

Splice variants of the relaxin and INSL3 receptors reveal unanticipated molecular complexity

Marco Muda^{1,4}, Chaomei He¹, Paolo G.V.Martini¹, Tania Ferraro², Sharon Layfield², Deanne Taylor¹, Colette Chevrier¹, Rene Schweickhardt¹, Christie Kelton¹, Peter L.Ryan³ and Ross A.D.Bathgate²

¹Serono Research Institute, One Technology Place, Rockland, MA, USA, ²Howard Florey Institute, University of Melbourne, Victoria, Australia and ³Department of Animal and Dairy Sciences, Mississippi State University, MS, USA

⁴To whom correspondence should be addressed at: Serono Research Institute, One Technology Place, Rockland, MA 02370, USA.
E-mail: marco.muda@serono.com

LGR7 and LGR8 are G protein-coupled receptors that belong to the leucine-rich repeat-containing G-protein coupled receptor (LGR) family, including the thyroid-stimulating hormone (TSH), LH and FSH receptors. LGR7 and LGR8 stimulate cAMP production upon binding of the cognate ligands, relaxin and insulin-like peptide 3 (INSL3), respectively. We cloned several novel splice variants of both LGR7 and LGR8 and analysed the function of four variants. LGR7.1 is a truncated receptor, including only the N-terminal region of the receptor and two leucine rich repeats. In contrast, LGR7.2, LGR7.10 and LGR 8.1 all contain an intact seven transmembrane domain and most of the extracellular region, lacking only one or two exons in the ectodomain. Our analysis demonstrates that although LGR7.10 and LGR8.1 are expressed at the cell surface, LGR7.2 is predominantly retained within cells and LGR7.1 is partially secreted. mRNA expression analysis revealed that several variants are co-expressed in various tissues. None of these variants were able to stimulate cAMP production following relaxin or INSL3 treatment. Unexpectedly, we did not detect any direct specific relaxin or INSL3 binding on any of the splice variants. The large number of receptor splice variants identified suggests an unforeseen complexity in the physiology of this novel hormone-receptor system.

Key words: GPCR/INSL3/LGR7/LGR8/relaxin/splice variants

Introduction

The leucine-rich repeat-containing G-protein coupled receptor (LGR) family comprises eight members in the human. The LGR family can be divided into three groups based on sequence similarity (Hsu *et al.*, 2000). One group consists of the glycoprotein hormone receptors, FSH receptor (FSHR), LH receptor (LHR), and thyroid-stimulating hormone receptor (TSHR). The orphan receptors LGR4, LGR5 and LGR6 form the second group whereas the third group includes LGR7 and LGR8, the recently identified relaxin and insulin-like peptide 3 (INSL3) receptors, respectively (Hsu *et al.*, 2002; Kumagai *et al.*, 2002).

Relaxin and INSL3 are key factors in the regulation of a variety of developmental processes related to reproduction. INSL3 is essential for the development of the gubernaculum during testis descent (Ivell and Bathgate, 2002) and recently was also demonstrated to be involved in germ cell maturation in males and females (Kawamura *et al.*, 2004). Relaxin is essential for the development of the reproductive tract and nipple during pregnancy and is also a key regulator of collagen turnover in reproductive and nonreproductive tissues (Bathgate *et al.*, 2003). Seven genes encoding relaxin family members are present in the human genome. The peptides encoded by these genes are relaxin-1, relaxin-2, INSL3 [also known as Leydig insulin-like peptide (LEY IL) or relaxin like factor (RLF)], the recently discovered relaxin-3 (Bathgate *et al.*, 2002), INSL4 (also known as placentin or early placenta insulin-like (EPIL) peptide; INSL4/Placentin/EPIL) (Chassin *et al.*, 1995; Koman *et al.*, 1996), INSL5 (Conklin *et al.*,

1999) and INSL6 (Hsu, 1999; Lok *et al.*, 2000). Relaxin-3 is predominantly expressed in the brain, is unlikely to be a circulating hormone and has no actions associated with the other relaxin genes (Bathgate *et al.*, 2003). Relaxin-1 and INSL4 orthologues are not present in non-primates (Bathgate *et al.*, 2002), hence throughout this article relaxin refers to the single relaxin in non-primates, whereas the two human relaxin peptides will be referred to as H1 and H2 relaxin. Recently, in addition to relaxin, LGR7 has been described as a specific receptor for relaxin-3 (Sudo *et al.*, 2003), although relaxin-3 will also interact with two receptors unrelated to LGRs, GPCR135 (Liu *et al.*, 2003a) and GPCR142 (Liu *et al.*, 2003b). Furthermore, INSL3 has been shown to be the selective ligand of the LGR8 receptor (Kumagai *et al.*, 2002) and consistently mice deficient in either INSL3 or LGR8 share a similar phenotype. Indeed, studies from INSL3 and LGR8 null mice demonstrated the critical role of this receptor ligand pair in the regulation of gubernacular differentiation (Gorlov *et al.*, 2002; Ferlin *et al.*, 2003). No other relaxin family peptide has been shown to interact with LGR7 or LGR8 (Hsu *et al.*, 2002; Ivell and Bathgate, 2002; Kumagai *et al.*, 2002; Bathgate *et al.*, 2003; Sudo *et al.*, 2003; Lin *et al.*, 2004). Although INSL5 has recently been demonstrated to be a specific ligand for GPCR142 (Liu *et al.*, 2005), the receptors for INSL4 and INSL6 are unknown. Relaxin and other relaxin family peptides have been implicated in a variety of biological functions not restricted to mammalian reproduction, suggesting a more widespread role for these hormones in the regulation of diverse functions in various organs such as lung, brain, kidney and heart.

LGR7 and LGR8, like other LGR family members, are unique GPCRs because of the presence of a very large extracellular domain (ectodomain). As has been previously shown for the glycoprotein hormone receptors, relaxin binds to the ectodomain of the LGR7 receptor. Hence, studies with an ectodomain only soluble LGR7 protein (7BP) have demonstrated it will bind relaxin (Hsu *et al.*, 2002). Furthermore, studies using chimeric LGR7/LGR8 receptors show that primary ligand binding is in the ectodomain and there is a secondary binding site in the transmembrane domains (Sudo *et al.*, 2003). While undertaking molecular cloning of LGR family members in our laboratory, it became clear that alternative splicing is a common occurrence within the LGR family. Previous reports from other laboratories, describing LGR splice variants, support this observation. Indeed splice variants were previously reported for all the glycoprotein hormone receptors, FSHR (Kraaij *et al.*, 1998; Tena-Sempere *et al.*, 1999), TSHR (Graves *et al.*, 1992), LHR (Loosfelt *et al.*, 1989; Misrahi *et al.*, 1996; Nakamura *et al.*, 2004), LGR4 and LGR6 (M.Muda and D.Taylor, unpublished data). Following the finding that one LHR receptor splice variant was able to modulate the functional property of the wild-type LHR receptor (Nakamura *et al.*, 2004), the identification of LGR7 and LGR8 splice variants was of particular interest. We report here the identification of 29 splice variants for LGR7 and three variants for LGR8. The variants can be grouped into two main classes: those that contain the transmembrane region, and those that encode only the truncated extracellular region. We have started analysing the function of some of the variants, namely LGR7.1, LGR7.2, LGR7.10 and LGR8.1.

