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Relaxin-like factor (RLF)/insulin-like peptide 3 (INSL3) is secreted from testicular Leydig cells as a monomeric protein comprising three domains B–C–A with full biological activity in boars

Itaru MINAGAWA*†, Masafumi FUKUDA‡, Hisako ISHIGE*, Hiroshi KOHRIKI*, Masatoshi SHIBATA§, Enoch Y. PARK¶, Tatsuo KAWARASAKI§[1] and Tetsuya KOHSAKA*†[2]

*Laboratory of Animal Reproduction and Physiology, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan, †Division of Animal Resource Production, The United Graduate School of Agricultural Science, Gifu University, Gifu 501-1193, Japan, ‡Theravalues Research Institute, Theravalues, Osaka 567-0085, Japan, §Shizuoka Swine and Poultry Experimental Station, Kikugawa 439-0037, Japan, and ¶Laboratory of Biotechnology, Graduate School of Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan

RLF (relaxin-like factor), also known as INSL3 (insulin-like peptide 3), is a novel member of the relaxin/insulin gene family that is expressed in testicular Leydig cells. Despite the implicated role of RLF/INSL3 in testis development, its native conformation remains unknown. In the present paper we demonstrate for the first time that boar testicular RLF/INSL3 is isolated as a monomeric structure with full biological activity. Using a series of chromatography steps, the native RLF/INSL3 was highly purified as a single peak in reverse-phase HPLC. MS/MS (tandem MS) analysis of the trypsinized sample provided 66 % sequence coverage and revealed a distinct monomeric structure consisting of the B-, C- and A-domains deduced previously from the RLF/INSL3 cDNA. Moreover, the N-terminal peptide was four amino acid residues longer than predicted previously. MS analysis of the intact molecule and PMF (peptide mass fingerprinting) analysis at 100 % sequence coverage confirmed this structure and indicated the existence of three site-specific disulfide bonds. RLF/INSL3 retained full bioactivity in HEK (human embryonic kidney)-293 cells expressing RXFP2 (relaxin/insulin-like family peptide receptor 2), the receptor for RLF/INSL3. Furthermore, RLF/INSL3 was found to be secreted from Leydig cells into testicular venous blood. Collectively, these results indicate that boar RLF/INSL3 is secreted from testicular Leydig cells as a B–C–A monomeric structure with full biological activity.

Key words: bioactivity, native conformation, relaxin-like factor/insulin-like peptide 3 (RLF/INSL3), tandem MS (MS/MS), testis.

INTRODUCTION

RLF (relaxin-like factor), also known as INSL3 (insulin-like peptide 3), is a novel member of the relaxin/insulin gene family that was originally discovered by screening a boar testicular cDNA library [1]. Since then, its cDNA has been cloned from the testes of a number of mammalian species [2]. RLF/INSL3 has been implicated in the regulation of testicular function: RLF/INSL3-knockout male mice exhibit cryptorchidism during fetal development because of a developmental abnormality in the gubernaculum, which results in abnormal spermatogenesis and infertility [3,4]. Furthermore, RLF/INSL3 suppresses male germ cell apoptosis in the rat testis [5], although its role in adults is still unclear.

In contrast with other peptides from the relaxin/insulin family, very little is known about the native conformation of RLF/INSL3. Nevertheless, based on predictions from the amino acid sequence derived from its cDNA, it has been presumed that RLF/INSL3 is biosynthesized as a pro-protein (pro-RLF/INSL3) containing: (i) a signal peptide that presumably permits the nascent protein access to the endoplasmic reticulum before being excised; and (ii) A- and B-domains connected by a C-domain [2,6]. Furthermore, pro-RLF/INSL3 is assumed to undergo proteolytic processing to remove the C-domain peptide and then to mature to an A–B heterodimer linked by two disulfide bonds to form an active hormone like the other members of this group of peptides [2,6]. On the basis of this prediction, a synthetic peptide that consists of an A–B heterodimer with site-specific sequential disulfide bonds has been produced in some species, including humans [7] and rats [8]. The synthetic peptide stimulates cAMP production through binding to its own receptor RXFP2 (relaxin/insulin-like family peptide receptor 2), originally called LGR8 (leucine-rich repeat-containing G-protein-coupled receptor 8) [9]. This is corroborated by only one previous study which reports that native RLF/INSL3 is isolated as an A–B heterodimer from bovine testis [10].

However, there is no evidence as to whether the native RLF/INSL3 of other species undergoes proteolytic processing to dissociate the C-domain and then mature to an A–B heterodimer. In fact, the RLF/INSL3 of tissue extracts from humans [11], rats [12], horse [13], deer [14] and goats [15], as revealed by Western blot analysis, appears to correspond in size with the pro-RLF/INSL3 deduced from the cDNA sequences. This implies that the native RLF/INSL3 of other species may not be processed to an A–B heterodimer. Clarifying the native conformation of RLF/INSL3 would be extremely meaningful not only for the development of a specific immunoassay system for measuring RLF/INSL3 in blood and body fluids, but also for conducting studies on the true physiological function of this hormone.

In the present study we purified native RLF/INSL3 from boar testes and demonstrated for the first time in any species that it was isolated as a B–C–A monomeric structure with site-specific disulfide bonds and full biological activity.

Abbreviations used: ACN, acetonitrile; DIG, digoxigenin; ECL, enhanced chemiluminescence; HEK, human embryonic kidney; INSL3, insulin-like peptide 3; MALDI, matrix-assisted laser desorption ionization; MS/MS, tandem MS; PC1/3, prohormone convertase 1/3; PMF, peptide mass fingerprinting; RLF, relaxin-like factor; RXFP2, relaxin/insulin-like family peptide receptor 2; TBST-milk, Tris-buffered saline containing Tween 20 with 2 % skimmed milk; TF, transferrin; TFA, trifluoroacetic acid; TR-FIA, time-resolved fluoroimmunoassay.

[1] Present address: Faculty of Agriculture, Tokai University, Kumamoto 869-1404, Japan.
[2] To whom correspondence should be addressed (email t-kohsaka@agr.shizuoka.ac.jp).

EXPERIMENTAL

Chemicals and general methods

HPLC grade ACN (acetonitrile) and TFA (trifluoroacetic acid) were purchased from Wako Pure chemicals and Peptide Institute respectively. Sephadex G-50 (fine) was obtained from GE Healthcare Bio-Sciences. All other chemicals were of guaranteed grade and were purchased from commercial sources. All DNA constructs were verified by DNA sequencing, which was performed by the sequencing service of Macrogen.

