Relaxin-3/Insulin-Like Peptide 5 Chimeric Peptide, a Selective Ligand for G Protein-Coupled Receptor (GPCR)135 and GPCR142 over Leucine-Rich Repeat-Containing G Protein-Coupled Receptor 7

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ABSTRACT

Relaxin-3, the most recently identified member of relaxin/insulin family, is an agonist for leucine-rich repeat-containing G protein-coupled receptor (LGR)7, GPCR135, and GPCR142. LGR7 can be pharmacologically differentiated from GPCR135 and GPCR142 by its high affinity for relaxin. Selective ligands that specifically activate GPCR135 or GPCR142 are highly desirable for studying their functional roles. We have created chimeric peptides that consist of the B-chain of human relaxin-3 in combination with various A-chains from other members of the relaxin/insulin family. Pharmacological characterization of these chimeric peptides indicates the A-chain from relaxin-1, relaxin-2, insulin-like peptide (INSL)3, and INSL6 does not change the pharmacological properties of relaxin-3 significantly. In contrast, substitution of the relaxin-3 A-chain with the A-chain from INSL5 results in a chimeric peptide that selectively activates GPCR135 and GPCR142 over LGR7. This study demonstrates that the A-chains among some of the insulin/relaxin family members are pharmacologically exchangeable. The relaxin-3/INSL5 chimeric peptide is a potential tool to study in vivo function of GPCR135. In addition, because of the substitution of a very hydrophobic peptide (the A-chain of relaxin-3) with a very hydrophilic peptide (the A-chain from INSL5), the radiolabeled ^{125}I-relaxin-3/INSL5 chimera is a suitable ligand (high-affinity, low-nonspecific binding) for receptor autoradiographic studies on tissue sections.

Relaxin-3 (Bathgate et al., 2002), the newest member of the relaxin (Hudson et al., 1983, 1984)/insulin superfamily, has been recently reported as a ligand for two related orphan G protein-coupled receptors (GPCRs), GPCR135 (Liu et al., 2003a) and GPCR142 (Liu et al., 2003a) in addition to leucine-rich repeat-containing G protein-coupled receptor (LGR) LGR7 (Hsu et al., 2000, 2002; Sudo et al., 2003). Both relaxin-3 and GPCR135 are predominantly expressed in the brain (Matsumoto et al., 2000; Bathgate et al., 2002; Burazin et al., 2002; Liu et al., 2003b) and are highly conserved among species from fish to humans (Bathgate et al., 2002;

Hsu et al., 2003; Liu et al., 2003b; Chen et al., 2005), suggesting that this ligand/receptor pair may play an important role in the central nervous system. The GPCR142 expression pattern is distinct from that of GPCR135 and relaxin-3 with an abundant peripheral tissue distribution in addition to expression in the brain (Liu et al., 2003a). GPCR142 is highly conserved among human, monkey, cow, and pig, but it is less conserved in the mouse, and a pseudogene in the rat despite extensive conservation of relaxin-3 genes in both rodent species (Chen et al., 2004), suggesting that GPCR142 has distinct function(s) from GPCR135 and diminished function(s) in the rodents. We recently reported that human insulin-like peptide 5 (INSL5) is a selective agonist for human GPCR142 (Liu et al., 2005). Recombinant human INSL5 activates human GPCR142 at high affinity but does not activate human

ABBREVIATIONS: GPCR, G protein-coupled receptor; LGR, leucine-rich repeat-containing G protein-coupled receptor; INSL, insulin-like peptide; CPRG, chlorophenol red-β-D-galactopyranoside; β-gal, β-galactosidase; PCR, polymerase chain reaction; R_3/R_1, chimera consisting relaxin-3 B-chain and relaxin-1 A-chain, R_3/R_2, chimera consisting relaxin-3 B-chain and relaxin-3 A-chain, R_3/I, chimera consisting relaxin-3 B-chain and insulin A-chain, R_3/I_3, chimera consisting relaxin-3 B-chain and INSL3 A-chain, R_3/I_4, chimera consisting relaxin-3 B-chain and INSL4 A-chain, R_3/I_5, chimera consisting relaxin-3 B-chain and INSL5 A-chain, R_3/I_6, chimera consisting relaxin-3 B-chain and INSL6 A-chain, R3/A, chimera consisting relaxin-3 B-chain and an A-chain with arbitrary sequence; HPLC, high-performance liquid chromatography; IGF, insulin-like growth factor.

GPCR135, LGR7, or LGR8. INSL5 is abundantly expressed in periphery (Conklin et al., 1999; Liu et al., 2005) and has similar tissue expression profile to that of GPCR142 (Liu et al., 2003a, 2005), suggesting that INSL5 is an endogenous ligand for GPCR142. Endogenous ligand/receptor pairs tend to evolve together (Goh et al., 2000). The fact that both INSL5 and GPCR142 are pseudogenes in rat further suggests that INSL5 is an endogenous ligand for GPCR142. The physiological functions of GPCR135 and GPCR142 remain to be elucidated. Although the physiological function of GPCR142 may be studied by in vivo administration of its specific ligand, INSL5, in vivo study of GPCR135 is potentially confounded by the lack of selective pharmacological tools for this receptor subtype. In vivo administration of relaxin-3 could also activate GPCR142 and LGR7 (Sudo et al., 2003), which is expressed in both the brain and periphery (Osheroff and Phillips, 1999; Tan et al., 1999; Hsu et al., 2000, 2002). GPCR142 is a pseudogene in rat, which makes the functional study of GPCR135 in that species a little simpler. However, the potential activation of LGR7 by relaxin-3 remains. Therefore, the creation of selective ligands for GPCR135 over LGR7 is greatly needed for studying the in vivo function of GPCR135.

In this report, we describe the creation of chimeric peptides consisting of relaxin-3 B-chain and A-chains from different members of the relaxin/insulin family and demonstrate that a chimeric peptide consisting of the relaxin-3 B-chain and the INSL5 A-chain is a selective, specific, and potent agonist for GPCR135 and GPCR142 but not for LGR7 and LGR8. Thus, this chimeric peptide is useful as a pharmacological tool for in vivo study of the central function of GPCR135, particularly in rat, in which GPCR142 is a pseudogene. In addition, the relaxin-3/INSL5 chimeric peptide is much more hydrophilic than relaxin-3, thus when labeled with iodine, it binds to the GPCR135- or GPCR142-expressing cells with a high signal-to-noise ratio. Therefore, this new peptide is a suitable tool for receptor autoradiography.

Materials and Methods

Materials. Synthetic human relaxin-3 B-chain and INSL3 (Adham et al., 1993) were purchased from Phoenix Pharmaceuticals (Belmont, CA). Porcine relaxin was purchased from the National Hormone and Peptide Program (Torrance, CA). Forskolin was purchased from Sigma-Aldrich (St. Louis, MO). Chlorophenol red-β-D-Galactopyranoside (CRGP) was purchased from Roche Diagnostics (Mannheim, Germany). African green monkey cell line COS-7 and human embryonic kidney cell line 293 were purchased from American Type Culture Collection (Manassas, VA). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Iodine-125 radionuclide was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Recombinant human relaxin-3 and ^{125}I-relaxin-3 were prepared as reported previously (Liu et al., 2003b).

Construction of Chimeric Peptide Expression Constructs. A series of chimeric peptides were prepared by creating gene cassettes that consisted of coding regions for an alpha peptide signal peptide, a FLAG tag, the human relaxin-3 B-chain, the human relaxin-3 C-chain, and an A-chain from one of the following: relaxin-1 (Hudson et al., 1983), relaxin-2 (Hudson et al., 1984), INSL3 (Adham et al., 1993), INSL4 (Koman et al., 1996), INSL5 (Conklin et al., 1999), INSL6 (Lok et al., 2000), insulin, or an artificial A-chain, in which only the Cys residues remain unchanged, whereas all the other amino acids residues were randomly assigned. The chimeric

peptide coding regions were PCR amplified from the human relaxin-3 expression vector (Liu et al., 2003b) as described below.