Materials and methods

Reagents and antibodies

Anti-FLAG M2 monoclonal antibody, biotinylated and peroxidase conjugated versions, was purchased from Sigma (St. Louis, MO, USA). [¹²⁵I]-Labelled streptavidin and peroxidase conjugated streptavidin were purchased from Amersham Biosciences (Piscataway, NJ, USA). Ez-Link Sulfo-NHS-LC-Biotin was purchased from Pierce (Rockford, IL, USA). Quantitation of cAMP was performed using Tropic chemiluminescent immunoassay system for 96-well microplates (Applied Biosystems, Bedford, MA, USA). Porcine relaxin was purified from pregnant sow ovaries as previously described (Sherwood and O'Byrne, 1974). Recombinant H2 relaxin was a gift from Bas Medical (San Mateo, CA, USA). Human INSL3 was synthesized as previously described (Fu *et al.*, 2004). Porcine relaxin and recombinant H2 relaxin for some of the studies were purchased from the National Hormone and Peptide Program (Torrance, CA, USA).

Identification and cloning of full-length LGR7.1

The NCBI database was searched using the human LGR7 nucleotide sequence (accession number NM021634) as query. One EST clone was identified encoding a partial sequence that corresponded to the published human LGR7 nucleotide sequence (accession number BG611610). The EST clone was purchased from Invitrogen (Carlsbad, CA, USA), and the complete DNA sequence determined using specific sequencing primers and the BigDye version 3.1 (Applied Biosystems, Foster City, CA, USA). DNA sequence determination was done on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The full LGR7.1 coding sequence was obtained as a PCR product of 562 bp using the following primers: sense primer 5'-ATGACATCTGGTTCTGTCTT-3', antisense primer 5'-CTTCATTTTTCAGAGCCATC-3'. Human placenta Marathon-ready cDNA (BD Biosciences Clontech, Palo Alto, CA, USA) was used as the template. PCR was performed in a final volume of 100 µl containing 1× Expand High Fidelity buffer, 200 µM dNTPs, 250 pmol each of cloning primers, 3.5 units of Expand High Fidelity enzyme mix (Roche, Indianapolis, IN, USA) and 2 µl of cDNA using a 9600 GeneAmp PCR System (Perkin Elmer, Torrance, CA, USA). Amplified product was cloned into dT-treated pBSK (Stratagene, La Jolla, CA, USA).

Cloning of LGR7, LGR8 and splice variants LGR7.2, LGR7.10 and LGR8.1

To identify novel splice variants, we utilized a full-length nested PCR approach using cDNA prepared from various tissues. We decided to focus on the following tissue cDNAs: adrenal, ovary, testis, pituitary, placenta and prostate (Marathon-Ready cDNA, BD Biosciences Clontech). Nested sets of primer were designed corresponding to the 5'- and 3'-untranslated sequences of LGR7 based on the genomic sequence: sense primer 5'-ACCAAGACCAAATTTGCTC-3', nested sense primer 5'-TGCTCACTTTCATTAATCAG-3', antisense primer 5'-TCCTCATGTCCTTGTAAAAT-3' and nested antisense primer 5'-CACAGTATTCTCTGCGAAGA-3'. Nested primers for LGR8 PCR were sense primer 5'-GCCTCAGATTGATTACAATGTTCTTCTAC-3', nested sense primer 5'-GATTACAATGTTCTTCTACTTCATTTCAT-3', antisense primer 5'-AGATGTCATCTTCCAAAAGCTGTCC-3' and nested antisense primer 5'-CTGTTTTAGGTAGTCCACTGAAAGTC-3'. Two sequential PCR were performed using Expand High Fidelity polymerase as suggested by the manufacturer in a final volume of 100 µl containing 1× Expand High Fidelity buffer, 200 µl dNTPs, 250 pmol each of 5' forward and 3' reverse primers, 3.5 units of Expand High Fidelity enzyme mix and 5 µl of cDNA. To obtain specific full-length product, we performed a second PCR using identical conditions with the nested primers. Amplified products were visualized using ethidium bromide on 1% agarose gels in 1× Tris/borate/EDTA buffer (Invitrogen). PCR products were gel purified and cloned into pCR4Blunt-Topo (Invitrogen). The restriction endonuclease *Pst*I was used to screen LGR7 clones, and *Spe*I and *Hind*III were used for screening the LGR8 clones. Following restriction digest analysis of up to 60 independent clones per tissue, DNA sequence analysis was performed to confirm novel sequences.

mRNA analysis and tissue distribution

To confirm mRNA tissue expression of LGR7 and LGR8 splice variants, we designed internal sets of primers that allowed the amplification of specific regions within the LGR7 and LGR8 coding sequence. One set of nested primers was used for the simultaneous detection of LGR8 and LGR8.1 mRNA expression. Sense and antisense primers were used for a first PCR reaction followed by a second PCR using nested sense and antisense primers, listed in Table I. Similarly, for LGR7 and its variants three nested sets of primer were used to allow detection of the regions spanning the novel exons splicing found in LGR7.1, LGR7.2 and LGR7.10. The sets of nested primers used allowed the simultaneous detection of LGR7 canonical sequence as well as the alternative splice variants, primers are listed in Table I. PCR conditions were as described above, and following the first round 1 µl of the first PCR reaction was used for the nested PCR and were run simultaneously using identical conditions. Following amplification, products were separated using 4% NuSieve 3:1 agarose gels (Cambrex Bio Science, Rockland, ME, USA) and detected using ethidium bromide.

Expression plasmids, cell culture, transfection and peptide treatment

For cellular expression, LGR7, LGR8 and their variants were subcloned into the pMT expression vector (Muda *et al.*, 1996). LGR7, LGR8 and variants were tagged via an N-terminal FLAG epitope tag as previously described (Hsu *et al.*, 2002). All receptor constructs were sequence verified.