Animals and tissue sampling

Testes were collected from mature fertile Duroc boars at the Shizuoka Swine and Poultry Experimental Station and at a local slaughterhouse. The testes were cut into small cubes, rapidly frozen in liquid nitrogen and stored at −80 °C until protein extraction. Some of the samples were either fixed in Bouin's solution and then paraffin-embedded for immunohistochemistry or frozen with Tissue-TeK O.C.T. embedding compound (Sakura Finetechnical) in liquid nitrogen for *in situ* hybridization. Testicular venous blood was also collected from the spermatic cord, whereas peripheral venous blood was taken from the jugular vein. The blood samples were centrifuged at 4 °C for 10 min at 1500 ***g***, and the serum was stored at −80 °C. Animal care and all experimental procedures were performed in accordance with the Health Guidelines for Care and Use of Experimental Animals at Shizuoka University.

Protein concentrations

Protein concentrations were determined using the method of Lowry–Folin [16], with BSA as a standard.

Anti-RLF/INSL3 antiserum

Anti-RLF/INSL3 antiserum (RLF-A-Ab808) used in the present study was generated previously by 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 RLF/INSL3 cDNAs. The specificity has been discussed in detail previously [15,17].

Purification of RLF/INSL3

Extraction

Boar testes (approximately 3400 g) were extracted according to the method of Kohsaka et al. [18], which minimizes proteolysis both by a low pH and by precipitating high molecular mass proteases. The testes were finely ground in a meat grinder while still frozen and homogenized at 4 °C in homogenizing medium [2 % (v/v) TFA, 5 % (v/v) formic acid, 1 % (w/v) sodium chloride and 1 M HCl] with an ULTRA-TURRAX® T25 Basic disperser (IKA). The homogenate was then centrifuged at 28000 ***g*** for 20 min at 4 °C, and the supernatant was successively filtrated through glass microfibre filters (934-AH; Whatman) and 0.45 μm Durapore membrane filters (Millipore) in an ice bath. The filtrate was pumped (Perista pump SJ-1211H; Atto) at 5 ml/min through Sep-Pak plus C_{18} Environmental Cartridges (Waters) that were connected in series in an ice bath. After the cartridges were washed at 4 °C with 10 % (v/v) ACN containing 0.1 % TFA, the absorbed protein was eluted with 80 % (v/v) ACN containing 0.1 % TFA. The eluate was reduced using a rotary evaporator at 40 °C, freeze-dried and stored at −80 °C until gel filtration.

Gel filtration

The freeze-dried extract was dissolved in 20 mM ammonium acetate buffer (pH 5.0) and applied to a Sephadex G-50 column (2.5 cm × 105 cm) equilibrated previously with the same buffer. The flow rate was maintained at 20 ml/h, and each 3-ml fraction collected was measured at an absorbance of 280 nm. The fractions containing RLF/INSL3 were detected by dot-blot analysis with anti-RLF/INSL3 antiserum, pooled and concentrated by freeze-drying.

Cation-exchange FPLC

RLF/INSL3-positive fractions were purified by FPLC (Pharmacia) on a TSKgel SP-5PW cation-exchange column (5 mm × 50 mm; Tosoh) equilibrated with 0.3 M NaCl in 25 mM ammonium acetate buffer (pH 5.0) and eluted over 80 min with a linear gradient of 0.3–0.9 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The eluted peptides were monitored at an absorbance at 280 nm, and 0.5-ml fractions were collected. The fractions containing RLF/INSL3 were detected by dot-blot analysis.

Reverse-phase HPLC

RLF/INSL3-positive fractions were further purified by HPLC (model 4200; Hitachi) on an YMC-Pack ODS-AM column (250 mm × 4.6 mm; YMC) using a linear gradient system. The solvents consisted of 0.1 % TFA (solvent A) and 80 % (v/v) ACN in 0.1 % TFA (solvent B). The column was equilibrated with 33 % solvent B, and the elution was carried out over 26 min with a 33–72 % 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 the fractions were manually collected and checked by dot-blot and Western blot analyses.

Recovery

RLF/INSL3 content recovered from each step of the purification was measured by TR-FIA (time-resolved fluoroimmunoassay) using anti-RLF/INSL3 antiserum and recombinant RLF/INSL3, which was expressed as proform of ~16 kDa in *Escherichia coli* as described previously [15,19], and was revealed as the EC_{50} values for the recombinant RLF/INSL3. The TR-FIA procedure was performed according to the method of Ogine et al. [20].

Dot-blot and Western blot analyses

Samples were spotted directly on to a nitrocellulose membrane (Bio-Rad Laboratories) using Bio-Dot SF (Bio-Rad Laboratories), or separated by SDS/PAGE (14 % gels) under non-reducing conditions and transferred on to nitrocellulose membranes using a semi-dry transfer cell (Bio-Rad Laboratories). The membranes were blocked in TBST-milk [TBS-T (Tris-buffered saline containing Tween 20; 20 mM Tris/HCl, pH 7.4, 140 mM NaCl and 0.1 % Tween 20) with 2 % (w/v) skimmed milk] and then incubated for 90 min with rabbit anti-RLF/INSL3 serum diluted 1:3000 in TBST-milk, followed by incubation for 1 h with peroxidase-conjugated goat anti-rabbit IgG (ICN/Cappel) diluted 1:8000 in TBST-milk at room temperature (25 °C). Specific signals were detected using an ECL (enhanced chemiluminescence) system (Amersham Biosciences). As a loading control for Western blot analysis of serum samples, TF (transferrin) was detected by sequential incubation with a polyclonal antibody against TF (Sigma), peroxidase-conjugated

goat anti-rabbit IgG (ICN/Cappel) and ECL (Amersham Biosciences). The absorbance was measured by densitometric scanning using the ImageJ software (http://rsb.info.nih.gov/ij/).

