Characterization of Novel Splice Variants of LGR7 and LGR8 Reveals That Receptor Signaling Is Mediated by Their Unique Low Density Lipoprotein Class A Modules*

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The relaxin and insulin-like peptide 3 receptors, LGR7 and LGR8, respectively, are unique members of the leucine-rich repeat-containing G-protein-coupled receptor (LGR) family, because they possess an N-terminal motif with homology to the low density lipoprotein class A (LDLa) modules. By characterizing several LGR7 and LGR8 splice variants, we have revealed that the LDLa module directs ligand-activated cAMP signaling. The LGR8-short variant encodes an LGR8 receptor lacking the LDLa module, whereas LGR7-truncate, LGR7-truncate-2, and LGR7-truncate-3 all encode truncated secreted proteins retaining the LGR7 LDLa module. LGR8-short and an engineered LGR7 variant missing its LDLa module, LGR7-short, bound to their respective ligands with high affinity but lost their ability to signal via stimulation of intracellular cAMP accumulation. Conversely, secreted LGR7-truncate protein with the LDLa module was able to block relaxin-induced LGR7 cAMP signaling and did so without compromising the ability of LGR7 to bind to relaxin or be expressed on the cell membrane. Although the LDLa module of LGR7 was N-glycosylated at position Asn-14, an LGR7 N14Q mutant retained relaxin binding affinity and cAMP signaling, implying that glycosylation is not essential for optimal LDLa function. Using real-time PCR, the expression of mouse LGR7-truncate was detected to be high in, and specific to, the uterus of pregnant mice. The differential expression and evolutionary conservation of LGR7-truncate further suggests that it may also play an important role in vivo. This study highlights the essential role of the LDLa module in LGR7 and LGR8 function and introduces a novel model of GPCR regulation.

Relaxin was initially named for its ability to relax the pubic symphysis in pregnant guinea pigs at parturition (1). Since then relaxin has been found to be involved in many physiological processes, including cervical ripening (2–5), inhibition of myometrial contractions in some mammals (6–8), uterine growth during pregnancy (9, 10), and nipple development for lactation (11–13). Most of the actions of relaxin are a direct result of its ability to stimulate the breakdown and remodeling of collagen fibers by inhibiting collagen type I and III synthesis and promoting matrix metalloproteinase expression and activation (14–16). Most mammalian species have relaxin; however, due to a gene duplication event, humans possess two relaxin genes, encoding H1 relaxin and H2 relaxin, with H2 relaxin being the major stored and circulating form (reviewed in Ref. 17). In pig, mouse, rat, and human, the primary source of relaxin is the corpus luteum (reviewed in Ref. 18), highlighting that the most pronounced roles of relaxin occur during pregnancy.

The relaxin receptor is a GPCR3 most recently named the RXFP1 receptor (relaxin family peptide receptor 1) (19), however, in this report it will be referred to by its original name, leucine-rich repeat-containing GPCR 7 (LGR7) (20). LGR7 has been highly conserved in vertebrate species throughout evolution and is related to the glycoprotein hormone receptors FSHR, TSHR, and LHR (21). The closest homolog of LGR7 is LGR8 (the RXFP2 receptor), whose specific ligand is insulin-like peptide 3 (INSL3) (22). INSL3 is the closest hormone relative of relaxin, acts to mediate testicular descent (23, 24), and is involved in germ cell maturation in rats (22). In addition to their seven transmembrane domains, LGR7 and LGR8 possess large extracellular ectodomains containing 10 leucine-rich repeats similar to those found in the glycoprotein hormone receptors. LGR7 and LGR8 are distinguished into LGR sub-family C, apart from the glycoprotein hormone receptors (class A) because of their unique N-terminal domains, which have homology to the LDL class A (LDLa) modules that make up the ligand binding repeats found in the LDLa receptor family (25). In this report these domains will be referred to as the LDLa modules of LGR7/8.

Alternative splicing of the LGR7 and LGR8 genes has been reported (26), as well as in other LGRs, such as FSHR (27, 28), TSHR (29), LHR (30–32), and LGR4 and LGR6 (26). We have

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3 The abbreviations used are: GPCR, G protein-coupled receptor; LGR7, leucine-rich repeat-containing G-protein-coupled receptor; INSL3, insulin-like peptide 3; LDLa, low density lipoprotein class A; HNK, human embryonic kidney; PBS, phosphate-buffered saline; GTPγS, guanosine 5′-3-O-(thio)triphosphate; TBS, Tris-buffered saline; LHR, luteinizing hormone receptor; Gpp(NH)p, guanosine 5′-O-[(β,γ-imido)triphosphate.]
previously reported the identification of an LGR7 splice variant in rodents that is missing exon 4 and named LGR7-truncate (33). LGR7-truncate is expressed in the reproductive tract of late stage pregnant mice and rats (33), and an expressed sequence tag clone (BF191498) from a pooled library of male and female pig reproductive organs encodes LGR7-truncate. Deletion of exon 4 causes a reading frameshift in the LGR7-truncate transcript, the appearance of a premature stop codon, and results in a splice variant encoding a protein comprising the unique LDLa module of LGR7. When co-transfected into human cells with LGR7, LGR7-truncate is able to reduce relaxin-induced LGR7 signaling through an unknown mechanism (33).

Here we report the identification of three novel human LGR splice variants, one LGR8 variant encoding a receptor missing its LDLa module, named LGR8-short, and two variants similar to LGR7-truncate, encoding secreted LDLa modules, named LGR7-truncate-2 and LGR7-truncate-3. Functional characterization of these splice variants revealed that the unique LDLa modules of LGR7 and LGR8 are crucial for mediation of receptor signaling upon ligand binding. Furthermore, LGR7-truncate expression is established as being specific to and prevalent in the uteri of pregnant mice, suggesting that the LDLa module of LGR7-truncate may play a regulatory role in vivo.

EXPERIMENTAL PROCEDURES

Reagents, Animals, RNA Extraction, PCR, and Cloning—H2 relaxin was kindly provided by BAS Medical (San Mateo, CA). INS/3 was synthesized by Dr. John Wade (34). All female mice (C57BL6) were housed in a controlled environment and maintained as described before (11). Pregnant female animals were killed at days 7.5, 13.5, and 17.5 of pregnancy for tissue collection. The cerebral cortices, uteri, and livers of three non-pregnant, three day-7.5 pregnant, three day-13.5 pregnant, and three day-17.5 pregnant female mice were dissected and snap frozen following the manufacturer’s protocol. RNA extraction was determined via spectrophotometric analysis in a spectrophotometer in a controlled environment. Total RNA in each was extracted following the manufacturer’s protocol. RNA concentration was determined via spectrophotometric analysis in an Exsor Spectrophotometer (Invitrogen), and the total RNA in each was extracted following the manufacturer’s protocol. RNA concentration was determined via spectrophotometric analysis in a spectrophotometer in a controlled environment. Total RNA in each was extracted following the manufacturer’s protocol. RNA concentration was determined via spectrophotometric analysis in an Exsor Spectrophotometer (Invitrogen), and the total RNA in each was extracted following the manufacturer’s protocol. RNA concentration was determined via spectrophotometric analysis in a spectrophotometer in a controlled environment.

