

The active form of goat insulin-like peptide 3 (INSL3) is a single-chain structure comprising three domains B-C-A, constitutively expressed and secreted by testicular Leydig cells

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The active form of goat insulin-like peptide 3 (INSL3) is a single-chain structure comprising three domains B-C-A, constitutively expressed and secreted by testicular Leydig cells

Abstract: Relaxin-like factor (RLF), also called insulin-like peptide 3 (INSL3), is a member of the insulin/relaxin gene family and is produced by testicular Leydig cells. While the understanding of its effects is growing, very little is known about the structural and functional properties of native INSL3. Here, we demonstrate that native INSL3 isolated from goat testes is a single-chain structure with full biological activity, and is constitutively expressed and secreted by Leydig cells. Using a series of chromatography steps, native INSL3 was highly purified as a single 12-kDa peak as revealed by SDS-PAGE. MS/MS analysis provided 81% sequence coverage and revealed a distinct single-chain structure consisting of the B-, C-, and A-domains deduced previously from the INSL3 cDNA sequence. Moreover, the N-terminal peptide was six amino acid residues longer than predicted. Native INSL3 exhibited full bioactivity in HEK-293 cells expressing the receptor for INSL3. Immunoelectron microscopy and Western blot analysis revealed that INSL3 was secreted by Leydig cells through the constitutive pathway into blood and body fluids. We conclude, therefore, that goat INSL3 is constitutively secreted from Leydig cells as a B-C-A single-chain structure with full biological activity.

Keywords: bioactivity; INSL3; MS/MS; native conformation; purification; subcellular localization.

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Introduction

Testicular Leydig cells produce a peptide hormone called relaxin-like factor (RLF), as well as steroid hormones. RLF, also known as insulin-like factor 3 (INSL3), is a novel member of relaxin/insulin gene family originally discovered by screening a boar testicular cDNA library (Adham et al., 1993). Its cDNA has been cloned from the testes of a number of species, including ruminants (Ivell and Bathgate, 2002). Although the properties and functions of INSL3 are still being elucidated, INSL3 has been implicated in the regulation of testicular function. Male mice with knockout of the gene encoding either INSL3 (Nef and Parada, 1999; Zimmermann et al., 1999) or its receptor relaxin family peptide receptor 2 (RXFP2) (Gorlov et al., 2002; Bogatcheva et al., 2003) [which was originally called leucine-rich repeat-containing G-protein-coupled receptor 8 (LGR8) (Hsu et al., 2002)], cause cryptorchidism during fetal development because of a developmental abnormality of the gubernaculum, which results in abnormal spermatogenesis and infertility. Moreover,

INSL3 can suppress male germ cell apoptosis in rat testes (Kawamura et al., 2004), although its role in adults is still unclear.

Unlike other peptides from the relaxin/insulin family, very little is known about the native conformation of INSL3. INSL3 is predicted from the cDNA sequence to be biosynthesized as a precursor protein (pro-INSL3) containing A- and B-domains connected by a C-domain and is assumed to undergo proteolytic processing to remove the C-domain peptide. The precursor then matures to an A-B heterodimer linked by two disulfide bonds to form an active hormone, as do the other members of this family of peptides (Ivell and Bathgate, 2002), although Ivell and Anand-Ivell (2009) have disputed the assumption that INSL3 is secreted only as an A-B heterodimer. This prediction has been supported by the findings that a synthetic INSL3 peptide, which consists of an A-B heterodimer with site-specific sequential disulfide bonds in some species, such as humans (Büllesbach and Schwabe, 1995) and rats (Smith et al., 2001), stimulates cAMP production by binding to its receptor RXFP2. Furthermore, this is corroborated by a report that native INSL3 exists as an A-B heterodimer when isolated from bovine testis (Büllesbach and Schwabe, 2002).

However, our recent study concerning native INSL3 purified from boar testes demonstrated for the first time that the native INSL3 exists as a monomer comprising three domains, B-C-A, with site-specific disulfide bonds and full biological activity (Minagawa et al., 2012). Therefore, there appears to be not only a bovine form of native INSL3 that exists as an A-B heterodimer (Büllesbach and Schwabe, 2002), but also a porcine form that exists as a B-C-A single-chain (Minagawa et al., 2012). In fact, the molecular mass of INSL3 detected by Western blot analysis in tissue extracts from humans (Hombach-Klonisch et al., 2000), rats (McKinnell et al., 2005), horses (Klonisch et al., 2003), deer (Hombach-Klonisch et al., 2004), and goats (Siqin et al., 2010a) corresponds to that of pro-INSL3 (B-C-A single-chain form) as deduced from their cDNA sequences. Hence, it is worth proving whether native INSL3 of other species, including goats, exists as a B-C-A single-chain that corresponds to a porcine model of native INSL3 but not the bovine model.

Here, we report the purification of native INSL3 from goat testes and demonstrate that it exists as a B-C-A single-chain, possesses full biological activity, and is constitutively expressed and secreted by Leydig cells. Goats are used worldwide as livestock to produce meat, milk, fiber, and hides. Because goats are one of the most fertile domestic species and are reputed to be precocious

in their sexual development among domestic ruminants (Deveson et al., 1992), they are thought to be a useful pilot animal for studying reproductive physiology in ruminants. Therefore, elucidating the native conformation and its functional characterization of INSL3 would be a major step toward conducting studies on its physiological roles in goats.

Results

Purification of goat INSL3

INSL3 was purified from goat testes using sequential chromatographic techniques. INSL3-containing fractions were identified using a dot-blot analysis with an anti-INSL3 peptide antibody, which was presumed to be monotypic and likely to recognize a single epitope in the A-domain. We used a synthetic A-B heterodimeric human INSL3 peptide (6292 Da; Phoenix Pharmaceuticals, Burlingame, CA) and recombinant INSL3 (His-tagged pro-RLF; ~16 kDa) (Kato et al., 2010; Siqin et al., 2010a) to determine whether an A-B heterodimer as well as precursors could be detected and quantitated using dot blots. The resulting calibration curves were linear from 2–16 ng (0.32–2.54 pmol) for synthetic human INSL3 (Figure 1A) and from 0.5–4 ng (0.03–0.25 pmol) for recombinant INSL3 (Figure 1B). Therefore, the dot-blot assay quantitates the A-B heterodimer; however, an approximately 10-fold higher pmol concentration of the A-B heterodimer is required to generate signals as intense as those of the B-C-A form. This suggests that the binding of the A-B heterodimers to the dot-blot matrix via the epitopes recognized by the antibody might prevent antibody binding.

A testicular extract (a reverse-phase Sep-Pak extract), which likely contained both precursors and putative A-B heterodimers, was chromatographed on a Sephadex G-50 column (Figure 2A); however, A-B heterodimers might have been eliminated. The INSL3-positive fraction was further processed by cation-exchange FPLC (Figure 2B), reverse-phase HPLC (Figure 2C), and rechromatography using reverse-phase HPLC (Figure 2D). The final INSL3 preparation was isolated as a single peak with a molecular mass of approximately 12 kDa, as revealed by SDS-PAGE and Western blotting (Figure 2D). The recoveries from the purification steps are summarized in Table 1. The loss of yield suggests that considerable INSL3 immunoreactivity was lost during the various chromatography steps, likely caused by loss of A-B heterodimers that might be present.