PCR Amplification of the Coding Regions for the Chimeric Peptides. Relaxin-3/Relaxin-1 (R_3/R_1) chimeric peptide consists of relaxin-3 B-chain and the relaxin-1 A-chain. The DNA coding region for this chimeric peptide was PCR-amplified using modified human relaxin-3 expression vector relaxin-3 RR (Liu et al., 2003) as template and using primers P1 and P2. Relaxin-3/Relaxin-2 (R_3/R_2) chimeric peptide DNA coding region was PCR-amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P4. Relaxin-3/INSL3 (R_3/I_3) chimeric peptide DNA coding region was PCR-amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P3. Relaxin-3/INSL4 (R_3/I_4) chimeric peptide DNA coding region was PCR amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P5. Relaxin-3/INSL5 (R_3/I_5) chimeric peptide DNA coding region was PCR-amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P6. Relaxin-3/INSL6 (R_3/I_6) chimeric peptide DNA coding region was PCR-amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P7. Relaxin-3/Insulin (R_3/I) chimeric peptide DNA coding region was PCR-amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P8. Relaxin-3/artificial A-chain (R_3/A) chimeric peptide DNA coding region was PCR- amplified using modified human relaxin-3 expression vector Relaxin-3 RR as template and using primers P1 and P9. All primer sequences are given in Table 1.

Construction of the Expression Vectors for Relaxin-3 Chimeric Peptides. The PCR products for relaxin-3 chimeric peptides coding regions were digested with DNA restriction enzymes PstI and BamHI and cloned into a mammalian expression vector pCMV sport1 (Invitrogen) between PstI and BamHI sites. The insert region for each construct was sequenced to confirm the sequence identity.

Expression and Purification of Chimeric Peptides. Different relaxin-3 chimera expression constructs were cotransfected with a human furin expression plasmid (Liu et al., 2003b) into COS-7 cells. Three days after transfection, the cell culture media were collected and run through an anti-FLAG affinity column (Sigma-Aldrich). The affinity-purified peptides were cleaved with enterokinase (Novagen, Madison, WI) to remove the N-terminal FLAG tag. The untagged chimeras were further purified by HPLC with a C18 column. Because the N-terminal Gln residue in INSL5 A-chain is converted to a pyro-Glu (<E) (Liu et al., 2005), the purified R_3/I_5 chimeric peptide was analyzed by N-terminal Edman degradation and mass spectrometry as described previously (Liu et al., 2003b) to determined whether a Gln to pyro-Glu conversion also occurs in R3/I5 A-chain.

Preparation of Radioligands. Human relaxin-3 was labeled with ^{125}I and Chloramine T (PerkinElmer Life and Analytical Sciences) as described previously (Liu et al., 2003b). Likewise,INSL3, and R_3/I_5 chimeric peptide were labeled with ^{125}I and Chloramine T. The labeled peptides were separated from the unlabeled peptides by HPLC using a C18 column. The freshly labeled peptides have a specific activity of 2200 Ci/mmol.

Molecular Cloning of LGR7 and LGR8. Human LGR7 cDNA containing the complete coding region was PCR-amplified from a human brain cDNA pool (BD Biosciences, Palo Alto, CA) using two primers [forward primer P10 and reverse primer P11 (Table 1)] designed according the published sequence (Hsu et al., 2000, 2002). Human LGR8 cDNA containing the complete coding region was PCR-amplified from a human testis cDNA pool (BD Biosciences) using two primers [forward primer P12 and reverse primer P13 (Table 1)] designed according to the published sequence (Hsu et al., 2002). The resulting cDNAs were cloned into the mammalian expression vector pCIneo (Promega, Madison, WI), and the insert regions were sequenced to confirm the sequence identities for LGR7 and LGR8, respectively.

Radioligand Binding Assays for GPCR135, GPCR142, LGR7, and LGR8. Radioligand binding assays for GPCR135 and GPCR142 were performed as described previously (Liu et al., 2003a,b). In brief, embranes from COS-7 cells transiently expressing GPCR135 or GPCR142 were incubated with ^{125}I-relaxin-3 at a final concentration 100 pM in 96-well plates. Peptides for competition binding studies were added to the binding mix (final volume 200 μl) in binding buffer (0 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% bovine serum albumin, 0.1% protease inhibitor cocktails; Sigma-Aldrich). The binding mixtures were incubated at room temperature for 1 h, filtered through GFC plates (PerkinElmer Life and Analytical Sciences) presaturated with 0.3% polyethylenimine (Sigma-Aldrich), and washed with cold washing buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA). Microscint-40 was added to each well, and the bound ^{125}I-relaxin-3 was counted in a scintillation counter (TopCount/NTX; PerkinElmer Life and Analytical Sciences).

Radioligand binding assays for LGR7 and LGR8 were performed using live COS-7 cells transiently expressing LGR7 or LGR8. In brief, COS-7 cells were transiently transfected with LGR7 or LGR8 expression plasmids in 15-cm culture dishes using LipofectAMINE (Invitrogen). Two days after transfection, the transfected cells were detached from the 15-cm culture dishes using phosphate-buffered saline plus 10 mM EDTA, seeded into 96-well opaque poly-D-lysine-coated culture plates and used for radioligand binding assays. For LGR7, ^{125}I-relaxin-3 was used as the tracer. For LGR8, ^{125}I-INSL3 (specific activity 2200 Ci/mmol) was used as the tracer. ^{125}I-labeled tracer was added in binding reactions at a final concentration of 100 pM in binding buffer containing Dulbecco's modified Eagle's medium plus 50 mM HEPES and 1% bovine serum albumin. Different unlabeled peptides at various concentrations were added to the binding assays as the competitors. The binding mixtures were incubated at room temperature for 1 h. The unbound radioligand was removed by aspiration of the binding buffer. The cells were washed with phosphate-buffered saline. The bound radioligand was counted in a scintillation counter (TopCount/NTX). The results were analyzed using Prism 3.0 program (GraphPad Software Inc., San Diego, CA). The IC$_{50}$ values represent the ligand concentrations that inhibit 50% of the maximum specific binding. The affinity of ^{125}I-relaxin-3 for LGR7 (K_d value) was determined using ^{125}I-relaxin-3/LGR7 binding assay with increasing concentration of ^{125}I-relaxin-3. Nonspecific binding was determined by performing the binding assay in the presence of 1 μM unlabeled relaxin-3. Likewise, the K_d value of ^{125}I-INSL3 to LGR8 was determined using ^{125}I-INSL3/LGR8 binding assay with increasing concentration of ^{125}I-INSL3. Nonspecific binding was determined by performing the binding assay in the presence of 1 μM unlabeled INSL3.

Functional Characterization of GPCR135, GPCR142, LGR7, and LGR8 Using Chimeric Peptides. Activations of GPCR135, GPCR142, LGR7, and LGR8 were measured in SK-N-MC/β-gal cells (Liu et al., 2001) stably expressing GPCR135, GPCR142, LGR7, or LGR8, respectively. SK-N-MC/β-gal cells harbor a β-galactosidase gene under control of cAMP-responsive element. An increase in intracellular cAMP concentration leads to increased β-galactosidase gene expression, whose activity can be measured using CPRG as the substrate. In brief, cells were seeded in 96-well tissue culture plate at a density of 30,000 cells/well in minimal essential medium plus 10% fetal serum, sodium pyruvate, penicillin, streptomycin, and G418 (400 mg/l). For cells expressing LGR7 or LGR8, intracellular cAMP was stimulated with different peptides at various concentrations. For cells expressing GPCR135 or GPCR142, forskolin was added to cells at a final concentration of 5 μM to stimulate intracellular cAMP accumulation. Different concentrations of ligands were added to the cells to inhibit the forskolin-induced cAMP accumulation. For all cells, after adding ligands, cells were incubated for six additional hours in 37°C incubator. The media were aspirated and the β-galactosidase activities, which represent the relative cAMP concentrations, were then measured as described previously (Liu et al., 2001). The results were analyzed by Prism 3.0 software. The EC$_{50}$ values represent the ligand concentrations that achieve 50% of the maximum inhibition of forskolin induced β-galactosidase activity (GPCR135 or GPCR142) or 50% of the maximum ligand induced β-galactosidase activity (LGR7 or LGR8), respectively.

