Pharmacological Characterization of Relaxin-3/INSL7 Receptors GPCR135 and GPCR142 from Different Mammalian Species

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ABSTRACT

Relaxin-3 has recently been identified as a ligand for two structurally related G-protein-coupled receptors, human GPCR135 and GPCR142. This current study reports the characterization of mouse and rat GPCR135 as well as GPCR142 from mouse, monkey, cow, and pig at the molecular and pharmacological levels. Mouse and rat GPCR135 exhibit high homology (85%) to the human GPCR135 and have very similar pharmacological properties to that of the human GPCR135. Human and mouse/rat relaxin-3 both bind to and activate mouse, rat, and human GPCR135 at high affinity with IC50 or EC50 values close to 0.5 nM. In contrast, the mouse GPCR142 is less well conserved (74% homology) with human GPCR142. The rat GPCR142 gene was found to be a pseudogene. We further cloned GPCR142 genes from monkey, cow, and pig and found that they are highly homologous (>84%) to human GPCR142. Pharmacological characterization of GPCR142 from different species demonstrated that relaxin-3 binds to GPCR142 from different species at high affinity (IC50 < 5 nM). However, relaxin-3 does not stimulate a Ca2+ response in cells coexpressing Go16 and mouse GPCR142, whereas it does for cells expressing GPCR142 from other species tested. Our results suggest that GPCR142 may have a diminished role as a receptor for relaxin-3 in rodents, or perhaps GPCR142 functions as a receptor for another ligand in nonrodents. Boels and Schaller recently reported bradykinin as a ligand for GPCR142 (also known as GPR100). In this report, we demonstrate that bradykinin activates neither GPCR135 nor GPCR142, whereas relaxin-3 does.

Relaxin, a polypeptide hormone that belongs to the insulin superfamily, is involved in a number of functions in mammals, including the stimulation of tissue growth, differentiation, and remodeling during pregnancy (Sherwood, 1994). In addition, relaxin is involved in the inhibition of uterine contraction as well as the regulation of fluid balance (Bani, 1997). Although there is only one relaxin gene that is found in the mouse, two relaxin genes, H1 and H2 (which share greater than 70% homology) have been identified in humans (Hudson et al., 1983, 1984). Unlike the insulin and insulin-like growth factors, which signal through single-transmembrane, growth factor/tyrosine kinase receptors (Ullrich et al., 1985, 1986), relaxin has been recently identified as a ligand for two highly related leucine-rich repeat-containing G-protein-coupled receptors (LGRs), LGR7 and LGR8 (Hsu et al., 2002). LGR7 and LGR8 belong to the hormone receptor G-protein-coupled receptor (GPCR) subfamily and share significant homology to the luteinizing hormone receptor. This subclass of GPCRs normally has a long N-terminal extracellular domain (>300 amino acids) and is linked to cAMP stimulation (Gudermann et al., 1992; Shenker et al., 1993; Laugwitz et al., 1996; Hsu et al., 2002). Among the insulin/relaxin family of peptides, relaxin is likely the endogenous ligand for LGR7 (Hsu et al., 2002). Although relaxin also activates LGR8 in vitro (Hsu et al., 2002), multiple lines of evidence indicate that INSL3 (Adham et al., 1993) in fact is the physiological ligand for LGR8 (Gorlov et al., 2002; Kuma-gai et al., 2002; Bogatcheva et al., 2003; Kawamura et al., 2004). Receptors for INSL4 (Koman et al., 1996), INSL5 (Conklin et al., 1999), and INSL6 (Lok et al., 2000) have yet to be identified. Relaxin-3 (also known as INSLL7), the most recently identified member of the superfamily (Bathgate et al., 2002), is predominantly expressed in the brain (Bathgate et al., 2002).
et al., 2002; Burzin et al., 2002; Liu et al., 2003b) and has been reported to be an additional ligand for the relaxin receptor LGRL7 (Sudo et al., 2003).

We recently purified and identified relaxin-3/INSL7 from porcine brain as a ligand for two related human neuropeptide-like GPCRs, GPCR135 (Liu et al., 2003b; also known as SALPR, Matsumoto et al., 2000) and GPCR142 (Liu et al., 2003a; also known as GPR100, Fredriksson et al., 2003). GPCR135 and GPCR142 are typical type I GPCRs, which are coupled to cAMP inhibition and show no significant homology to LGR7 and LGR8. They are distantly related to peptide receptors such as somatostatin receptors and the angiotensin II receptor AT1. Pharmacological studies indicate that relaxin-3 binds and activates both human GPCR135 and human GPCR142 with high affinity and is the only known member of the relaxin/insulin superfamily that activates GPCR135 (Liu et al., 2003b). The predominant brain expression of both relaxin-3 and GPCR135, combined with pharmacological specificity of relaxin-3 as the only known ligand for GPCR135, strongly suggest that relaxin-3 is the endogenous ligand for GPCR135. Recently, the human GPCR142 (also known as GPR100) was reported to be a bradykinin receptor (Boels and Schaller, 2003).