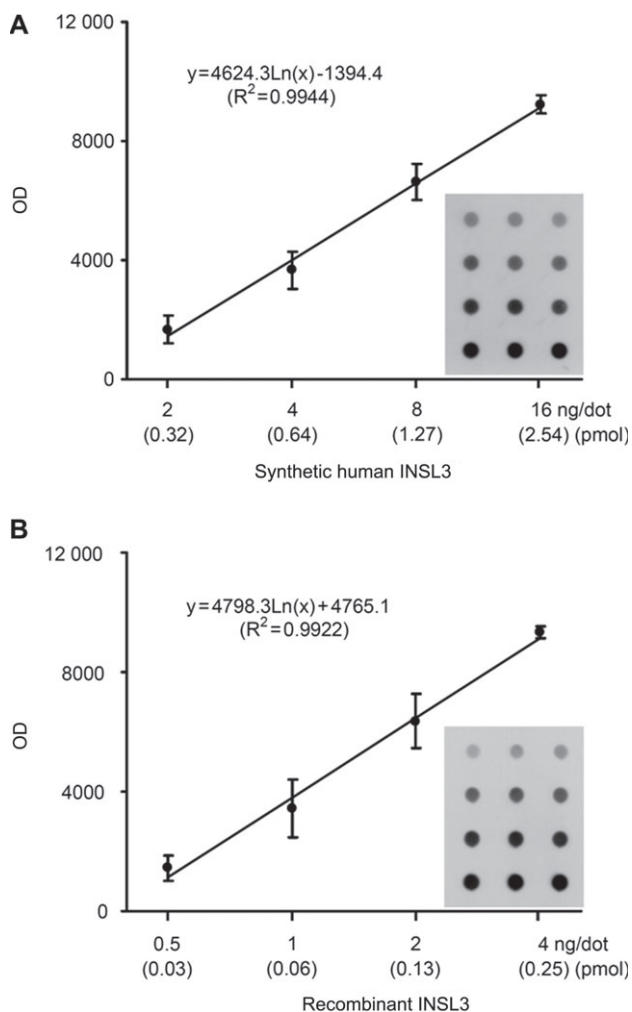


Figure 1 Comparison of dot-blot calibration curves for the synthetic A–B heterodimeric INSL3 peptide (6.3 kDa) and recombinant INSL3 (His-tagged pro-INSL3; ~16 kDa).

Samples were spotted directly onto a nitrocellulose membrane in triplicate. (A) From top to bottom, synthetic human INSL3 was 2, 4, 8, and 16 ng, corresponding to 0.32, 0.64, 1.27, and 2.54 pmol, respectively. (B) From top to bottom, recombinant INSL3 was 0.5, 1, 2, and 4 ng, corresponding to 0.03, 0.04, 0.13, and 0.25 pmol, respectively. Regression lines were calculated using three measurements for each point. When compared with (A) and (B), an approximately 10-fold higher pmol concentration of A–B heterodimer was required to detect the signal intensity comparable with that of the B–C–A form. Optical density (OD) indicates the densitometric analysis of the dot-blot.

MS/MS analysis of purified goat INSL3

Purified goat INSL3 was digested with trypsin, and the resultant peptide mixtures were analyzed directly by LC-MALDI-MS/MS. When all MS/MS data were analyzed using ProteinPilot Software, the goat pro-INSL3 sequence was distinctly assigned as the top hit with 81% sequence

coverage, including matches to the C-terminal peptide (Figure 3A). The assigned peptides corresponded to the B-, C-, and A-domains of the goat pre-pro-INSL3 deduced from the cDNA sequence (EMBL: AAF60301.1) (Hombach-Klonisch et al., 1999) (Figure 3A). The MS/MS spectrum of the M-H⁺ precursor ion at m/z 2360.17 was identified as the N-terminal peptide of the B-domain, which is six residues longer in the N-terminus than the predicted B-domain (Figure 3B). This was quite close to the theoretical mass of 2360.18, which was calculated from the deduced sequence from cDNA. This extension (Ile-Ala-Leu-Gly-Pro-Ala) was assigned previously as part of the signal peptide deduced from the cDNA sequence (Figure 3A and B). We thus concluded that native goat INSL3 is a single-chain structure comprising 26 residue A-, 38 residue B-, and 49 residue C-domains, in which C-domain does not undergo proteolytic cleavage, and the A- and B-domains are likely linked by three disulfide bonds (Figure 3C), as reported previously (Büllesbach and Schwabe, 2002; Minagawa et al., 2012).

Biological activity of purified INSL3

A bioassay system based on the cAMP production in a mouse INSL3 receptor RXFP2-expressing HEK-293 cells was used to examine the biological activity of the purified INSL3. Heterologous assay systems, such as that used here, have been employed by our group and others. We have used the mouse RXFP2-expressing cells for evaluating purified boar INSL3 (Minagawa et al., 2012). Furthermore, Kumagai et al. (2002) demonstrated that treating HEK-293 cells expressing human RXFP2 with synthetic human, ovine, or rat INSL3 induced similar dose-dependent increases in cAMP production. In addition, Anand-Ivell et al. (2006) also demonstrated that synthetic human INSL3 activated cAMP production in HEK-293 cells transfected with either mouse or human RXFP2. When HEK-293 cells were transiently transfected with a mouse RXFP2 cDNA construct, the cell-surface expression of RXFP2 was observed histochemically with a FLAG-tag antibody (Figure 4A). Treatment of RXFP2-expressing HEK-293 cells with the INSL3 resulted in a dose-dependent increase in intracellular cAMP production with an EC₅₀ value of approximately 7 nM (Figure 4B). This was comparable with the EC₅₀ values determined for the synthetic A-B heterodimeric human INSL3 peptide (Figure 4B). In contrast, native and synthetic INSL3 preparations had no effect on cells transfected with an empty vector (Figure 4B). These results indicated that purified goat INSL3 was as active as synthetic human INSL3.

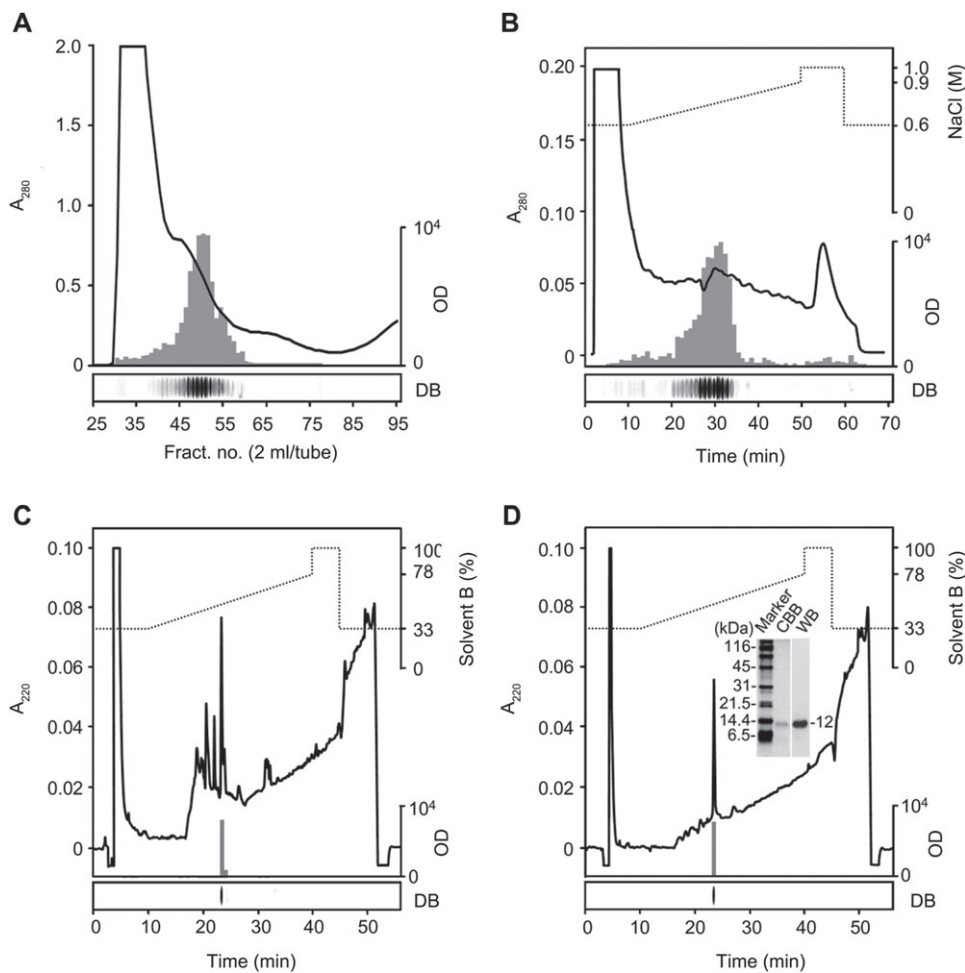


Figure 2 Purification of goat testicular INSL3.