HEK 293T cells were grown under 5% CO₂ in Dulbecco's modified Eagles medium (DMEM) 10% FBS supplemented with 1× penicillin/streptomycin (Invitrogen). The day before transfection cells were seeded at 2 × 10⁶ cells per 100 mm Petri dishes with 10 ml of growth media. The next day, cells were transfected with plasmid DNA using Fugene (Roche) according to the manufacturer's instructions. In all transfections constant amounts of plasmid DNA were used by complementing when needed to 10 µg with pBSK. After 48–72 h cells were lifted using Dulbecco's phosphate buffered saline (PBS) 2.7 mM KCl, 1.4 mM KH₂PO₄, 4.3 mM NaH₂PO₄, 137 mM NaCl, containing 5 mM EDTA and 5 mM EGTA. Cells were then collected by centrifugation, at 1000 rpm for 4 min, resuspended, counted and plated into 96-well plates at 3 × 10⁴ cells per well in 50 µl assay media (DMEM no phenol red, 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma) 0.1% bovine serum albumin (BSA, Fraction V, Sigma), 1× penicillin/streptomycin (Invitrogen) and incubated at 37°C for 1 h. Dilutions of porcine relaxin, H2 relaxin or human INSL3 were then added to

Table I. Sequence of the nested sets of primers used for mRNA detection

LGR7.1 (198 bp)	Sense: 5'-GGAGCTTGACTGTGATGAAA-3' Nested sense: 5'-GAATTATCATGATCTTCAGAAGCTG-3'	Antisense: 5'-CGACTGAGGTGATTATCTTCAATTA-3' Nested antisense: 5'-TTCAAAAACACCCGGCTTCA-3'
LGR7.2 (206 bp)	Sense: 5'-AAGGCAACCATATCCATAAT-3' Nested sense: 5'-TTGACTTTTATTTCCTGCAG-3'	Antisense: 5'-TTGCCAAGAGATTCTCTAGA-3' Nested antisense: 5'-CCACAGTACTGGAATTTCTTAA-3'
LGR7.10 (285 bp)	Sense: 5'-ACCAAGACCAAATTTGCTC-3' Nested sense: 5'-TGCTCACTTTCATTAATCAG-3'	Antisense: 5'-CAGCTTCTGAAGATCATGATAATTC-3' Nested antisense: 5'-TTTCATCACAGTCAAGCTCC-3'
LGR8.1 (278 bp)	Sense: 5'-CAAAGACTTACATCAGCTAAC-3' Nested sense: 5'-CATCAGCTAACTGGCTAAT-3'	Antisense: 5'-GGAGCAGTATCGAAAGTTTT-3' Nested antisense: 5'-AAAAGGTGAGGTGATAGCTC-3'

each well and incubated for 12–16 h at 37 C. Following stimulation cells were lysed, and total cAMP was measured.

Receptor surface expression FLAG detection

Twenty-four hours after Fugene transfection cells were lifted, as described above, and re-suspended in DMEM 10% FBS media at 1×10^6 cells per ml. Two millilitres of cell suspensions were transferred into six-well poly-L-lysine-coated plates (Biocoat, Becton Dickinson, Palo Alto, CA, USA). Plates were then incubated at 37°C overnight to allow cells to attach. Cells were washed twice with 1 ml PBS before incubating with 1 ml of biotinylated M2 antibody at 5 µg/ml in PBS 5% BSA at room temperature for 30 min with agitation. Cell monolayers were washed three times with 2 ml PBS, and 400,000 counts per minute (cpm) of [¹²⁵I]-labelled streptavidin were added in PBS 5% BSA. After 30 min of incubation with agitation at room temperature, plates were washed three times with PBS. Antibody complexes were recovered using 1 M NaOH, and samples were counted with an auto-gamma counter Cobra II (Packard, Meriden, CT, USA). Triplicate wells for each splice variant were tested in parallel to LGR7 and/or LGR8 and empty vector in each assay and tested at least three times.

Biotinylation of cell surface proteins

Biotinylation of cell-surface forms of LGRs was performed using the protocol described previously (Bai *et al.*, 1998). 293 T cells were transfected as described above, 48 h after transfection cells were rinsed with PBS and incubated with Sulfo-NHS-LC-Biotin (Pierce) 1 mM in PBS. After quenching with 1 ml Tris, 0.5 M, pH 7.5 at room temperature, cells were lysed at 4°C in 1 ml lysis buffer [10 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP40, Protease Inhibitors Mix (Roche), Iodoacetamide 100 mM]. Insoluble material was removed by centrifuging at 15,000 r.p.m. for 15 min at 4°C. FLAG-tagged receptors were immunoprecipitated from solubilized lysates using 60 µl of M2 agarose beads (50% suspension equilibrated in lysis buffer). FLAG-tagged receptors were eluted from the beads in 100 µl of 2× sodium dodecyl sulphate (SDS) sample buffer with 100 mM dithiothreitol (DTT) by incubating at 60°C for 30 min.

Eluted samples were separated on 7 or 12% (LGR7.1) SDS-polyacrylamide gels and transferred to PVDF membranes. Blotted membranes were blocked with 5% non fat milk in 1× Tris-buffered saline (TBS) 0.1% Tween-20 and then incubated with streptavidin horse-radish peroxidase (HRP) conjugated for 1 h at room temperature (1:5000) (5% milk in 1× TBS 0.1% Tween-20) to identify the biotinylated form of the receptors. In parallel, identical blots were incubated with anti-FLAG M2 HRP conjugated (diluted 1:1000) for 1 h at room temperature with 5% milk in 1× TBS/0.1% Tween-20 to reveal total immunoprecipitated FLAG-tagged receptors. Following five washes using 1× TBS/0.1% Tween-20, membranes were developed using an enhanced chemiluminescence (ECL) detection system following the manufacturer's instructions (Amersham Biosciences).

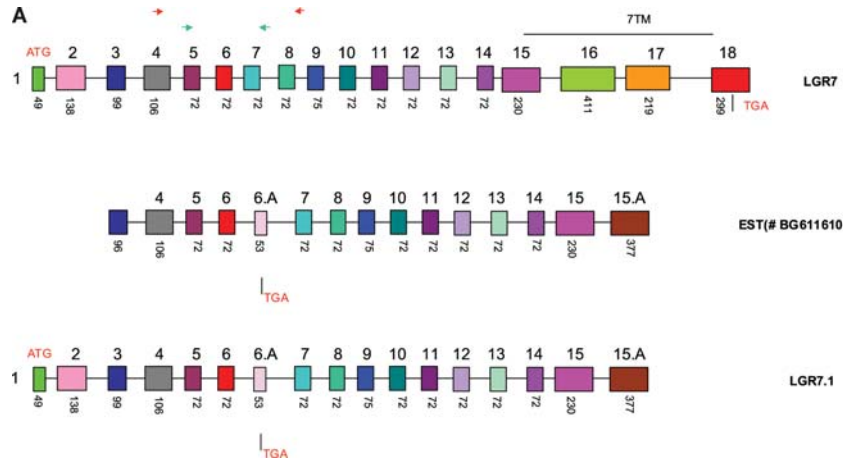
Binding assays

pMT constructs encoding LGR7, LGR8 and LGR7/LGR8 splice variants were transfected into HEK 293T cells in 75 cm flasks as previously described

(Sudo *et al.*, 2003). After 24 h the cells were lifted in EDTA phosphate solution and then plated in 24- or 6-well poly-L-lysine coated plates for relaxin and INSL3 binding or cell surface binding assays, respectively. Assays were performed in parallel 24 h later. Relaxin binding studies were performed as previously described (Sudo *et al.*, 2003) using two concentrations (100 pM and 500 pM) of [³³P]-labelled H2 relaxin. [¹²⁵I]-Labelled human INSL3 was obtained from Perkin-Elmer (Boston, MA, USA), and binding studies were performed using a concentration of 100 pM of radioligand. Specific binding was determined in the presence and absence of an excess of H2 relaxin or human INSL3 (1 µM). Cell surface binding was tested in parallel for each transfected construct in six-well plates as described above. Splice variants were tested in parallel to LGR7 and/or LGR8 and empty vector transfected cells in triplicate wells within each assay. Each variant was tested in at least three separate assays. Specific binding data (total binding minus nonspecific binding) have been pooled from the individual experiments and plotted as mean ± SEM cpm.