Receptor Autoradiography on Rat Brain Sections. All the experiments described in this study have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Adult male Sprague-Dawley rats (150–200 g) were asphyxiated by carbon dioxide inhalation. Rat brains were immediately removed from the skull and rapidly frozen in dry ice. Thick sagittal sections (20 μm) were cut using a Cryostat-microtome (Microm HM505E; Mikron, San Diego, CA) and thaw-mounted on adhesive microscope slides (Superfrost$^+$ Plus; VWR, West Chester, PA). The sections were kept at −70°C until use. Sections were thawed and dried under a cold airstream and then preincubated for 15 min at room temperature in incubation buffer (20 mM HEPES, pH 7.4, 120 mM NaCl$_2$, 0.22 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 0.8 mM MgSO$_4$) by immersing sections in a 400-ml jar. Sections were dried again under a cold airstream and

TABLE 1
Primer sequences

| Primer	Sequence
P1	5′-ACT AGA CTG CAG GCC GCC ATG CTG ACC GCA GCG T-3′
P2	5′-ACT AGA GGA TCC TCA GCA ATA TTT AGC AAG AGA CCT TTT GGT ACA ACC AAT TAG GCA ACA TTT CTC AAA CAG TGC CAC GTA GGG TCG TCG TCG GCC CCG AAG AAC CCC AGG-3′
P3	5′-ACT AGA GGA TCC TCA GCA AAA TCT AGC AAG AGA TCT TTT GGT ACA ACC AAC ATG GCA ACA TTT ATT AGC CAA TGC ACT GTA GAG TTG TCG TCG GCC CCG AAG AAC CCC AGG-3′
P4	5′-ACG ATA GGA TCC TCA CAG GGT CAG CAG GTC CTG CTG GGT GCA GCC GGA CAG GCA GCA GTA GCG GGC GGG GTT TCG TCG GCC CCG AAG AAC CCC AG-3′
P5	5′-ATG ACA GGA TCC TCA GCA CAG CTT CAC GGA GGT GCC GTC GTC GCA GAT CAC CTC GCA GCA GAA GGG GTC GAA TCG TCG GCC CCG AAG AAC CCC AGG-3′
P6	5′-ATA GAA GGA TCC TTA GCA AAG AGC ACT CAA ATC AGT CAT GGA ACA GCC ATC AGT GCA ACA CAA AGT TTG TAA ATC TTG TCG TCG GCC CCG AAG AAC CCC AGG TCC-3′
P7	5′-ATG ACA GGA TCC TCA GCA GGC GAT GGA CAG CTC CTC CTT GGT GCA GCC GGT CAG GCA GCA CTT CTC GGA GTA GCC TCG TCG GCC CCG AAG AAC CCC AG-3′
P8	5′-ACG ATA GGA TCC TCA GCA GTA GTT CTC CAG CTG GTA CAG AGG GCA GAT GGA GGT GCA GCA CTG CTC CAC GAT GCC TCG TCG GCC CCG AAG AAC CCC AG-3′
P9	5′-ACG ATA GGA TCC TCA GCA GGC CTT CTC GAT CTC GTA GTC GTC GCA GAT GGA CAG GCA GCA GTT CTC GTA CAG GCC TCG TCG GCC CCG AAG AAC CCC AG-3′
P10	5′-AGA TGA GAA TTC GCC ACC ATG ACA TCT GGT TCT GTC TTC TTC TAC-3′
P11	5′-TAG AGA GCG GCC GCT CAT GAA TAG GAA TTG AGT CTC GTT GA-3′
P12	5′-TAG ACA GAA TTC GCC ACC ATG ATT GTT TTT CTG GTT TTT AAA CAT CTC-3′
P13	5′-ATG ATA GCG GCC GCC TAG GAA ACT GGT TTC ATT ATA CTG TC-3′

incubated for 60 min with 7 pM ^{125}I-relaxin-3 (specific activity 2200 Ci/mmol) or ^{125}I-R$_3$/I$_5$ (specific activity 2200 Ci/mmol) in incubation buffer containing 0.5% bovine serum albumin and protease inhibitor cocktail (Sigma-Aldrich). Nonspecific binding was determined in the presence of 100 nM unlabeled human relaxin-3. After incubation, the excess radioligand was washed off by immersing the slides in the jar containing incubation buffer at 4°C (3 times 10 min) followed by a quick immersion in water. Sections were dried and exposed to Fuji-film Imaging plates (BAS-MS 2025) for 48 h. The imaging plates were scanned using the Fuji Bio-Imaging Analyzer System (BAS-5000) and visualized using ImageGauge version 3.12 software.

Results

Relaxin-3 B-Chain Is an Agonist for GPCR135 and GPCR142 but Not for LGR7 or LGR8. We have shown previously that synthetic human relaxin-3 B-chain alone, but not A-chain, is an agonist for both GPCR135 and GPCR142 (Liu et al., 2003a,b). We repeated these studies in a different cell background, which allows easy measurement of cAMP stimulation and inhibition. Our results indicate that relaxin-3 B-chain inhibited forskolin-stimulated β-galactosidase activity in SK-N-MC/β-gal cells expressing GPCR135 (with an EC$_{50}$ value of 88 nM; Fig. 1A) or GPCR142 (with an EC$_{50}$ value of 125 nM; Fig. 1B) in dose-dependent manner, which is consistent with our previous reports (Liu et al., 2003a,b). In a parallel experiment, human relaxin-3 B-chain did not activate either LGR7 (Fig. 1C) or LGR8 (Fig. 1D)-expressing cells, whereas porcine relaxin and human relaxin-3 stimulated β-galactosidase activity in LGR7-expressing cells (with EC$_{50}$ values of 0.25 and 2.1 nM, respectively; Fig. 1C), and porcine relaxin and human INSL3 induced β-galactosidase activity in LGR8-expressing cells (with EC$_{50}$ values of 1.5 and 0.16 nM, respectively; Fig. 1D).