(A) Gel filtration of a testicular extract on a Sephadex G-50 column (2.0×62 cm) in 20 mM ammonium acetate buffer (pH 5.0). The fractions containing INSL3 immunoreactivity (dark area) were revealed by dot-blot analysis. (B) Cation-exchange FPLC of the G-50 fraction using a TSKgel SP-5PW column (5×50 mm), which was eluted for 40 min with a linear gradient of 0.6–0.9 M NaCl in 25 mM ammonium acetate buffer (pH 5.0) at a flow rate of 0.5 ml/min. INSL3 immunoreactivity, as revealed by dot-blot analysis, was detected exclusively in the dark area of the effluent curve. (C) Reverse-phase HPLC of the active fraction from cation-exchange FPLC on a YMC-Pack ODS-AM column (4.6×250 mm) using a 33–78% linear gradient of solvent B at a flow rate of 1 ml/min. INSL3-positive peaks were revealed by dot-blot analysis. (D) Rechromatography of INSL3-positive peaks using the same HPLC conditions described above. INSL3 was isolated as a single peak with a molecular mass of ~12 kDa, as revealed by Coomassie brilliant blue-stained SDS-PAGE (CBB) and Western blot analysis (WB). DB, dot-blot analysis. OD, the densitometric analysis of the dot-blot.

Table 1 Summary of the purification of testicular INSL3.

Purification step	Total protein (μg)	Immunoactivity (ng/ml)	Immunoactivity (nM)	Yield (μg)	Recovery (%)	Purification (fold)
Recombinant INSL3	–	21.12	1.34	–	–	–
Testicular extract	483 600	33 457	2124	305.28	100	1
Gel filtration	59 237	9339	593	133.96	44	4
Cation-exchange FPLC	294	309	19.62	20.09	7	108
Reverse-phase HPLC	10.75	21.12	1.34	10.75	4	1584

The preparation was taken from 400 g of frozen tissues as a starting material. The immunoactivity of INSL3 (ng/ml and nM) at each step of the purification was measured by TR-FIA and was expressed as EC_{50} values for the recombinant INSL3, which was expressed as a ~16 kDa protein (calculated mass of 15751) in *Escherichia coli* by using a construct that expresses the His-tagged proINSL3 sequence containing the B-C-A domain inserted into the pCold I vector, as described by us (Kato et al., 2010; Siqin et al., 2010a). Similar results were obtained with two independent experiments.

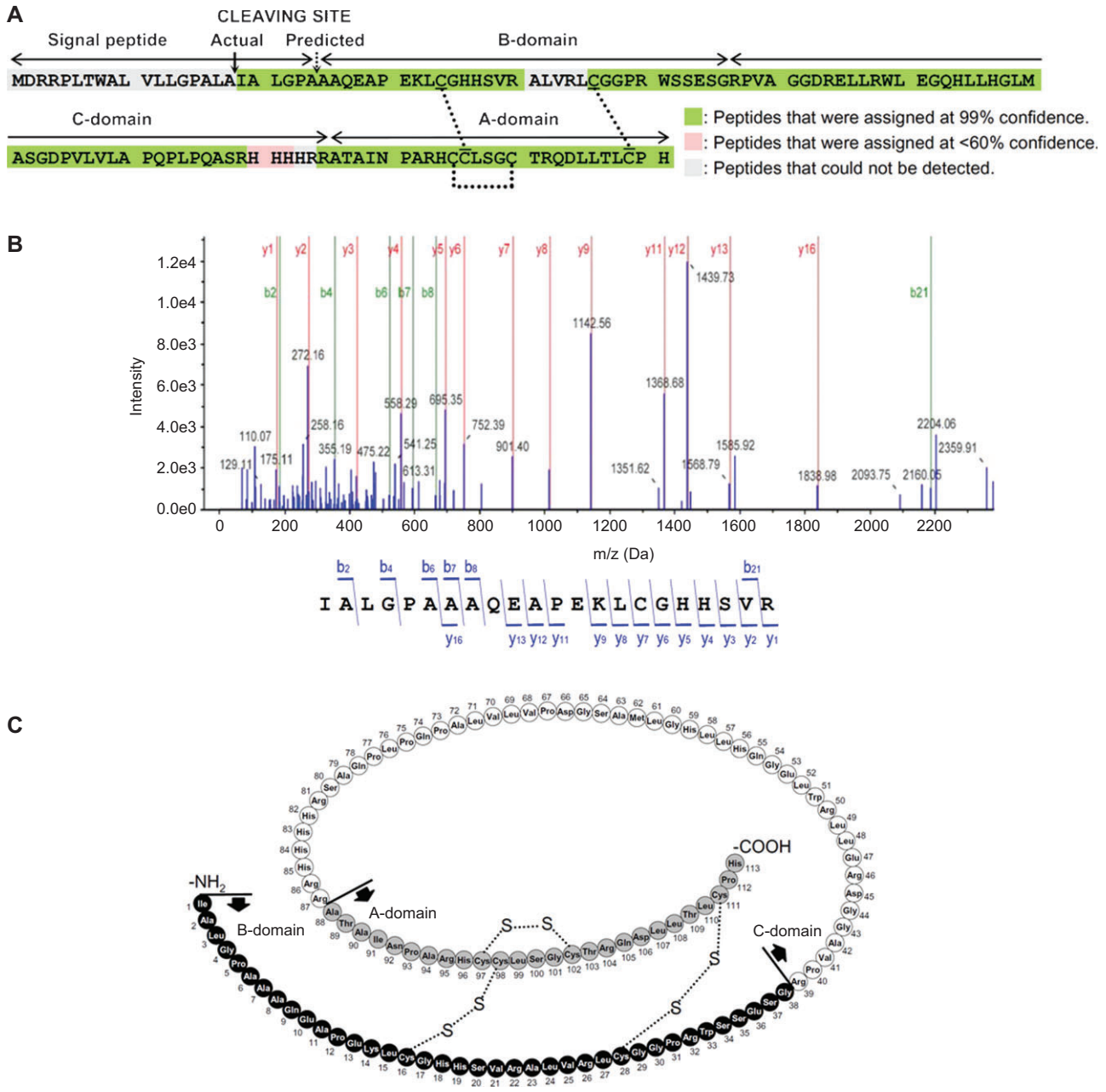


Figure 3 MS/MS analysis of purified goat INSL3.