Results

Using human LGR7 and LGR8 nucleotide sequences (accession number NM021634 and accession number NM130806) as queries, we searched the NCBI database and identified one EST clone encoding a partial nucleotide sequence with some identity to the LGR7 sequence. We obtained the corresponding EST clone (accession number BG611610) and completed its sequence. This analysis revealed that this partial cDNA sequence represents a bona fide novel alternative spliced mRNA of LGR7. Indeed, this cDNA clone encodes two novel exons and a characteristic poly-A tail preceded by a canonical upstream poly-A signal sequence, AAUAAA. As the coding sequence in the EST clone lacked the start codon, we completed its sequence using PCR and cDNAs from human placenta. Assembly of the LGR7.1 coding region with the 3' sequence from the EST clone sequence generated a cDNA sequence of 1775 bp. Using the software program SIM4 (Florea *et al.*, 1998), we aligned and compared the LGR7 and LGR7.1 sequences with the human genomic sequence, Celera Release 27. Of the 18 exons encompassing the coding sequence of LGR7 (Figure 1A), exon 1 encodes most of the putative signal sequence, exon 2 encodes the low-density lipoprotein class A (LDLa) module, and a short connecting sequence (exon 3) precedes the cysteine rich region flanking the leucine rich repeats (LRRs). The 10 LRRs, present in the extracellular domain, span exons 5–14 and the seven transmembrane domain is encoded by the last four exons, 15–18 (Figure 1A). In contrast, LGR7.1 is encoded by 17 exons, which are identical from exon 1 to exon 6 to LGR7 then a novel exon, named 6.A, is used. This exon introduces a short amino acid sequence, SRAVKDGEK, and a stop codon, suggesting that LGR7.1 encodes a protein of 190 amino acids characterized by a N-terminal portion of the extracellular domain of LGR7 (Figure 1A and B). After exon 6.A, LGR7.1 sequence is identical to LGR7 from exons 7–15 (Figure 1A),



B

LGR7.1 novel exon boundaries

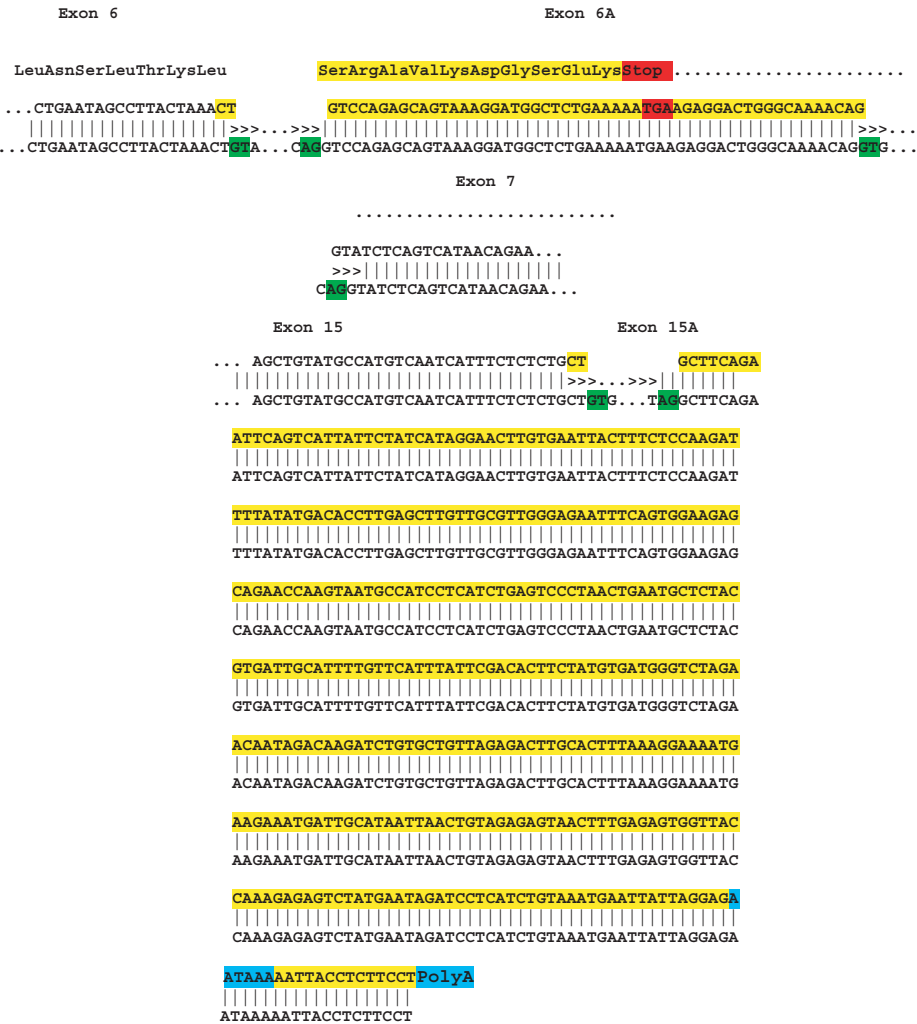


Figure 1. LGR7.1 exon structure. (A) Schematic representation of the exon structure of LGR7, the partial sequence from the LGR7 EST clone (accession number BG 611610) and the deduced complete cDNA sequence of LGR7.1. Novel exons are designated 6.A and 15.A. The position of the nested set of sense and antisense primers used for mRNA detection is shown. (B) Alignment of the 7.1 cDNA with the human genomic sequence. Exon/intron boundaries were determined using the SIM4 software. Canonical mRNA splicing consensus sequences for the donor and acceptor splice site are highlighted green. The novel nucleotide and amino acid sequences present in LGR7.1 are in yellow. mRNA polyadenylation signal present on exon 15A is blue.

then a second novel exon, named 15.A encoding an alternative poly-A signal sequence is used (Figure 1B). Following addition of a poly-A tail LGR7.1 is predicted to be expressed as a mRNA of approximately 1.9 Kb. Importantly, and consistent with this, previously reported northern blot analysis of LGR7 mRNA expression revealed two main bands, one of which is consistent with the molecular size predicted for LGR7.1 mRNA (Hsu *et al.*, 2000).

