INSL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent

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Key words: INSL3, relaxin, LGR, G protein-coupled receptors, cryptorchidism, orphan receptor.

Running title: INSL3 is the cognate ligand for LGR8

This study was supported by NIH Grant HD23273.

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Abbreviations: INSL3, insulin-like 3; LGR, leucine-rich repeat-containing G protein-coupled receptor; GPCR, G protein-coupled receptor; IBMX, 3-isobutyl-1-methyl xanthine; FBS, fetal bovine serum; HRP, horseradish peroxidase.
Summary
Several orphan G protein-coupled receptors homologous to gonadotropin and thyrotropin receptors have recently been identified and named as LGR4-8. INSL3, also known as Leydig insulin-like peptide or relaxin-like factor, is a relaxin family member expressed in testis Leydig cells and ovarian theca and luteal cells. Male mice mutant for INSL3 exhibit cryptorchidism or defects in testis descent due to abnormal gubernaculum development whereas overexpression of INSL3 induces ovary descent in transgenic females. Because transgenic mice missing the LGR8 gene are also cryptorchid, INSL3 was tested as the ligand for LGR8. Here, we show that treatment with INSL3 stimulated cAMP production in cells expressing recombinant LGR8, but not LGR7. In addition, interactions between INSL3 and LGR8 were demonstrated following ligand receptor cross-linking. Northern blot analysis indicated that the LGR8 transcripts are expressed in gubernaculum whereas treatment of cultured gubernacular cells with INSL3 stimulated cAMP production and thymidine incorporation. The present study identified the ligand for an orphan GPCR based on common phenotypes of ligand and receptor null mice. Demonstration of INSL3 as the ligand for LGR8 facilitates understanding of the mechanism of testis descent and allows studies on the role of INSL3 in gonadal and other physiological processes.

Introduction:
During fetal development, the sexual dimorphic position of the gonads in mammals is dependent on the differential development of two ligaments. In males, growth of the gubernaculum and regression of the cranial suspensory ligament results in transabdominal descent of the testes. Impaired testicular descent (cryptorchidism) is a prevalent congenital abnormality in humans, found in 2% of male births. INSL3, also known as Leydig insulin-like peptide or relaxin-like factor (RLF), is one of the seven relaxin-like genes in humans known to be expressed in Leydig cells of fetal and adult testes as well as in theca and luteal cells of the postnatal ovary (1). Male mice mutant for INSL3 exhibit bilateral abdominal cryptorchidism (2, 3) whereas female mice overexpressing INSL3 showed ovary descent and displayed bilateral inguinal hernia (4). Although INSL3 binds to gubernacular homogenates (5, 6) and induces...
growth of rat gubernaculum in whole organ cultures (7), the exact nature of the INSL3 receptor is unknown.

A recent study indicated that transgene integration in crsp mice resulted in a 550-kb deletion located upstream of the Brca2 gene, leading to defective testis descent. Because a candidate gene encoding a G protein-coupled receptor homologous to human LGR8 was deleted in these mice (8), we tested if INSL3 is the cognate ligand for LGR8 based on the observed common phenotypes of potential ligand-receptor pairs in null mice. Here, we report that INSL3 is capable of binding LGR8, leading to the stimulation of cAMP production and thymidine incorporation in the gubernaculum.

**Experimental procedures:**

Ovine and rat INSL3 were chemically synthesized and characterized as described (7, 9). Human INSL3 and biotinylated ovine INSL3 were prepared similarly with the ovine INSL3 containing a single biotin molecule on the N-terminus of the A chain. The National Hormone and Pituitary Program (NIDDK, National Institutes of Health, Bethesda, MD) supplied porcine relaxin. ¹²⁵I-Streptavidin and streptavidin conjugated to horseradish peroxidase (HRP) were purchased from Amersham Biosciences, Inc (Piscataway, NJ), whereas foskolin, glucagon, collagenase and trypsin were from Sigma Chemical Co. (St. Louis, MO). Sprague-Dawley rats were obtained from Simonsen Laboratories (Gilroy, CA). Animals were anesthetized and killed using CO₂.

Animal care was consistent with institutional and NIH guidelines.