(A) The predicted amino acid sequence of goat pre-pro-INSL3 deduced from the cDNA sequence and the peptides assigned by MALDI-MS/MS analysis of purified native goat INSL3. Assigned peptides were recognized with a high score of 81% sequence coverage of the A-, B-, and C-domains deduced from the cDNA (EMBL: AAF60301.1) (Hombach-Klonisch et al., 1999). (B) MALDI-MS/MS spectra of the M-H⁺ precursor ion at m/z 2360.17. Sequential product ions were successfully detected in both the b- and y-series. The assigned peptide was the N-terminal peptide of the B-domain, which is six residues longer at the N-terminus than at the B-domain, deduced from the cDNA sequence. (C) Structural features of goat INSL3 determined by MS/MS analysis.

Subcellular localization of INSL3 in Leydig cells

INSL3 protein was visualized only in Leydig cells within the testis (Figure 5A and B). No signal was observed in seminiferous tubules that are known as the site of

spermatogenesis (Figure 5A). The ultrastructural features of Leydig cells are characterized by a large quantity of smooth endoplasmic reticulum (SER), which are observed as a network of anastomosing or interconnecting tubules, mitochondria with tubular cristae, and lipid droplets. Secretory granules were not observed in the cytoplasm

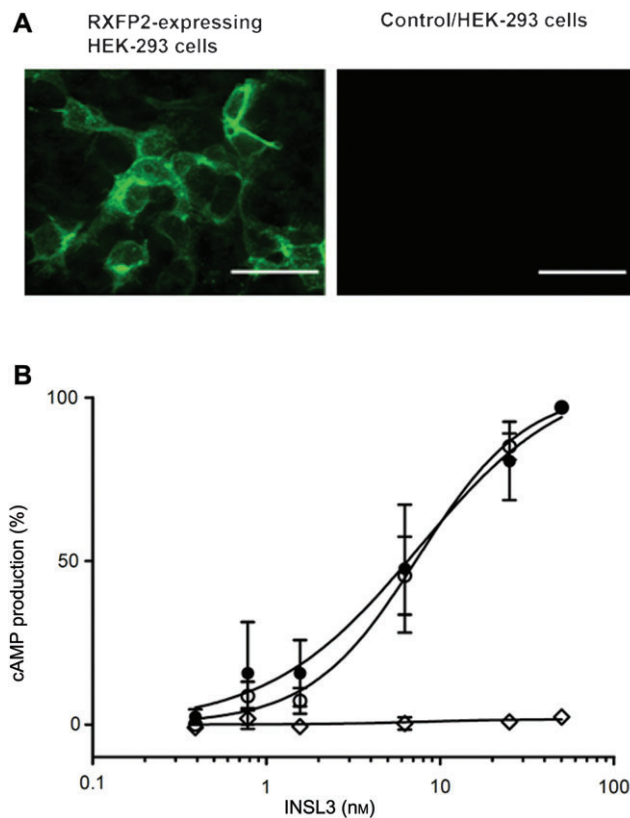


Figure 4 Biological activity of purified INSL3 based on cAMP production by RXFP2-expressing HEK-293 cells.

(A) The INSL3 receptor RXFP2 was transfected into HEK-293 cells for detecting the biological activity of INSL3. RXFP2 was expressed on the cell surface in RXFP2-expressing HEK-293 cells, but is not detected in the cells transfected with an empty vector (control/HEK-293 cells). Scale bars = 50 μ m. (B) Stimulation of cAMP production by INSL3 in RXFP2-expressing HEK-293 cells. Purified goat INSL3 (●) stimulated dose-dependent cAMP production with EC_{50} values comparable with those of the synthetic A-B heterodimeric human INSL3 (○) indicates the retention of full bioactivity. In contrast, purified INSL3 did not stimulate cAMP production in the control/HEK-293 cells (◇). The production of cAMP is expressed as a percentage of the maximum native goat INSL3 or synthetic human INSL3 peptide response for RXFP2. Results are presented as the mean \pm SEM for three independent experiments. The data were analyzed using the non-linear curve fitting functions in GraphPad PRISM version 5.0 software.

(Figure 5C–E). When we examined the subcellular location of INSL3 within the Leydig cells by the protein A-gold method, gold particles indicating the localization of INSL3 could be visualized mainly in the trans-Golgi network (TGN) (Figure 5C). No gold labeling was observed in the other organelles, such as mitochondria, SER, and lipid droplets (Figure 5C and D). Immunolabeling was blocked sufficiently by incubation with INSL3 antibody preabsorbed with purified INSL3 (Figure 5E).

Secretion of INSL3 from Leydig cells into blood and body fluids

Using a synthetic human A-B heterodimeric INSL3 peptide (Phoenix Pharmaceuticals), we first determined the appropriate conditions for separating the A-B form without loss caused by its elution from the gels. The INSL3 peptide could be successfully transferred to a membrane and quantitatively detected with our anti-INSL3 antibody using Western blot analysis (Figure 6A). This allowed further studies of INSL3 secretion. When we compared the calibration curves generated from Western blot analysis performed simultaneously for synthetic human INSL3 and recombinant INSL3 (His-tagged pro-INSL3), we found a marked difference in sensitivity. The calibration curves for synthetic human INSL3 peptide and recombinant INSL3 were linear in the ranges of 16–64 ng (2.5–10 pmol) (Figure 6B) and 0.25–1 ng (0.016–0.06 pmol) (Figure 6C), respectively. Therefore, using Western blotting, an approximately 160-fold higher pmol concentration of the A-B heterodimer is required to detect a signal as intense as that generated by the corresponding level of the B-C-A form. These findings suggest that the A-B heterodimer either bound to the membrane through the same residues that serve as epitopes, thereby preventing antibody binding, or did not tightly bind to the membrane.

Under these conditions, Western blot analysis of testicular venous plasma and peripheral plasma clearly detected the 12-kDa INSL3 species (Figure 7A). Furthermore, the relative level of INSL3 detected in testicular venous plasma was significantly three times higher ($p < 0.01$) than in peripheral plasma (Figure 7B). Western blot analysis of seminiferous tubular fluid, body fluids within the lumen of the seminiferous tubules, also demonstrated the presence of the 12-kDa band (Figure 7C). Therefore, when our results are considered together, they indicate that INSL3 is secreted by Leydig cells into the circulation and body fluid as a 12-kDa A-B-C single-chain. However, we cannot exclude the possibility that some of the circulating INSL3 might exist as an A-B heterodimer because the A-B form is difficult to detect using the monoclonal antibody used to probe Western blots.

Discussion

Until now, goat INSL3 is predicted to be biosynthesized as a precursor protein (pro-INSL3) containing A- and B-domains connected by a C-domain and is assumed to be an A-B heterodimer to form an active hormone