We then decided to verify if other variants were expressed in human tissues. Using commercially available human cDNAs as templates, we have identified and cloned 29 alternative LGR7 variants and three alternative LGR8 variants (Tables II and III). Remarkably, in our analysis, splice variants of LGR7 and LGR8 were more represented than the canonical LGR7 and LGR8 sequences.

Splice variants were named by a numerical suffix to the original gene in the order of isolation. Similar to LGR7.1 analysis, each of the novel identified sequence variants was analysed using the SIM4 program by comparing it with the human genomic sequence. Two LGR7 splice variants and one LGR8 splice variant, named LGR7.2, LGR7.10 and LGR8.1, were chosen for further studies.

LGR7.2 was cloned twice from ovary and prostate cDNAs and its sequence is identical to LGR7 except that it lacks two LRRs corresponding to exons 12 and 13 close to the seven transmembrane domain which starts at exon 15 (Figure 2A). In addition to this deletion, because of the novel exon junction, LGR7.2 contains a single amino acid substitution, phenylalanine in place of leucine (Figure 2B). Subsequent analysis revealed that LGR7.2 mRNA lacking exons 12 and 13 might be broadly expressed (see below). Consistent with this, the commercially available clone for LGR7, from a full-length cDNA library collection (clone number AB3603-B02, Origene, Rockville, MD, USA), when fully sequenced was found to correspond to LGR7.2.

Table II. LGR7 clones analysed

Tissue	Clones		Total
	LGR7	LGR7 variants (number of variants)	
Ovary	10	19 (11)	29
Uterus	19	12 (3)	31
Placenta	12	4 (2)	16
Prostate	13	12 (8)	25
Testis	0	60 (4)	60
Pituitary	12	48 (7)	60
Adrenal	13	0	13
Total	79	155	234

Full DNA sequence analysis was used to confirm identity and/or identify novel splice variants. Twenty-nine variants were found for LGR7 from seven tissues analysed. Like LGR7.2, cloned from ovary and prostate cDNA, several splice forms were also cloned multiple times from separate tissues.

Table III. LGR8 clones analysed

Tissue	Clones		Total
	LGR8	LGR8 variants (number of variants)	
Uterus	22	38 (1)	60
Adrenal	21	37 (2)	58
Total	43	75	118

Three distinct variants were identified for LGR8 from two tissues. LGR8.1 was the most represented sequence from uterine cDNA (38 of 60) clones analysed. LGR8.1 was not among the 58 clones obtained from adrenal gland cDNA.

LGR7.10 was also cloned from ovary cDNA, but in contrast to LGR7.2 lacks only one exon, exon 3 (Figure 2A). Although LGR7.10 has been described previously (Hsu *et al.*, 2000), no characterization other than its inability to increase cAMP production in response to relaxin was reported (Hsu *et al.*, 2002).

We cloned LGR8.1 from uterine cDNA, and remarkably 38 of 60 clones isolated from this tissue contained LGR8.1 rather than LGR8. No LGR8.1 was found in 58 clones obtained from adrenal gland (Table III). Consistent with this, LGR8.1 mRNA expression was detected in the uterus but not in seven other tissues examined (see below). The LGR8.1 form is identical in sequence to LGR8 with the exception of one missing LRR encoded by exon 11 (Figure 2C). In contrast to LGR7.2 and LGR7.10, LGR8.1 has no amino acid substitution because of the alternative splicing event (Figure 2D).

To further confirm and validate the expression of the different splice forms, we used a second PCR-based analysis using primers that were designed to simultaneously amplify the variant form and the canonical sequences. Using this method, we confirmed the expression of all the alternative exon structures previously identified by full-length cloning (Figure 3). In our analysis, using primer sets that span three separate regions of the coding sequence, we detected a band corresponding to canonical LGR7 sequence in seven of eight tissues. LGR7.1 and LGR7.2 mRNA were broadly expressed and detected in seven of eight tissues examined, whereas LGR7.10 was detected only in four of the tissues analysed (Figure 3). Finally, in contrast to LGR7 and its variants, mRNA expression of LGR8 and LGR8.1 were more restricted and detected in only two tissues (Figure 3). In conclusion, our analysis demonstrated the expression of distinct splice variants and that several splice forms are typically expressed in the same tissue.

Previous studies on the LGR7 signalling mechanism revealed that a single amino acid substitution mimicked the naturally occurring gain-of-function mutation of the LH receptor which leads to constitutive cAMP production (Hsu *et al.*, 2000). Subsequent studies showed that cells expressing LGR7 increase cAMP production in response to relaxin or H3 relaxin stimulation (Sudo *et al.*, 2003). Similarly, LGR8 was recently shown to mediate cAMP production following relaxin or INSL3 stimulation (Kumagai *et al.*, 2002). Therefore, the interaction of relaxin peptides with LGR7 or LGR8 induces a conformational change that results in the activation of cAMP synthesis, most likely through the Gs type of heterotrimeric G-proteins (Hsu *et al.*, 2002). In early studies, a gain-of-function mutant of LGR7.10 displayed constitutive activity (Hsu *et al.*, 2000), in contrast to LGR7 wild type. Later work, consistent with our results found that LGR7.10 did not respond to relaxin stimulation (Hsu *et al.*, 2002). To better understand these apparently contradictory results, we reanalysed the responses to relaxin and INSL3 of LGR7.10 and the other splice variants as well as their membrane localization. In our hands, consistent with previous results, porcine or recombinant H2 relaxin were both active on LGR7 and LGR8 receptors (Figure 4a), whereas they did not activate LGR7.10. Surprisingly, but similar to LGR7.10 none of the variants identified was able to increase either basal cAMP synthesis or induce cAMP production following relaxin stimulation (Figure 4a). Furthermore, although the LGR8 transfected cells responded to INSL3 stimulation, none of the splice variants responded to INSL3 (Figure 5a).

We then monitored the binding of [³³P]-labelled H2 relaxin and [¹²⁵I]-labelled INSL3 to LGR7, LGR8 and splice variants using transfected 293 T cells. Consistent with previous reports, our analysis revealed that both LGR7 and LGR8 bound [³³P]-labelled H2 relaxin with affinity in the low nanomolar range, $K_d \sim 0.1$ nM and ~ 1 nM, respectively. We then tested the splice variants for specific binding

using two concentrations of [³³P]-labelled H2 relaxin. Unexpectedly, our analysis demonstrated that none of the splice variants tested showed specific binding for [³³P]-labelled H2 relaxin, at both concentrations tested (Figure 4b).

We next tested INSL3, the proposed physiological ligand of LGR8. Consistent with previous results (Kumagai *et al.*, 2002; Sudo *et al.*, 2003), [¹²⁵I]-labelled INSL3 bound LGR8 with high affinity ($K_d \sim 0.1$ nM) whereas it did not bind to LGR7 (Figure 5b).

