Relaxin-like factor (RLF) serum concentrations and gubernaculum RLF receptor display in relation to pre- and neonatal development of rats

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Deletion of the relaxin-like factor (RLF) gene in mice causes retention of testicles and infertility. The development of a synthetic RLF has made it possible to investigate the events that connect the genomic event and the basic biological responses that cause gonadal positioning. Anti-RLF antibodies were raised against synthetic RLF, allowing determination of RLF concentrations during the critical period, testing for RLF receptors on the gubernaculum and exploration of the temporal relationship between receptor display and migration of the testes in developing rats. In male rat pups, serum RLF concentrations were high at day 2 before parturition (2.4 ng ml⁻¹) and decreased sharply just before parturition. Thereafter, males and females had the same low serum concentrations until RLF concentrations began to increase in males only, starting at day 10 after parturition and continuing until adult RLF concentrations (0.6 ng ml⁻¹) were reached on day 39 after parturi-

tion. The testicles are descending into the scrotum during this phase of increasing RLF concentrations and are descended fully by day 19–21 after parturition, before adult hormone concentrations are established. The high prenatal serum RLF concentration coincides with high expression of RLF receptors in the gubernaculum tissue. Competitive binding of RLF per mg of membrane protein prepared from rat gubernacula at various developmental stages showed no increase in receptor density as sexual maturity was reached. Gubernaculum cells in primary culture showed an increased uptake of 5-bromo-2'-deoxyuridine in the presence of RLF compared with controls. These studies demonstrate that the synthetic RLF is biologically active and indicate that the cryptorchid phenotype *INSL3*^{-/-} is a direct consequence of defective gubernaculum growth, caused by the absence of RLF during early phases of development.

Introduction

The failure of testes to descend into the scrotum is one of the most common disorders of sexual differentiation in humans. This condition, known as cryptorchidism, occurs in 3.5% of newborn boys, but decreases to 0.5% at 1 year of age (Scorer, 1964; John Radcliffe Hospital Cryptorchidism Study Group, 1986; Toppari and Kaleva, 1999). Retention of the testes in the abdomen results in infertility and an increased risk of testicular cancer. Surgical intervention prevents infertility but the risk of testicular cancer appears to persist (Pike *et al.*, 1986; Strader *et al.*, 1988; Whitaker, 1988; Møller *et al.*, 1996). In men, the relaxin-like factor (RLF) serum concentrations are higher than in children and post-pubertal females (Büllesbach *et al.*, 1999). This finding is in accordance with the relative amounts of RLF mRNA observed in gonadal tissue in mice (Zimmermann *et al.*, 1997).

Adham *et al.* (1993) discovered an mRNA in pig Leydig cells that encodes an insulin-like structure, Leydig insulin-like peptide (Ley I-L), and the coding gene was denoted *INSL3* (Burkhardt *et al.*, 1994). After chemical synthesis of this peptide in our laboratory it became apparent that the structure was similar to that of relaxin and that 'relaxin-like factor' (RLF) would be a more fitting name for the protein encoded by this gene (Büllesbach and Schwabe, 1995).

There are strong indications that RLF plays a major role in testicular development, transport and maintenance. *RLF*^{-/-} male mice develop cryptorchidism and are sterile (Nef and Parada, 1999; Zimmermann *et al.*, 1999). The mechanism of action of RLF in females is not known.

Variable levels of RLF mRNA have been detected in ovaries and expression of RLF is cycle-dependent (Bathgate *et al.*, 1996; Roche *et al.*, 1996; Zimmermann *et al.*, 1997). The sites of expression of RLF, which are the corpora lutea (Tashima *et al.*, 1995; Bathgate *et al.*, 1996; Roche *et al.*, 1996) and theca cells (Bathgate *et al.*, 1996; Roche *et al.*, 1996), as well as the location of RLF receptors (Büllesbach and Schwabe, 1995, 1999a), imply a potential physiological

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role for RLF in reproduction and also a functional relatedness to relaxin.

The RLF molecule, synthesized chemically according to the mRNA (Pusch *et al.*, 1996; Zimmermann *et al.*, 1997), allowed investigation of the physiological events and the molecular interactions required for testicular descent in laboratory rats. The use of anti-RLF antibodies raised against synthetic RLF to determine endogenous RLF concentrations in rat sera and the use of ^{125}I -labelled mouse RLF to search for gubernaculum RLF receptors is reported. The relationship between gubernaculum growth, circulating RLF concentrations and migration of testes during fetal and neonatal development of rats is also reported.