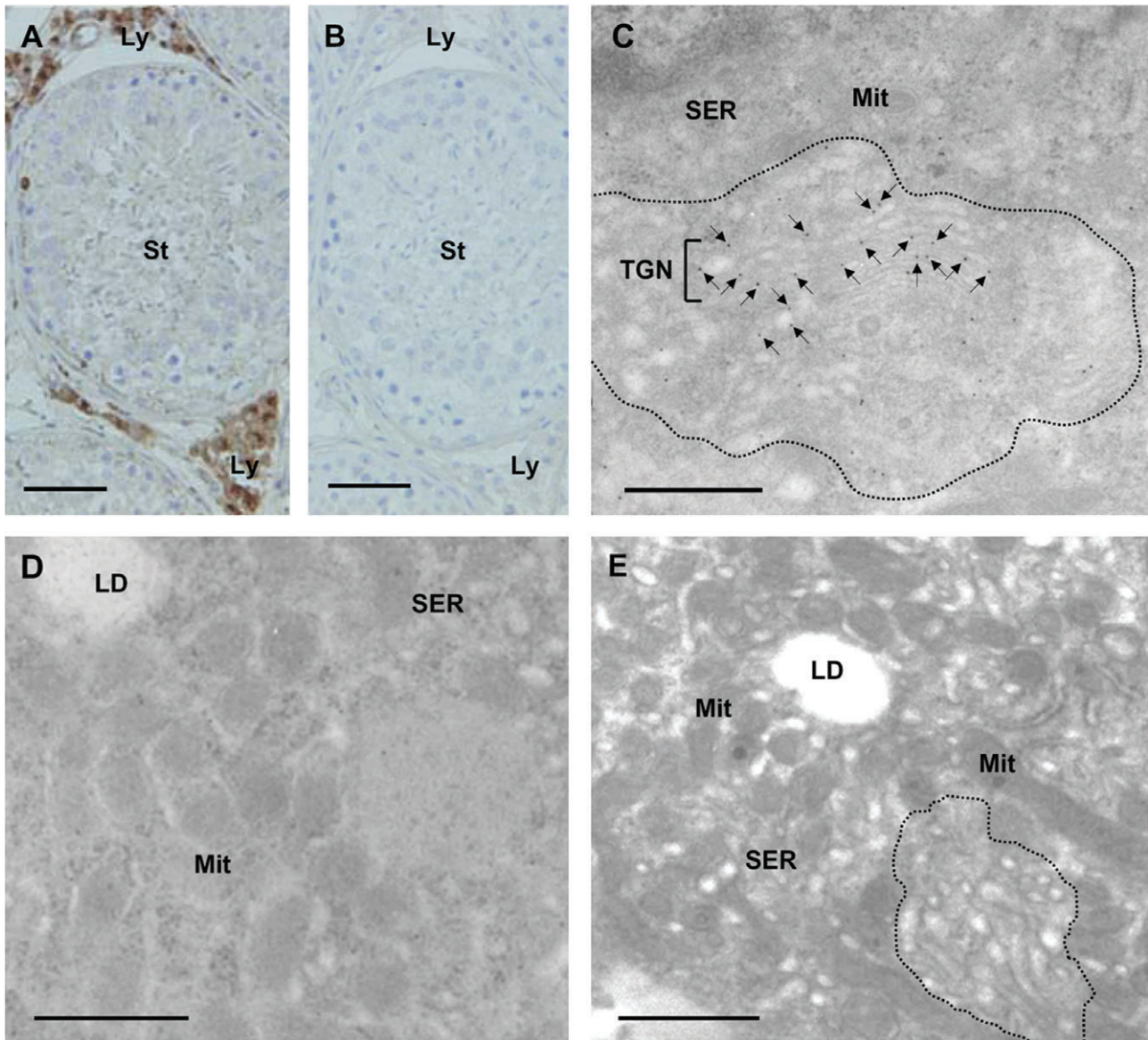


Figure 5 Subcellular localization of INSL3 in Leydig cells.

(A) Immunolocalization of INSL3 in the testis. Using anti- INSL3 antibody, signals of immunoreactive INSL3 were restricted to Leydig cells. Ly, Leydig cells; St, seminiferous tubule. (B) Verification by preabsorbed antibody. Staining of Leydig cells was abolished by preabsorbing the antibody with purified goat INSL3. (C) Subcellular localization of INSL3 in the Leydig cells labeled with anti- INSL3 antibody IgG fraction followed by protein A-gold. The gold particles (arrows) indicating INSL3 immunoreactivity could be observed mainly in the trans-Golgi network (TGN). The area surrounded by a dashed line shows Golgi field. Mit, mitochondria; SER, smooth endoplasmic reticulum; LD, lipid droplet. (D) Gold labeling of the other subcellular organelles except TGN within the Leydig cells. There was no gold labeling of the other subcellular organelles, such as the Mit, SER, and LD. (E) Verification by preabsorbed antibody. Immunolabeling was blocked sufficiently by incubation with INSL3 antibody preabsorbed with purified INSL3. The area surrounded by a dashed line shows the Golgi field. Scale bars in A,B =50 μ m; Bars in C–E =500 nm.

(Hombach-Klonisch et al., 1999). However, we succeeded in isolating and purifying biologically active INSL3 from goat testes and demonstrated for the first time that it exists as a single-chain protein, and is constitutively expressed and secreted by Leydig cells.

In the present study, when we purified the protein using a series of chromatography steps and examined the

native conformation by MS/MS analysis, we determined that the native goat INSL3 is a 12-kDa protein with a B-C-A single-chain structure, in which the C-domain does not undergo proteolytic processing. Only limited evidence is available concerning bovine and porcine forms of native INSL3 isolated from testes: native bovine INSL3 has been characterized as an A-B heterodimer either by protein

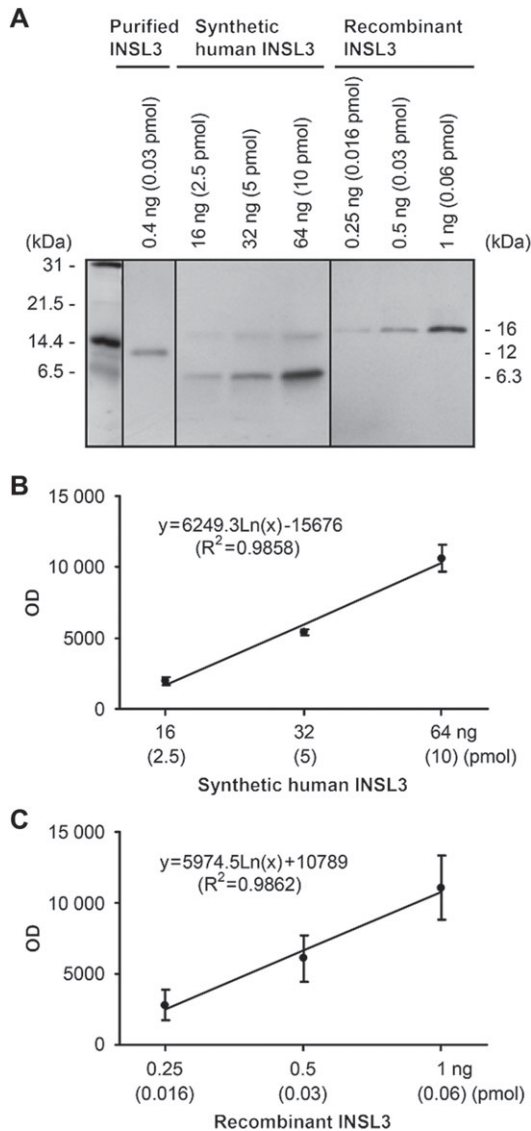


Figure 6 Comparison of Western blot calibration curves of a synthetic A–B heterodimeric human INSL3 peptide (6.3 kDa) and recombinant INSL3 (His-tagged pro-INSL3; ~16 kDa). (A) A representative Western blot analysis. Samples were separated by SDS-PAGE on a 14% gel under non-reducing conditions and transferred to nitrocellulose membranes. The loads were 16, 32, and 64 ng (corresponding to 2.5, 5, and 10 pmol, respectively) for the synthetic human INSL3, and 0.25, 0.5, and 1 ng (corresponding to 0.016, 0.03, and 0.06 pmol, respectively) for the recombinant INSL3. (B) Calibration curve for synthetic human INSL3. (C) Calibration curve for recombinant INSL3. Regression lines were calculated using three measurements for each band. Comparison of (B) and (C) shows that an approximately 160-fold higher pmol concentration of the A–B heterodimer is required to detect signal intensity comparable to that of the A–B–C form.

sequencing or by amino acid composition (Büllesbach and Schwabe, 2002), whereas the primary structure of native porcine INSL3 determined by MS and MS/MS analyses is