Identification of Human LGR Splice Variants—Human uterine cDNA was screened for LGR8 splice variants using PCR and an exon 1–7 spanning primer pair (forward primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’; reverse primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’) containing a SalI restriction enzyme site at its 5’-end and a reverse primer (5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’) containing an Xhol site at its 5’-end. The LGR8-short pGEM-T plasmid was used as the template DNA for a PCR following the conditions: 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 150 s repeated 25 times. The resultant product was cloned into pcDNA3.1/Zeo, in the process the insert was ligated to a bovine prolactin signal sequence and N-terminal FLAG tag as described previously (35). A FLAG-tagged LGR8-short equivalent mammalian expression construct (LGR7 missing the sequence derived from exon 2) was generated in the same way using a different primer pair (forward primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ EcoRI enzyme site, and a reverse primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ Xhol enzyme site), and human LGR7 pcDNA3.1 plasmid as the PCR template, following the conditions: 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 150 s repeated 25 times. A FLAG-tagged Mouse LGR7-truncate pcDNA3.1/Zeo plasmid was cloned in a similar manner using the primer pair (forward primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ EcoRI enzyme site, and a reverse primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ Xhol enzyme site) using pregnant mouse uterine cDNA as the PCR template, following the conditions: 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 45 s repeated 25 times. A mouse LGR7-truncate pcDNA3.1/Zeo plasmid was cloned without a FLAG tag and with its native signal peptide was generated as described above, using a different forward primer (5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ BamHI enzyme site) and reverse primer (5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ Xhol enzyme site) using human mouse uterine cDNA as the PCR template following the conditions: 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s repeated 25 times. A FLAG-tagged human LRG7-truncate (mouse LGR7-truncate equivalent) pcDNA3.1/Zeo plasmid was also generated using a specific primer pair (forward primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ EcoRI enzyme site, and a reverse primer, 5’-CAT GTG AGA TGT TTT TCT TTG AGG AAC ATC T-3’, containing a 5’ Xhol enzyme site) using human LGR7 pcDNA3.1/Zeo pcDNA3.1/Zeo plasmid as a PCR template following the same conditions as the mouse LGR7-truncate PCR outlined above. pcDNA3.1/Zeo plasmids
containing human LGR7-truncate-2 and human LGR7-truncate-3 were generated using the same forward primer (5'-CAT CAT GGA TCC GCC ACC ATG ACA TCT GGT TCT GTC TTC TTC-3' containing a 5' BamHI enzyme site) and specific reverse primers (LGR7-truncate-2, 5'-CAT CAT CTC GAG TTA AGT CCT ACT GAA GTG ACC GCA-3', and LGR7-truncate-3, 5'-CAT CAT CTC GAG TCA CTC ACC ACA GGT GTC CTC ATC-3', each containing a 3' XhoI enzyme site) using the human LGR7-truncate-2 and LGR7-truncate-3 containing pGEM-T plasmids generated above as templates for site-directed mutagenesis (36) or LGR8-short and pCRE-galactosidase reporter plasmid (36) for 24 h later. Co-transfected cells were treated with 5 μM forskolin and increasing concentrations of H2 relaxin. After 6 h, the cell's media was aspirated, and the cells were frozen at -80°C overnight. The amount of cAMP-driven β-galactosidase expression in each well was determined by lysing the cells in 10 mM Na2PO4, pH 8.0, 0.2 mM MgSO4, 0.01 mM MnCl2 for 10 min. 100 μl of assay buffer was added to the wells (100 mM Na2PO4, pH 8.0, 2 mM MgSO4, 0.1 mM MnCl2, 0.5% Triton X-100, 40 μM β-mercaptoethanol) for a further 10 min before the addition of 25 μl of enzyme substrate solution (1 mg/ml chlorophenol red β-D-galactopyranoside (Roche Applied Science) in 100 mM Na2PO4, pH 8.0, 2 mM MgSO4, 0.1 mM MnCl2). The absorbance of each well was monitored at 570 nm using a Ceres UV900C plate reader (Bio-Tek Instruments). When the absorbance readings were between 0.3 (no forskolin or relaxin treatment) and 1.0 (5 μM forskolin treatment) the final readings were recorded. Ligand-induced inhibition of forskolin-stimulated adenylate cyclase activity was expressed as a percentage of 5 μM forskolin-induced cAMP accumulation. Data points were measured in triplicate, and each experiment was repeated three times.

**Analysis of LGR7 Signal Transduction in the Presence of LGR7 Truncate—HEK 293T cells at ~80% confluency in 48-well plates were transfected with 0.5 μg of the pcDNA3.1/Zeo plasmid containing the receptor of interest in Lipofectamine 2000 (Invitrogen) and Opti-MEM serum-free media (Invitrogen). At least 24 h later, the transfection media was aspirated, and each well was washed with 0.5 ml of PBS. The transfected cells were treated with solutions with or without hormones in RPMI 1640 media containing 0.25 mM 3-isobutyl-1-methylxanthine (Sigma) for 30 min before aspiration of the treatment solutions. Cell lysis and measurement of intracellular cAMP accumulation was performed using the cAMP Biotrak enzyme immunoassay kit (Amersham Biosciences) following the manufacturer’s protocol. Each treatment point was measured in triplicate, and data are represented as the mean ± S.E. of three independent experiments. Data analysis was performed using GraphPad Prism 4 (GraphPad Software).