Materials and Methods

Animals

All procedures were performed under an institutionally approved protocol in accordance with the NIH Guide for Care and Use of Laboratory Animals. Holtzman rats at known stages of pregnancy (Harlan Sprague Dawley Inc., Frederick, MD) were used. Rats obtained at day 15 of pregnancy were housed individually in standard 27 cm $\times$ 48 cm clear plastic cages at 22°C under a 12 h light:12 h dark cycle with water and rat chow available *ad libitum*. The day of parturition was designated day 0 and the litter and mother were kept together until the young were weaned at day 21; thereafter, males and females were separated and housed in groups ($n = 4$ per group). Animals were killed by CO_2 anoxia, decapitated, and trunk blood and gubernaculum tissues were collected for radioimmunoassay and receptor-binding experiments, respectively.

Hormones and tracers

Human RLF (Büllesbach and Schwabe, 1995), human relaxin (Büllesbach and Schwabe, 1991) and rat relaxin (Büllesbach and Schwabe, 1996) were synthesized chemically. For control synthetic mouse RLF B-chain, synthetic mouse RLF A-chain containing the intrachain disulphide bond, natural bovine insulin (Sigma, St Louis, MO) and recombinant human insulin-like growth factor I (IGF-I; Sigma) were used. The method for human RLF synthesis (Büllesbach and Schwabe, 1995) was used to produce mouse RLF, 3-(4-hydroxyphenyl)propionyl(A1) (N(in)formyl) mouse RLF (for tracer preparations), an inactive desB(27-31) human RLF and rat RLF. In general, these RLF molecules are produced by solid-phase synthesis of A- and B-chains, followed by site-specific sequential disulphide bond formation. Selectivity is achieved by protecting sulphhydryl groups with reagents that can be removed under different conditions. The synthesis of a tracer required a slight deviation as described below.

The 3-(4-hydroxyphenyl)propionyl(A1) (N(in)formyl) mouse RLF analogue (10 μg) was dissolved in 10 μl of 250 mmol phosphate buffer l^{-1} at pH 7.5 and chilled on ice.

Na^{125}I (1.8 μl , 0.9 mCi) was added, followed by 5 μl chloramine T (2 mg ml^{-1} in 250 mmol phosphate buffer l^{-1} at pH 7.5). The reaction was performed for 1 min on ice and the excess of oxidant was reduced with sodium thiosulphate pentahydrate (50 mg ml^{-1} in 250 mmol phosphate buffer l^{-1} at pH 7.5). The N(in)formyl group was removed by exposure to piperidine (5 μl) for 2 min; the reaction was acidified with 10 μl glacial acetic acid, diluted with 20 μl of water and separated by HPLC on an Aquapore 300 (C_8 , 2.1 mm $\times$ 30 mm) column. The solvent system was 0.1% (v/v) trifluoroacetic acid in water (solvent A) and acetonitrile–water–trifluoroacetic acid 80:20:0.1 (v/v/v) (solvent B). Separation was achieved by a 60 min linear gradient from 23% to 34% solvent B at a flow rate of 100 $\mu\text{l min}^{-1}$. Proteins were detected by UV absorbance at 230 nm, collected into Eppendorf vials containing 100 μl of 1% (w/v) aqueous BSA solution, and a 2 μl aliquot was assessed in a gamma counter. The calculated molar activity of this preparation is 2.1 Ci μmol^{-1} . Trichloroacetic acid precipitation (13% (w/v) final concentration) of tracer in 1% (w/v) BSA followed by centrifugation at 12 000 g for 10 min revealed that < 3% of the radioactivity was found in the supernatant. The mouse RLF tracer was used for the original critical experiments to determine the relative utility of a homologous radioimmunoassay for our studies. It would also be of use for mouse RLF tissue distribution work. In our system it provided no advantage over a previously synthesized human RLF tracer so that routine measurements were done with the easily accessible human RLF tracer labelled in the A9 position (Büllesbach and Schwabe, 1999a).