Protein digestion and nano-LC separation

RLF/INSL3 purified by a series of chromatography steps was prepared to a concentration of approximately 2 μM in 20 mM ammonium acetate (pH 6.0). Trypsin (1 μl at 100 ng/μl) (Trypsin Gold, MS grade; Promega) in 1 mM HCl was added to 20 μl of the RLF/INS3 solution, and the sample was incubated at 37 °C for 6 h. The digestion was terminated by the addition of 1 μl of 50 % (v/v) acetic acid. The digest solution (4 μl) was submitted to a Prominence nano-LC System (Shimadzu) coupled to an AccuSpot LC spotting system (Shimadzu). The mobile phases were solvent A [0.1 % TFA in water/ACN (95:5, v/v)] and B [0.1 % TFA in water/ACN (10:90, v/v)]. Peptide digests were adsorbed and desalted on the precolumn (Monolith, 0.2 mm × 100 mm; Monotech) with 0.1 % TFA in water at a 35 μl/min flow rate (0–5min). Peptide digests were then separated using an analytical column (Monolith, 0.1 mm × 250 mm; Monotech) at a 1 μl/min flow rate with a gradient in which the amount of solvent B was changed as follows: 5–8 min, 10 %; 22 min, 40 %; 23 min, 95 %; 43 min, 95 %; 44 min, 10 %; and 59 min, 10 %. The eluate was recorded at 210 nm, mixed with the MALDI (matrix-assisted laser desorption ionization) matrix solution [5 mg/ml CHCA (α-cyano-4-hydroxycinnamic acid) in 60 % (v/v) ACN containing 0.1 % TFA], and then directly spotted on to a 192-well MALDI plate (Applied Biosystems).

MALDI-MS and MS/MS (tandem MS) analysis

MS and MS/MS analysis were performed using a 4700 Proteomics Analyzer (Applied Biosystems). MS/MS analysis of an offline spotted peptide digest sample was performed automatically with a Plate Model external calibration. All MS/MS spectra were combined, processed and database-searched with ProteinPilot™ Software (version 2.0.1; Applied Biosystems). For PMF (peptide mass fingerprinting) analysis, a digested sample that had not undergone the column purification process was mixed with the matrix solution at a ratio of 1:3 (v/v) and analysed with external calibration. For MS analysis of intact RLF/INSL3, a HPLC-purified sample solution [~10 μM in 10 % (v/v) ACN containing 0.1 % (v/v) TFA] was mixed with matrix solution at a ratio of 1:1 (v/v) and analysed in linear mode with internal calibration.

RLF/INSL3 bioassay based on the cAMP production in receptor RXFP2-expressing HEK (human embryonic kidney)-293 cells

The full-length cDNA encoding receptor RXFP2 (GenBank® accession number NM_080468) was generated by an RT (reverse transcription)–PCR-based cloning method. Briefly, total RNA was extracted from the mouse uterus using ISOGEN (Nippon Gene) and reverse-transcribed by reverse transcriptase (Promega). The PCR product (2225 bp) was amplified using the mRxfp2-F primer 5′-AGCTGAATTCGCCGCAATGTGGCTCCTACTTC-3′ and the mRxfp2-R primer 5′-AGCTGAATTCCTACGGGG-AGACCGGCTTCAT (EcoRI sites underlined) with Vent DNA polymerase (New England BioLabs) and cloned into a pT7-Blue vector (TaKaRa Bio). Amplification was performed using a Dice PCR Thermal Cycler (TaKaRa Bio) with the following programme: initial denaturation for 2 min at 94 °C, followed by 35 cycles of denaturation for 10 s at 98 °C and annealing and extension for 2 min at 68 °C. The FLAG-tag sequence at the N-terminus of RXFP2 was inserted by invert PCR. The RXFP2 cDNA bearing the FLAG sequence was subcloned with EcoRI into a pdE vector derived from pdEGFP (BD Biosciences Clontech) to generate the expression construct (2250 bp).

HEK-293 cells were maintained in DMEM (Dulbecco's modified Eagle's medium; Sigma) supplemented with 10 % (v/v) FBS (fetal bovine serium; Biofill Australian Pty), 1 % (v/v) penicillin/streptomycin (Sigma) and 2 mM L-glutamine under a humidified environment containing 5 % CO_2 at 37 °C. When the cells that were seeded in 96-well plates (Techno Plastic Product) were 90 % confluent (2×10^5), transient transfection of the expression construct or empty vector was performed by Lipofectamine™ 2000 (Invitrogen). After 24 h, cells were incubated for 1 h in the presence of 0.05 mM IBMX (isobutylmethylxanthine; Sigma) with or without isolated RLF/INSL3 or synthetic human RLF/INSL3 peptide (R&D Systems). Intracellular cAMP was detected with the cAMP Biotrak enzyme immunoassay kit (GE Healthcare) and expressed as a percentage of the maximum ligand response for receptor RXFP2. All experiments were repeated at least three times using cells from independent transfections.

In situ hybridization

Extraction of total RNA and first-strand cDNA synthesis for boar testis were performed as described above. The partial sequence (324 bp) of boar RLF/INSL3 cDNA (GenBank® accession number X68369) was PCR-amplified using forward (5′-CAGGAGGCGCCAGAGAAGCTGT-3′) and reverse (5′-GGGACAGAGGGTCAGCAAGTCTTG-3′) primers with Taq DNA polymerase (New England BioLabs). cRNA probes for *in situ* hybridization were prepared according to the method of Kohsaka et al. [21]. Briefly, the RLF/INSL3 construct in pGEM-T Easy (Promega) was digested with NocI and SpeI, and then the product was used as a template for SP6 and T7 RNA polymerase (Promega) to generate antisense and sense cRNA probes, which were labelled with DIG (digoxigenin)-11-UTP (Roche Diagnostics). The specificity of the DIG-labelled cRNA probes was verified by Northern blot analysis, in which the anti-sense probe hybridized to a 0.9-kb RLF/INSL3 mRNA, whereas the sense probe yielded no signal. Testicular cryosections (6 μm) were cut, and *in situ* hybridization was performed as described previously [21].

Immunohistochemistry

For the localization of RLF/INSL3 in the testis, deparaffinized testicular sections (4 μm) were immunostained with anti-RLF/INSL3 antiserum followed by a goat anti-rabbit IgG polymer conjugated to peroxidase (DakoCytomation), as described previously [19]. In contrast, for the localization of receptor RXFP2 in HEK-293 cells transfected with a RXFP2 cDNA construct, the cells were immunostained with anti-FLAG M2 monoclonal IgG (Sigma) followed by an Alexa Fluor® 488-conjugated goat anti-mouse IgG (Invitrogen). Signals were examined under a BX50 Olympus microscope equipped with a CCD (charge-coupled device) camera (DP50; Olympus).