Since limited information has been accumulated thus far for relaxin-3, GPCR135, and GPCR142, their functional roles in mammalian physiology remain unclear. Further characterization of relaxin-3, GPCR135, and GPCR142 from different mammalian species is necessary to elucidate the functional roles for these ligand/receptor systems. In this study, we report the cloning of the mouse and rat GPCR135 genes, as well as the monkey, bovine, porcine, and mouse GPCR142 genes. We also provide pharmacological evidence to support that relaxin-3 can function as a ligand for GPCR135 and GPCR142 from different species. In addition, we investigated whether bradykinin can activate GPCR135 or GPCR142.

Materials and Methods

General Polymerase Chain Reaction (PCR) Conditions. All PCRs, unless described otherwise, were performed using TaqDNA polymerase (Expand High Fidelity DNA Polymerase; Roche Diagnostics, Indianapolis, IN) at a condition of 94°C for 30 s for denaturing, 65°C for 30 s for annealing, and 72°C for 3 min for extension for 40 cycles.

Molecular Cloning of Mouse and Rat GPCR135 Genes. The mouse GPCR135 coding region was PCR-amplified from BALB/c mouse genomic DNA using the forward primer, 5′ AGG ATA CTC GAG GCC ACC ATG CAG GTG GCT TCT TCAACC CCC GCC GCG 3′ and reverse primer, 5′ ACT AGA TCT AGA TCA GTA GCC AGA GCT ACT AGG GAG CAG GT 3′. The rat GPCR135 gene has two putative translation starting codons (ATG). The first ATG in the rat GPCR135 gene is unique to the rat and leads to a seven-amino acid addition at the N terminus compared with human and mouse GPCR135; thus, the putative rat GPCR135 receptor that uses the first ATG as the translation starting codon is designated as rat GPCR135L. The second ATG in the rat GPCR135 gene is conserved among human, mouse, and rat and corresponds to the apparent translation starting codon for human and mouse genes. The putative rat GPCR135 receptor protein derived from using the second ATG as the translational starting codon is designated as rat GPCR135. The rat GPCR135L was PCR-amplified from Sprague-Dawley rat genomic DNA using the forward primer, 5′ AGG ATA CTC GAG GCC ACC ATG CAG GTG GCT TCT TCA ACC CCC GCC AAA GCC CAAC CTG AGC ATG CAA GT 3′ and reverse primer, 5′ AGG ATA TCT AGA TCA GTA GCC AGA GCT GT 3′.

GPCR135 and GPCR142 from different species. In addition, we investigated that relaxin-3 can function as a ligand for GPCR135 and as well as the monkey, bovine, porcine, and mouse GPCR142.

In mammalian physiology remain unclear. Further characterization of relaxin-3, GPCR135, and GPCR142, their functional roles

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Molecular Cloning of Mouse and Rat GPCR135 Genes. The mouse GPCR135 coding region was PCR-amplified from BALB/c mouse genomic DNA using the forward primer, 5′ AGG ATA CTC GAG GCC ACC ATG CAG GTG GCT TCT TCA ACC CCC GCC GCG 3′ and reverse primer, 5′ ACT AGA TCT AGA TCA GTA GCC AGA GCT ACT AGG GAG CAG GT 3′. The rat GPCR135 gene has two putative translation starting codons (ATG). The first ATG in the rat GPCR135 gene is unique to the rat and leads to a seven-amino acid addition at the N terminus compared with human and mouse GPCR135; thus, the putative rat GPCR135 receptor that uses the first ATG as the translation starting codon is designated as rat GPCR135L. The second ATG in the rat GPCR135 gene is conserved among human, mouse, and rat and corresponds to the apparent translation starting codon for human and mouse genes. The putative rat GPCR135 receptor protein derived from using the second ATG as the translational starting codon is designated as rat GPCR135. The rat GPCR135L was PCR-amplified from Sprague-Dawley rat genomic DNA using the forward primer, 5′ AGG ATA CTC GAG GCC ACC ATG CAG GTG GCT TCT TCA ACC CCC GCC AAA GCC CAAC CTG AGC ATG CAA GT 3′ and reverse primer, 5′ AGG ATA TCT AGA TCA GTA GCC AGA GCT GT 3′. The rat GPCR135 coding region was PCR-amplified from Sprague-Dawley rat genomic DNA using the forward primer, 5′ AGG ATA CTC GAG GCC ACC ATG CAG GTG GCT TCT TCA ACC CCC GCC GCA 3′ and reverse primer, 5′ AGG ATA TCT AGA TCA GTA GCC AGA GCT GT 3′. These PCR products were cloned into the mammalian expressing vector pcIneo (Promega, Madison, WI) between Xhol and Xbal sites, separately, and were sequenced to confirm the sequence identities.

Molecular Cloning of GPCR142 from Different Species. The monkey GPCR142 gene containing the complete coding region, the 5′-untranslated region (UTR), and the 3′ UTR was PCR-amplified from Macaca nemestrina monkey genomic DNA using two primers (forward primer, 5′ AGG TGG TGG GTG GTG CTT TCA ACC ACA 3′; reverse primer, 5′ CTG AAG GAT CCT ACA TCT GTG G 3′) designed according to the human GPCR142 gene sequence. The PCR was performed as described above with the exception of using an annealing temperature of 55°C. The monkey GPCR142 coding region sequence was obtained by direct sequencing of the PCR products. The monkey GPCR142 coding region was then PCR-amplified from monkey genomic DNA using the forward primer (5′ ACT AGA GAA TTC GCC ACC ATG CCC ACA CTC AAT ACT TCT TCT GCC T 3′) and reverse primer (5′ ACT AGA GCC GCC GCT TAC CCG GGC GTC CTT CCG TCC AGG A 3′) designed according to the newly assembled monkey GPCR142 sequence. The PCR product was cloned into the mammalian expression vector pcIneo between the EcoR1 and NotI sites. The insert region of the plasmid was sequenced to confirm the sequence identity.