**Anti-FLAG Cell-surface Expression Assay—**FLAG-tagged receptor-transfected cells were plated into 6-well poly-l-lysine-coated plates (1,000,000 transfected cells per well). 24 h later the cells were washed with 1 ml of PBS before incubation in 1 ml of a 5% bovine serum albumin, PBS solution containing 5 μg/ml Anti-FLAG M2 antibody (Sigma) for 1 h at room temperature on a gently tilting platform. Cells were lifted from their wells in this solution and resuspended in 1.5-ml tubes (Axygen), centrifuged for 2 min at 14,000 rpm in a Heraeus Biofuge 13 (Sepatech), and the antibody solution was aspirated. The cell pellet was washed twice in 1 ml of ice-cold PBS, before being resuspended in 200 μl of an ice-cold solution of 5% bovine serum albumin/PBS containing 2,000,000 cpm/ml of 125I-labeled anti-mouse-IgG sheep antibody (Amersham Biosciences) and incubated on ice for 2 h. After 2 cycles of centrifugation, aspiration and washing with 1 ml of 1% bovine serum albumin/PBS, the resultant pellet was counted in a Cobra Auto-Gamma liquid scintillation analyzer (Packard). Empty vector-transfected cells were used to determine the nonspecific binding in the cell-
Co-culture Experiments—175-cm² flasks of 80% confluent 293T cells were transfected with either pcDNA3.1/Zeo plasmids encoding FLAG-tagged LGR7, mouse LGR7-truncate, or empty vector. After 24 h the cells were lifted in EDTA phosphate solution, washed in PBS twice, counted, and then plated in 96-well plates (10,000 LGR7-transfected cells) for cAMP accumulation assays, 24-well poly-L-lysine-coated plates (100,000 LGR7-transfected cells) for relaxin binding assays or 6-well poly-L-lysine-coated plates (1,000,000 LGR7-transfected cells) for cell-surface-binding assays. Into each well of the plates described above already containing LGR7-transfected cells, either LGR7-truncate or empty vector-transfected cells were co-plated at a 1:2.5 or 1:5 ratio of LGR7-transfected cells to LGR7-truncate or empty vector-transfected cells. LGR7 signaling, relaxin binding assays, and FLAG cell-surface binding assays were performed in parallel 24 h later as described above. Data were plotted, and statistical differences were determined using Student t-tests in GraphPad Prism 4.

Expression and Purification of Recombinant LGR7 Truncate Protein—Multiple 175-cm² flasks of 80% confluent HEK 293T cells were washed with PBS and transfected with a pcDNA3.1/Zeo plasmid encoding the FLAG-tagged LGR7-truncate of interest using as described above. After 24 h the transfected cells were lifted with EDTA phosphate solution and resuspended into 500-cm² plates in RPMI 1640 media for another 24 h before replacement of the RPMI 1640 media with 50 ml of Xten Eo serum-free media (Thermotrace) for 3 days. Collected media were filtered with 500 ml of 0.45-µm sterile bottle top filters (Nalgene), and CaCl₂ was added to 2 mM. The resultant solution was then passed through a pre-packed and equilibrated column containing Anti-FLAG M1 affinity Gel (Sigma) five times at 4 °C, washed with 36 ml of TBS containing 2 mM CaCl₂, and eluted with 10 lots of 1 ml of TBS (2 mM EDTA). The eluant (10 ml) was then washed with 100 ml of TBS containing 2 mM CaCl₂ and concentrated in 5,000 Da molecular mass cut off Amicon Ultra 15 centrifugal filtration units (Millipore) at 4,000 × g in a swinging bucket centrifuge (Beckman).

Affinity-purified protein was applied to SDS-PAGE and analyzed by Western blotting using the Anti-FLAG M2 antibody (Sigma), following the manufacturer’s recommended protocol. The activity of affinity-purified LGR7-truncate was accessed by preincubating 50 µl of TBS solutions containing various amounts of either affinity-purified LGR7-truncate or a mock Anti-FLAG M1 affinity column eluant on 293T cells (10,000) stably expressing LGR7 in 48-well plates for 15 min before treatment with 1 nM relaxin. Relaxin-induced LGR7 signaling was determined as above.

Determination of LGR7 Truncate Specificity—A plasmid encoding a mutant mouse LGR7-truncate plasmid (C44A) was generated with the following primer pair (5′-CGG ATG GCT CCC CTC GGC TTC TCT-3′ and 5′-GGG AGC ACT GAC ATC GTG GAC ATC ATC-3′) using a QuikChange PCR mutagenesis kit (Stratagene). PCR was undertaken following the manufacturer’s recommended protocol, and the resultant product was treated with 1 µl of DPN1 for 1 h at 37 °C and transformed into the supplied competent cells. The resultant plasmid was sequenced on both strands to confirm generation of the missense mutation. Co-cultures of LGR7-transfected cells with either LGR7-truncate, LGR7-truncate (C44A), or empty vector were prepared as above and co-cultured at a 1:5 cell number ratio. Relaxin-induced cAMP signaling was determined as above but in the presence or absence of 100 ng/ml pertussis toxin (cells pre-treated with pertussis toxin for 16 h). Wells containing 50,000 Chinese hamster ovary cells stably expressing the relaxin-3 receptor (GPCR135), which couples to inhibitory G proteins (37), were used as a positive control for pertussis toxin activity. These cells were pre-treated with or without pertussis toxin before stimulation with 100 nM human relaxin-3. Data were plotted, and statistical differences were determined using Student t-tests in GraphPad Prism 4.

Prediction and Identification of an N-Linked Glycosylation Site in LGR7-truncate—An N-linked glycosylation site was predicted in LGR7 and LGR7-truncate using NetNGlyc 1.0^a at amino acid position 14. Thus the codon encoding asparagine 14 in human LGR7-truncate was mutated to encode glutamine using a QuikChange PCR mutagenesis kit (Stratagene) and a designed oligonucleotide primer pair (5′-CCCC CTG TGG GCA AAT CAC AAA GTG C-3′ and 5′-GCT AGT GCT CCC ACA GGG G-3′) as above. FLAG-tagged human LGR7-truncate or FLAG-tagged human LGR7-truncate (N14Q) proteins were immunoprecipitated with the Anti-FLAG M2 antibody from transfected cells, and these samples were used for Western blotting. 80% confluent 293T cells in 75-cm² flasks were transfected with FLAG-tagged human LGR7-truncate or FLAG-tagged human LGR7-truncate (N14Q) as described. 48 h later, the media from each flask was collected, and the cells were washed and resuspended in 3 ml of lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and frozen overnight. The resultant cell lysates were centrifuged at 8000 rpm for 3 min in a Heraeus Biofuge 13 (Sepatcel), and 1 ml of each resultant supernatant was added to 20 µl of pre-washed Anti-FLAG M2 affinity agarose gel (Sigma). 2 ml of collected media from each flask was also added to 20 µl of pre-washed Anti-FLAG M2 affinity agarose gel. Tubes containing the Anti-FLAG M2 affinity gel mixes were incubated for 16 h at 4 °C with continuous gentle inversion. To recover the FLAG-tagged LGR7-truncate proteins, the tubes were centrifuged at 8000 rpm for 1 min at 4 °C, and the supernatant was discarded. The affinity gel pellet was washed three times with 4 °C PBS. Finally each pellet was resuspended in 20 µl of 2× SDS-PAGE loading buffer, heated to 100 °C for 3 min, and centrifuged at 7.6 relative centrifugal force for 1 min. 20 µl of each supernatant was separated using SDS-PAGE and transferred to a 0.22-µm polyvinylidene difluoride membrane for Western blot analysis using the Anti-FLAG M2 peroxidase-conjugated antibody (Sigma) following the manufacturer’s protocol.