Statistical analysis

Values are presented as the means ± S.E.M. Results were analysed 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.

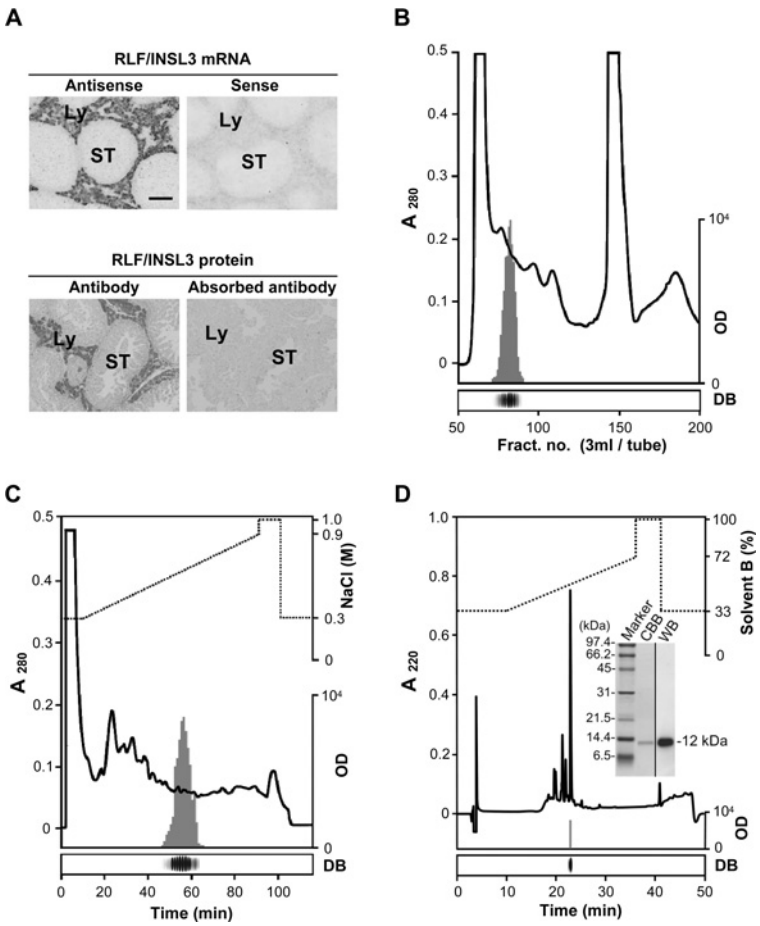


Figure 1 Expression and purification of boar testicular RLF/INSL3

(**A**) Verification of RLF/INSL3 expression. *In situ* hybridization and immunohistochemistry revealed that RLF/INSL3 mRNA and protein were localized in the Leydig cells. Scale bar = 100 μm. Ly, Leydig cells; ST, seminiferous tubule. (**B**–**D**) Purification of testicular RLF/INSL3. (**B**) Gel filtration of boar testicular extract on a Sephadex G-50 column (2.5 cm × 105 cm) in 20 mM ammonium acetate buffer (pH 5.0). The fractions containing RLF/INSL3 immunoactivity (dark area) were revealed by dot-blot analysis. (**C**) Cation-exchange FPLC of the G-50 fraction on a TSKgel SP-5PW column (5 mm × 50 mm) eluted for 80 min with a linear gradient of 0.3–0.9 M NaCl in 25 mM ammonium acetate buffer (pH 5.0) at a flow rate of 0.5 ml/min. RLF/INSL3 immunoactivity, as revealed by dot-blot analysis, was found exclusively in the dark area of the effluent curve. (**D**) Reverse-phase HPLC of the active fraction from cation-exchange FPLC on an YMC-Pack ODS-AM column (250 mm × 4.6 mm) by a 33–72 % linear gradient of solvent B at a flow rate of 1 ml/min. RLF/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 indicates the densitometry of the dot-blot analysis.

RESULTS

Expression and purification of boar testicular RLF/INSL3

RLF/INSL3 mRNA and protein were visualized in Leydig cells in the boar testis using DIG-labelled cRNA probes complementary to boar RLF/INSL3 mRNA and anti-RLF/INSL3 antiserum respectively (Figure 1A). By using an extraction procedure that minimized proteolysis, followed by gel filtration (Figure 1B), cation-exchange FPLC (Figure 1C) and reverse-phase HPLC (Figure 1D), we isolated RLF/INSL3 from boar testes as a single peak. The RLF/INSL3 eluted at each purification step was detected by dot-blot and/or Western blot analysis. The molecular size of the isolated RLF/INSL3 was ~12 kDa, as observed by Western blotting (Figure 1D). The recoveries in the purification steps are summarized in Table 1.

Structural determination of isolated boar RLF/INSL3

The isolated RLF/INSL3 was digested at pH 6 to minimize disulfide bond scrambling, which typically occurs during enzymatic digestion if the pH is too high (pH $\geqslant$8). The resultant peptide mixtures were then directly analysed by LC-MALDI-MS/MS. When the MS/MS data were subjected to ProteinPilot Software, boar pro-RLF/INSL3 sequence was distinctly assigned as the top hit with 66 % sequence coverage, including matches to the C-terminal peptide (Figure 2A). The assigned peptides corresponded to the B-, C- and A-domains of the boar pre-pro-RLF/INSL3 deduced from the cDNA sequence [1]. The MS/MS spectrum of the M-H$^+$ precursor ion at a m/z value of 1264.65 was identified as the N-terminal peptide of the B-domain, which was four residues longer at the N-terminus than the B domain deduced from the cDNA sequence (Figures 2A and 2B). This amino acid extension (Arg-Ala-Pro-Ala) was assigned previously to be a part of the signal peptide deduced from the cDNA sequence. Because nano-LC-MS/MS analysis could have failed to detect additional N-terminal amino acids, MALDI-MS analysis of the intact isolated RLF/INSL3 was performed in linear mode. Only one prominent peak was detected with external calibration (results not shown). The observed mass of the isolated RLF/INSL3 with internal calibration was 12031.25 (M-H$^+$, average mass) (Figure 2C). This was quite close to the theoretical mass of 12031.80 (M-H$^+$, average mass: $C_{521}H_{812}N_{171}O_{144}S_8$), which was calculated from the deduced sequence from cDNA without the signal peptide (residues

Table 1 Summary of the purification of testicular RLF/INSL3

The preparation was from 300 g of frozen tissue as a starting material. The immunoactivity of RLF/INSL3 at each step of the purification was measured by TR-FIA and was expressed as the EC_{50} values (ng/ml) for the recombinant RLF/INSL3. Similar results were obtained with two independent experiments.