Human 293T cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. When 70-80% confluent, cells were transfected with 10 µg of plasmid using the calcium phosphate precipitation method (10). After 18-24 h of incubation, media were replaced with DMEM/F12 containing 10% FBS. Forty-eight hours after transfection, cells (10⁵/ml) were preincubated at 37°C for 30 min in the presence of 0.25 mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma Chemical Co.) before treatment with or without hormones for 12 h. Total cAMP content was measured in triplicate by a specific radioimmunoassay (11). All experiments were repeated at least four times using cells from independent transfections.
To estimate INSL3 binding, transfected cells were washed twice with D-PBS and collected in D-PBS before centrifugation at 400 x g for 5 min. Cells pellets were resuspended in D-PBS containing 1 mg/ml BSA and incubated with increasing doses of the rat INSL3 at 4 °C for 24 h in the presence of biotinylated INSL3 (5 nM/tube). After incubation, cells were centrifuged and washed twice with 1% BSA/PBS before incubation with 125I-Streptavidin (400,000 cpm/tube) for 1 h at 4 °C. After washing the cells three times, radioactivity in the pellets was determined. For protein blotting, transfected cells were incubated with biotinylated INSL3 (50 nM/tube) with or without an excess of rat INSL3 (1 µM/tube). After washing, pellets were incubated in D-PBS with disuccinimidyl suberate (0.5 mM) for 30 min. at room temperature. The cross-linked INSL3-LGR8 complexes were solubilized with 100 µl 1% Triton X-100 in 50 mM Tris-HCl. The lysates were denatured with SDS and 2-beta-mercaptoethanol, and fractionated using SDS-PAGE. After blotting onto nitrocellulose membranes (Hybond-P, Amersham) and blocking with a 5% milk solution, the blots were incubated for 2 h at room temperature with streptavidin (1:10,000 dilution) before development using enhanced chemiluminescence solution (ECL, Amersham Life Science). In addition, epitope-tagged LGR8 was extracted with 1% Triton X-100 from cells transfected with the LGR8 expression plasmid and incubated with the M1 antibody for 1 h. Protein G-Sepharose was subsequently added to precipitate the M1-tagged receptor protein. The precipitate was further fractionated using SDS-PAGE followed by immunoblotting using the M1 antibody.

Total RNA from different rat tissues were extracted using the RNeasy purification kits (QIAGEN Inc. Chatsworth, CA) before Northern blotting. Rat orthologs for LGR7 and LGR8 were identified in the GenBank (accession number AC098607 and AC098990, respectively). These sequences were used in reverse transcription-PCR to yield LGR8 and LGR7 probes of 230 and 226 bp, respectively.

Gubernacular cells were isolated by modifying an earlier method (5). Tissues were removed from one-week-old rats and cut into 1 mm pieces, and dissociated for 2 h at 37°C in DMEM/F12 with 0.1% collagenase. Cell debris was removed by passage through a sterile filter and cells were collected by centrifugation. After suspension in DMEM/F12 with 10% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, cells were cultured for 24 h in 5% CO2 incubator at 37°C. The cells were then washed once with serum-free medium and treated in DMEM/F12 containing IBMX with or without hormones and reagents. After 16 h of
incubation, total cAMP was measured in triplicates as described above. For thymidine incorporation studies, gubernacular cells (2 x 10^5 cells/500 µl) were cultured in 5 ml polypropylene Falcon tubes (Becton Dickinson, Franklin Lakes, NJ) with or without hormones together with 1 µCi/tube of [methyl-3H]thymidine (Amersham Pharmacia Biotech). After 24 h of culture, cells were washed once and resuspended with ice-cold PBS before centrifugation at 2000 x g for 3o min. at 4°C. Radioactivities in the washed cell samples were determined using a β-photomultiplier.

Results
INSL3 is the cognate ligand for LGR8
Although INSL3 binds to gubernacular homogenates (5, 6), and induces growth of rat gubernaculum in organ cultures (7), the exact nature of the INSL3 receptor is unknown. Human fetal kidney 293T cells were transfected with expression vectors encoding human LGR8 or the related LGR7 for testing of INSL3 signaling. In cells expressing LGR8 (Fig. 1A), treatment with synthetic human, ovine, or rat INSL3 led to dose-dependent increases in cAMP production. Although treatment with biotinylated-ovine INSL3 or porcine relaxin (RLX) was also effective, treatment with glucagon did not increase cAMP production. In contrast, cells expressing LGR7 responded only to relaxin treatment whereas treatments with INSL3 from different species or human glucagon were ineffective (Fig. 1B). These results indicated that INSL3 is a specific ligand for LGR8.