Characterization of N14Q LGR7—The codon encoding LGR7 asparagine 14 was mutated to encode glutamine (N14Q) using the same primer pair and conditions as above. The ³²P-labeled H2 relaxin binding to, and receptor cell-surface expres-

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4 R. Gupta, E. Jung, and S. Brunak (2004) NetNGlyc 1.0, www.cbs.dtu.dk/services/NetNGlyc/.
LGR7 and LGR8 LDLa Modules Mediate Receptor Signaling

In 293T cells transfected with LGR7 (N14Q) was assessed as described above. Receptor cAMP signaling was assessed using a cAMP reporter gene assay. 293T cells in 96-well plates were co-transfected with either LGR7 and a pCRE-β-galactosidase reporter plasmid (36) or LGR7 (N14Q) and pCRE-β-galactosidase to assess the cAMP signaling response to H2 relaxin of the N14Q mutant receptor. 24 h later co-transfected cells were incubated with increasing concentrations of H2 relaxin or 5 μM forskolin for 6 h, and the resultant intracellular cAMP accumulation was measured as above. H2 relaxin-induced receptor cAMP signaling was expressed as a percentage of 5 μM forskolin-induced cAMP accumulation. Data points were measured in triplicate, and each experiment was repeated three times.

Quantitative Real-time PCR Analysis of LGR7-truncate Expression—RNA from individual tissue samples was reverse-transcribed using TaqMan reverse transcription reagents (Applied Biosystems) and random hexamer primers into three sets of cDNA samples. The first set was for control 18 S amplification and used 30 ng of RNA as a template, the second was for LGR7/LGR7-truncate amplification and used 1.7 μg of RNA as a template, and the final set comprised the standards at 56, 7, 0.87, 0.44, 0.015, and 0.007 ng of RNA in each. Two primer pairs were designed with a common forward primer (5′-CGG AGC TGA TGA GGA CCA CT-3′) and 5′-FAM-labeled sense probe (5′-CGG AGA CAA CAA TGG GTG GTC TCT GC-3′), paired with either a reverse primer complimentary to a region in exon 4 of LGR7 (which will not amplify LGR7-truncate, 5′-CAA ACA ATG CAT GGG CAC AG-3′) or an LGR7-truncate specific reverse primer complimentary to the sequence spanning the unique exon 3–5 boundary (5′-GCA GGG ACA GCA TTC GGA-3′). An 18 S primer pair and probe were used to standardize the cDNA samples (forward primer, 5′-CGG AGA CAA CAA TGG GTG GTC TCT GC-3′), reverse primer, 5′-GCT GGA ATT ACC GCG GCT 3′, and 5′-FAM-labeled probe, 5′-TGG CAC CAG ACT TGC CCT C-3′). Real-time PCR was undertaken using 2.5 μl of cDNA in a 25-μl reaction using TaqMan Universal Master Mix (Applied Biosystems) in a 7700 Sequence Detector (Applied Biosystems) using optical 96-well plates (Applied Biosystems) and optical caps (Applied Biosystems). The standard reactions were amplified on each plate to ensure standardization for cross-plate comparisons. Data were retrieved using Sequence Detector Software (Applied Biosystems) and plotted and analyzed for statistical differences by Student t-tests using GraphPad Prism 4.

RESULTS

Cloning of LGR7/8 Splice Variants—Alternate splicing of the LGR7 and LGR8 genes has been previously reported (26, 33). Some of these splice variants encode receptors with shortened extracellular domains (LGR7.2, LGR7.10, and LGR8.1), whereas other variants, such as mouse LGR7-truncate, encode potentially secreted proteins. Mouse LGR7-truncate is of particular interest because it encodes the most unique domain of the mosaic LGR7 protein, the LDLa module. Here we aimed to identify LGR7-truncate equivalent splice variants derived from human LGR7 and LGR8 genes. Reverse transcription-PCR was performed using oligonucleotide primer pairs spanning the first 5 exons of LGR7 and LGR8 with human uterus and brain cDNA as the reaction templates. Smaller than expected LGR7 and LGR8 products were amplified from human brain and uterus cDNA and cloned into pGEM-T vector (Promega). These products were sequenced, identifying a new LGR8 splice variant and two novel LGR7 splice variants (Fig. 1).

The LGR8 splice variant identified was missing exon 2, the exon that encodes the receptors unique LDLa module. The exclusion of this exon does not induce any frame shifts in the LGR8 mRNA, resulting in a transcript that encodes a receptor without its LDLa module (residues 1–45 in LGR8, Fig. 1), thus this variant was termed LGR8-short.

The first LGR7 splice variant identified was missing exons 3 and 4, causing a reading frameshift and premature stop codon at the beginning of exon 5 (Fig. 1). The second variant identified was missing exons 3 and 4 but contained an extended exon 2 containing the first 53 nucleotides of the second intron of LGR7. Comparison of the sequences of both splice variants to the human genome sequence highlighted the presence of consensus mRNA splicing sequences at the donor and acceptor sites (Fig. 1) and the presence of the exon 2 extension in the human LGR7 intron 2 as expected. This extension of exon 2 caused a premature stop codon 6 nucleotides into the novel exonic sequence (Fig. 1). These two human LGR7 variants were named LGR7-truncate-2 and LGR7-truncate-3 because of their similarity to the previously identified LGR7-truncate, which is devoid of exon 4. The deletion of this extra exon (and additional intronic sequence in the case of LGR7-truncate-3) caused the appearance of a premature stop codon closer to the end of exon 2 (22 nucleotides for LRG7-truncate 2, 8 nucleotides for LRG7-truncate 3) compared with LGR7-truncate (124 nucleotides).

Thus LGR7-truncate-2 and LGR7-truncate-3 transcripts encoded 68 and 64 amino acid proteins, respectively, comprising only the LDLa module of LGR7, whereas LRG7-truncate proteins (102 amino acids) comprise the LDLa module with additional sequence from the N-terminal leucine-rich repeat caps of LGR7 (Fig. 1).

Characterization of LGR8-short and LGR7-short—The discovery of these naturally occurring splice variants with modifications centered on the unique LDLa module of LGR7 and LGR8 presented an opportunity to elucidate the function of this highly conserved domain.