The mouse GPCR142 coding region was PCR-amplified from BALB/c mouse genomic DNA using the forward primer, 5′ AGC ATA GAA TTC GCC ACC ATG GCC ACA TCA TCT TCT GCC TCC ATG CC 3′ and reverse primer, 5′ AGC ATA GGC GCC GCC GCT CAC ACT CCT TCT GCC GGC GAC ACA GCA G 3′, and the PCR product was cloned into the mammalian expression vector pcIneo between the EcoR1 and NotI sites. The insert region of the plasmid was sequenced to confirm the sequence identity.

The bovine and porcine GPCR142 genes were PCR-amplified from bovine and porcine genomic DNAs, respectively, using two primers (forward primer, 5′ ACC AAT CTC TGA TGC CCT GGC 3′; reverse primer, 5′ GAG TTG GGG ATC AAA GAT CAG ACT 3′), which are designed according to the human GPCR142 gene 5′ end and 3′ end UTRs. The PCRs were performed using 55°C as the annealing temperature. The PCR products were sequenced, and then the coding regions for the bovine and porcine GPCR142 genes were assembled.

The bovine GPCR142 complete coding region was then PCR-amplified from bovine genomic DNA using two primers (forward primer, 5′ ACT AGA GAA TTC GCC ACC ATG CCC ACC ACC AAC TCT TGC GC 3′; reverse primer, 5′ ACT AGA GGC GCC GCC CAC GAA GAG GAG GGG GTT TAA CTT GC 3′), which were designed according to the newly assembled bovine GPCR142 sequence, and the PCR product was cloned into pcIneo. Similarly, the porcine GPCR142 complete coding region was PCR-amplified from porcine genomic DNA using two primers (forward primer, 5′ ACT AGA GAA TTC GCC ACC ATG CCC ACA CTC AAT ACT TCT TCT GCC TCC ATG CC 3′; reverse primer, 5′ ACT AGA GGC GCC GCC CAC AAA GAG GAG GGG GTT TAA CTT GC 3′), which were designed according to the newly assembled porcine GPCR142 sequence and cloned into pcIneo. The inserts of the resulting clones were sequenced to confirm the identities, respectively.

Cloning, Expression, and Purification of Mouse/Rat Relaxin-3. The mouse and rat relaxin-3 mature peptides are identical and designated as mouse/rat (m/r) relaxin-3. The m/r relaxin-3 was produced recombinantly similar to human relaxin-3, as previously reported (Liu et al., 2003b). We used a two-step strategy to construct the relaxin-3 pro-peptide coding region with a mutation between the C-chain and the A-chain coding regions to create a preferred furin cleavage site for complete processing of the cleavage between the C-chain and the A-chain. The 5′ end of the rat relaxin-3 pro-peptide coding region that covers the B-chain and C-chain was PCR-amplified from rat brain cDNA (BD Biosciences, San Jose, CA) using two primers with the forward primer (P1), 5′ ACT GAG AAT TCG...
ATG ACG ACG ATA AGA GGC CCG CGC CCT AG
and reverse primer (P2), TCT CCG GCC CCG AAC CAC TGG CTA A.
The 3′ end was PCR-amplified from rat brain cDNA (BD Biosciences) using two primers (forward primer (P3), 5′ GAG TGG TTC GGG GGA GAG ATG T; reverse primer (P4), 5′ ATC GCG GAT CCC TAG AAC CTA 3′). The second step PCR was conducted using the PCR products of the resulting 5′ and 3′ ends as the template and the two primers P1 and P4 described above. The final PCR product was cloned into the modified expressing vector pCMV-sport1 containing an alpha peptide signal sequence and a FLAG-tag sequence as described (Liu et al., 2003b). The recombinant m/r relaxin-3 secreted into the culture medium was purified as described for human relaxin-3 (Liu et al., 2003b).

Reverse Transcriptase-PCR Detection of mRNA Tissue Expression Profiles for Mouse GPCR135, GPCR142, and Relaxin-3 and Rat GPCR135 and Relaxin-3. Two primers (forward primer, 5′ TGC TGG GCT TCC TGC TGC CGC TGA GCA TCA TCA 3′; reverse primer, 5′ CTG CGG GCC AGG TGG TCT GTA TTG GCT TCA 3′) designed according to the conserved regions between the mouse and rat GPCR135 coding regions were used to PCR-amplify the mouse and rat GPCR135 cDNA, respectively, using cDNA sources from different mouse and rat tissues (BD Biosciences). To detect the mouse and rat relaxin-3 mRNA expression in different tissues, two primers (forward primer, 5′ GAG TGG TTC GGG GCC GGA GAG ATG T; reverse primer, 5′ ATC GCG GAT CCC TAG CAC AAG CTG CTA 3′) designed according to the conserved regions of the mouse and rat relaxin-3 coding regions to amplify the mouse and rat relaxin-3 cDNA from different tissues (BD Biosciences), respectively. The mouse GPCR135 mRNA detection was performed using two primers (forward primer, 5′ GTG TGG CCG GCC TGT CTT CTG ATC AATA CCT TGT 3′; reverse primer, 5′ AGG GCA CGA TGA AGG CCA GAA CTA CCT TCT 3′) to amplify mouse cDNA from different tissue sources (BD Biosciences). As internal controls, mouse G3PDH primers and rat G3PDH primers (BD Biosciences) were used to PCR amplify (annealing temperature, 55°C) the mouse and rat G3PDH cDNA from different mouse and rat cDNA sources, respectively. The PCR products were run in a 2% agarose gel, stained with ethidium bromide, and then visualized under UV irradiation.

