy balance in rodents.

While the synthetic peptide dY-bombesin is a high-affinity ligand for human BRS-3, surprisingly it possesses low affinity for the rat BRS-3. Although current technologies do not allow direct comparison of the affinity and activation potency of heterologously expressed BRS-3 to those of BRS-3 in native neurons, species-dependent pharmacological properties have been documented for some NK1 receptor ligands both in vitro and in vivo (*21*). Through mutational analysis, we found that the substitution of the third extracellular loop in the rat BRS-3 with the corresponding human BRS-3 sequence can confer high affinity to dY-bombesin. Such a species-dependent pharmacology is specific to the synthetic dY-bombesin since three natural peptides tested here possess low affinity for both human and rat BRS-3. Furthermore, substitution of only three residues in the third extracellular loop is not sufficient to confer high-affinity binding to dY-bombesin binding to the rat BRS-3. While the present data do not prove a direct interaction between dY-bombesin and the third extracellular loop, the specificity of the observed effects pertaining to only dY-bombesin but not other natural peptides suggests that the third loop is at least intimately connected to the peptide binding site. Additional residues near the third extracellular loop will likely be part of the binding site. This model is consistent with the observation that two residues in the top portion of helix 6 and helix 7 are critical for NMB and GRP binding (*22*, *23*). However, BB1 receptor and BB2 receptor do not share similar sequence in the E3 region with human BRS-3, suggesting that the high-affinity binding of dY-bombesin to BB1 or BB2 receptor is not dependent on the E3 loop.

In summary, we have shown that the rat BRS-3 possesses different pharmacological properties when compared to the human BRS-3. Rat BRS-3 has substantially lower affinity for dY-bombesin than human BRS-3. Such a drastic difference results from the variations in the amino acid sequence of the third extracellular loop of the receptor. These data indicate that other as yet unidentified ligands may also interact differently with BRS-3 depending on the species origin of BRS-3.

ACKNOWLEDGMENT

We thank Anna Sirotina-Meisher, Dr. Robert C. Boltz, and Cathy Huang for technical assistance, and Dr. James Battey at the National Institutes of Health for the human BRS3 plasmid.

REFERENCES

1. Anastasi, A., Erspamer, V., and Bucci, M. (1971) Isolation and structure of bombesin and alytesin, 2 analogous active peptides from the skin of the European amphibians *Bombina* and *Alytes*. *Experientia 27*, 166−167.
2. Erspamer, V. (1988) Discovery, isolation, and characterization of bombesin-like peptides. *Ann. N.Y. Acad. Sci. 547*, 3−9.
3. Battey, J., and Wada, E. (1991) Two distinct receptor subtypes for mammalian bombesin-like peptides. *Trends Neurosci. 14*, 524−528.
4. Fathi, Z., Corjay, M. H., Shapira, H., Wada, E., Benya, R., Jensen, R., Viallet, J., Sausville, E. A., and Battey, J. F. (1993) BRS-3: a novel bombesin receptor subtype selectively expressed in testis and lung carcinoma cells. *J. Biol. Chem. 268*, 5979−5984.
5. Pradhan, T. K., Katsuno, T., Taylor, J. E., Kim, S. H., Ryan, R. R., Mantey, S. A., Donohue, P. J., Weber, H. C., Sainz, E., Battey, J. F., Coy, D. H., and Jensen, R. T. (1998) Identification of a unique ligand which has high affinity for all four bombesin receptor subtypes. *Eur. J. Pharmacol. 343*, 275−287.
6. Kroog, G. S., Jensen, R. T., and Battey, J. F. (1995) Mammalian bombesin receptors. *Med. Res. Rev. 15*, 389−417.
7. Ryan, R. R., Weber, H. C., Hou, W., Sainz, E., Mantey, S. A., Battey, J. F., Coy, D. H., and Jensen, R. T. (1998) Ability of various bombesin receptor agonists and antagonists to alter intracellular signaling of the human orphan receptor BRS-3. *J. Biol. Chem. 273*, 13613−13624.
8. Ohki-Hamazaki, H., Watase, K., Yamamoto, K., Ogura, H., Yamano, M., Yamada, K., Maeno, H., Imaki, J., Kikuyama, S., Wada, E., and Wada, K. (1997) Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature 390*, 165−169.
9. Whitley, J. C., Moore, C., Giraud, A. S., and Shulkes, A. (1999) Molecular cloning, genomic organization and selective expression of bombesin receptor subtype 3 in the sheep hypothalamus and pituitary. *J. Mol. Endocrinol. 23*, 107−116.
10. Weber, H. C., Hampton, L. L., Jensen, R. T., and Battey, J. F. (1998) Structure and chromosomal localization of the mouse bombesin receptor subtype 3 gene. *Gene 211*, 125−131.
11. Liu, J., Gallagher, M., Horlick, R. A., Robbins, A. K., and Webb, M. L. (1998) A time-resolved fluorometric assay for galanin receptors. *J. Biomol. Screen. 3*, 199−206.
12. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Mannual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Guan, X.-M., Yu, H., and Van der Ploeg, L. H. T. (1998) Evidence of altered hypothalamic pro-opiomelanocortin/neuropeptide Y mRNA expression in tubby mice. *Mol. Brain Res. 59*, 273−279.
14. Fong, T. M. (1999) in *Structure−function analysis of G protein coupled receptors* (Wess, J., Ed.) pp 1−20, Wiley-Liss, New York.
15. Berridge, M. J., Dawson, M. C., Downes, C. P., Helson, J. P., and Irvine, R. F. (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochem. J. 212*, 473−482.
16. Schoneberg, T., Liu, J., and Wess, J. (1995) Plasma membrane localization and functional rescue of truncated forms of a G protein-coupled receptor. *J. Biol. Chem. 270*, 18000−18006.
17. Wess, J. (1999) *Structure−Function Analysis of G Protein-Coupled Receptors*, John Wiley, New York.
18. Ohki-Hamazaki, H., Wada, E., Matsui, K., and Wada, K. (1997) Cloning and expression of the neuromedin B receptor and the third subtype of bombesin receptor genes in the mouse. *Brain Res. 762*, 165−172.
19. Gorbulev, V., Akhundova, A., Grzeschik, K. H., and Fahrenholz, F. (1994) Organization and chromosomal localization of the gene for the human bombesin receptor subtype expressed in pregnant uterus. *FEBS Lett. 340*, 260−264.
20. Gorbulev, V., Akhundova, A., Buchner, H., and Fahrenholz, F. (1992) Molecular cloning of a new bombesin receptor subtype expressed in uterus during pregnancy. *Eur. J. Biochem. 208*, 405−410.
21. Fong, T. M., Yu, H., and Strader, C. D. (1992) Molecular basis for the species selectivity of the neurokinin-1 receptor antagonist CP-96,345 and RP67580. *J. Biol. Chem. 267*, 25668−25671.
22. Akeson, M., Sainz, E., Mantey, S. A., Jensen, R. T., and Battey, J. F. (1997) Identification of four amino acids in the gastrin-releasing peptide receptor that are required for high affinity agonist binding. *J. Biol. Chem. 272*, 17405−17409.
23. Sainz, E., Akeson, M., Mantey, S. A., Jensen, R. T., and Battey, J. F. (1998) Four amino acid residues are critical for high affinity binding of neuromedin B to the neuromedin B receptor. *J. Biol. Chem. 273*, 15927−15932.

BI0202777