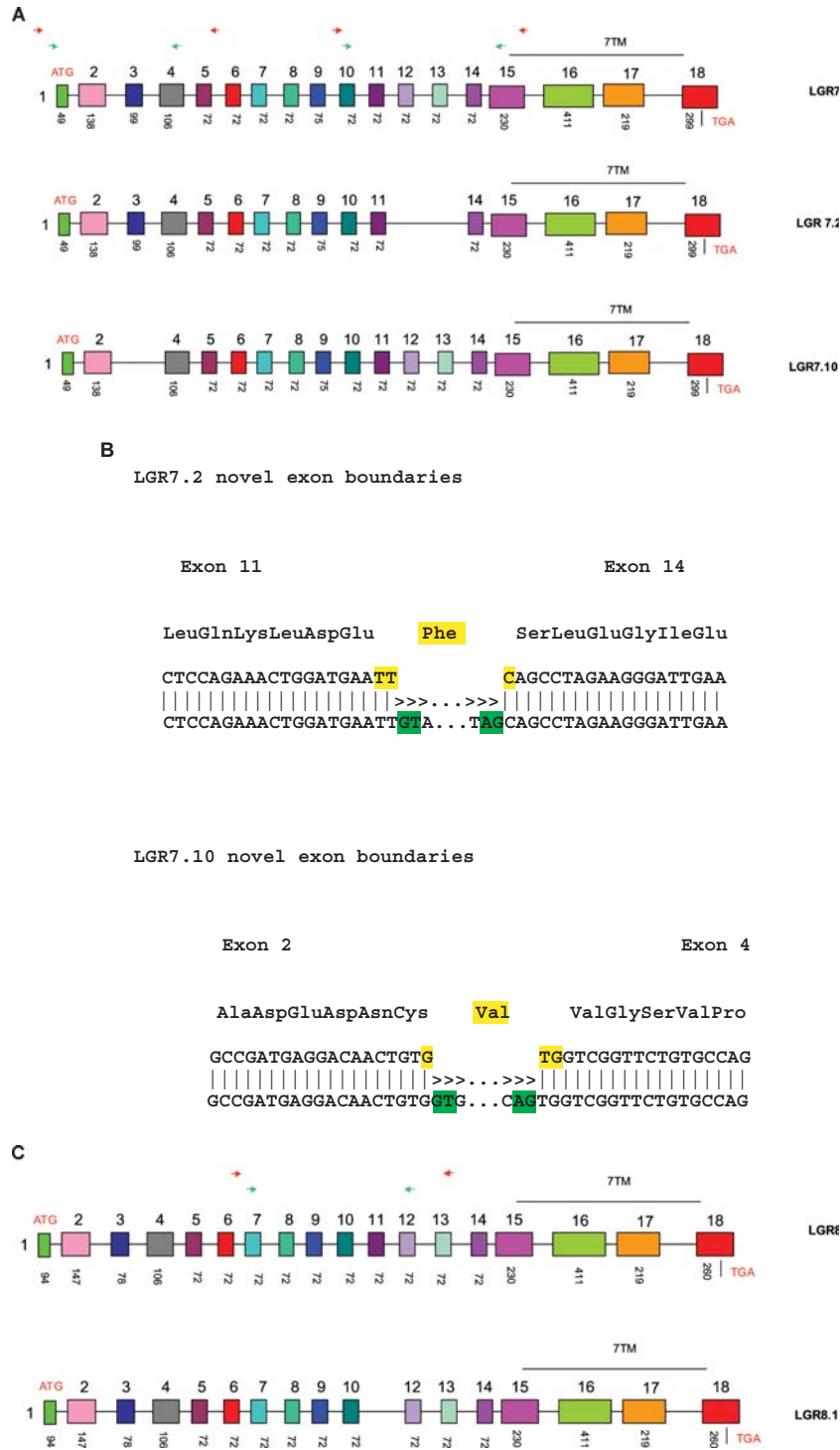


Figure 2. LGR7.2, LGR7.10 and LGR8.1 exons structure. (A) Schematic representation of the exon sequence encoding LGR7.2 and LGR7.10 matched up with the exons of LGR7. The two exons missing in LGR7.2 encode two leucine rich repeat (LRR) domains next to the transmembrane region. Exon number three missing in LGR7.10 encodes the region flanking the low-density lipoprotein class A (LDL) motif (exon 2), whereas exon 4 encodes the region flanking the LRR. The position of the nested set of sense and antisense primers used for mRNA detection is shown. (B) Alignment of the novel exon junctions in the 7.2 and 7.10 cDNAs with the human genomic sequence. (C) Schematic representation of the exon structure of LGR8.1 and comparison with the LGR8 exon structure. Only one LRR repeat, encoded within exon 11, is missing in LGR8.1. The position of the nested set of sense and antisense primers used for mRNA detection is shown. (D) Alignment of the novel exon junctions in the 8.1 cDNAs with the human genomic sequence. Canonical mRNA splicing consensus sequences for the donor and the acceptor site are highlighted in green. Novel codon sequences generated by splicing and corresponding novel amino acid sequence are in yellow.