Saturation Binding Assay. Membranes from cells expressing relaxin-3 receptors were used for saturation binding assays using human [125I]relaxin-3 as the radioligand as described (Liu et al., 2003b). The results were analyzed by PRISM software (GraphPad Software Inc., San Diego, CA).

Competition Binding Assay. The assays were performed using human [125I]relaxin-3 (2200 Ci/mmol) as previously described (Liu et al., 2003a,b). Briefly, cell membranes from COS-7 cells transiently expressing GPCR135 or GPCR142 from different species were incubated with human [125I]relaxin-3 (final concentration, 100 pM) in the presence of various concentrations of unlabeled human relaxin-3. The reaction was incubated at room temperature for 1 h, and the membranes were filtered through the 96-well GPC plates (PerkinElmer Life and Analytical Sciences, Boston, MA) presaturated with 0.3% polyethylenimine (Sigma-Aldrich, St. Louis, MO) using a cell harvester (PerkinElmer Life and Analytical Sciences). The filter was washed with the ice-cold washing buffer (50 mM Tris-HCl, pH 7.5). Fifty microliters of Microscint 40 (PerkinElmer Life and Analytical Sciences) was added to each well, and the plates were counted on a Topcount NTX (PerkinElmer Life and Analytical Sciences). The results were analyzed using the PRISM soft-
ware (GraphPad Software Inc.). The IC$_{50}$ value is the concentration of unlabeled relaxin-3 producing 50% inhibition of specific human [${}^{125}$I]relaxin-3 binding.

**GTP$\gamma$S Binding Assays.** GTP$\gamma$S binding assays were performed using membranes from CHO-K1 (ATCC no. CCL-61) cells transiently expressing either GPCR135 or GPCR142 receptors from different species as previously reported (Liu et al., 2003a,b). The assays were carried out in 96-well plates in a final volume of 200 $\mu$L. Different ligands in various concentrations were added to cell membranes and incubated at room temperature for 20 min. [${}^{35}$S]GTP$\gamma$S (1250 Ci/mol; PerkinElmer Life and Analytical Sciences) was then added to each well at a final concentration of 200 pM. The reaction was further incubated at room temperature for 1 h and filtered through 96-well GFC plates using a cell harvester. The filters were washed with the ice-cold washing buffer (50 mM Tris-HCl, pH 7.5, and 10 mM MgCl$_2$). Fifty microliters of Microscint 40 (PerkinElmer Life and Analytical Sciences) was added to each well, and the plates were counted on a Topcount NTX (PerkinElmer Life and Analytical Sciences). The results were analyzed using the PRISM software (GraphPad Software Inc.). The EC$_{50}$ value is the concentration of ligand that induces 50% of the maximum specific [${}^{35}$S]GTP$\gamma$S incorporation.

**Ca$^{2+}$ Mobilization Assays.** HEK293 (ATCC no. CRL-1573) cells or CHO-K1 cells were transiently transfected with different receptor expression constructs using the transfection reagent Lipofectamine (Invitrogen). Two days after transfection, the transfected cells were seeded in 96-well black plates (BD Biosciences Discovery Labware, Bedford, MA) loaded with the Ca$^{2+}$-dye Fluo-3 (TEFLABS, Austin, TX) and stimulated with different concentrations of human relaxin-3 or human bradykinin (Bachem California, Torrance, CA). The ligand-stimulated Ca$^{2+}$ mobilizations were monitored using a FLIPR instrument (Molecular Devices, Sunnyvale, CA). The results were analyzed using the PRISM software (GraphPad Software Inc.). The EC$_{50}$ value is the concentration of ligand that induces 50% of the maximum Ca$^{2+}$ response.

**Results**

**GPCR135 is Highly Conserved among Different Species.** We previously reported that by searching the mouse and rat National Center for Biotechnology Information genomic sequence database, we identified putative mouse...
and rat GPCR135 genes (Liu et al., 2003a). In this report, we cloned both mouse and rat GPCR135 genes and confirmed their open reading frames. Both the mouse and rat GPCR135 genes appear to be intronless. The deduced receptor protein sequences are shown in Fig. 1. The putative mouse and rat GPCR135 receptors have 85% and 86% sequence identity to the human GPCR135, respectively, whereas they share higher homology (94%) with each other. Among the putative seven transmembrane domains (TM), TM2, TM3, and TM6 are conserved among human, mouse, and rat GPCR135. Between mouse and rat GPCR135, all but TM4 are completely conserved. The rat GPCR135 cDNA has two putative translational starting codons (ATG). The first ATG in the rat GPCR135 gene is unique to the rat and leads to a seven-amino acid (MPKAHLS) addition at the N terminus compared with that of human and mouse GPCR135; thus, the putative rat GPCR135 receptor that uses the first ATG as the translational starting codon is designated as rat GPCR135L. The second ATG in the rat GPCR135 gene is conserved among human, mouse, and rat and corresponds to the appa-
ent translation start site for human and mouse genes. The putative rat GPCR135 receptor protein derived from using the second ATG as the translational starting codon is designated as rat GPCR135. Both human GPCR135 and rat GPCR135 have 469 amino acids, whereas mouse GPCR135 has 472 amino acids with three extra amino acids between TM5 and TM6 (Fig. 1). The DNA sequences for mouse GPCR135, rat GPCR135, and rat GPCR135L have been deposited in GenBank (GenBank accession nos. AY633762, AY633763, and AY633764, respectively).