Expression and Purification of Relaxin-3 Chimeras. Different relaxin-3 chimera expression DNA constructs en-
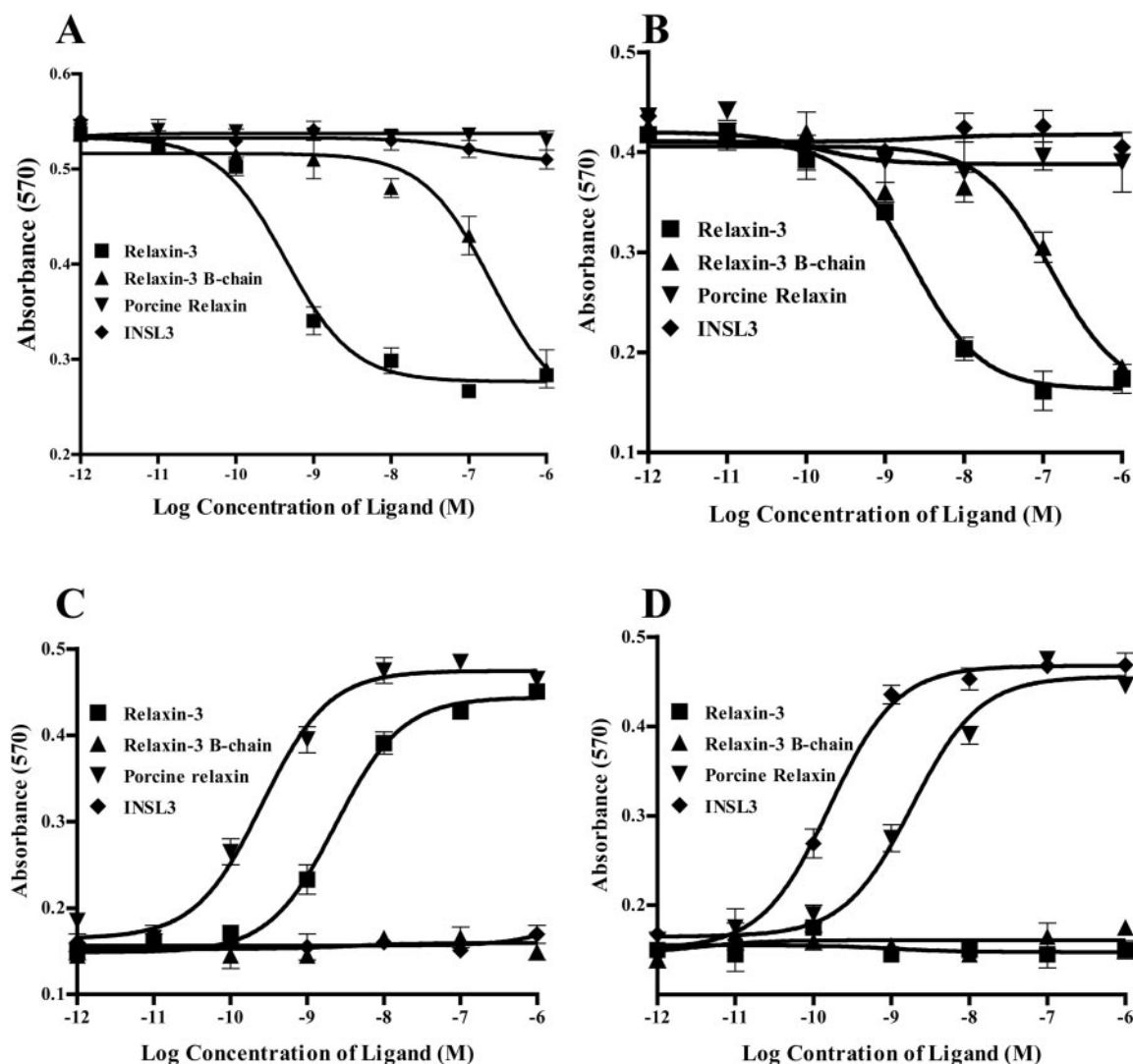
Fig. 1. Relaxin-3 B-chain activates GPCR135 and GPCR142 but not LGR7 or LGR8. SK-N-MC/β-gal cells expressing human GPCR135 (A) and GPCR142 (B) were stimulated with relaxin-3, relaxin-3 B-chain, porcine relaxin, or INSL3 at various concentrations to inhibit the forskolin stimulated β-galactosidase expression. Forskolin was added to cells at a final concentration of 5 μM to stimulate the β-galactosidase expression. SK-N-MC/β-gal cells expressing human LGR7 (C) and LGR8 (D) were stimulated with relaxin-3, relaxin-3 B-chain, porcine relaxin, and INSL3 at various concentrations to induce the β-galactosidase expression. The intracellular β-galactosidase activities were measured by a colorimetric assay using CPRG as the substrate, reading the absorbance at a wavelength of 570 nm. The EC$_{50}$ values of different peptides for GPCR135, GPCR142, LGR7, or LGR8 are given under *Results*.

coding peptides with the B-chain from relaxin-3 and an A-chain from different members of the insulin/relaxin family were constructed similarly to that of the recombinant expression of the wild type relaxin-3 (Liu et al., 2003b). Each construct contains the coding regions for a signal peptide for secretion, a FLAG tag for affinity purification, the B-chain and the C-chain of relaxin-3, and an A-chain from one of other members of the insulin/relaxin family, including relaxin-1 (R_3/R_1), relaxin-2 (R_3/R_2), INSL3 (R_3/I_3), INSL4 (R_3/I_4), INSL5 (R_3/I_5), INSL6 (R_3/I_6), and insulin (R_3/I). In addition, a chimera (R_3/A) construct encoding an A-chain with the conserved cystines and arbitrarily assigned amino acids at the other positions was also created. The junction of the C-chain and the A-chain for each construct contains an artificial furin cleavage site (RGRR) for efficient in vivo cleavage when coexpressed with furin (Hosaka et al., 1991; Liu et al., 2003b). The predicted B-chain and A-chain sequences for different chimeras are shown in Fig. 2. The expression constructs for the different relaxin-3 chimeras were coexpressed with furin in COS-7 cells. The recombinant peptides secreted into the conditioned medium of transfected cells were affinity purified with an anti-FLAG affinity column, cleaved with enterokinase to remove the N-terminal FLAG tag, and then further purified by reversed phase HPLC. The purified peptides were characterized by SDS-polyacrylamide gel electrophoresis under nonreducing conditions to verify the purity. The protein expression levels for R_3/R_2, R_3/I_3, R_3/I_5, and R_3/I_6 were comparable with the production of relaxin-3 wild-type peptides (Liu et al., 2003b), which was about 1 mg/l. The production levels of R_3/R_1 and R_3/I_4 were lower at approximately 200 mg/l. Attempts to make R_3/I and R_3/A chimeras resulted in no detectable peptides, when analyzed by SDS-polyacrylamide gel electrophoresis (data not shown). The N-terminal Gln of INSL5 A-chain is converted to a pyro-Glu residue (Liu et al., 2005). To investigate whether this conversion also occurs in R_3/I_5 chimera, purified R_3/I_5 peptide was analyzed by Edman degradation and mass spectrometry. The Edman degradation resulted in only one sequence (RAA-PYGV—), which matches the B-chain sequence of R_3/I_5, suggesting the N terminus of the A-chain is blocked from Edman degradation. Mass spectrometry showed that the chimeric peptide has a molecular mass of 5240 Da, which matches the predicted molecular mass of R_3/I_5 with the Gln to pyro-Glu conversion and is 17 Da smaller than the predicted molecular mass (5257 Da) of R_3/I_5 without the Gln to pyro-Glu conver-

sion, indicating that the N-terminal Gln of R_3/I_5 A-chain is converted to a pyro-Glu.

Pharmacological Characterization of Relaxin-3 Chimeric Peptides by Radioligand Binding. The different chimeric peptides were tested as ligands for GPCR135 (Fig. 3A), GPCR142 (Fig. 3B), LGR7 (Fig. 3C), and LGR8 (Fig. 3D) in radioligand binding assays to evaluate their receptor binding properties. ^{125}I-Relaxin-3 was used as the tracer to characterize the chimeras for their receptor binding properties for GPCR135, GPCR142, and LGR7. For LGR8, ^{125}I-INSL3 was used as the radioligand. The K_i values of different chimeras for GPCR135, GPCR142, LGR7, and LGR8 are listed in Table 2. Our results show that all chimeras, except R_3/I_4, bind both GPCR135 and GPCR142 with high affinity with slight differences in potency. R_3/I_4 only demonstrated some marginal binding for both GPCR135 and GPCR142 at the highest concentration (1 μM). Chimeras R_3/R_1, R_3/R_2, and R_3/I_3 bind to LGR7 with high affinity, with K_i values in low nanomolar range, which is similar to that of relaxin-3. R_3/I_6 binds LGR7 with slightly lower affinity with a K_i value of 12 nM. It is interesting that R_3/I_5, which demonstrated high affinity for GPCR135 and GPCR142, showed very low affinity for LGR7 with a K_i value of about 0.5 μM. R_3/I_4 is totally inactive for LGR7 even at the highest concentration (1 μM) tested. None of the chimeras bind LGR8 with high affinity. R_3/I_4, R_3/I_5, R_3/I_6 showed no binding affinity for LGR8, whereas chimeras R_3/R_1, R_3/R_2, and R_3/I_3 each demonstrated modest binding affinity for LGR8.