Polyclonal antibodies

Antibodies were produced in the Antibody Facility, Medical University of South Carolina. Two rabbits were each injected s.c. with 50 μg mouse RLF at monthly intervals. The antibody titre in the serum was determined by radioimmunoassay using goat anti-rabbit IgG conjugated to cellulose to precipitate the tracer–antibody complex.

Serum RLF concentrations in pre-and neonatal rats

Trunk blood was collected from male rats at selected stages of development, stored on ice and assayed within 24 h of collection. Each experiment required 250 μl rat serum, which was obtained by combining sera from two appropriately age-matched litters of pre- and postnatal male rats up to 4 days of age, one litter of males aged between 10 and 23 days and one male rat aged > 23 days. Each assay was repeated two more times starting with the same number of animals. Serum was used either undiluted or diluted 1:9 (v/v) with radioimmunoassay buffer (PBS supplemented with 1% (w/v) BSA in the presence of 0.01% (w/v) sodium azide). Data points obtained in duplicate were compared with a dose–response curve of mouse RLF. For these assays, rabbit anti-mouse antiserum 9956 was used with ^{125}I -labelled human RLF (20 000 c.p.m.) as radioligand.

(Büllesbachet *et al.*, 1999). The data from the three independent experiments were pooled and are presented as mean $\pm$ SEM.

Gubernaculum receptor binding assay

Gubernaculum tissue was collected from male rats at various stages of development and dropped immediately into ice-cold Hepes buffer (25 mmol Hepes l^{-1} , 0.14 mol NaCl l^{-1} , 5.7 mmol KCl l^{-1} , pH 7.5, supplemented with 0.25 mol sucrose l^{-1} , 0.2 mmol phenylmethylsulfonylfluoride (PMSF) l^{-1} and 8 mg soybean trypsin inhibitor l^{-1}). Gubernaculum tissues of 12 males (two age-matched litters) of pre- or postnatal rats from 2 days before birth to 4 days after birth were pooled into 300 μl buffer for analysis. In addition, several experiments were done, each of which used the gubernacula of one litter ($n = 6$ rats) of males aged 10–23 days. The gubernacula were pooled into 600 μl buffer and the gubernacula of three male rats aged > 23 days were also pooled into 600 μl buffer.

The tissues were homogenized with a handheld Polytron (PT1200; Brinkmann Instruments, Westbury, NJ) homogenizer at maximum speed for 20 s. The homogenate was centrifuged in an Eppendorf centrifuge (Brinkmann Instruments) at 2000 g for 10 min at 4°C ; the pellet was discarded and the supernatant was collected and centrifuged at 14 000 g for 30 min at 4°C . The second pellet was resuspended in sucrose-free buffer and centrifuged as before. The resulting pellet was resuspended in binding buffer consisting of 25 mmol Hepes l^{-1} , 0.14 mol NaCl l^{-1} , 5.7 mmol KCl l^{-1} , 2.8 mmol glucose l^{-1} , 25 μmol MgCl_2 l^{-1} , 1.5 mmol MnCl_2 l^{-1} , 1.6 mmol CaCl_2 l^{-1} at pH 7.5, 0.2 mmol PMSF l^{-1} and 1% (w/v) BSA. Gubernaculum cell membranes from rats aged ≤ 4 days were suspended in 300 μl binding buffer and membrane suspensions from rats aged > 4 days were reconstituted into 600 μl binding buffer. For each assay, 20 μl tracer (approximately 100 000 c.p.m.), 40 μl buffer or buffer containing 0.2 μg RLF, and 40 μl membrane suspension were used. The membrane preparation was incubated for 1 h at room temperature. Thereafter, the reaction was quenched with 1 ml ice-cold wash buffer (25 mmol Hepes l^{-1} , 0.14 mol NaCl l^{-1} , 5.7 mmol KCl l^{-1} , pH 7.5, supplemented with 0.2% (w/v) BSA) and centrifuged in an Eppendorf centrifuge for 10 min at 14 000 g at room temperature. The supernatant was discarded and the tip of the vial was cut and radioactivity was counted in a gamma counter. Gubernaculum RLF receptor densities were determined in duplicate in rats aged ≤ 4 days and in triplicate from rats aged > 4 days. For each data point, at least three independent experiments, starting with the age-matched rats, were performed at weekly intervals. The data were pooled and are presented as means $\pm$ SEM.