**GPCR142 Is Highly Conserved among Human, Monkey, Cow, and Pig; Less Conserved in Mouse; and a Pseudogene in Rat.** The mouse and rat GPCR142 genes were identified by searching the mouse and rat National Center for Biotechnology Information genomic sequence database, respectively, using human GPCR142 as the query (Liu et al., 2003a). In this report, the mouse GPCR142 gene was cloned, and its open reading frame was confirmed by DNA sequencing. The complete coding region of the mouse GPCR142 was deposited in GenBank (GenBank accession no. AY633765). Overall, the mouse GPCR142 shares 74% sequence identity to that of human. Human GPCR142 has 374 amino acids, whereas the mouse equivalent is longer with 414 amino acids. The biggest difference between human and mouse GPCR142 is located at the C termini, where the mouse GPCR142 has a different and much longer C-terminal tail (Fig. 2A). Searching the rat genomic database, the existence of a rat gene corresponding to GPCR142 was confirmed. Although the rat GPCR142 gene is highly homologous to mouse GPCR142 (Fig. 2B), its coding region is disrupted by multiple deletions and insertions. Since rat GPCR142 does not have an open reading frame to encode a functional GPCR, we conclude that rat GPCR142 is a pseudogene.

**Pharmacological Characterization and Comparison of Human, Mouse, and Rat GPCR135.** Previously, it was demonstrated that human relaxin-3 binds to and activates both human GPCR135 and GPCR142 with high affinity (Liu et al., 2003a,b). We tested whether human relaxin-3 also binds and activates recombinant mouse, rat GPCR135, and rat GPCR135L. In radioligand competition binding assays using human [125I]relaxin-3 as the tracer, human relaxin-3 specifically binds to both mouse and rat GPCR135 with high affinity. The observed IC50 values for recombinant mouse and rat GPCR135 are 0.52 ± 0.14 and 0.65 ± 0.21 nM, respectively (Fig. 3A). Rat GPCR135L displays an almost...
identical binding affinity for relaxin-3 compared with rat GPCR135 (data not shown). Similarly, in the same experiment, human relaxin-3 demonstrated an IC_{50} value of 0.42 ± 0.09 nM for human GPCR135. No specific binding was detected in mock-transfected control cells (data not shown).

Mouse and rat GPCR135 were further characterized using functional assays, including GTPγS binding and Ca^{2+} mobilization assays. The results show that relaxin-3 is a potent agonist for both receptors. In the GTPγS binding assay, human relaxin-3 demonstrated high agonist potency with EC_{50} values of 0.35 ± 0.04 nM for mouse GPCR135 and 0.49 ± 0.06 nM for rat GPCR135, respectively (Fig. 3B). In the same experiment, human relaxin-3 stimulated human GPCR135 with an EC_{50} value of 0.24 ± 0.05 nM. Rat GPCR135L was also evaluated in the GTPγS binding assay for its response to relaxin-3 stimulation and was observed to behave very similarly to that of rat GPCR135 (data not shown). In Ca^{2+} mobilization assays, relaxin-3 stimulates Ca^{2+} mobilization in the HEK293 cells coexpressing Gq5 and mouse or rat GPCR135 in a dose-dependent manner with EC_{50} values close to 5 to 10 nM (Fig. 3C), similar to those of human GPCR135.

**Pharmacological Characterization of GPCR142 from Different Species.** Competition binding experiments were

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**Fig. 3.** A, characterization of mouse, rat GPCR135 in comparison to human GPCR135 in radioligand competition binding assays. Membranes from COS-7 cells transiently expressing mouse or rat GPCR135 were incubated with 100 pM [125I]relaxin-3 with increasing concentrations of unlabeled human relaxin-3 as competitors. Specific binding was determined by subtracting the nonspecific binding from the total binding. Membranes from COS-7 cells expressing the human GPCR135 were included in the assays for comparison. B, functional characterization of the mouse, rat, and human GPCR135 using GTPγS binding assays. Membranes from CHO cells transiently expressing GPCR135 from different species were stimulated with various concentrations of human relaxin-3 in the presence of [35S]GTPγS. Membranes from CHO cells transfected by pcDNA were used as negative control. C, relaxin-3 stimulates Ca^{2+} mobilization in HEK293 cells coexpressing Gq5 and mouse or rat GPCR135. HEK293 cells coexpressing Gq5 and GPCR135 from mouse and rat, respectively, were seeded in 96-well black plates, loaded with Ca^{2+} dye Fluo-3, and then stimulated with various concentrations of human relaxin-3. The ligand-stimulated Ca^{2+} mobilizations were monitored using a FLIPR instrument. HEK293 cells expressing Gq5 alone, or coexpressing Gq5 and human GPCR135, were used as the negative and positive controls, respectively. Data represent the mean ± S.E.M. of duplicated experiments.
performed to compare the ligand binding properties for recombinant monkey, mouse, bovine, and porcine GPCR142 versus that of the human GPCR142. The result show that GPCR142 from all species tested binds human relaxin-3 with high affinity with IC_{50} values of 0.87 ± 0.64 nM for bovine, 1.34 ± 0.32 nM for monkey, 1.45 ± 0.22 nM for human, 1.59 ± 0.45 nM for porcine, and 5.45 ± 1 nM for mouse (Fig. 4A). No specific binding was observed in mock-transfected COS-7 cells (data not shown).