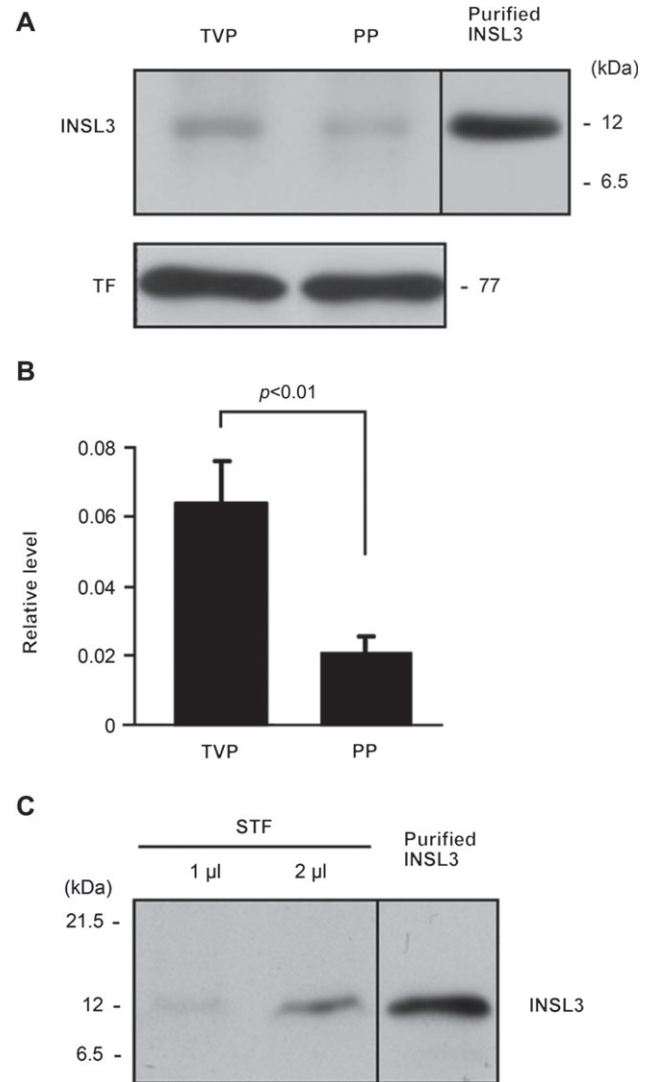


Figure 7 Secretion of INSL3 from Leydig cells into blood and body fluids as revealed by Western blot analysis.

(A) A representative Western blot analysis of testicular venous plasma (TVP) and peripheral plasma (PP). For each lane, 2.5 µl of plasma and 3 ng of purified INSL3 were applied. (B) The relative level of each message normalized to that of transferrin (TF). Values represent the means ± SEM from three animals. The relative level of INSL3 was significantly three-fold higher ($p < 0.01$) in TVP than that in PP. (C) A representative Western blot analysis of seminiferous tubular fluid (STF), body fluids within the lumen of the seminiferous tubules. For each lane, 1 and 2 µl of STF and 3 ng of purified INSL3 were applied.

a single-chain peptide comprising three domains B–C–A with site-specific disulfide bonds (Minagawa et al., 2012). Our present findings are consistent with the structural features of porcine native INSL3 (Minagawa et al., 2012) but quite different from those of native bovine INSL3 (Büllesbach and Schwabe, 2002) in that the C-domain does not undergo processing. The data provided by Büllesbach

and Schwabe (2002) revealed a substantial amount of specific immunoreactivity in the exclusion volumes from their chromatography columns, so that they only targeted the A-B heterodimer and likely would have been unable to detect a B-C-A single-chain form, as stated by Ivell et al. (2011). Furthermore, we demonstrated unequivocally that the goat INSL3 stimulated dose-dependent cAMP production by a mouse RXFP2-expressing HEK-293 cells with an EC_{50} value of approximately 7 nM. This value is close to that of the EC_{50} of the synthetic A-B heterodimeric human INSL3 peptide, indicating that bioactivity resides fully in the isolated INSL3. Moreover, Western blot analysis of testicular venous and peripheral plasma and also of seminiferous tubular fluid indicated that the INSL3 secreted from testicular Leydig cells into blood and body fluids migrated as the 12-kDa species. However, we were not able to exclude the possibility that some circulating INSL3 might represent the 6-kDa A-B heterodimer because of the insensitivity of Western blotting used for its detection. These results establish that goat INSL3 is secreted from testicular Leydig cells into blood and body fluids as a biologically active single-chain peptide. Therefore, the native goat INSL3 is quite similar to insulin-like growth factors (LeRoith and Roberts, 2003), as well as native porcine INSL3 (Minagawa et al., 2012), in that the proforms are not processed into two-chain peptides and exert full bioactivity.

In addition to the B-C-A single-chain structure, we determined that the B-domain of the native goat INSL3 possesses an additional six residues at the N-terminus compared with the predicted sequence from the cDNA (Hombach-Klonisch et al., 1999). The structures of native porcine (Minagawa et al., 2012), bovine (Büllesbach and Schwabe, 2002), and goat INSL3 are similar in this regard. Signal peptides control the entry of virtually all proteins into the secretory pathway and are cleaved from their precursors during transport of nascent proteins into the rough endoplasmic reticulum (RER). The common structural features of signal peptides are as follows: a positively charged N-terminus, a central hydrophobic region, and a C-terminal region with small and neutral amino acids at positions -3 and -1 (relative to cleavage site) (von Heijne, 1986; Nielsen et al., 1997). Based on this -3 and -1 rule, it would be reasonable to conclude that the predicted signal peptide is cleaved between two alanine residues (in bold text and solidus) within the sequence GPA/AA. However, the six-residue extension of the B-domain is generated by cleavage between alanine and isoleucine residues (in bold text and solidus) within ALA/IALG-PAAA, where the -3 and -1 residues are small and neutral, which matches the consensus criteria for cleavage sites

(von Heijne, 1986; Nielsen et al., 1997). Thus, it is clear that the signal peptidase recognized this part as a cleavage site.

It is important to note what secretory pathways are responsible for regulating INSL3 production and why INSL3 is secreted as a single-chain peptide. It is now accepted that endocrine cells producing peptide hormone possess regulated or constitutive secretory pathways, and that most secretory proteins that are synthesized as prohormones on the RER are transported to the *cis*-Golgi network and then through the TGN (Halban and Irminger, 1994). In the regulated secretory pathway, prohormones are sorted into secretory granules for processing and storage before their release. In contrast, the constitutive secretory pathway is thought to be the default pathway by which prohormones exit the TGN and are rapidly released without storage (Kelly, 1985; Halban and Irminger, 1994). Moreover, the major proteolytic enzymes mediating protein processing are prohormone convertase 1/3 (PC1/3) in the secretory granules in the regulated pathway (Halban and Irminger, 1994; Jutras et al., 1997; Steiner, 1998) and furin in the TGN in the constitutive pathway (Halban and Irminger, 1994; Nakayama, 1997; Steiner, 1998). For example, insulin and relaxin, which belong to the same family as INSL3, are stored in secretory granules (Kohsaka et al., 1992, 1993a; Rorsman and Renström, 2003), and they undergo processing by PC1/3 (Marriott et al., 1992). Here, we determined that INSL3 localizes to the TGN within the Leydig cells, where secretory storage granules were undetectable. Together with findings that INSL3 mRNA and/or protein seem to be constitutively expressed in rodent Leydig cells (Balvers et al., 1998; Sadeghian et al., 2005; Anand-Ivell et al., 2009), our immunoelectron microscopic data strongly suggest that goat INSL3 is not produced by the regulated secretory pathway but by the constitutive secretory pathway in Leydig cells. If this is the case, furin, rather than PC1/3, would mediate processing of INSL3 in goat testes. However, goat INSL3 did not actually have an RXK/RR sequence for cleavage catalyzed by furin (Nakayama, 1997). Therefore, it is reasonable to conclude that goat testicular INSL3 is regulated by the constitutive secretory pathway and that the A-B heterodimeric INSL3 is not produced due to a lack of the RXK/RR motif at the furin cleavage site required for processing pro-INSL3.