For Scatchard analyses (Scatchard, 1949), crude membranes from pooled gubernaculum tissue from five litters of 12–15-day-old males were prepared as described above. The membranes were suspended in 1.5 ml binding buffer. Assays were run in duplicate or triplicate using ^{125}I -

labelled Tyr(A9)human RLF as tracer. Non-specific binding was determined in the presence of 1 μg unlabelled human RLF. Protein concentrations were determined using the modified Lowry method adopted for membrane proteins (Markwellet *et al.*, 1981). Three sets of animals were used for these assays.

Primary cell culture

Primary cell cultures from gubernaculum tissue were initiated according to Visser and Heyns (1995). In brief, gubernacula from 3-day-old rats were removed by microdissection and placed into Dulbecco's modified Eagle's medium (DMEM) supplemented with an enzyme solution containing type 1 collagenase (1 mg ml^{-1}) and DNase-1 (0.13 mg ml^{-1}). The suspension was incubated for 1 h at 37°C with gentle trituration every 15 min. After removal of the enzymes, the cells were filtered through a 25 μm filter to remove large cell clumps and transferred to culture flasks. The cultures were grown in 5% CO_2 and 95% air in DMEM supplemented with fetal calf serum (FCS: 10% v/v) and 10 000 U penicillin–streptomycin l^{-1} . After 1 week, 50 000 cells were transferred into each well of a 12-well culture plate, allowed to attach and grown to confluency (2 days).

Receptor binding was measured on intact cells after removing the medium and treating cells with 100 μl binding buffer with and without 2 μg human RLF, followed by addition of tracer (approximately 2×10^5 c.p.m.). The cells were incubated for 1 h at room temperature, the buffer was removed, the cells were lysed with 0.1 mol NaOH l^{-1} and the lysates were transferred into counting vials. In each experiment, six data points of total and six data points of non-specific binding were averaged. Three independent experiments were performed, each of which started with a new preparation of primary culture.

Gubernaculum cell growth

BrdU (5-bromo-2'-deoxyuridine) incorporation in cultured gubernaculum cells was measured to examine the growth factor potential of RLF. Twelve 3-day-old male rats were killed by CO_2 inhalation and the gubernacula were excised and collected in DMEM (low glucose and 100 U penicillin–streptomycin ml^{-1} ; Gibco, Grand Island, NY). The tissues were cut into 1 mm pieces with a sterile scalpel, dispersed in a spinner flask and incubated for 1 h at 37°C with sterile filtered medium containing 15 ml low glucose DMEM, 15 mg collagenase type I (Worthington Biochemical Corporation, Lakewood, NJ) and 1.95 mg DNase I (Sigma). Debris was removed by passage through sterile nylon filters and the cells were collected by centrifugation in a Dynac II (Becton-Dickson, Franklin Lakes, NJ) swinging bucket centrifuge at 300 g for 10 min. The dispersion media were removed and the cells were rinsed once with culture medium (high glucose DMEM with 10% (v/v) FBS and 100 U penicillin–streptomycin ml^{-1}) (Gibco). Gubernaculum cells (100 000 cells per ml of culture medium) were plated

Fig. 1. Primary structure of mouse and human relaxin-like factor (RLF). Boxed-in sequences show the differences between mouse and human RLF, whereas circles indicate residues that differ between rat and mouse RLF.

Fig. 2. Radioimmunoassay of rabbit anti-mouse relaxin-like factor (RLF) antibody 9956 using ^{125}I -labelled (A9)human RLF as tracer and mouse RLF as competitor. ●: Mouse RLF in radioimmunoassay buffer ($n = 4$; $\pm$ SEM), ED_{50} value = 1.1 ± 0.2 ng ml^{-1} . ○: Mouse RLF in female rat serum ($n = 3$; $\pm$ SE), ED_{50} value = 1.1 ± 0.39 ng ml^{-1} . ■: Rat RLF ($n = 3$; $\pm$ SE). □: Mouse RLF B chain ($n = 2$; $\pm$ SEM). ◆: Mouse RLF A chain ($n = 2$; $\pm$ SEM). ◇: Rat relaxin ($n = 2$; $\pm$ SEM). ▲: Bovine insulin ($n = 2$; $\pm$ SEM). △: Human insulin-like growth factor I ($n = 2$; $\pm$ SEM). B:Bo: bound:free ligand.