An LGR8-short equivalent LGR7-short sequence was artificially constructed. Both LGR7-short and LGR8-short were cloned into mammalian expression vectors with N-terminal FLAG tags. The extracellular domain of LGR7 and LGR8 is known to possess the primary binding sites of their respective ligands (35, 39). To access the effect of losing the LDLa module in LGR7-short and LGR8-short, radioligand binding assays were performed (Fig. 2, A and B). LGR8 short was able to bind 33P-labeled H2 relaxin, although its affinity for human INSL3 (LGR8-short pIC50 = 8.87 ± 0.07 versus LGR8 pIC50 = 9.56 ± 0.23) and H2 relaxin (LGR8-short pIC50 = 8.22 ± 0.07 versus LGR8 pIC50 = 8.92 ± 0.16) was slightly lower (both p < 0.05) than the wild-type receptor. LGR7-short was also able to bind 33P-labeled H2 relaxin, and demonstrated unchanged affinity for H2 relaxin compared with wild-type LGR7 (LGR7-short pIC50 = 8.90 ± 0.21 versus LGR7 pIC50 = 8.99 ± 0.07). The lack...
of change in the affinity of LGR7-short for H2 relaxin compared with LGR7, and the small change in the ligand affinity of LGR8-short compared with wild-type LGR8 implies that the LDLa module is not involved in primary ligand binding.

Strong evolutionary conservation of the LDLa module in LGR7 and LGR8 indicates that it is likely an integral part of these receptors. To determine if the LDLa module is involved in the mediation of receptor signaling, LGR7-short- and LGR8-short-transfected cells were treated with their respective ligands, and intracellular cAMP accumulation was determined as a measure of hormone-induced receptor signaling. Remarkably, neither LGR7-short nor LGR8-short were able to induce cAMP signaling in response to H2 relaxin and INSL3, respectively, at concentrations up to 1 μM (Fig. 2, C and D). Additionally, LGR8-short was not responsive to H2 relaxin.

**FIGURE 1.** Exon structure of LGR7 and LGR8 compared with the novel splice variants LGR8-short, LGR7-truncate, LGR7-truncate-2, and LGR7-truncate-3. LGR7 and LGR8 are made up of 18 exons with the extracellular domain of the receptors being encoded in the first 15 exons. The four splice variants of these genes are represented schematically, with the resultant proteins they encode. LGR8-short is missing exon 2 and encodes a full-length receptor missing its LDLa module. LGR7-truncate is missing exon 4, causing a reading frameshift and premature stop codon and encodes the LDLa module of LGR7 plus 33 specific LGR7 residues before 7 novel residues as a result of the reading frameshift. LGR7-truncate-2, discovered in human, is missing exons 3 and 4, resulting in a reading frameshift and premature stop codon, encoding the LDLa module of LGR7 and 6 novel residues as a result of the reading frameshift. LGR7-truncate-3 is missing exons 3 and 4, but has an extended exon 2 containing 53 nucleotides from the second intron of LGR7, resulting in a reading frameshift, premature stop codon, and a transcript encoding the LDLa module and two novel residues as a result of the frameshift. The novel exon boundaries are shown with the canonical mRNA splicing consensus sequences for the donor and acceptor sites boxed and intronic sequence in bold.

**FIGURE 2.** The [33P]-labeled H2 relaxin binding and H2 relaxin-induced cAMP signaling in HEK 293T cells transfected with LGR7-short and LGR8-short. A, competition of [33P]-labeled H2 relaxin binding to LGR7 and LGR7-short with H2 relaxin. B, competition of [33P]-labeled H2 relaxin binding to LGR8 and LGR8-short with INSL3. C, H2 relaxin-induced cAMP production mediated by LGR7 and LGR7-short. D, INSL3-induced cAMP production mediated by LGR8 and LGR8-short. Data are the mean ± S.E. of data from at least three independent experiments performed in triplicate.
relaxin stimulation (up to 1 μM, data not shown). Characterization of LGR7-short and LGR8-short has demonstrated that the LDLa module is involved in the mediation of receptor signaling upon ligand binding.

The inability of these variant receptors to signal through the cAMP pathway does not indicate that they are completely unable to signal. G protein coupling is a requirement for high affinity ligand binding to many GPCRs, which can be unveiled by treating receptors with non-hydrolyzable GTP analogs and detecting any changes in ligand affinity (40, 41). If high affinity hormone binding to LGR7-short and LGR8-short is influenced by such GTP analogs, then G proteins can couple to these receptors and thus they may signal through other pathways. Membrane preparations containing these receptors were treated with the non-hydrolyzable isoforms of GTP, Gpp(NH)p, and GTPγS. The [33P]relaxin binding assays showed that neither Gpp(NH)p nor GTPγS treatment altered the affinity of LGR7-short or LGR8-short for H2-relaxin or INSL3, respectively (data not shown). Importantly, Gpp(NH)p or GTPγS treatment failed to alter the affinity of native LGR7 or native LGR8 for H2 relaxin or INSL3, respectively, indicating that the affinity of all of these receptors for their ligands is independent of G protein coupling.

We have previously shown that the LGR8 receptor can signal through inhibitory G proteins to block adenylate cyclase activity in the testis and ovary (42). We investigated whether LGR7-short or LGR8-short were able to signal through inhibitory G proteins to decrease forskolin-induced stimulation of intracellular cAMP accumulation. Treatment of cells transfected with LGR7-short or LGR8-short with 5 μM forskolin resulted in a large increase in intracellular cAMP accumulation, indicating that the adenylate cyclase signaling system is not affected by the presence of these variant receptors. Up to 1 μM relaxin treatment of these cells did not result in any decrease in the intracellular cAMP accumulation caused by 5 μM forskolin treatment (data not shown). Similarly, up to 1 μM INSL3 treatment did not inhibit forskolin-induced stimulation of adenylate cyclase in cells transfected with LGR8-short (data not shown). Thus LGR7-short and LGR8-short are unable to signal through inhibitory G proteins to reduce intracellular cAMP accumulation.