To demonstrate the direct binding of INSL3 to LGR8, cells expressing LGR8 were incubated with biotinylated INSL3 with or without increasing doses of non-biotinylated INSL3. Following incubation at 4 C for 24 h, cells were washed and incubated further with I^{125}-labeled streptavidin to estimate the levels of cell-bound biotinylated INSL3. As shown in Fig 2A, specific binding of biotinylated INSL3 to LGR8 could be competed by non-biotinylated INSL3 in a dose-dependent manner with an ED_{50} of 12 nM (filled circles). In contrast, 293T cells without LGR8 expression did not exhibit specific binding (open triangles). We further estimated the formation of the LGR8-INSL3 complexes following cross-linking and protein blotting before signal detection using avidin-horseradish peroxidase (HRP). As shown in Fig 2B, biotinylated INSL3 cross-linked with LGR8 could be detected as a high MW band (~84 KDa) whereas a 20-
fold excess of non-biotinylated INSL3 decreased signal intensity (lanes 2 and 3). In contrast, the free biotinylated INSL3 migrated at 6.5 KDa (Fig. 2B, lane 1) and the epitope-tagged LGR8 extracted from transfected cells migrated at ~75 KDa when monitored using the M1 antibody after immunoprecipitation with the same antibody (Fig. 2B, lane 4).

Expression of LGR8 in gubernaculum and INSL3 stimulation of gubernacular functions

Northern blotting analyses demonstrated the expression of the LGR8 transcript in the gubernaculum of one-week-old immature rats and testis of adult rats, but not in diaphragm (Fig. 3A). In the gubernaculum, a single transcript of ~2.5 kb was evident whereas an additional transcript of a higher size was found in the testis. In addition, treatment of gubernacular cells with INSL3 led to dose-dependent increases in cAMP production (Fig. 3B) to levels comparable to cells treated with forskolin (FS), a diterpene adenyl cyclase activator. Although glucagon treatment was ineffective, treatment with relaxin also stimulated cAMP production by these cells, consistent with its ability to activate LGR8 (12). For diaphragm cells, none of the hormones tested elicited cAMP production despite the stimulatory effects of forskolin (Fig. 3B). Because an increase in gubernacular cell division is believed to be needed during testis descent, the ability of INLS3 to stimulate thymidine incorporation by cultured gubernacular cells was tested. As shown in Fig. 3C, treatment with INSL3 led to dose-dependent increases in thymidine incorporation by these cells. In addition, treatment with relaxin and forskolin, but not glucagon, was also effective.

Discussion

The present findings demonstrate that INSL3 is the cognate ligand for LGR8. The observed expression of LGR8 transcripts in the gubernaculum and the INSL3 stimulation of cAMP production by these cells are consistent with the common cryptorchid phenotypes of this ligand-receptor pair in earlier transgenic mouse studies (2, 3, 8). Although the large 550 kb DNA deletion induced in transgenic mice following random insertional mutagenesis includes genes other than the mouse LGR8 ortholog (8), present findings of the ligand-receptor relationship for INLS3 and LGR8 supports the hypothesis that deletion of this receptor gene alone is responsible for the cryptorchid phenotype. Despite the bilateral cryptorchidism found in male INSL3 null
mice as a result of developmental abnormalities of the gubernaculum, most studies indicated that INSL3 gene mutations are not associated with cryptorchidism in patients (13-17). Two putative mutations, R49X and P69L, were identified in the connecting peptide region of the precursor INSL3 protein (18). Because the frequency of these INSL3 gene mutations is low (1.4%), their potential influence on testis descent waits further testing. The present identification of LGR8 as the receptor for INSL3 raised the possibility that partial or complete loss-of-function mutations in the LGR8 gene could be associated with cryptorchidism, the most frequent congenital abnormalities in humans.

A recent study demonstrated that the classical hormone relaxin activates LGR7 and, with lower efficacy, LGR8 (12). In contrast, INSL3 specifically activate LGR8, but not LGR7 (12). A total of seven relaxin members are present in the human genome. Relaxin H1 and H2 are clustered together with INSL4 and INSL6 in chromosome 9p23-24 whereas INSL3 is located together with relaxin 3 in 19p13 (19). The present findings provide the basis to test the receptor binding specificity of other relaxin paralogs, thus allowing a better understanding of the evolution and physiology of the relaxin ligand gene family. Based on the divergent receptor specificity of relaxin and INSL3, future chimeric receptor studies on the ligand specificity of LGR7 and LGR8 are also of interest.

During fetal development, the sexual dimorphic position in mammalian gonads is dependent on the differential development of two ligaments. In males, growth of the gubernaculum and regression of the cranial suspensory ligament result in transabdominal descent of the testes. Circulating INSL3 concentrations increase in male rats starting at day 10 of age and continuing until INSL3 concentrations reached adult levels at day 39 after parturition. The testicles are descending into the scrotum during this phase of increasing INSL3 concentrations (5). INSL3 is expressed in Leydig cells of the fetal and postnatal testis and also in theca and luteal cells of the postnatal ovary (20), whereas LGR8 is expressed in multiple tissues including testis, brain, kidney, muscle, thyroid, uterus, peripheral blood cells, and bone marrow (12). In addition to its endocrine role in testis descent mediated by LGR8 in gubernaculum, INSL3 could also have important endocrine or paracrine roles in other tissues. Although defective spermatogenesis found in INSL3 or LGR8 null mice could be the secondary effects of cryptorchidism, Leydig cell-derived INSL3 could play a paracrine role in the testis because LGR8 is also expressed in the testis (12). In females, INSL3 is expressed in the luteal cells of the
ovary through the cycle, and during pregnancy and (20). Because female INSL3 null mice have impaired fertility associated with deregulation of the estrous cycle (2), the present findings could facilitate understanding of the paracrine role of INSL3 in the ovary in addition to providing understandings on the physiological roles of LGR8 in non-gonadal tissues such as brain, thyroid, and uterus (12).