out into six-well culture plates and left for 24 h in a CO_2 incubator at 37°C . The cells were rinsed once with serum-free culture medium and were treated with 1 ng ml^{-1} of hRLF, des-(B27-31)hRLF or human relaxin; control wells received no additions. BrdU (Calbiochem, San Diego, CA) was added to a final concentration of 10 $\mu\text{mol l}^{-1}$ and the cells were incubated for an additional 18 h. The cells were taken up with trypsin-EDTA (Gibco), attached to poly-L-lysine-coated (Sigma) slides, and the BrdU incorporation was measured using a staining kit from Zymed Laboratories Inc. (San Francisco, CA). Starting with the preparation of the primary cell culture, BrdU uptake was measured in seven independent experiments. Each data point was collected in duplicate and data were normalized to the control experiment (= 100%).

Statistical analysis

Statistical significance was evaluated by paired analysis using the t test for correlated samples. Statistical significance of the RLF concentration in serum and receptor

Fig. 3. Relaxin-like factor (RLF) concentrations in 100 μl male rat serum during development. Rabbit anti-mouse antiserum 9956 was used in combination with ^{125}I -labelled human RLF as radioligand. Each data point was collected in duplicate and three independent assays were averaged. Data are mean $\pm$ SEM. The data were compared with a mouse RLF standard curve (see Fig. 2).

concentrations at day 2 before parturition was evaluated by one-way ANOVA followed by Newman-Keuls multiple comparison test.

Results

The differences in the sequences of RLFs between species are not as great as the variability in relaxin sequences. The primary structures of human and mouse RLF are shown (Fig. 1). It was important to determine whether the structural differences, however small, would cause functional differences in the mouse system if human and mouse RLF were used interchangeably. Mouse RLF was synthesized by the methods used for human RLF (Büllesbach and Schwabe, 1995). As mouse RLF does not contain tyrosine, the mouse RLF A-chain was synthesized with a des-aminotyrosyl extension at the N-terminal and combined with a B-chain in which the Trp(B27) had been protected against the oxidative iodination conditions. Radioactive iodine was introduced by the chloramine-T method (Hunter and Greenwood, 1962) and the tryptophan-protecting formyl group was removed to complete the mouse RLF tracer synthesis. It was important to purify RLF tracers by HPLC as

Fig. 4. Representative experiment of equilibrium saturation binding of human relaxin-like factor (RLF) tracer on crude membranes from rat gubernacula from 12–15-day-old male rats. Each data point represents duplicates. $\square$: Total binding; $\circ$: non-specific binding; $\bullet$: specific binding. For total and non-specific binding, bars indicate standard errors. Inset: corresponding Scatchard plot. B/F: bound/free. For evaluation of data, three Scatchard analyses were performed and normalized to the protein content. Dissociation constant K_D : 138 ± 34.9 pmol; $B_{\max}$ = 5.67 ± 1.3 pmol l^{-1} ; 2.2 fmol RLF receptors per mg membrane protein.

reported previously for human RLF to achieve high sensitivity (Büllesbach and Schwabe, 1995). Unlabelled, mono-iodinated and di-iodinated RLF was separated and the mono-iodination product only was used for these studies. Subsequent investigations have shown that mouse RLF and human RLF tracers are indistinguishable when used for receptor binding studies on crude membranes of mouse or rat tissues.

Antibodies raised in rabbits against synthetic mouse RLF gave rise to a typical sigmoidal binding curve when incubated with increasing concentrations of mouse RLF (Fig. 2). The dose–response curves determined in radioimmunoassay buffer and in the presence of female rat serum are identical, resulting in an ED_{50} value of 1.1 ± 0.2 ng ml^{-1} (Fig. 2). The sensitivity of the assay allows quantitative measurements up to the ED_{80} value (0.25 ng ml^{-1}). The antiserum is specific for RLF and does not bind to rat relaxin, bovine insulin, IGF-I or mouse RLF A-chain. It binds only very weakly to mouse RLF B-chain (Fig. 2), indicating that it interacts with a non-continuous epitope. When the antibody was used in combination with human RLF tracer, the radioimmunoassay was improved further to an ED_{50} value of 0.5 ± 0.1 ng ml^{-1} ($n = 3$) and the limit of detection was increased to 0.1 ng ml^{-1} . Rat RLF, synthesized according to the recently published cDNA sequence (Spiess *et al.*, 1999), showed a parallel dose–response curve within the critical region. This antibody recognized a natural component in rat sera and was used to determine RLF concentrations in the sera of pre- and postnatal male rats. Circulating RLF concentrations varied sharply with the developmental stage of the pups, showing high serum concentrations at 2 days before birth followed by a sharp decrease 1 day before