Currently, it remains largely unknown what role INSL3 actually exerts in goats. In the present study, however, single-chain INSL3 seemed to be physiologically relevant, because INSL3 is constitutively secreted from testicular Leydig cells into blood and body fluids

as a single-chain molecule with biological activity. The INSL3 secreted into the circulation is likely to function as an endocrine factor (Ferlin and Foresta, 2005), although its extra-testicular targets are undetermined. In contrast, receptor RXFP2 mRNA and/or protein have recently been shown to be expressed in Leydig cells and seminiferous germ cells in adult testes of some species (Kawamura et al., 2004; Anand-Ivell et al., 2006; Feng et al., 2007; Filonzi et al., 2007; Koeva et al., 2008), including goats (Siqin et al., 2010b). Taken together with previous findings, single-chain INSL3 secreted into body fluids may function as an autocrine and/or paracrine factor in Leydig cells and seminiferous germ cells in goat testes. In fact, there is evidence that RLF/RNSL3 suppresses germ cell apoptosis in the rat testis (Kawamura et al., 2004), whereas INSL3 stimulates the proliferation of the mouse Leydig cell-line TM3 *in vitro* (Feng et al., 2007). In any event, clarification of native goat INSL3 and its functional characterization in the present study will facilitate development of a specific immunoassay system for measuring INSL3 in blood and body fluids, whereby the yet unidentified paracrine and/or autocrine aspects of INSL3 can be explored, thereby giving insight into the potential role of INSL3 in goat testicular function.

In conclusion, we isolated native INSL3 from goat testes and demonstrated that it is constitutively secreted from Leydig cells as a B-C-A single-chain molecule with full biological activity.

Materials and methods

Reagents and chemicals

Glutaraldehyde, osmium tetroxide, and LR-White resin were purchased from TAAB Laboratories Equipment (Berkshire, UK), Nisshin EM (Tokyo, Japan), and London Resin Company (Berkshire, UK), respectively. Sephadex G-50 (fine), cAMP Biotrak ELISA kit and enhanced chemiluminescence (ECL) were purchased from GE Healthcare (Buckinghamshire, UK). Peroxidase-conjugated donkey anti-rabbit IgG was purchased from Chemicon International (Temecula, CA, USA), and the Vectastain Universal Elite ABC kit was from Vector Laboratories (Burlingame, CA, USA). Protein A-gold (10 nm) was from BBInternational (Cardiff, UK). Alexa Fluor 488-conjugated goat anti-mouse IgG, Lipofectamine 2000, and Opti-MEM serum-free medium were purchased from Invitrogen (Carlsbad, CA, USA). FBS was from Nichirei Biosciences (Tokyo, Japan), and DMEM, 3-isobutyl-1-methyl xanthine (IBMX) and anti-FLAG M2 monoclonal IgG were from Sigma (Saint Louis, MO, USA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Wako Pure chemicals (Osaka, Japan) and Peptide Institute Inc. (Osaka, Japan), respectively. All other chemicals were of appropriate grade and were purchased from commercial sources.

Animals and tissue sampling

Adult male Saanen goats fed at the Nagano Station of National Livestock Breeding Center were used during breeding seasons. For purification study, testes were obtained by castration or just after slaughter. After removal of the epididymis, testes were rapidly frozen in liquid nitrogen and stored at -80°C . For electron microscopy, testes with spermatic cord were removed at castration from adult goats and processed as described (Lunstra et al., 1986; Kohsaka et al., 1992, 1993a). Briefly, the testes were perfused immediately with Zamboni solution (picric acid-paraformaldehyde containing 1% glutaraldehyde in 50 mM phosphate buffer, pH 7.2) via the testicular artery of the spermatic cord for 90 min at 4°C . The perfused testes were cut into small pieces and further fixed for 10 h with the same fixative at 4°C . Tissues were washed in 10 mM PBS with subsequent post-fixation in 0.5% osmium tetroxide in PBS for 1 h at 4°C , dehydrated in a graded ethanol series, and embedded in LR-White resin at 4°C . Ultrathin sections were cut with a diamond knife (Diatome, Biel, Switzerland) with an LKB 2088 ultramicrotome (LKB, Bromma, Sweden) and mounted on uncoated nickel grids (Nisshin EM). Testicular venous plasma was also collected from the spermatic cord, while peripheral venous plasma was taken from the jugular vein. In addition, seminiferous tubular fluid, body fluids within the lumen of the seminiferous tubules, was taken from the parenchyma of the testis, as described by others (Gustafsson et al., 2002; Anand-Ivell et al., 2009). The blood and body fluid samples were stored at -80°C . Animal care and all experimental procedures were performed in accordance with the Health Guideline for Care and Use of Experimental Animals at Shizuoka University.

Protein concentration

Protein concentrations were determined by the method of Lowry-Folin (Hess et al., 1978) with BSA as a standard.

Anti- INSL3 antibody

The anti- INSL3 antiserum (RLF-A-Ab808) used here was previously generated in our laboratory in New Zealand White rabbits against the synthetic peptide of 15 amino acid residues of the A-domain, which shared 100% amino acid homology among boar, bovine, sheep and goat INSL3 cDNAs. The specificity of the antibody has been described in detail elsewhere (Siqin et al., 2010a,b; Minagawa et al., 2012).

Purification of INSL3

Extraction

Goat testes (about 3600 g) were extracted according to our method (Kohsaka et al., 1993b), which minimized proteolysis by using a low pH and by precipitating high molecular mass proteases. Testes were finely ground and extracted at 4°C in homogenizing medium (2% TFA, 5% formic acid, 1% sodium chloride, and 1 M HCl) with an ULTRA-TURRAX™ disperser (T25 Basic; IKA, Staufen, Germany). The

homogenate was then centrifuged at 28000 *g* for 20 min at 4°C, and the supernatant was successively filtrated through glass microfiber filters (Whatman 934-AH; GE Healthcare) and 0.45 µm Durapore membrane filters (Millipore, Billerica, MA, USA) in an ice bath. The filtrate was pumped (Perista pump SJ-1211H; Atto, Tokyo, Japan) at 5 ml/min through Sep-Pak plus C18 Environmental Cartridges (Waters, Milford, MA, USA) that were connected in series in an ice bath. After the cartridges were washed at 4°C with 10% ACN containing 0.1% TFA, the absorbed protein was eluted with 80% ACN containing 0.1% TFA. The eluate was concentrated using a rotary evaporator at 40°C, lyophilized, and stored at -80°C until gel filtration.

Gel filtration

Gel filtration was performed using a Sephadex G-50 column (2.0×62 cm) equilibrated with 20 mM ammonium acetate buffer (pH 5.0). The flow rate was maintained at 13.3 ml/h, and each 2-ml fraction collected was measured by the absorbance at 280 nm. The fractions containing INSL3 were detected by dot-blot analysis with anti-INSL3 antiserum.