Purification step	Total protein (μg)	Immunoactivity (ng/ml)	Yield (μg)	Recovery (%)	Purification (fold)
Recombinant RLF/INSL3	–	22.2	–	–	–
Sep-Pak C_{18} extract	284 500	34 407	184	100	1
Gel filtration	27 300	8707	70	38	4
Cation-exchange FPLC	199	170	26	14	202
Reverse-phase HPLC	11	22.2	11	6	1546

1–20), but with the three disulfide bonds. PMF analysis was then performed to improve the sequence coverage and provide information on the site-specific disulfide bonds. The mass peaks in the PMF spectrum corresponded to 100 % sequence coverage for the deduced sequence from cDNA without the signal peptide and indicated the existence of the three predicted disulfide bonds, namely, one inter-peptide disulfide bond on T5–T12 and one inter-peptide and one intra-peptide disulfide bond on T3-T11 (Figures 2A and 2D). The RMS (root mean square) mass error of the monoisotopic peaks was 21 p.p.m. Other peaks that represent disulfide bond scrambling were not observed in the spectra (Figure 3). The T3–T5 (*m/z* 1567.78), T5–T11 (*m/z* 1578.68), T3–T12 (*m/z* 2005.00) and T11–T12 (*m/z* 2015.90) pairings indicated scrambling, but were observed only in the PMF spectra acquired from the sample trypsinized in the basic buffer condition (20 mM ammonium bicarbonate, pH 8.0, for 2 or 6 h) (Figure 3). Therefore the three assigned disulfide bond pairings, which were acquired from the sample digested at pH 6 for 6 h, are considered to be the original linkage pattern. These observations from the PMF spectrum at 100 % sequence coverage were consistent with the obtained molecular mass of the isolated RLF/INSL3 without any other post-translational modifications. We thus conclude that the primary structure of boar testicular RLF/INSL3 is a monomeric structure consisting of the 26-residue A-, 36-residue B- and 49-residue C-domains, in which the C-domain does not undergo proteolytic dissociation, and the A- and B-domains are site-specifically linked by three disulfide bonds (Figure 2E).

Biological activity of isolated RLF/INSL3

HEK-293 cells were transfected with a RLF/INSL3 receptor RXFP2 cDNA construct (RXFP2-expressing HEK-293 cells) to examine the biological activity of the isolated RLF/INSL3. The cell-surface expression of RXFP2 was observed immunohistochemically with a FLAG-tag antibody (Figure 4A). Treatment of the RXFP2-expressing HEK-293 cells with the isolated boar RLF/INSL3 resulted in a dose-dependent increase in intracellular cAMP production, with an EC_{50} value of approximately 5 nM (Figure 4B). This was comparable with the EC_{50} values of the synthetic A–B heterodimeric human RLF/INSL3 peptide (Figure 4B). In contrast, the isolated RLF/INSL3 and synthetic RLF/INSL3 (results not shown) had no effect on cells transfected with an empty vector (Figure 4B). These results indicated that full bioactivity was present in the isolated RLF/INSL3.

Secretion of RLF/INSL3 from Leydig cells into blood

Western blot analysis of testicular venous serum and peripheral serum clearly demonstrated the 12 kDa immunoreactive RLF/INSL3 protein corresponding with the molecular mass of the RLF/INSL3 isolated from the testis (Figure 5A). Furthermore, the relative level of RLF/INSL3 detected in testicular venous serum was significantly 9-fold higher ($P < 0.01$) than that in peripheral serum (Figure 5B). These results indicate that RLF/INSL3 is secreted into the circulation by Leydig cells.

DISCUSSION

The present study demonstrates for the first time in any species that RLF/INSL3 is secreted from testicular Leydig cells as a monomeric protein with biological activity.

On the basis of the predictions from the cDNA sequences, the structure of RLF/INSL3 has been assumed to be an A–B heterodimer [2,6]. However, there is only one report concerning a bovine model of a native RLF/INSL3 that was purified from testes and characterized as an A–B heterodimer either by protein sequencing or by amino acid composition [10]. In the present study, after purifying the protein using a series of chromatography steps and performing MS and MS/MS analysis, we demonstrated that the native RLF/INSL3 isolated from boar testes is a 12 kDa monomeric structure comprising three domains B–C–A, in which the C-domain does not undergo proteolytic processing. This finding is consistent with the molecular size of RLF/INSL3 in other species corresponding with pro-RLF/INSL3, as deduced from their cDNA sequences [11–15]. However, it is not clear why boar RLF/INSL3 is present as a B–C–A monomeric form, unlike an A–B heterodimeric form of bovine RLF/INSL3. One explanation is that there are differences in the post-translational regulatory events between these species. Insulin and relaxin, which belong to the same family as RLF/INSL3, are known to undergo processing of prohormone by cleavage of the B-chain/C-peptide junction by PC1/3 (prohormone convertase 1/3), which is one of the processing enzymes [22,23]. Recently, we found that no PC1/3 transcripts were expressed in boar testes at any of the developmental stages [19]. Therefore it is reasonable to suggest that the A–B heterodimeric RLF/INSL3 is not produced in the boar testis due to a lack of the enzyme required for processing the pro-RLF/INSL3, unlike the situation in bovines.