GPCR142 receptors from different species were compared in the functional assays, including GTP\_\gamma\_S binding and Ca^{2+} mobilization assays. In GTP\_\gamma\_S binding assays, human relaxin-3 potently stimulated all GPCR142 receptors with EC_{50} values of 0.45 ± 0.07 nM for bovine, 0.96 ± 0.15 nM for porcine, 0.98 ± 0.23 nM for human, 1.1 ± 0.2 nM for monkey, and 3.5 ± 0.76 nM for mouse, respectively (Fig. 4B). Previously, it was shown that relaxin-3 stimulates Ca^{2+} signaling in cells expressing human GPCR142 and G_{\alpha\_16} (Liu et al., 2003a). In this report, it was demonstrated that monkey, bovine, and porcine GPCR142, when coexpressed with G_{\alpha\_16}, behave similarly to that of human GPCR142 with the EC_{50} values ranging from 50 to 100 nM. However, relaxin-3 did not stimulate detectable Ca^{2+} response in cells expressing mouse GPCR142 and G_{\alpha\_16} (Fig. 4C). To investigate whether mouse recombinant GPCR142 is actually expressed in HEK293 cells, saturation binding assays were conducted using cells expressing mouse GPCR142 and G_{\alpha\_16} in comparison with cells expressing human GPCR142 and G_{\alpha\_16}. The results show that the expression levels of recombinant mouse GPCR142 in HEK293 cells are similar to that of human.

![Fig. 4.](image)
GPCR142, with expression density of approximately 13,000 fmol receptor/mg membrane protein for mouse GPCR142 and approximately 15,000 fmol receptor/mg membrane protein for human GPCR142.

Pharmacological Comparison of Mouse/Rat Relaxin-3 versus Human Relaxin-3. Relaxin-3 is highly conserved in the mouse, rat, human, and pig (Bathgate et al., 2002; Liu et al., 2003b). The mouse and rat relaxin-3 peptides are identical (designated as m/r relaxin-3) and share 92% sequence identity to human relaxin-3. Among the 51 amino acids that constitute the B-chain and A-chain of the relaxin-3, which are linked by two disulfide bridges, only four amino acids are different between the human and the m/r relaxin-3. The recombinant m/r relaxin-3 was produced and tested for its ligand activity at GPCR135 and GPCR142 from different species using GTPγS binding assays. The results indicate that overall the m/r relaxin-3 behaved very similarly to the human relaxin-3. It potently activated human, mouse, and rat GPCR135 with EC50 values of 0.34 ± 0.05, 0.55 ± 0.16, and 0.67 ± 0.21 nM, respectively (Fig. 5A). The m/r relaxin-3 also stimulated bovine GPCR142 (~0.5 nM), human, monkey, and porcine GPCR142 (1–2 nM each) and mouse GPCR142 (~5 nM) with the EC50 values shown in the parentheses, respectively (Fig. 5B).

Characterization of GPCR135 and GPCR142 Using Bradykinin as the Ligand. Recently, Boels and Schaller (2003) reported that bradykinin is a ligand for GPCR142 (also known as GPR100). In that report, Boels and Schaller (2003) reported that bradykinin stimulated a Ca2+ response in a CHO cell line stably expressing human GPCR142. In this study, we show that bradykinin stimulates detectable Ca2+ response in untransfected CHO cells and consistently stimulates much stronger Ca2+ responses in CHO cells transiently expressing human bradykinin receptor 2 (BK2), whereas it does not stimulate any significant Ca2+ responses that are specifically related to the expression of human GPCR135 or GPCR142 (Fig. 6A). In the same experiment, relaxin-3 stimulated strong Ca2+ responses in CHO cells that are specific to the coexpression of Gα16 and GPCR135 or Gα16 and GPCR142 (Fig. 6B). Similar results were obtained in experiments conducted in HEK293 cells. Bradykinin stimulates stronger Ca2+ response in control HEK293 cells compared with the control CHO cells, presumably due to the higher endogenous bradykinin receptor expression in HEK293 cells. Compared with the control HEK293 cells, HEK293 cells transiently expressing human bradykinin receptor BK2 responded to bradykinin stimulation with much stronger Ca2+ signals. In the same experiment, cells transiently expressing human GPCR135 or GPCR142 responded to bradykinin but not significantly different from that of the control cells (Fig. 6C). However, they responded robustly to relaxin-3 stimulation (Fig. 6D).