In addition to the elucidation of the INSL3-LGR8 ligand-receptor pair, the present results provide the basis for ligand receptor pairing of other relaxin family members without a known receptor as well as for the discovery of ligands for the remaining orphan LGRs 4-6. Ligands for orphan GPCRs have been identified based on the purification of endogenous ligands from tissue extracts (21-23), or following screening of ligand libraries (24, 25). Instead, the present approach is based on common phenotypes of ligand and receptor null mice. The characterization of gene function has expanded from the single gene knockout method to random or targeted gene trapping approaches (25). A secretory trap method has allowed the generation of many mouse lines with a deletion of genes encoding membrane and secreted proteins, including those for polypeptide ligands and plasma membrane receptors (26). The present approach is valuable for future pairing of other orphan ligands and receptors as the phenotypes of more null mice are being characterized.
ACKNOWLEDGEMENTS Supported by NIH grant HD23273. We thank C. Spencer for editorial assistance, and the National Hormone & Peptide Program for the cAMP antiserum.

Figure Legends

Fig. 1 Activation of LGR8 but not LGR7 by INSL3. Cells expressing recombinant human LGR8 or LGR7 were treated with INSL3 from different species, or with biotinylated-ovine INSL3 (Biotin-INSL3), porcine relaxin (RLX) or glucagon. Ligand signaling was estimated based on extracellular cAMP production. A) LGR8. B) LGR7.

Fig. 2 Direct binding of biotinylated INSL3 to LGR8. A) Ligand binding assays. Cells expressing LGR8 were incubated with 5 nM of biotinylated ovine INSL3 with or without increasing levels of rat INSL3. Specific INSL3 binding to LGR8 was estimated using labeled strepavidin. B) Cross-linking of INSL3 to LGR8. Cells expressing LGR8 were incubated with biotinylated INSL3 (Biotin-INSL3) with or without a 20-fold excess INSL3 before cross-linking. Complexes of biotinylated INSL3 and LGR8 were detected using the avidin-HRP following SDS-PAGE and protein blotting. Lane 1, biotin-INSL3 alone; lane 2, INSL3-LGR8 complexes; lane 3; competition with excess non-biotinylated INSL3; lane 4, recombinant LGR8 detected using the M1 antibody.

Fig. 3 Expression of LGR8 transcripts in the gubernaculum and INSL3 stimulation of cAMP production and thymidine incorporation by cultured gubernacular cells. A) Northern blot analyses. G: gubernaculum; D: diaphragm; T, testis. B) Stimulation of cAMP production in primary cultures of gubernacular cells treated with rat INSL3, porcine relaxin (RLX) or glucagon (Glu). Some cells were treated with foskolin (FS) served as positive controls whereas diaphragm muscle cells served as negative controls. C) Stimulation of thymidine incorporation by cultured gubernacular cells treated with different hormones for 24 h.
REFERENCES

Fig. 1

(A)  

- Human INSL3
- Ovine INSL3
- Rat INSL3
- Biotin-INSL3
- Porcine RLX

Concentration (nM)

0 0.1 0.3 1 3 10 30

Glucagon

cAMP (pmole/well)

0 100 200 300

(B)  

- Porcine RLX
- Human INSL3
- Ovine INSL3
- Rat INSL3
- Biotin-INSL3
- Glucagon

Concentration (nM)

0 0.03 0.3 1 3 10 30

0 100 200 300
Fig. 2

(A) 125I-Streptavidin Bound (Cpm/tube) vs INSL3 (nM)
- LGR8 in 293T cells
- 293T cells

(B) Streptavidin-HRP vs M1-Ab
MW
-15
-10
-6
1. Biotin-INSL3
2. Biotin-INSL3 + LGR8
3. Biotin-INSL3 + LGR8 + INSL3
4. IP LGR8

MW
-105
-75
-50

IgG heavy chain
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J. Biol. Chem. published online July 11, 2002

Access the most updated version of this article at doi: 10.1074/jbc.C200398200

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