Characterization of LGR7-truncate Variants—Instead of encoding a receptor missing its LDLa module, the LGR7-truncate splice variants primarily encode a soluble LDLa module (33). We have previously reported that mouse LGR7-truncate can reduce relaxin-induced cAMP signaling when co-transfected into cells with LGR7 (33). However, it was unclear in this study if the antagonistic effect of LGR7-truncate co-transfection was due to an intracellular or extracellular action. To ensure LGR7-truncate is not acting on LGR7 intracellularly, 293T cells were transfected with plasmids containing either mouse LGR7-truncate or FLAG-tagged human LGR7 separately. After transfection, LGR7-truncate transfected cells were co-cultured together with LGR7-expressing cells. Whole cell relaxin binding assays and anti-FLAG cell-surface assays were performed in parallel to cAMP accumulation assays to assess whether LRG7-truncate was influencing LGR7 function. Co-cultures of LGR7 and LGR7-truncate cells exhibited no difference in relaxin binding (Fig. 3A) or cell-surface expression (Fig. 3B).
induced cAMP signaling in co-cultures of LGR7 and LGR7-truncate-transfected cells (Fig. 3C) was severely diminished compared with the control co-cultures. The presence of LGR7-truncate transfected cells at 2.5:1 and 5:1 ratios with LGR7 cells resulted in 75% ± 6.3% (p = 0.0003) and 45% ± 5.6% (p < 0.0001) of the H2 relaxin-induced cAMP signaling seen in the empty vector-transfected cell: LGR7 cell co-cultures (Fig. 3C). This set of experiments clearly established that secreted LGR7-truncate can block relaxin-induced LGR7 signaling and does so without disrupting the relaxin binding integrity of LGR7, or the level of cell-surface expression of the receptor. Similar experiments with human LGR7-truncate-2 and LGR7-truncate-3 yielded equivalent results (data not shown).

HEK 293T cells were transfected with a plasmid encoding FLAG-tagged mouse LGR7-truncate. Mouse LGR7-truncate protein was then purified from the cell media using an anti-FLAG affinity column, and its presence was determined using Western blotting. Affinity-purified mouse LGR7-truncate was assessed for its ability to antagonize H2 relaxin-induced LGR7 cAMP signaling. Varying amounts of affinity-purified mouse LGR7-truncate protein was incubated on LGR7-transfected 293T cells in a total of 100 µl of treatment solution (TBS) with 100 µl of RPMI media, for 15 min before 1 nM H2 relaxin treatment. Compared with mock affinity-purified protein (from the media of empty vector-transfected cells) and TBS controls, affinity purified mouse LGR7-truncate solution was able to significantly reduce H2 relaxin-induced intracellular cAMP accumulation in LGR7-transfected cells in a dose-dependent manner. LGR7 cells exposed to 5, 10, 25, and 50 µl of affinity-purified LGR7-truncate suppressed LGR7 signaling to 90%, 76%, 72%, and 57% that of the control groups (Fig. 4). This experiment demonstrates that secreted LGR7-truncate containing the LDLa module is capable of specifically inhibiting H2 relaxin-induced LGR7 cAMP signaling.

To determine the specificity of LGR7-truncate antagonism of LGR7 signaling, the second cysteine in its LDLa module was replaced with alanine. This mutation perturbs the structure of the LDLa module by not allowing the formation of the second disulfide bond. This bond is essential for proper folding of the LDLa module and is likely to result in production of an inactive protein (43). Thus we tested whether the disruption of the LDLa module structure in LGR7-truncate disrupts its ability to inhibit cAMP accumulation upon relaxin stimulation of LGR7. Using the co-culture paradigm described previously, replacement of cysteine 44 in LGR7-truncate corresponded to the loss of its ability to inhibit LGR7 signaling (Fig. 5). This indi-
cates that the LGR7 antagonism we are observing in our assays is a specific effect caused by properly folded LGR7-truncate protein.

To establish that LGR7-truncate is acting to inhibit LGR7 signaling through Goi and not by stimulating the switching of LGR7 G protein coupling to inhibitory G proteins, we used pertussis toxin to block inhibitory G proteins. Co-cultures of LGR7-transfected cells and LGR7-truncate or LGR7-truncate (C44A)-expressing cells were pre-treated with 100 ng/ml pertussis toxin before 1 nM H2 relaxin treatment. This pretreatment had no effect on LGR7-truncate antagonism of LGR7, indicating that inhibitory G proteins are not involved in this process (Fig. 5). Not surprisingly, pertussis toxin did not have any effect on relaxin-induced LGR7 signaling in the control co-cultures of LGR7- and LGR7-truncate (C44A)-transfected cells (Fig. 5).

N-Linked Glycosylation of the LDLa Module of LGR7—The affinity-purified mouse LGR7-truncate protein used above was analyzed using Western blotting and found to be ~16 kDa in size (data not shown), which is larger than the predicted molecular mass of 9.84 kDa. Thus mouse and human LGR7 and LGR7-truncate protein sequences were screened for potential N-linked glycosylation sites using the NetNGlyc 1.0 program. An asparagine at position 14 in all LGR7 and LGR7-truncate variants was predicted as a strong candidate for N-linked glycosylation (data not shown). This residue was mutated to glutamine in a FLAG-tagged human LGR7-truncate and the wild-type human LGR7 plasmids. Human LGR7-truncate and human LGR7-truncate (N14Q) plasmids were expressed in 293T cells, and the resultant proteins were immunoprecipitated and analyzed by Western blotting in parallel. A shift in size from ~16 to 12 kDa was observed indicating that LGR7-truncate is a secreted glycoprotein (Fig. 6A).

The importance of N-linked glycosylation at Asn-14 of human LGR7 was assessed by measuring receptor cell-surface expression of 33P-labeled relaxin binding to, and H2 relaxin-induced cAMP signaling in, LGR7 (N14Q)-transfected 293T cells. LGR7 (N14Q) was expressed on the cell surface at the same level as wild-type LGR7 (Fig. 6B). LGR7 (N14Q) demonstrated unchanged affinity for H2 relaxin compared with LGR7 (LGR7 (N14Q) pIC50 = 8.96 ± 0.17 versus LGR7 pIC50 = 8.99 ± 0.07) (Fig. 6C). Importantly, LGR7 (N14Q) exhibited the same efficacy for stimulating cAMP accumulation with increasing H2 relaxin concentrations (LGR7 (N14Q) pEC50 = 10.53 ± 0.14 versus LGR7 pEC50 = 10.24 ± 0.08), although the maximum cAMP responses elicited were slightly lower than the native LGR7 responses (Fig. 6D). It is therefore unlikely that glycosylation at amino acid Asn-14 of the LDLa module of LGR7 is essential for optimal LDLa-mediated signal transduction.