Discussion

The recent identification of relaxin as a ligand for LGR7 and LGR8 (Hsu et al., 2002), which demonstrated that a relaxin/insulin family of peptides could signal through a GPCR, has opened a new chapter for the research of relaxin and its related peptides. Although relaxin is likely the endogenous ligand for LGR7 (Hsu et al., 2002), a number of recent studies indicate that INSL3 is the endogenous ligand for LGR8. In addition to the fact that INSL3 activates LGR8 but not LGR7 (Kumagai et al., 2002), LGR8 has been shown to be the only receptor for INSL3 (Gorlov et al., 2002; Bogatcheva et al., 2003). The recent identification of relaxin-3 as a ligand for GPCR135 and GPCR142 (Liu et al., 2003a,b), which are typical type I GPCRs, has added complexity to the field, since relaxin-3 has also been shown to bind and activate LGR7 (Sudo et al., 2003). This apparent selection promiscuity is made even more confusing by possible species variations in the ligands and receptors. It remains to be determined whether the apparent cross talk between those ligands and receptors represents true physiological complex-

![Fig. 5. Mouse/rat relaxin-3 stimulates GTPγS incorporation in cells expressing GPCR135 (A) and GPCR142 (B) from different species. Membranes from CHO cells transiently expressing GPCR135 or GPCR142 from different species were stimulated with increasing concentrations of m/r relaxin-3. [35S]GTPγS was used as the tracer. Mock-transfected cells were used as the negative control. Data represent the mean ± S.E.M. of duplicated experiments.](image-url)
ity or whether it is just a phenomenon of in vitro testing. Regardless, it is clear that before one can begin to understand the true physiological role(s) of these ligands and receptors, one must first understand the pharmacology in the relevant species. In this report, we cloned the mouse and rat GPCR135, as well as the monkey, mouse, bovine, and porcine GPCR142, and provided an initial pharmacological characterization of those receptors. GPCR142 was cloned and characterized from monkey, cow, and pig in addition to the mouse because GPCR142 is a pseudogene in the rat. In addition, compared with GPCR135, mouse GPCR142 shows significant sequence difference from the human and behaves quite different from human GPCR142 in the in vitro pharmacological studies. We wondered whether GPCR142 is conserved in other mammals and whether the lack of a functional GPCR142 gene in the rat and the less conserved GPCR142 gene in the mouse is only represented in rodents.

Overall, pharmacologically the rat and the mouse GPCR135 receptors have very similar properties compared with that of human GPCR135 in terms of their relaxin-3 ligand binding affinity as well as signal transduction, suggesting that the functions of GPCR135 are highly conserved among species. The sequence comparison of GPCR135 among different species indicates that it is a very well conserved gene from human to Fugu fish. It has been reported that there are five copies of relaxin-3 gene in the Fugu genome (Hsu, 2003). Using the human GPCR135 as the query, we searched the Fugu fish genome and found five copies of genes (GenBank accession nos. CAAB01000018, CAAB01001312, CAAB01001814, CAAB01003934, and CAAB01004008) (Fig. 7A) that are highly homologous to the human GPCR135 (Fig. 7B). Like mammalian GPCR135, the DRY signature sequence appearing after the third transmembrane domain of many GPCRs is replaced with TRY in four copies of the Fugu fish

**Fig. 6.** Characterization of bradykinin as a possible ligand for GPCR135 and GPCR142. CHO cells (A and B) or HEK293 cells (C and D) transiently expressing bradykinin receptor 2 (BK2) or coexpressing G_{q,16} and GPCR135 or GPCR142 were stimulated either with bradykinin (A and C) or with human relaxin-3 (B and D). The ligand-stimulated intracellular Ca^{2+} mobilization was monitored using a FLIPR instrument. Untransfected cells or cells expressing G_{q,16} alone were used as the negative controls. Data represent mean ± S.E.M. of duplicated experiments.
Fig. 7. A, GPCR135-like sequences found from Fugu rubripes genome. The numbers (CAAB01000018, CAAB01001312, CAAB01001814, CAAB01003934, and CAAB01004008) indicate Genbank accession numbers for Fugu fish genomic sequences that contain the GPCR135-like genes. The putative transmembrane domains are underlined. The TRY or VRY sequences in Fugu GPCR135-like proteins, corresponding to the DRY signature sequence in many GPCRs, are highlighted in bold letters. B, homology degrees, expressed in percentage amino acid sequence identity, among human GPCR135 and GPCR142, and different GPCR135-like receptors found in Fugu fish.
genes (CAAB01000018, CAAB01001312, CAAB01003934, and CAAB01004008). In another copy of Fugu fish gene (CAAB01001814), the DRY sequence is replaced with VRY.

Previously, we have shown that relaxin-3 and GPCR135 are both predominantly expressed in the human brain, suggesting that relaxin-3 is the cognate ligand for GPCR135. In this study, supporting our hypothesis, we show that both relaxin-3 and GPCR135 have very similar tissue expressing profiles in both mouse and rat. In addition, we demonstrated that both GPCR135 and relaxin-3 from different species are highly conserved from genetic sequences to biological functions, which adds additional support that this ligand receptor pair is playing an important physiological role.