Fig. 5. Relaxin-like factor (RLF) receptor concentrations in crude membrane preparations of rat gubernacula during development. The determination of radioactivity (counts per mg membrane protein) in duplicate in three independent experiments. The data were pooled and the mean $\pm$ SEM is shown.

parturition ($P < 0.05$). The low RLF concentrations persisted up to day 10 after parturition and were followed by a steady increase until day 39 when adult concentrations of 0.6 ng ml^{-1} were reached (Fig. 3). RLF was barely detectable in the sera of female pups at day 2 before parturition and in postpartum female rats at day 30.

The next series of experiments was designed to determine whether the gubernaculum carried RLF-specific binding sites. Gubernaculum tissues from 12–15-day-old animals were dissected carefully and used to prepare crude membrane preparations for binding studies. Binding of ^{125}I -labelled RLF to gubernaculum membranes reached saturation and non-specific binding was linear at about 60% of total binding (Fig. 4). The results were subjected to Scatchard analysis (inset in Fig. 4) and yielded a dissociation constant of 138 ± 34.9 pmol and a $B_{\max}$ of 5.67 ± 1.3 pmol l^{-1} . There are 2.2 fmol RLF receptors per mg membrane protein, which is within the order of magnitude reported for RLF and relaxin receptors in mouse uterus (Yang *et al.*, 1992; Büllesbach and Schwabe, 1999a).

Determination of the number of gubernaculum RLF receptors as a function of development showed relatively high concentrations per mg membrane protein at day 2 before parturition, and a sharp decrease after parturition. The number of receptors per mg of tissue decreased further up to day 39 after parturition, the endpoint of this study (Fig. 5). The question of specificity of receptor distribution was addressed by testing for RLF receptors in the neighbouring inguinal canal and the testis. Inguinal tissues from 4-day-old rats and testis from 30-day-old rats were dissected carefully and membranes were prepared for RLF receptor binding assays. No receptors were found in inguinal tissue and the testis, indicating that the RLF response is limited to the gubernaculum (Table 1).

Cultures of rat gubernaculum cells prepared from collagenase-treated tissues of 3-day-old rats showed specific RLF binding. The complete set of experiments,

Table 1. Relaxin-like factor (RLF) receptor binding on different male rat tissues

Tissue	Age (days)	Total binding (c.p.m.)	Non-specific binding (c.p.m.)	P value
Gubernaculum	30	1671 ± 51	1240 ± 45	< 0.001
Gubernaculum cell culture*	3**	2401 ± 113	1544 ± 60	< 0.001
Inguinal canal	4	1253 ± 92	1176 ± 67	ns
Testis	30	11 835 ± 446	11 102 ± 188	ns

Values are mean ± SE.

Each experiment was performed in triplicate.

*Whole cell assay.

**Tissue collection for culture.

Fig. 6. The uptake of 5-bromo-2'-deoxyuridine (BrdU) in primary cultures of rat gubernaculum cells. Experiments were done in duplicate. The mean ± SE of a representative experiment is shown. *Only synthetic RLF (hRLF) stimulated BrdU uptake above control values ($P < 0.005$, $n = 7$). des(B27-31)hRLF lacks the major receptor binding residues ($n = 3$) and human relaxin (hRlx; $n = 3$) is inactive.

starting with gubernacula, was repeated three times with the same results. One representative experiment indicating that the primary cell culture contains RLF receptor-bearing cells is shown (Table 1). When these cells were exposed to RLF, accelerated BrdU incorporation was observed only in cells that received media containing 1 ng RLF ml⁻¹ ($P < 0.005$, $n = 7$). This effect was not observed when human relaxin ($n = 3$) or inactive (desB(27-31)) human RLF ($n = 3$) was substituted for the intact RLF (Fig. 6).