Co-expression of LGR7-short and LGR7-truncate—Because LGR7-short and LGR8-short lose their ability to signal without their LDLa modules, we attempted to rescue signaling in these receptors by adding back an LDLa module in the form of secreted LGR7-truncate proteins. 293T cells were transfected with LGR7-short and either empty vector, LGR7-truncate, LGR7-truncate-2, or LGR7-truncate-3. 24 h later relaxin binding to, and H2 relaxin-induced cAMP signaling in, the co-transfected cells were measured. No significant difference in H2 relaxin binding or H2 relaxin-induced signaling was observed between any of the groups of co-transfected cells (data not shown). These data indicated that co-expression of LGR7-short with a secreted LDLa module protein is insufficient to rescue receptor signaling, and the proper orientation of the LDLa module in the intact LGR7 molecule is needed for signal transduction.
Expression of Mouse LGR7-truncate mRNA in the Pregnant Uterus—Previously, we demonstrated that mouse LGR7-truncate mRNA expression is localized to the reproductive tract of pregnant mice using reverse transcription-PCR (33). Here we employed quantitative real-time PCR to determine the mRNA expression levels of both LGR7 and LGR7-truncate in the uterus, cerebral cortex, and liver of non-pregnant female mice and pregnant mice at three different stages of pregnancy. The amount of LGR7 mRNA in the cerebral cortices of female mice was demonstrated to be high and to remain relatively stable throughout pregnancy, with a slight decrease in receptor expression in the cerebral cortex as pregnancy progressed (Fig. 7A). By direct comparison, the level of LGR7-truncate mRNA expression in the cerebral cortices at all stages of pregnancy was very low (~3% compared with LGR7) (Fig. 7A). Serving as a negative control, no significant LGR7 or LGR7-truncate mRNA expression was detected in the liver of any mice (data not shown). The highest expression of LGR7 mRNA was observed in the uterus of day-7.5 pregnant mice, being twice the expression observed in the cerebral cortex (p = 0.002, Fig. 7B). As pregnancy progressed LGR7 mRNA expression was seen to decrease substantially. As opposed to its expression level in the cerebral cortex, LGR7-truncate mRNA levels are comparable to that of LGR7 mRNA in the uterus at day 7.5 of pregnancy (Fig. 7B). In non-pregnant animals, LGR7 and LGR7-truncate mRNA expression in the cerebral cortex was comparable to their expression at day 7.5 of pregnancy, with LGR7-truncate mRNA expression being very low compared with LGR7. In the uterus of non-pregnant mice, LGR7-truncate expression was at the same level as LGR7, displaying ~5% of the level that LGR7 mRNA was expressed in the non-pregnant cerebral cortex (Fig. 7). This pronounced, tissue-specific expression indicates that it is likely that LGR7-truncate plays a functional role in the uterus of mice during pregnancy.

DISCUSSION

The first insights into the molecular mechanisms of action of LGR7 were revealed when soluble LGR7 extracellular domain protein (7BP) was seen to bind to relaxin and antagonize its actions in vivo (20). Chimeric LGR7/LGR8 receptors were used to further establish that the extracellular domains of LGR7 and LGR8 contain the primary ligand binding sites, whereas the extracellular loops of the seven transmembrane domains harbor a second, lower affinity binding site (35, 39). The proposed model of LGR7 activation involves a two-step process, with relaxin interacting with a high affinity site within the leucine-rich repeats of the ectodomain resulting in a conformational change and secondary interactions between bound relaxin and the transmembrane domain of LGR7 mediating receptor signaling (39).

By characterizing two types of naturally occurring LGR7 and LGR8 splice variants we have unveiled another important step involved in the activation of these receptors. LGR8-short, a splice variant lacking the LDLa module of LGR8 and LGR7-short, an engineered LGR8-short equivalent, were unable to confer cAMP signaling in response to INSL3 or H2 relaxin treatment even though their affinities for INSL3 or relaxin binding were comparable to that of the wild-type receptors. Complementary to this, we demonstrated that secreted LGR7 LDLa module-containing proteins such as mouse LGR7-truncate can specifically disrupt the ability of LGR7 to stimulate intracellular cAMP accumulation in response to H2 relaxin treatment.

This potential involvement of the LDLa module of LGR7 in signal mediation has been postulated previously, when decreased LGR7 signaling was observed in cells co-transfected with LGR7 and LGR7-truncate, an LGR7 splice variant encoding a secreted protein comprising the LDLa module (33). However, the results gained from LGR7/LGR7-truncate co-transfections could have been due to intracellular or extracellular disruption of LGR7 receptor integrity or trafficking of receptors within the cell. A splice variant of the luteinizing hormone receptor (LHR) missing exon 9 (LHR(exon 9)) is able to modulate the expression of wild-type LHR and thus its activity when co-expressed in the same cell (32). In this report we proved that LGR7-truncate antagonism of LGR7 is completely different to that of wild-type receptors. By transfecting separate cell cultures with LGR7 or LGR7-truncate and then co-culturing these cells we eliminated the

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**FIGURE 7. Expression of LGR7 and LGR7-truncate mRNA during pregnancy in the mouse.** Quantitative real-time PCR with LGR7-specific and LGR7-truncate-specific primer pairs. A, expression of LGR7 and LGR7-truncate in the cerebral cortex at three stages of pregnancy (preg) and in non-pregnant (non preg) animals. a, significantly different from b (p < 0.015); c is not significantly different from a or b. B, expression of LGR7 and LGR7-truncate in the uterus at three stages of pregnancy and in non-pregnant animals. a, significantly different from b (p < 0.0002), c (p < 0.0015), and d (p < 0.0033), b, significantly different from c (p < 0.0003) and d (p < 0.0046). c, significantly different from d (p < 0.0001). Data are the mean ± S.E. of data derived from the tissues of at least three separate animals, measured in triplicate.
possibility of any intracellular effects of LGR7-truncate expression in LGR7-expressing cells. By analyzing cell-surface expression of LGR7 and relaxin binding to these co-cultures we therefore demonstrated that LGR7-truncate is not affecting the trafficking of LGR7 to the cell surface or its ability to bind to relaxin. However, both the highly significant reduction in H2 relaxin-induced signaling in H2 relaxin-induced signaling in LGR7-transfected cells exposed to increasing concentrations of affinity-purified mouse LGR7-truncate protein, proves that LGR7-truncate is acting on LGR7 to specifically block receptor signaling. The inability of a structurally perturbed mutant LGR7-truncate protein to inhibit LGR7 signaling further demonstrates that LGR7-truncate action is specific. Furthermore, pertussis toxin had no effect on LGR7-truncate antagonism, indicating that its action is to block normal LGR7 signaling mechanisms through Gα coupling, not by switching receptor signaling through Gαi coupling.

Western blotting and bioinformatic analysis of LGR7-truncate revealed that the LDLa module of LGR7 contains an N-linked glycosylation site at asparagine 14. When this glycosylation site was removed through mutagenesis, the resultant receptor, LGR7 (N14Q), demonstrated similar properties to the wild-type receptor. It was expressed at the cell surface at the same level as LGR7 and responded to H2 relaxin with identical affinity and efficacy. It is therefore unlikely that glycosylation of Asn-14 in the LDLa module is essential for the function of LGR7.