Cation-exchange FPLC

Next, INSL3-positive fractions were purified by FPLC (Pharmacia, Uppsala, Sweden) on a TSKgel SP-5PW cation-exchange column (5×50 mm; TOSOH, Tokyo, Japan) equilibrated with 0.6 M NaCl in 25 mM ammonium acetate buffer (pH 5.0) and were eluted over 40 min with a linear gradient of 0.6–0.9 M NaCl in the same buffer, at a flow rate of 0.5 ml/min. The eluted peptides were monitored by their absorbance at 280 nm, and 0.5-ml fractions were collected. The fractions containing INSL3 were detected by dot-blot analysis.

Reverse-phase HPLC

INSL3-positive fractions were further purified by reverse-phase HPLC (Hitachi model 4200; Hitachi, Tokyo, Japan) on a YMC-Pack ODS-AM (4.6×250 mm; YMC, Kyoto, Japan) using a linear gradient system. The solvents used were 0.1% TFA in water (solvent A) and 0.1% TFA in 80% ACN (solvent B). The column was equilibrated with 33% solvent B, and elution was carried out over 30 min with a 33–78% linear gradient of solvent B at a flow rate of 1 ml/min. The eluted peptides were monitored by their absorbance at 220 nm, and fractions were manually collected and checked by dot-blot and Western blot analyses.

Rechromatography

INSL3-positive peaks were pooled, lyophilized, and further purified by rechromatography using the reverse-phase HPLC conditions mentioned above, and fractions were manually collected and analyzed by dot-blot and Western blot analyses.

Recovery

INSL3 content recovered from each step of the purification was determined using time-resolved fluoroimmunoassay (TR-FIA) with the anti-INSL3 antiserum and europium (Eu)-labeled recombinant INSL3. The TR-FIA procedure was performed according to our protocol (Ogine et al., 1999), and the recombinant INSL3 was labeled using Eu-labeling reagent (PerkinElmer, Boston, MA, USA). The recombinant INSL3 (~16 kDa, calculated mass of 15751) was expressed in *Escherichia coli* by using a construct that expresses the His-tagged proINSL3 sequence containing the B-C-A domain inserted into the pCold I vector, as described previously (Kato et al., 2010; Siqin et al., 2010a). The immunoreactivity of INSL3 was revealed as EC₅₀ values for the recombinant INSL3 as described elsewhere (Minagawa et al., 2012).

Dot-blot and Western blot analyses

Samples, synthetic A-B heterodimeric human INSL3 peptide and recombinant INSL3 (His-tagged pro-INSL3) were spotted directly onto a nitrocellulose membrane (BioRad, Hercules, CA, USA) using Bio-Dot SF (BioRad) or separated by SDS-PAGE on a 14% gel under non-reducing conditions and transferred to nitrocellulose membranes by using a semi-dry transfer cell (BioRad). The membranes were immunostained with anti-INSL3 antiserum and peroxidase-conjugated donkey anti-rabbit IgG, and the signals were detected using an ECL. As a loading control for Western blot analysis of plasma samples, transferrin (TF) was detected by sequential incubation with a polyclonal antibody against TF (Inter-Cell Technologies, Hopewell, NJ, USA), peroxidase-conjugated donkey anti-rabbit IgG, and ECL. The optical density was measured by densitometric scanning using Image J software (<http://rsb.info.nih.gov/ij/>).

Protein digestion and nano-LC separation

Purified INSL3 was digested with trypsin as described previously (Minagawa et al., 2012), and peptide digests were separated on a direct nanoflow LC system (DiNa; KYA Technologies, Tokyo, Japan) using a reverse-phase trap column (HiQ-Sil C18-3, 0.8×3 mm; KYA Technologies) and an analytical column (HiQ-Sil C18-3, 0.15×50 mm; KYA Technologies) with a linear 0–50% gradient of solvent B (70% ACN in 0.1% TFA) for 12.5 min followed by a 50–100% gradient of solvent B for 5 min at a flow rate of 300 nl/min. The column effluent was recorded at 214 nm, mixed with the MALDI matrix solution (4 mg/ml α -cyano-4-hydroxycinnamic acid) in 70% ACN in 0.1% TFA, and then spotted directly onto an Opti-TOF™ 384-well plate (Applied Biosystems SCIEX, Framingham, MA, USA).

MALDI- MS/MS analysis

MS/MS analysis of offline spotted peptide samples were performed automatically using a 4800 Plus MALDI-TOF/TOF Analyzer (Applied Biosystems SCIEX) with a Plate Model external calibration. All MS/MS spectra were combined and processed with ProteinPilot™ Software (Ver.2.01, Applied Biosystems SCIEX).

INSL3 bioassay based on the cAMP production in HEK-293 cells expressing RXFP2

A mouse RXFP2 cDNA expression construct was generated previously by our group (Minagawa et al., 2012). HEK-293 cells were maintained in DMEM supplemented with 10% FBS, a 1:100 dilution of a stock penicillin-streptomycin solution (5000 units penicillin-G and 5 mg streptomycin per ml; Sigma), and 2 mM L-glutamine under a humidified environment containing an atmosphere of 5% CO₂ at 37°C. When cells were 80% confluent (3×10⁵/ml), they were seeded in 96-well plates (TPP Techno Plastic Product, Trasadingen, Switzerland). Transient transfection of the expression construct or empty vector was performed using Lipofectamine 2000 in Opti-MEM serum-free media. After 24 h, the transfection media were aspirated, and each well was washed with DMEM. The transfected cells were incubated for 1 h in DMEM containing 200 μM IBMX with or without purified INSL3 or synthetic human INSL3 peptide (Phoenix Pharmaceuticals). Intracellular cAMP was detected using the cAMP Biotrak ELISA kit and expressed as a percentage of the maximum ligand response for RXFP2. All experiments were repeated at least three times using cells from independent transfections. The EC₅₀ was calculated employing the non-linear curve fitting functions in GraphPad PRISM version 5.0 software (San Diego, CA, USA).

Immunohistochemistry

For localization of INSL3 in the testis, deparaffinized testicular sections (4 μm) were immunostained with the anti-INSL3 antibody IgG fraction (0.5 μg/ml) or the antibody preabsorbed with purified INSL3 (25 μg/ml) followed by a Vectastain Universal Elite ABC kit as described previously (Siqin et al., 2010a,b). In contrast, for the localization of receptor RXFP2 in HEK293 cells transfected with a RXFP2 cDNA construct, the cells were evaluated by immunofluorescence staining with an anti-FLAG M2 monoclonal IgG followed by an Alexa Fluor 488-conjugated goat anti-mouse IgG. Signals were observed us-

ing a BX50 Olympus microscope equipped with a CCD camera (DP50; Olympus, Tokyo, Japan).

Electron-microscopic immunocytochemistry

Detection of INSL3 by electron-microscopic immunocytochemistry was performed by a protein A-gold method (Kohsaka et al., 1992, 1993a). In brief, ultrathin sections on nickel grids were preincubated in a saturated aqueous solution of sodium metaperiodate for 1 h at room temperature. Subsequently, the sections were incubated with anti-INSL3 antibody IgG fraction (0.5 μg/ml) or the antibody preabsorbed with purified INSL3 (25 μg/ml) for 12 h at 4°C, washed in PBS containing 1% BSA (BSA-PBS), and incubated for 30 min at room temperature in protein A-gold complex, at a 1:30 dilution. Sections were then thoroughly washed in PBS, rinsed in distilled water, and dried. The sections were counterstained for 5 min in 4% uranyl acetate and examined using a JEM-1200EX electron microscopy (JEOL, Tokyo, Japan) at 80 kV.

Statistical analysis

Values are presented as the means±SEM. Results were analyzed by one-way ANOVA, together with Fisher's Protected Least Significant Difference multiple range test to compare the means of different groups. $p < 0.01$ was considered statistically significant.

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