In addition to the B–C–A monomeric structure, we revealed that the B-domain of the isolated boar RLF/INSL3 had an additional four residues at the N-terminus compared with that deduced from the cDNA sequence [1]. A similar finding was also seen in bovine RLF/INSL3, but only 20 % of the B-chains had a five-residue extension at the N-terminus [10]. Signal peptides control the entry of virtually all proteins into the secretory pathway and are cleaved off during the transport of the nascent proteins into the endoplasmic reticulum. The common structural features of signal peptides are 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 the cleavage site) [24,25]. On the basis of this $-3, -1$ rule, it was thought that the RLF/INSL3 signal peptide deduced

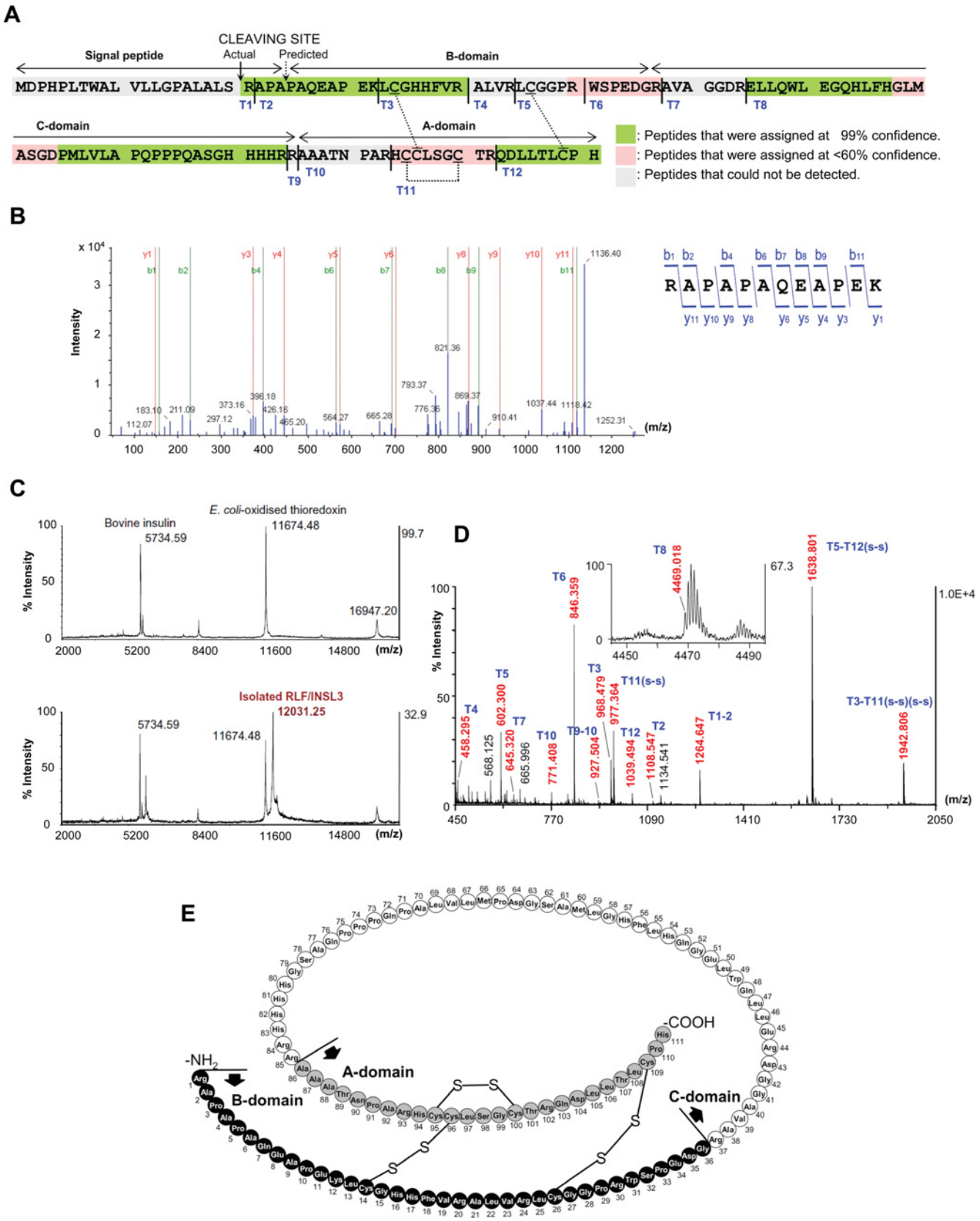
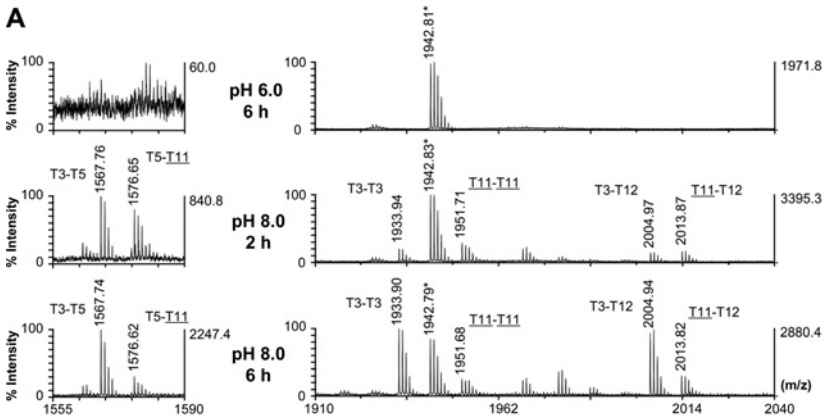


Figure 2 MS and MS/MS analysis for the structural determination of isolated boar RLF/INSL3

(**A**) Amino acid sequence of boar pre-pro-RLF/INSL3 deduced from the cDNA sequence and the peptides assigned by MALDI-MS/MS analysis of the isolated boar RLF/INSL3. Assigned peptides were recognized with a high score of 66 % sequence coverage of the A-, B- and C-domains deduced from the cDNA [1]. (**B**) MALDI-MS/MS spectra of the *M*-H$^+$ precursor ion at a *m/z* value of 1264.65. Sequential product ions in both the *b*- and *y*-series were successfully observed. The assigned peptide was the N-terminal peptide of the B-domain, which was four residues longer at the



B

Condition	Predicted		Unpredicted				Dimers				total
	T5-T12 1638.81	T3-T11 1942.84	T3-T5 1567.77	T5-T11 1576.66	T3-T12 2004.99	T11-T12 2013.88	T5-T5 1201.59	T3-T3 1933.95	T11-T11 1951.73	T12-T12 2076.03	
pH 6.0, 6 h	84.6%	15.4%	–	–	–	–	–	–	–	–	100%
pH 8.0, 2 h	86.8%	6.0%	1.4%	1.1%	0.9%	1.0%	–	1.1%	1.7%	–	100%
pH 8.0, 6 h	66.0%	6.4%	5.9%	1.7%	7.0%	2.3%	1.2%	7.5%	2.0%	–	100%