It is, however, clear that the LDLa module is essential for LGR7 signaling. The introduced exogenous LDLa module, in the form of LGR7-truncate, may result in competition with the receptor’s own LDLa module, thus interfering with the conformational changes required for LGR7 and LGR8 activation, independent of primary ligand binding. Thus we propose a third step in the current model of LGR7 and LGR8 activation. Primary ligand binding occurs in the leucine-rich repeats of the ectodomain followed by a secondary binding interaction in the transmembrane exoloops. These binding events likely lead to conformational changes in the receptor, which allow the LDLa module to confer receptor signaling via an unknown mechanism (Fig. 8). The location of the LDLa module at the N-terminal end of the ectodomain makes it difficult to predict what it may be interacting with to mediate receptor activation. The similar binding affinities of the receptors lacking the LDLa module do not support participation of the LDLa module in

FIGURE 8. Three-step model of LGR7 activation. 1, relaxin initially binds to the primary ligand binding site in the leucine-rich repeats of the receptor. 2, the conformational changes induced in the receptor upon primary ligand binding allow the accommodation of interactions between the ligand and the extracellular loops of the receptor. 3, the LDLa module of LGR7 drives receptor activation and intracellular cAMP accumulation via an unknown mechanism.
LGR7 and LGR8 LDLa Modules Mediate Receptor Signaling

LGR7 and LGR8 LDLa modules form binding sites for many ligands, including apoE, proteases, and protease/protease inhibitor complexes, albumin, lactoferrin, and many others (reviewed in Ref. 44). Singular LDLa modules can also form protein interaction sites, for example a single LDLa module in the avian sarcoma and leukosis virus protein EnvA receptor, Tva, mediates the interaction with its cognate ligand EnvA (45). Thus it is likely that the LDLa module in LGR7 and LGR8 mediates intramolecular interactions that are needed for the coordination of G-protein signaling. Future determination of the structure of LGR7 or LGR8 will greatly assist in understanding the molecular mechanisms underlying the role of the LDLa module in ligand signaling. The fact that we could not rescue ligand-induced LGR7-short and LGR8-short signaling by co-expressing these receptors with LGR7-truncate implies that the LDLa interaction is complex and requires a receptor-tethered LDLa module to elicit an active receptor.

LGR7, LGR8, and the homologous snail and Drosophila LGRs are the only known GPCRs with LDLa modules (25), hence the use of this domain to direct receptor signaling is unique within the GPCR family. Furthermore, if active in vivo, the ability of LGR7-truncate protein to specifically disrupt signaling of LGR7 would be a novel mode of GPCR regulation via a splice variant. The probability of LGR7-truncate playing such a role was strengthened by the discovery of LGR7-truncate homologous transcripts in human uterus and brain. Human LGR7-truncate-2 and LGR7-truncate-3 differ from mouse, rat, and pig LGR7-truncate (which are missing exon 4) in that exon 3 and exon 4 are missing in both. The LGR7-truncate-3 transcript also contains 53 nucleotides of intron 2. These alternate splicing events cause reading frame shifts and premature stop codons causing LGR7-truncate-2 and LGR7-truncate-3 transcripts to encode 68 and 64 amino acid proteins, respectively, comprising only the LDLa module of LGR7. In several mammals (mouse, rat, and human) we have found an LGR7 transcript encoding a putatively secreted LDLa module-containing protein. This evolutionary conservation suggests that LGR7-truncate transcripts are functionally important.

We have previously demonstrated via reverse transcription-PCR that mouse LGR7-truncate is highly expressed in the cervix/vagina, whole uterus, as well as myometrium and endometrium during late pregnancy, whereas there was only weak expression in non-reproductive tissues (33). The present study demonstrated that LGR7-truncate is differentially expressed in reproductive tissues during pregnancy. Hence we used real-time PCR to compare LGR7-truncate to LGR7 expression in the cerebral cortex, non-pregnant uterus, and pregnant uterus throughout pregnancy. LGR7 expression was detected to be high in the cerebral cortex of all animals, whereas LGR7-truncate expression was barely detectable. In comparison both LGR7 and LGR7-truncate expression were at their highest at the beginning of pregnancy (day 7.5), with both transcripts being increased compared with the non-pregnant animals. The high level of LGR7-truncate expression specifically in the uteri of pregnant mice implies that LGR7-truncate expression is important during pregnancy. Dose-dependent inhibition of LGR7 signaling by LGR7-truncate protein (Fig. 4) indicates that LGR7-truncate is able to antagonize relaxin over a wide concentration range, suggesting that its expression levels in the uterus may result in enough LGR7-truncate protein to effect the actions of relaxin. However, it is difficult to postulate a specific role for an endogenous regulator of LGR7 activity throughout pregnancy in the mouse. Relaxin has numerous important roles in the mouse reproductive tract during pregnancy (18). Throughout pregnancy relaxin has an important role as an inhibitor of spontaneous myometrial contractions as well as a role in the development of the reproductive tract. At the end of pregnancy relaxin is essential for the lengthening of the pubic symphysis and is also involved in cervical ripening. Clearly, to establish whether LGR7-truncate plays a functional role during pregnancy, it would be necessary to establish that an LGR7-truncate protein is indeed produced in vivo.

Although the functional significance of LGR8-short is unknown, it should not be dismissed as an irrelevant artifact of LGR8 mRNA processing. We have demonstrated that it cannot signal through the Gaα/adenylate cyclase/cAMP signaling pathway, or through coupling to inhibitory G Proteins. However, it may still couple to other G proteins or signal through other mechanisms. We know that the affinity of these receptors for their ligands is independent of G protein coupling, because artificial tethering of their extracellular domains to single transmembrane domains results in no loss of ligand affinity (39). Thus we cannot assume that LGR7-short and LGR8-short are still able to couple to G proteins, because they bind their ligands with high affinity. Our experiments did not definitely show that LGR8-short was unable to couple to G proteins because of the fact that hormone binding to LGR7 and LGR8 is independent of G protein coupling. This is the case for many GPCRs (38, 46), and thus alternative methods will need to be used to determine if any signaling pathways can be stimulated by LGR8-short.

In summary, through characterizing two types of naturally occurring LGR7 and LGR8 splice variants, we have demonstrated that the unique N-terminal LDLa modules of LGR7 and LGR8 are part of a novel mechanism of GPCR activation. We propose a three-step model of LGR7 and LGR8 activation. The respective hormone initially binds to the primary ligand binding site in the leucine-rich repeats of the receptor allowing the accommodation of interactions between the ligand and the receptor’s extracellular loops followed by receptor signaling mediated by the LDLa module. LDLa module-containing proteins derived from LGR7-truncate splice variants are able to interfere with LGR7 activation at the third step of this model. The antagonistic action of LGR7-truncate is also a novel mode of GPCR signal regulation, and its evolutionary conservation and high differential expression in the uterus of pregnant mice suggest that this splice variant may undertake a unique regulatory role during pregnancy.

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