Pharmacological Characterization of Relaxin-3 Chimeric Peptides in Functional Assays. We tested the different chimeric peptides for their ability to inhibit forskolin-induced β-gal expression in SK-N-MC/β-gal cells stably expressing GPCR135 (Fig. 4A) or GPCR142 (Fig. 4B). The EC_{50} values of different chimeras to activate GPCR135 or GRPCR142 are listed in Table 3. Our results demonstrated that all chimeras tested act as agonists for both GPCR135 and GPCR142. Chimeras including R_3/R_1, R_3/R_2, R_3/I_3, and R_3/I_5 have similar potency compared with that of the wild-type relaxin-3 with EC_{50} values in the low nanomolar range. Chimera R_3/I_6 is slightly less potent for both GPCR135 and GPCR142 with EC_{50} values around 10 nM. R_3/I_4 only stimulated GPCR135 or GPCR142 at the highest concentration (1 μM) tested. Relaxin-3 chimeras were also tested for their agonist activity for LGR7 and LGR8 in the SK-N-MC/β-gal cells expressing LGR7 (Fig. 4C) or LGR8 (Fig. 4D). The EC_{50}

	B-chain	**A-chain**
Relaxin-3	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	DVLAGLSSS**CC**KWG**C**SKSEISSL**C**
R3/R1	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	RPYVALFEK**CC**LIG**C**TKRSLAKY**C**
R3/R2	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	QLYSALANK**CC**HVG**C**TKRSLARF**C**
R3/I3	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	ATNPARY**CC**LSG**C**TQQDLLTL**C**
R3/I4	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	FDPF**CC**EVI**C**DDGTSVKL**C**
R3/I5	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	<EDLQTL**CC**TDG**C**SMTDLSAL**C**
R3/I6	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	GYSEK**CC**LTG**C**TKEELSIA**C**
R3/I	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	GIVEQ**CC**TSI**C**SLYQLENY**C**
R3/A	RAAPYGVRL**C**GREFIRAVIFT**C**GGSRW	GLYEN**CC**LSI**C**DDYEIEKA**C**

Fig. 2. Amino acid sequences of human relaxin-3 and relaxin-3 chimeras. All chimeras have the identical B-chain from human relaxin-3. The chimeras R_3/R_1, R_3/R_2, R_3/I_3, R_3/I_4, R_3/I_5, R_3/I_6, and R_3/I possess A-chain sequences from human relaxin-1, relaxin-2, INSL3, INSL4, INSL5, INSL6, and insulin, respectively. R3/A possesses an A-chain with arbitrarily assigned amino acid sequence. All A-chains are arbitrarily designed to end with a Cys at the C terminus. The A-chain of R_3/I_3 is two amino acids (two Ala residues) shorter at the N terminus than the natural INSL3 A-chain. The two Ala residues were removed to decrease the hydrophobicity of R_3/I_3 chimera. The A-chain sequences of R_3/I_4 and R_3/I_6 are designed based on predicted A-chain sequences of INSL4 and INSL6, respectively. A Gln to pyro-Glu (<E) conversion that is found at the N terminus of the INSL5 A-chain (Liu et al., 2004) is predicted to occur in R_3/I_5 A-chain.

values of the different chimeras to activate LGR7 or LGR8 are also listed in Table 3. Our results indicated that R_3/R_1, R_3/R_2, and R_3/I_3 have high potency with EC_{50} values around 1 to 2 nM for LGR7. R_3/I_6 has slightly lower potency for LGR7 with an EC_{50} value of 13.7 nM. R_3/I_5, a potent agonist for GPCR135 and GPCR142, demonstrated very low potency for LGR7 (with an EC_{50} value of ~400 nM). For LGR8, R_3/R_1 demonstrated some marginal activity at the highest concentration tested (1 μM). R_3/R_2 and R_3/I_3 showed slightly higher potency with EC_{50} values of about 600 and 300 nM, respectively. Other chimeras showed no activity for LGR8. Control SK-N-MC/β-gal cells without expressing recombinant GPCR135, GPCR142, LGR7, or LGR8 did not respond to any peptides (data not shown).

R_3/I_5 Binds to GPCR135-Expressing Cells with Low Nonspecific Binding. Relaxin-3 is a very hydrophobic peptide. When labeled with ^{125}I and applied to receptor binding, ^{125}I-relaxin-3 generates high nonspecific binding. When used as radioligand, ^{125}I-relaxin-3 still produces reasonably good signal-to-noise ratios for cells expressing recombinant GPCR135, GPCR142, and LGR7 because in the recombinant system, the receptor expression level is very high. However,

the high nonspecific binding creates a big obstacle for radioligand autoradiographic studies, in which the endogenous receptors, normally expressed at much lower levels compared with the recombinant receptors, are studied. By creating the relaxin-3 chimera R_3/I_5, we replaced the native A-chain of relaxin-3, which is very hydrophobic, with the INSL5 A-chain, which is much more hydrophilic, thus increasing the overall hydrophilicity of the peptide. We labeled R_3/I_5 chimera with ^{125}I and characterized its receptor binding properties for recombinant GPCR135 and LGR7. In addition, we compared the results of radioligand autoradiography on rat brain slide using either ^{125}I-relaxin-3 or ^{125}I-R_3/I_5 as the radioligand. Our results showed that although both ^{125}I-relaxin-3 (Fig. 5A) and ^{125}I-R_3/I_5 (Fig. 5B) bind to GPCR135 expressing cells with high affinity, ^{125}I-R_3/I_5 produces much lower nonspecific binding than ^{125}I-relaxin-3. In addition, ^{125}I-R_3/I_5 did not demonstrate significant binding to LGR7, whereas ^{125}I-relaxin-3 was bound with high affinity (Figs. 3A and 5A). When ^{125}I-relaxin-3 was used as the radioligand on rat brain sections, virtually no difference between total binding and nonspecific binding (determined in the presence of 100 nM of unlabeled relaxin-3) was observed (Fig. 5C). In
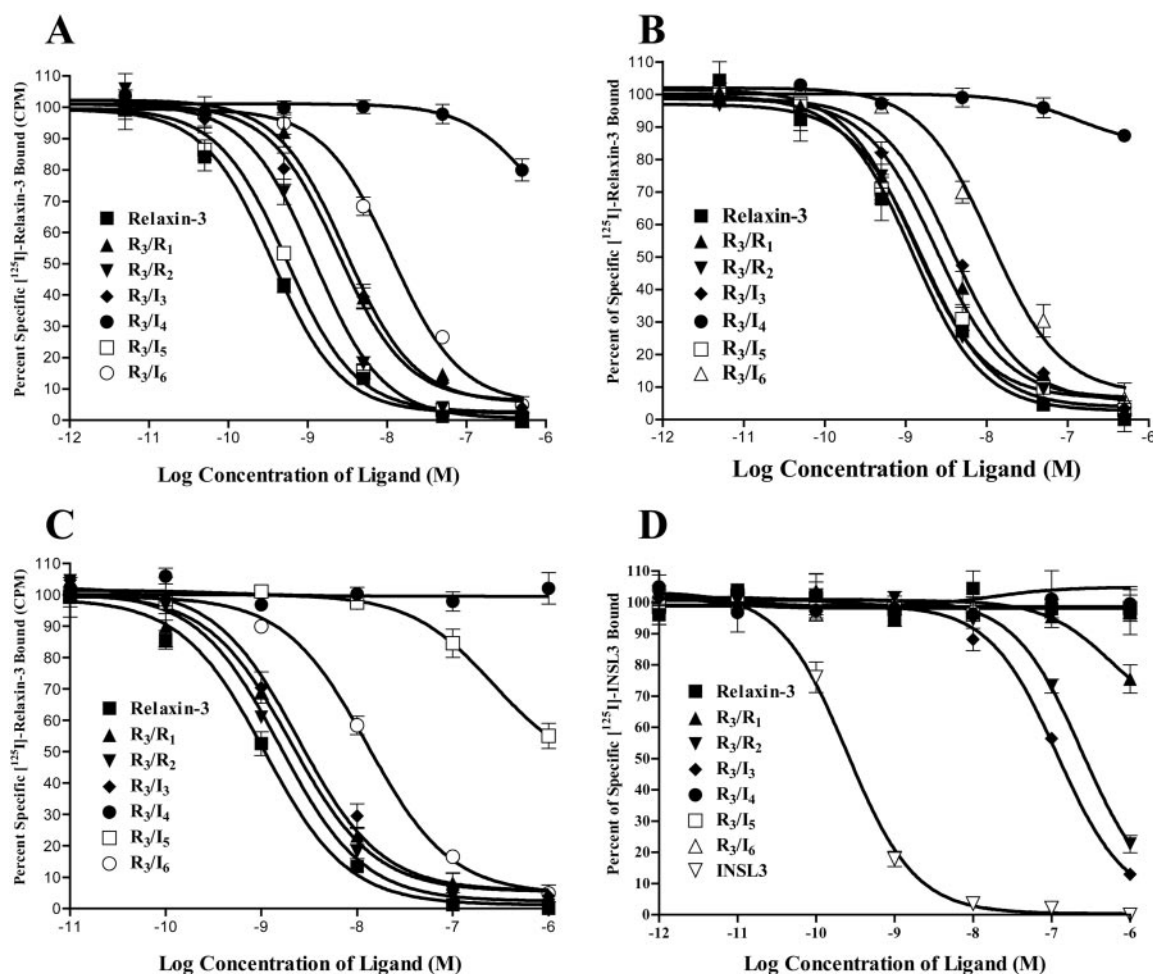
Fig. 3. Characterization of relaxin-3 chimeras using radioligand binding assays. Different relaxin-3 chimeras were characterized for their receptor binding properties for human recombinant GPCR135 (A), GPCR142 (B), LGR7 (C), or LGR8 (D). For GPCR135, GPCR142, and LGR7, ^{125}I-relaxin-3 was used as the radioligand at a final concentration of 100 pM. Different unlabeled peptides were used in the binding assay as the competitors. For LGR8, ^{125}I-INSL3 was used as the radioligand at a final concentration of 100 pM. Different unlabeled peptides including relaxin-3 chimeras at various concentrations were added to the binding assay as the competitors. The K_i values of different chimeras to different receptors are listed in Table 2. Data represent mean ± S.E.M. ($n = 3$).