Figure 3 Scrutiny of disulfide bond scrambling in the digestion of isolated RLF/INSL3 under basic buffer conditions

(**A**) PMF spectra of the samples trypsinized in 20 mM ammonium acetate (pH 6.0) (top panel) and 20 mM ammonium bicarbonate (pH 8.0) (middle and bottom panels). The underlined peptide (T11) represents an intra-peptide disulfide bond. Predicted mass values are indicated with asterisks. (**B**) Peak intensities of the observed disulfide-bond-containing peptides in PMF spectra. Percentages were calculated using each peak intensity (peak height). Mass values in the table are calculated *m/z* (M-H$^+$).

from the cDNA would be cleaved between alanine and proline residues (in bold) within the sequence AP**AP**AQE [1]. However, the four-residue extension of the B-domain was produced by cleavage between serine and arginine residues (in bold) within AL**SR**APAPAQE. The -3 and -1 residues in ALSRAPAPAQE are small and neutral, which matches the criteria for cleavage [24,25]. Thus it is clear that the signal peptidase recognized this part as a cleavage site.

It is also important to demonstrate whether site-specific disulfide bonds reside in the isolated RLF/INSL3. The high pH conditions (pH $\geqslant$8) most frequently used in enzymatic digestion can promote disulfide scrambling [26], and the positively charged amino acids located close by or adjacent to the cysteine residues can greatly enhance disulfide exchange reactions in random-coil proteins up to 1×10^6-fold in rate constant [27]. In fact, the enzymatic digestion of certain peptides containing such cysteine residues in basic buffer conditions instantly causes disulfide bond scrambling before the peptide begins to be digested, thereby requiring enzymatic digestion under neutral or acidic buffer conditions for the determination of disulfide pairings [28]. In the present study, when the primary structure analysis was performed under digestion conditions at pH 6.0 using ammonium acetate, the PMF analysis resulted in the exclusive detection of two predicted disulfide-linked peptides, namely, T3–T11 and T5–T12. Meanwhile PMF analysis of the sample trypsinized at pH 8.0 using 20 mM ammonium bicarbonate recognized several disulfide bond scrambling. However, these unpredicted peptides and dimers were not observed under digestion conditions at pH 6.0, but only under those at pH 8.0, with the peak intensity incrementally increasing in a time-dependent manner (See Figure 3). It is therefore reasonable to conclude that the two predicted peptides without the peak intensity increments are the original disulfide-linked peptides. As in boars, bovine RLF/INSL3 was shown by HPLC analysis to have two prominent peaks corresponding with disulfide-linked peptides confirmed using synthetic peptides, as reported by Büllesbach and Schwabe [10], who carried out enzymatic digestion under basic buffer conditions using 50 mM ammonium bicarbonate for 30 min. Taken together with the present finding that the original disulfide linkages of the isolated boar RLF/INSL3 were still retained predominantly at least up to 2 h after digestion at pH 8.0, the two disulfide-linked peptides obtained from the shorter digestion time in the bovine RLF/INSL3 [10] appear to reveal the original disulfide-linked peptides. Moreover, the disulfide linkage pattern of T3–T11 (one inter- and one intra-peptide disulfide bond) could be considered identical with that determined experimentally in bovine RLF/INSL3 [10] because of the structural similarity of the relaxin/insulin family peptides.

RLF/INSL3 mediates its effect by stimulating cAMP production through the cell-surface receptor RXFP2 [9]. Using a reliable bioassay system based on cAMP production in

N-terminus than the B-domain deduced from the cDNA sequence. (**C**) Linear mode MALDI-MS spectra of standard proteins (upper panel) and isolated RLF/INSL3 with internal calibration (lower panel). The calibrants were bovine insulin (M-H$^+$: $C_{254}H_{378}N_{65}O_{75}S_6$) and *E. coli*-oxidized thioredoxin (M-H$^+$: $C_{528}H_{837}N_{132}O_{159}S_3$). (**D**) PMF of isolated RLF/INSL3. The acquired sequence coverage was 100% for the deduced sequence from cDNA without the signal peptide (residues 1–20). The two peptides peaks with disulfide bonds corresponded with the predicted information, namely, T5–T12 (one inter-peptide) and T3–T11 (one inter- and one intra-peptide) (see **A**). T3, T5, T12 and T11 were attributed to the peptides containing sulfhydryl groups resulting from disulfide bond cleavage by in-source fragmentation during the MALDI process [34]. (**E**) Primary structure of boar RLF/INSL3 determined by MS/MS and MS analyses.