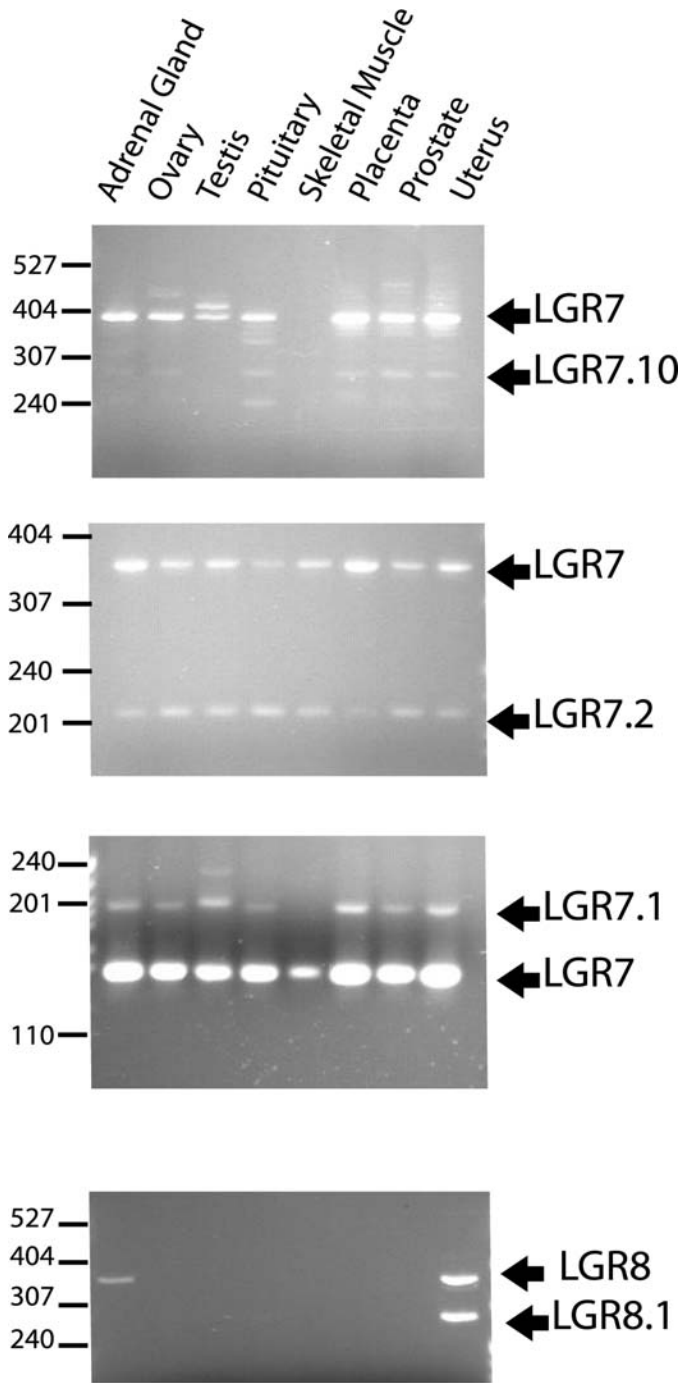


Figure 3. mRNA tissue distribution of the various LGR7 and LGR8 forms. RT-PCR mRNA analysis of the tissue expression of LGR7, LGR8 and the splice variants: LGR7.1, LGR7.2, LGR7.10 and LGR8.1. PCR conditions are described in Materials and methods. The primers used have the capacity to simultaneously detect expression of both the full-length as well as variant forms. PCR-products were visualized using ethidium bromide. This picture is representative of two separate experiments. This analysis revealed that splice variants are not expressed by themselves but instead are expressed simultaneously in several tissues.

affects the processing of the full-length LGR7 form. Finally, defining the role of LGR7.1 was difficult in part due to the renowned problematic expression in heterologous systems of the truncated (ectodomain only) versions of LGRs. Indeed, in our hands, only a small fraction of total LGR7.1 protein expressed was secreted in the

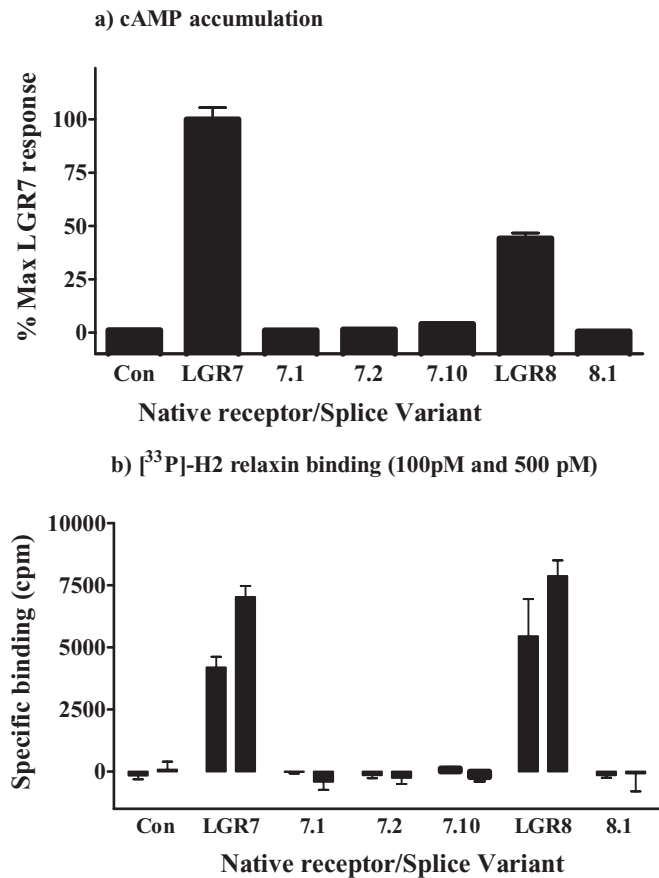


Figure 4. (a) Analysis of cAMP responses following treatment with porcine relaxin (200 nM) in transfected 293T cells expressing recombinant full-length receptors LGR7, LGR8 and splice variants LGR7.1, LGR7.2, LGR7.10 and LGR 8.1 compared to mock transfected cells (Con). Results are expressed as a percentage of the LGR7 response run in parallel. Data are represented as mean \pm SE of the three experiments performed in triplicate. (b) Direct binding of [³³P]-labelled H2 relaxin. Whole cell binding assays using two concentrations of [³³P]-labelled H2 relaxin 100 and 500 pM to ensure that low affinity interaction would be detected. Nonspecific binding was determined using an excess of H2 relaxin (1 mM). Specific binding was normalized to cells transfected with empty vector and compared to binding to LGR7 and LGR8. Data are mean \pm SE of at least three experiments performed in triplicate.

media, and detection of LGR7.1 by western analysis in conditioned media was dependent on the use of a very strong promoter element (data not shown). In spite of a lack of strong experimental evidence, we still think that LGR7.1 may represent a bona fide secreted version of LGR7, and future analysis using different expression systems might be able to tackle this issue.

In conclusion, our work established that several splice variants of LGR7 and LGR8 are expressed in human tissues. It is tempting to speculate that some of the splice variants identified might affect LGR7, LGR8 as well as each other's function via direct intramolecular interactions as was reported for LHR (Nakamura *et al.*, 2004). Moreover, the LH receptor itself was shown to self-associate (Tao *et al.*, 2004) and, given the structural similarity among LGRs receptors, we put forward the hypothesis that some of the variants identified may have similar functions. The large number of variants identified drastically underscores the importance of alternative splicing in the physiology of the LGR family of G-protein coupled receptors.