contrast, when ^{125}I-R$_3$/I$_5$ was used as the radioligand, the nonspecific binding was dramatically reduced (Fig. 5D). GPCR135-like binding sites were clearly observed in cortex, olfactory bulb, and superior colliculus areas (Fig. 5D).

TABLE 2
K_i values for relaxin-3 chimeras to binding human GPCR135, GPCR142, LGR7, and LGR8

K_i (mean $\pm$ S.E.M., $n = 3$) values were calculated based on IC$_{50}$ values using the Cheng and Prusof (1973) formula (K_i = IC$_{50}$/[1 + S/K_d]). IC$_{50}$ values are the ligand concentrations that inhibit 50% of the maximum specific binding. S, the radioligand concentration used in the binding assays, is 0.1 nM. The K_d values of relaxin-3 for GPCR135 and GPCR142 are 0.31 and 1.9 nM as determined previously (Liu et al., 2003a,b), respectively. The K_d value of ^{125}I-relaxin-3 for LGR7 is 2.7 nM as determined by saturation binding assay. The K_d value of ^{125}I-INSL3 to LGR8 is 0.2 nM as determined by saturation binding assay

	GPCR135	GPCR142	LGR7	LGR8
		nM		
Relaxin-3	0.30 $\pm$ 0.04	1.1 $\pm$ 0.19	2.0 $\pm$ 0.24	Inactive
R$_3$/R$_1$	2.2 $\pm$ 0.26	2.3 $\pm$ 0.27	4.9 $\pm$ 0.75	>1000
R$_3$/R$_2$	0.92 $\pm$ 0.21	1.6 $\pm$ 0.32	1.4 $\pm$ 0.36	410 $\pm$ 37
R$_3$/I$_3$	1.8 $\pm$ 0.25	3.1 $\pm$ 0.41	2.2 $\pm$ 0.34	317 $\pm$ 21
R$_3$/I$_4$	>1000	>1000	Inactive	Inactive
R$_3$/I$_5$	0.52 $\pm$ 0.1	1.2 $\pm$ 0.31	696 $\pm$ 80	Inactive
R$_3$/I$_6$	7.3 $\pm$ 1.3	9.9 $\pm$ 2.0	12.4 $\pm$ 1.8	Inactive

Discussion

Recent identification of GPCR135 as a receptor for relaxin-3 opened up a new field for the physiological study of relaxin-related peptides in the central nervous system. However, elucidation of the physiological role(s) of their receptors is potentially confounded by cross-reactivity of these peptide ligands to different receptors. Creation of new tool that can selectively characterize GPCR135 in the brain is necessary to understand the physiological role of GPCR135. The hallmark of the insulin/relaxin family of peptides is that each of them consists of two peptide subunits (A-chain and B-chain) connected by three pairs of conserved disulfide bridges. The unique dipeptide structure of the peptides in the insulin/relaxin family, in combination to our observation that the relaxin-3 B-chain alone was sufficient to activate GPCR135 (Liu et al., 2003b), let us to construct a series of chimeric peptides. These studies have led to creation of pharmacological tools and a better understanding of the structural requirement for receptor activation by insulin/relaxin-related peptides.
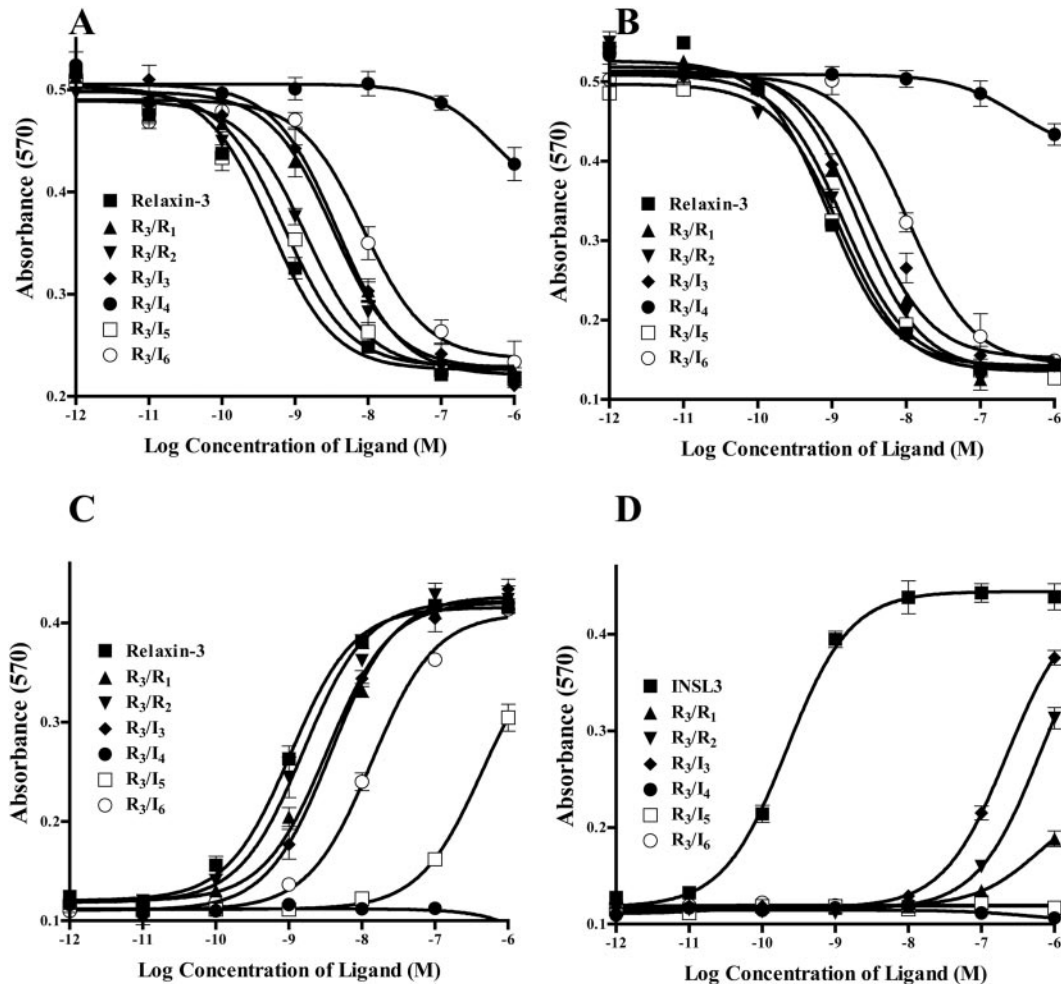
Fig. 4. Evaluation of the agonist activity of relaxin-3 chimeras in the biofunctional assays. Different chimeras at various concentrations were added to SK-N-MC/β-gal cells expressing human GPCR135 (A) or GPCR142 (B) to inhibit forskolin stimulated β-galactosidase expression. Human relaxin-3 was used as the positive control. In parallel, chimeras at various concentrations were added to SK-N-MC/β-gal cells expressing human LGR7 (C) and LGR8 (D) to stimulate β-galactosidase expression. Relaxin-3 and INSL3 were used as the positive controls for LGR7 and LGR8, respectively. Intracellular β-galactosidase activity was measured by a colorimetric assay using CPRG as the substrate and with reading the absorbance at wavelength of 570 nm. The EC$_{50}$ values of different chimeras to the four receptors are listed in Table 3. Data represent mean $\pm$ S.E.M. ($n = 3$).