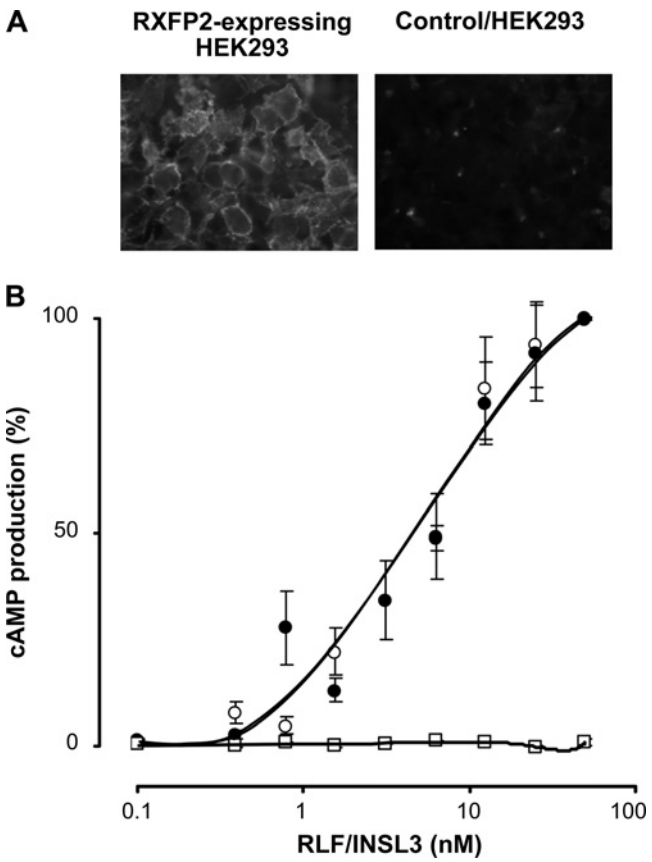


Figure 4 Biological activity of isolated RLF/INSL3

(**A**) The RLF/INSL3 receptor RXFP2 was transfected into HEK-293 cells for detecting the biological activity of RLF/INSL3. RXFP2 is expressed on the cell surface in RXFP2-expressing HEK-293 cells, but is not detected in the cells transfected with an empty vector (Control/HEK293). (**B**) Stimulation of cAMP production by RLF/INSL3 in RXFP2-expressing HEK-293 cells. Isolated RLF/INSL3 (●) stimulated dose-dependent cAMP production with EC_{50} values comparable with those of the synthetic A–B heterodimeric human RLF/INSL3 (○), indicating the retention of full bioactivity. In contrast, isolated RLF/INSL3 did not stimulate cAMP production in the control/HEK293 (□). cAMP production was expressed as a percentage of the maximum isolated RLF/INSL3 or synthetic human RLF/INSL3 peptide response for RXFP2. Results are means ± S.E.M. for three independent experiments.

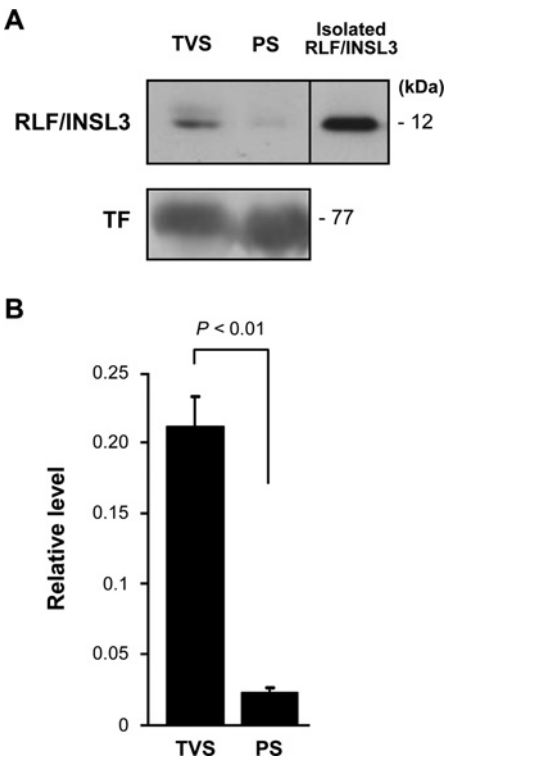


Figure 5 Secretion of RLF/INSL3 from Leydig cells into blood as revealed by Western blot analysis

(**A**) A representative Western blot analysis of testicular venous serum (TVS) and peripheral serum (PS). For each lane, 12.5 μl of the serum was applied. (**B**) The relative level of each message normalized to that of TF. The relative level of the RLF/INSL3 content was 9-fold higher ($P < 0.01$) in testicular venous serum than that in peripheral serum. Results are means ± S.E.M. for four animals.

RXFP2-expressing HEK-293 cells, we were able to show that the isolated RLF/INSL3 stimulated dose-dependent cAMP production with an EC_{50} value of approximately 5 nM. This was quite similar to the EC_{50} value of the synthetic A–B heterodimeric human RLF/INSL3 peptide, revealing that bioactivity fully resides in the isolated RLF/INSL3. Furthermore, Western blot analysis of testicular venous serum and peripheral serum demonstrated that RLF/INSL3 secreted was present as the 12 kDa of molecular nature in serum, revealing that RLF/INSL3 is not processed in the circulation after secretion. Therefore RLF/INSL3 differs from insulin, in which the prohormone undergoes proteolytic processing to form the active hormone, but is quite similar to IGFs (insulin-like growth factors), in which the proforms are not processed and have full bioactivity [29]. In addition, boar RLF/INSL3 differs from porcine relaxin in the point that is not processed into two-chain peptides, although a recombinant primate pro-relaxin has full biological activity [30].

The present study revealed that monomeric RLF/INSL3 produced by Leydig cells is secreted into the circulation by showing the evidence that RLF/INSL3 levels in the testicular vein were much higher than those in the peripheral blood. This finding is in good agreement with that of human RLF/INSL3, as shown by Ferlin and Foresta [31], who revealed that RLF/INSL3 is an endocrine factor. In contrast, receptor RXFP2 mRNA and/or protein have been shown to be expressed in Leydig cells and seminiferous tubule epithelial cells in adult testes of some species, including humans [5,17,32,33]. There is also evidence that RLF/INSL3 suppresses germ cell apoptosis in the rat testis [5], whereas the treatment of mouse Leydig TM3 cells with RLF/INSL3 *in vitro* induces cell proliferation [33]. Collectively, these findings suggest that the RLF/INSL3 produced by Leydig cells functions as an autocrine and/or paracrine factor in Leydig cells and seminiferous tubule epithelial cells. Unfortunately, it remains presently unknown what effects RLF/INSL3 actually exerts in boars. However, the monomeric RLF/INSL3 seems to be physiologically relevant since boar RLF/INSL3 is indeed secreted from testicular Leydig cells as a monomeric structure with biological activity. In the present study, therefore, the clarification of the native conformation of RLF/INSL3 will lead a development of a specific immunoassay system measuring the monomeric RLF/INSL3 in blood and body fluids, whereby the yet unidentified paracrine, endocrine and/or autocrine aspects of RLF/INSL can be explored and provide insight into the potential role of RLF/INSL3 in boar testicular function.

In conclusion, we have isolated native RLF/INSL3 from boar testes and demonstrated for the first time that the native RLF/INSL3 is a B–C–A monomeric structure with full biological activity.

AUTHOR CONTRIBUTION

Itaru Minagawa performed the expression analysis and the purification of boar testicular RLF/INSL3. Masafumi Fukuda performed the MS, MS/MS and PMF analyses for the structural determination of RLF/INSL3, and analysed the data. Hisako Ishige and Hiroshi Kohriki performed the cell-based bioassay. Tatsuo Kawarasaki and Masatoshi Shibata participated in the experimental design and contributed to the sampling. Enoch Park participated in project oversight and data interpretation. Tetsuya Kohsaka conceived the project, analysed the results and wrote the paper with the assistance of Masafumi Fukuda.

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