In contrast, GPCR142 receptors from different species show significant discrepancies. In binding assays, recombinant mouse GPCR142 consistently yielded much lower specificity binding compared with GPCR142 from other species. This is possibly due to its lower affinity for human relaxin-3, which is used as the radioligand. In GTPγS binding assays, the relaxin-3-stimulated signal was also consistently lower for mouse GPCR142 compared with GPCR142 from other species. Although the bovine GPCR142 demonstrated highest specificity binding in both radioligand binding as well as GTPγS binding assays, human, monkey, and porcine GPCR142 produced almost identical results. Pharmacologically, the most striking difference between mouse GPCR142 and GPCR142 from other species is its inability to couple with Gα16. Coexpression of Gα16 and GPCR142 from different species in HEK293 cells results in a marked Ca$^{2+}$ signal in response to relaxin-3. However, coexpression of mouse GPCR142 with Gα16 produces no Ca$^{2+}$ response in the Ca$^{2+}$ mobilization assays.

The lack of Ca$^{2+}$ signal in cells expressing mouse GPCR142 could be due to the poor coupling between the mouse GPCR142 and Gα16 in the artificial system. It is worth noting that mouse GPCR142 has a significantly longer and different C-terminal tail than GPCR142 from the other species (Fig. 2A). Genetically, compared with GPCR135, GPCR142 appears to be less conserved among species. We searched the Fugu rubripes genome database for GPCR142 related sequences and were unable to find any distinct genes other than the five GPCR135-like genes. Given that the TRY sequence after the third transmembrane domain of the Fugu fish receptors is conserved with that of human GPCR135 but not with that of human GPCR142 (which has ARY instead of DRY or TRY), together with their higher homology to GPCR135 (Fig. 7B), we speculate that the Fugu fish has five copies of GPCR135 gene and no GPCR142 gene. Molecular characterization of GPCR142 from different mammals reveals significant GPCR142 sequence differences between mammalian species. Nowhere is this more apparent than the clear difference between rat and mouse. Although we were able to find an open reading frame for GPCR142 in the mouse, GPCR142 appears to be a pseudogene in the rat (Fig. 2B). The weak activity demonstrated by the mouse GPCR142 in response to relaxin-3 may in fact support this hypothesis, or it is possible that GPCR142 may not play an important role in rodents. The molecular cloning and functional characterization of GPCR142 from monkey, cow, and pig, in addition to human, suggest that GPCR142 is likely to play a significant role for mammals other than rodents. Additionally, the mRNA expression pattern of GPCR142 is drastically different from that of GPCR135 and relaxin-3 (peripheral versus central). This also supports the potential existence of a distinct ligand for GPCR142 other than relaxin-3.

There is a recent publication reporting that bradykinin is a ligand for GPCR142 (also known as GPR100; Boels and Schaller, 2003) because bradykinin stimulated Ca$^{2+}$ response in a CHO cell line stably expressing GPR100. However, they were not able to demonstrate that bradykinin activates GPR100 in transiently transfected cells. In our hands, both control (untransfected) CHO and HEK293 cells responded to bradykinin stimulation, suggesting that CHO and HEK293 cells express endogenous bradykinin receptors. In response to bradykinin stimulation, CHO or HEK293 cells transiently expressing GPCR135 or GPCR142 did not show any significant difference compared with the control cells. In contrast, as a positive control, CHO or HEK293 cells transiently expressing recombinant human bradykinin receptor 2 (BK2) responded to bradykinin stimulation with robust Ca$^{2+}$ mobilization, which is much higher than that from the control cells. Similar to cells expressing recombinant BK2, we also observed very strong Ca$^{2+}$ mobilization in HEK293 cells transiently expressing human recombinant bradykinin receptor 1 (BK1) upon stimulation with bradykinin (data not shown). The fact that, in the same experiment, relaxin-3 potently stimulated Ca$^{2+}$ responses in CHO or HEK293 cells transiently transfected with either GPCR135 or GPCR142 but not in control cells, strongly indicates that both GPCR135 and GPCR142 are expressed in our recombinant system but are not just responding to the bradykinin stimulation. Therefore, we have to conclude that if there is another ligand for GPCR142, it is not likely to be bradykinin.

In summary, we have characterized GPCR135, GPCR142, and relaxin-3 from different species, including human, mouse, and rat, at molecular and pharmacological levels. Our results indicated that GPCR135 and relaxin-3 are highly conserved in sequence and function from different species, strongly suggesting that this ligand/receptor pair plays a very important role in the physiological system and a very conserved role in different species. Compared with GPCR135, GPCR142 is less conserved in rodents but is highly conserved in human, monkey, cow, and pig, suggesting that the functional role of GPCR142 in rodents is either different from that in the humans or is diminished. The lack of GPCR142 in the rat and less conserved GPCR142 in the mouse, despite highly conserved relaxin-3 genes in both rat and mouse, suggest that relaxin-3 may not be the ligand for GPCR142 under the physiological conditions. The potential endogenous ligand for GPCR142, which is not bradykinin, could be still a peptide that is related to the insulin/relaxin family. We are currently evaluating peptides (other than relaxin-3) in the insulin/relaxin family as well as other peptides as the possible additional ligand for GPCR142. The pharmacological characterization of GPCR135 and GPCR142 from different species has provided useful information for future in vitro and in vivo study of relaxin and relaxin-3 systems.

References


Boels K and Schaller HC (2003) Identification and characterisation of GPR100 as a


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