Chimeric Peptide R_3/I_5 Is a Suitable Tool for in Vivo Functional Studies and Receptor Autoradiography Studies of GPCR135. GPCR135 is predominantly expressed in the brain. In vivo administration of relaxin-3 in the brain to activate GPCR135 is a useful means to understand the physiological role of GPCR135. However it could be potentially confounded by the activation of LGR7, which is also expressed in the brain (Tan et al., 1999; Hsu et al., 2000, 2002). Selective ligands that specifically activate GPCR135 but not LGR7 are highly desirable to study the in vivo function of GPCR135. Therefore, our creation of the relaxin-3 chimeric peptide R_3/I_5, a peptide that has very high affinity for GPCR135 (K_i of ~0.5 nM) but very low affinity for LGR7
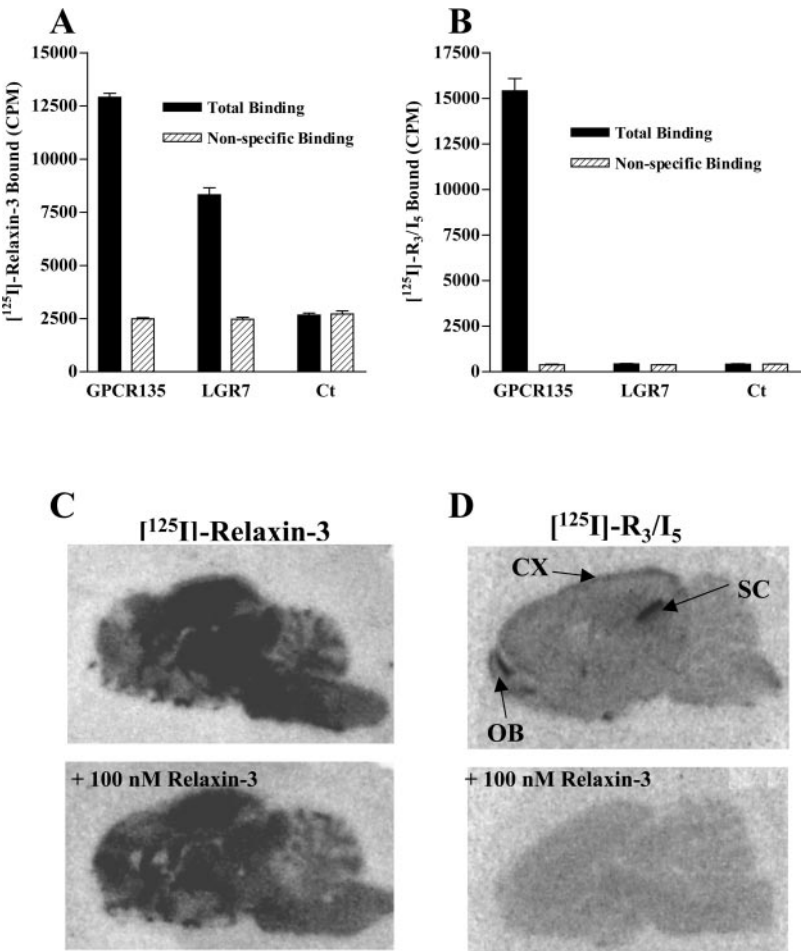
($K_i > 0.5 \mu M$), will greatly assist the in vivo functional study of GPCR135, particularly in rat, which does not have GPCR142.

Radioligand autoradiography is an important and useful tool to study the receptor protein in vivo distribution, which can be used to predict the potential function of the receptor and guide in vivo studies. The GPCR135 binding sites in the brain have not been studied. Attempts to use ^{125}I-relaxin-3 as radioligand in rat brain section autoradiography have not been successful. In addition to the fact that ^{125}I-relaxin-3 also labels LGR7, ^{125}I-relaxin-3 is very hydrophobic and produces a very high background when applied in the radioligand autoradiography studies. Addition of the INSL5 A-chain to the B-chain of relaxin-3 resulted in a molecule that had two desirable properties. First, it is selective for GPCR135. Second, it has reduced hydrophobicity, which resulted in 5- to 10-fold lower nonspecific binding. Direct application of ^{125}I-R_3/I_5 to rat brain sections in radioligand autoradiography clearly showed specific binding sites. Because ^{125}I-R_3/I_5 applied in trace concentrations practically does not bind LGR7, ^{125}I-R_3/I_5 is a very useful tool to selectively study GPCR135 receptor autoradiography in the rat brain, which has no GPCR142.

The Chimeric Peptide Studies Shed Light on the Molecular Structure of the Insulin/Relaxin-Related Peptides. Chimeric peptides R_3/R_1, R_3/R_2, R_3/I_3, R_3/I_4, R_3/I_5, and R_3/I_6 were successfully produced in our mammalian expression system, whereas R_3/I and R_3/A were not, indicating

TABLE 3

EC_{50} values of relaxin-3 chimeras to activate human GPCR135, GPCR142, LGR7, and LGR8

EC_{50} values (mean ± S.E.M., $n = 3$) for GPCR135 and GPCR142 were ligand concentrations that achieve 50% of the maximum ligand-induced inhibition of forskolin stimulated β-galactosidase expression. EC_{50} values (mean ± S.E.M., $n = 3$) for LGR7 and LGR8 were ligand concentrations that induce 50% of the maximum ligand-stimulated β-galactosidase expression.

	GPCR135	GPCR142	LGR7	LGR8
	nM			
Relaxin-3	0.42 ± 0.05	0.91 ± 0.12	1.5 ± 0.17	Inactive
R_3/R_1	1.8 ± 0.27	2.2 ± 0.31	4.5 ± 0.51	>1000
R_3/R_2	1.1 ± 0.14	1.4 ± 0.26	1.1 ± 0.16	575 ± 65
R_3/I_3	2.2 ± 0.31	2.9 ± 0.28	2.5 ± 0.3	258 ± 42
R_3/I_4	>1000	>1000	Inactive	Inactive
R_3/I_5	0.45 ± 0.1	0.91 ± 0.21	412 ± 74	Inactive
R_3/I_6	8.4 ± 1.6	11.2 ± 2.5	13.7 ± 1.9	Inactive

Fig. 5. ^{125}I-R_3/I_5 specifically binds GPCR135 but not LGR7 with low nonspecific binding. COS-7 cells in six-well culture dishes were transfected with human GPCR135 and LGR7, respectively. Mock-transfected COS-7 cells were used as the negative controls. ^{125}I-relaxin-3 (A) or ^{125}I-R_3/I_5 (B) was added to cells at a final concentration of 100 pM either in the absence or presence of 1 μM unlabeled relaxin-3 to determine the total and the nonspecific bindings. C, digitized computer images of ^{125}I-relaxin-3 binding in rat brain section (sagittal section). Nonspecific binding was determined in the presence of 100 nM unlabeled relaxin-3. D, digitized computer images of ^{125}I-R_3/I_5 binding in rat brain section (sagittal section). Nonspecific binding was determined in the presence of 100 nM unlabeled relaxin-3. CX, cortex; OB, olfactory bulb; SC, superior colliculus.

that the A-chains from many members of the insulin/relaxin family, although bearing limited sequence conservation, are interchangeable for protein folding, secretion, and stability maintenance. The failure to produce R₃/I and R₃/A suggests that certain structural requirements have to be met; thus, a compatible A-chain has to pair with the relaxin-3 B-chain. In an effort to understand the successful pairing of the relaxin-3 B-chain with the A-chains from relaxin, INSL3, INSL4, INSL5, or INSL6, but not the A-chain from insulin, we compared the amino acid sequence of all known members in the family (Fig. 6). Amino acid sequence comparison indicates that except for insulin, IGF1 and IGF2, the B-chains from all other members of the family have two conserved positively charged amino acids (Arg or Lys) corresponding to the Arg[8] and Arg[16] of the relaxin-3 B-chain. It is possible that relaxin, INSL3, INSL4, INSL5, INSL6, and relaxin-3 have a similar B-chain/A-chain pairing mechanism that is significantly different from that in insulin and IGFs.