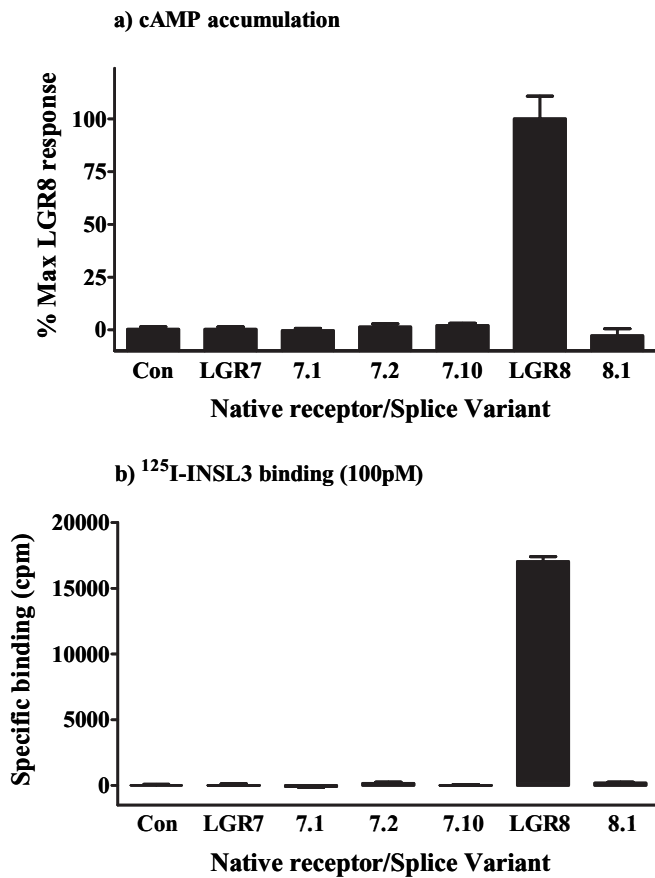


Figure 5. (a) Analysis of cAMP responses following treatment with 100 nM INSL3 in transfected 293T cells expressing LGR7, LGR8, splice variants LGR7.1, LGR7.2, LGR7.10 and LGR8.1. Results are expressed as a percentage of the LGR8 response run in parallel. Data are represented as mean \pm SE of three experiments performed in triplicate. (b) Direct binding of [¹²⁵I]-labelled human INSL3 to LGR7 and LGR8 and splice variants. Whole cell binding assays were performed using 100 pM [¹²⁵I]-labelled human INSL3, nonspecific binding was determined using an excess of INSL3 (1 μ M). Specific binding was normalized to cells transfected with empty vector and compared to binding to LGR7 and LGR8. Data are mean \pm SE of at least three experiments performed in triplicate.

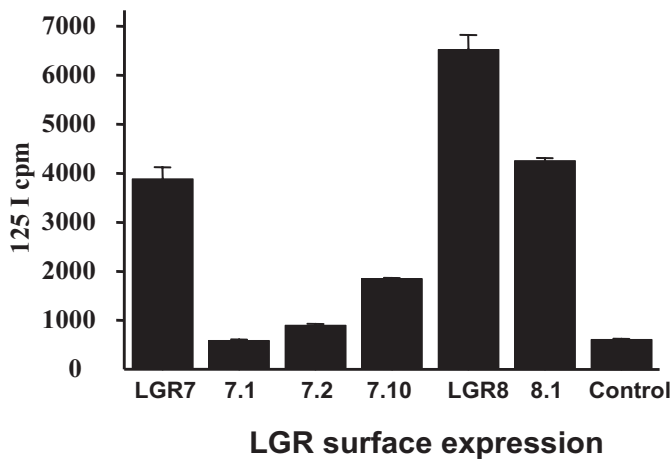


Figure 6. Bar graph of cell-surface expression of LGR7, LGR8 and splice variants. Cell surface expression of distinct receptors is based on the specific counts from intact 293T cells. Cells transfected with empty vector (control) or vector encoding different splice variants were labelled using FLAG antibody followed by incubation with [¹²⁵I]-conjugated streptavidin. Values are mean \pm SE of a representative experiment repeated three times.

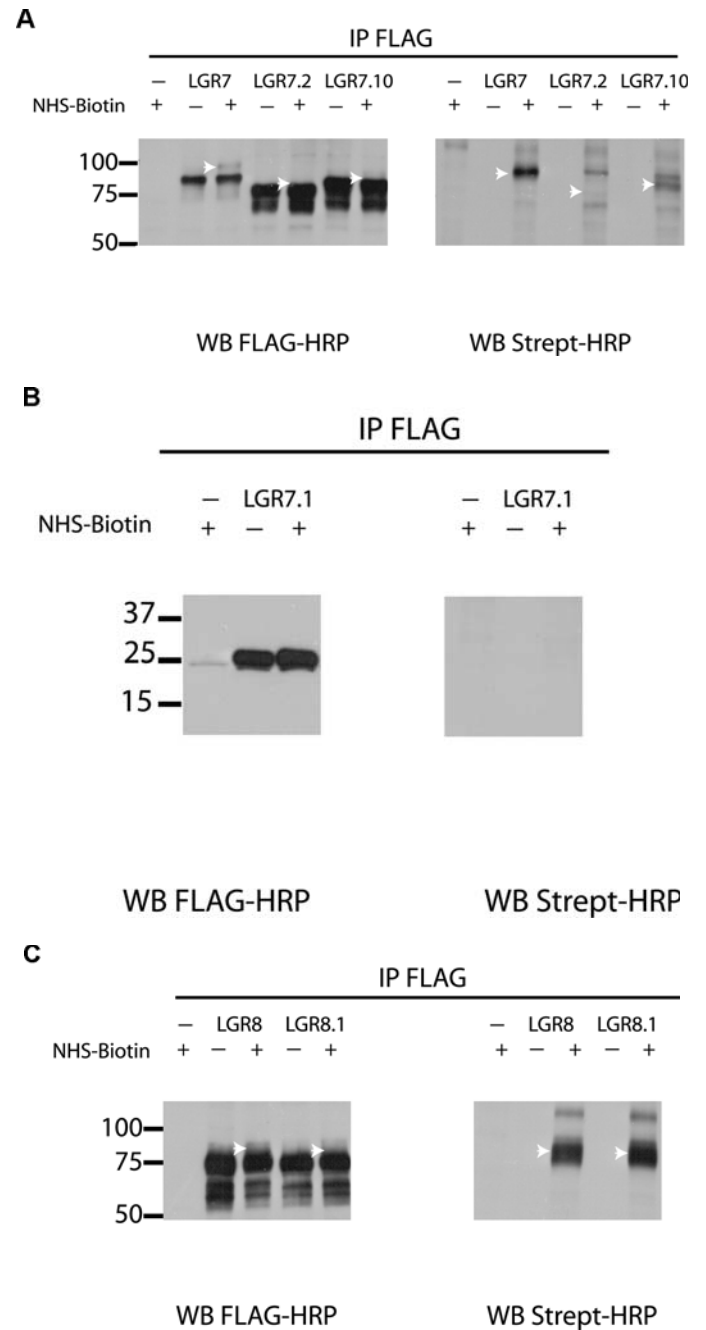


Figure 7. Biotinylation analysis of the surface expression of LGR7 and LGR8 splice variants. Cells transfected with either empty vector or FLAG-tagged LGR7, 7.2 and 7.10. LGR7s were labelled using *N*-hydroxysuccinimide (NHS) biotin or mock treated in parallel. As shown, incorporation of NHS biotin results in a small size shift of protein bands. (A) Following immunopurification using FLAG-M2 beads, surface biotinylated forms of LGR7 were detected using peroxidase conjugated streptavidin, and in parallel total immunopurified LGR7 forms were detected using FLAG-M2 antibodies conjugated with peroxidase. White arrows indicate the corresponding positions of the biotinylated band on the two blots. (B) Cells were transfected with either empty vector (lane 1) or FLAG-tagged LGR7.1 and treated as above, no biotinylated bands were detected from cell expressing LGR7.1. (C) Cells were transfected with either empty vector (lane 1) or FLAG-tagged LGR8 or LGR8.1.

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