The Receptors for INSL4 May Be Distinct from GPCR135, GPCR142, LGR7, and LGR8, Whereas the Receptor for INSL6 May Be a Close Neighbor of LGR7 and LGR8. Our results indicate that chimeras including R₃/R₁, R₃/R₂, and R₃/I₃ have almost identical agonist properties compared with that of relaxin-3 wild-type peptide in terms of their ligand activity at GPCR135, GPCR142, and LGR7. The relaxin-3 B-chain alone is sufficient to activate GPCR135 and GPCR142, suggesting that the B-chain of relaxin-3 may have the necessary amino acid residues that directly interact with GPCR135 or GPCR142. With the addition of A-chain from other members of the family, we anticipated that some of the chimeric peptides would have high affinity for GPCR135 and GPCR142. Becauserelaxin-1, relaxin-2, relaxin-3, and INSL3 are natural ligands for either LGR7 (Hsu et al., 2002; Sudo et al., 2003), LGR8 (Kumagai et al., 2002; Bogatcheva et al., 2003), or both (Hsu et al., 2002), it is not surprising to see that R₃/R₁, R₃/R₂, and R₃/I₃ display a very similar pharmacological profile to relaxin-3 (i.e., potent ligands for GPCR135, GPCR142, and LGR7). It is worth noting that although relaxin-3 is not a ligand for LGR8 (Sudo et al., 2003), R₃/R₁, R₃/R₂, and R₃/I₃ have demonstrated ligand activities for LGR8, albeit with very low potency. Because those chimeras have A-chains from the natural ligands of LGR8, it is possible that the A-chains in those chimeras interact with and stimulate LGR8. Because the B-chains of relaxin and INSL3 also play important roles in the ligand receptor interaction (Bullesbach et al., 1992; Bullesbach and Schwabe, 1999, 2000; Tan et al., 2002), our results strongly suggest that LGR7 and LGR8 interact with

both the B-chain and A-chain of their ligands. In addition, our results demonstrated a trend that chimeras with relaxin-3 B-chain and an A-chain from a natural ligand for LGR7 or LGR8 tend to have higher affinity for LGR7 and/or be active for LGR8. On the other hand, relaxin-3 chimeric peptides with A-chains from family members that are not ligands for LGR7 or LGR8 have lower or no affinity for LGR7 or LGR8; however, they may still retain high affinity for GPCR135 and GPCR142. INSL5 is not a ligand for LGR7 or LGR8 (Liu et al., 2003b, 2005). R₃/I₅ retains very high affinity for GPCR135/GPCR142 but displays very low affinity for LGR7 and is totally inactive at LGR8. Chimeric peptide R₃/I₆ has demonstrated reasonably high affinity (EC₅₀ ~10 nM) for GPCR135/GPCR142, and LGR7, but it is totally inactive for LGR8, suggesting that the cognate receptor for INSL6 may be a close neighbor of LGR7; however, it may not be LGR8. Chimeric peptide R₃/I₄ has no activity for LGR7 and LGR8, suggesting that INSL4 is not a ligand for LGR7 or LGR8. We expected that R₃/I₄ would retain a high affinity for GPCR135/GPCR142. What surprised us is that R₃/I₄ demonstrated no improved activity compared with the relaxin-3 B-chain alone. Given that INSL4 is only found in human (Koman et al., 1996; Hsu et al., 2003), INSL4 may be a unique member of the relaxin subfamily and has distinct structure. A recent report shows that INSL4 peptide lacks tertiary conformation (Lin et al., 2004). It is possible that the lack of appropriate structural support from INSL4 A-chain in R₃/I₄ leads to the inactivity of R3/I4, which may also account for the reduced production level of R₃/I₄ in the recombinant system because proteins without tertiary structure are often secreted less efficiently and tend to degrade faster.

In summary, we created a relaxin-3 chimeric peptide, R₃/I₅, as a selective ligand for GPCR135 and GPCR142 over LGR7. This chimeric molecule opens a new avenue for future in vivo studies of the physiological function of GPCR135 particularly in rat, which has no functional GPCR142. In addition, ^{125}I-R₃/I₅ is a much more hydrophilic peptide compared with the relaxin-3 wild-type peptide and binds GPCR135-expressing cells with a much greater signal-to-noise ratio; therefore, it is a useful tool for radioligand autoradiographic studies. Also in this report, by expression and functional characterization of chimeric peptides with relaxin-3 B-chain and A-chains from different members of the relaxin subfamily, we have demonstrated that the A-chains from many members are structurally and functionally exchangeable. These results offer very useful information for the future production of modified peptides for relaxin-related peptides. Functional analysis of the chimeric peptides has provided valuable information for the

	B-chain	**A-chain**
Relaxin-1:	KWKDDVI**K**LCGRELV**R**AQIAICGMSTWS	RPYVALFEKCCLIGCTKRSLAKYC
Relaxin-2:	DSWMEEVI**K**LCGRELV**R**AQIAICGMSTWS	QLYSALANKCCHVGCTKRSLARFC
Relaxin-3:	RAAPYGV**R**LCGREF**I**RAVIFTCGGSRW	DVLAGLSSSCCKWGCSKSEISSLC
INSL3:	PTPEMRE**K**LCGHHF**V**RALVRVCGGPRWSTEA	AAATNPARYCCLSGCTQQDLLTLCPY
*INSL4:	ESLAAEL**R**GCGPRF**G**KHLLSYCPMPEK	FDPFCCEVICDDGTSVKLCT
INSL5:	KESV**R**LCGLEYI**R**TVIYICASSRW	<EDLQTLCCTDGCSMTDLSALC
*INSL6:	ISSAR**K**LCGRYLV**K**EIEKLCGHANWSQF	GYSEKCCLTGCTKEELSIACLPYIDF
Insulin:	FVNQHLCGSHLVEALYLVCGERGFFYTPKT	GIVEQCCTSICSLYQLENYCN
IGF1:	GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSA	
IGF2:	AYRPSETLCGGELVDTLQFVCGDRGFYFSRP–ASRVSRRSR---GIVEECCFRSCDLALLETYCATPAKSE	

Relaxin Subfamily: Relaxin-1, Relaxin-2, Relaxin-3, INSL3, *INSL4, INSL5, *INSL6

Insulin Subfamily: Insulin, IGF1, IGF2

Fig. 6. Amino acid sequence comparison of known members of the insulin/relaxin family. The two conserved positive charged amino acids in the B-chains of relaxin-1, relaxin-3, relaxin-3, INSL3, INSL4, INSL5, and INSL6 are highlighted in bold letters. * B-chain and A-chain termini for INSL4 and INSL6 are based on predictions.

future study of the structure/function relationship of the relaxin-related peptides. In addition, chimeric peptide studies indicate that GPCR135, or GPCR142 may only directly interact with the B-chain of relaxin-3, whereas LGR7 and LGR8 interact with both the B-chain and A-chain of their ligands. Finally, relaxin-3 chimeric peptide studies suggest that the receptors for INSL4 may be distinct from GPCR135, GPCR142, LGR7, and LGR8, whereas the receptor for INSL6 may be a close neighbor of LGR7 or LGR8.

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