Discussion

The mechanisms by which the testes are moved through the abdomen toward the genital region and eventually through the inguinal canal into the scrotum are unclear. However, it is generally accepted that normal testis descent relies on structural changes in the gubernaculum (Hutson *et al.*, 1997). In rats, the gubernaculum develops before birth and before testis migration occurs. It is a mesenchymal structure that extends from the testes located in the middle of the abdominal cavity to the inguinal area and the scrotum. Several changes that occur in the gubernaculum are described by the two-phase model of testicular descent

(Hutson, 1985). Accordingly, outgrowth of the gubernaculum tissue is caused by cell division and by changes in various matrix components. Firstly, the gubernaculum widens to dilate the inguinal canal and then the outgrowth distal to the inguinal canal shortens and pulls the testis towards the inguinal passageway. During the second phase, the gubernaculum undergoes regression, caused mainly by changes in extracellular matrix (Fentener van Vlissingen *et al.*, 1989; Heyns *et al.*, 1990), and the testes pass through the area formerly occupied by the gubernaculum, into the scrotal compartment. RLF plays a crucial role in the overall process (Nef and Parada, 1999; Zimmermann *et al.*, 1999; Adham *et al.*, 2000) and the experiments performed in the present study were designed to investigate the involvement of RLF. Although knockout experiments left no doubt that male mice devoid of RLF develop cryptorchidism, there was no evidence linking RLF directly to the gubernaculum changes or to indicate that the gubernaculum has RLF receptors.

The synthesis of mouse RLF and the murine-specific tracer, as well as the production of a specific antibody, provided powerful tools to explore the role of RLF in testis descent. The binding curve indicates that the radio-immunoassay for RLF is sensitive and specific, which can be attributed in part to HPLC purification of all tracers. While this study was in progress, the rat RLF sequence was published, which showed differences from mouse RLF sequence at positions A2 (Ala/Val) and B17 (Ala/Thr) (Spiess *et al.*, 1999). Rat RLF was synthesized in the present study and in the heterologous assay it was confirmed that both mouse and rat RLF produce dose-response curves that are parallel within the critical region.

High RLF concentrations were detected by radio-immunoassay in sera from male rat fetuses at day 2 before parturition; this may be preparatory, a trigger event for the development of the gubernaculum (at day 2 before parturition females have very low serum RLF concentrations). RLF decreases to very low concentrations at birth, indicating that the prepartum increase in RLF concentration may be physiologically important. Furthermore, the movement of testes and the descent into the scrotum are completed before RLF concentrations increase again in adult rats, indicating that postpartum RLF concentrations are not critical for testicular positioning. This 'critical time'

hypothesis would explain why human males with a history of cryptorchidism usually have normal adult RLF serum concentrations (C. Schwabe, unpublished). In future, testing of newborns for RLF concentrations may shed light on this observation. Clearly, other factors, such as the RLF receptor, may contribute to cryptorchidism in humans and our experimental system provides a method to elucidate these factors.

RLF receptors are very prominent in the differentiating gubernaculum tissue as early as they can be detected. The initial display of receptors also supports the contention that the prenatal serum RLF concentrations may have provided the crucial starting signal for gubernaculum growth in preparation for testicular migration. Further support comes from gene deletion experiments, which show that the gubernaculum in RLF^{-/-} animals does not even begin to undergo organization (Nef and Parada, 1999; Zimmermann *et al.*, 1999). Finally, our observation of high prenatal RLF serum concentrations supports the results of experiments showing that RLF mRNA is expressed at high concentrations in the testes of rat embryos (Spiess *et al.*, 1999).

The binding constant for rat gubernaculum receptor was within the same order of magnitude as that of mouse uterine RLF receptor (Büllesbach and Schwabe, 1999a). At an infinite concentration of free ligand there appears to be 2.2 fmol receptor per mg membrane protein, which compares well with about 7 fmol mg⁻¹ uterine tissue (Büllesbach and Schwabe, 1999a). Transiently active hormones such as relaxin and RLF appear to have significantly fewer receptors in their target tissues than does, for example, insulin.

Experiments involving BrdU indicate that RLF may have a mild growth factor activity, which is not unexpected given that the first phase of gonadal movement is associated with induction and growth of the gubernaculum. A similar observation was made by Emmen *et al.* (2000), who described an increasing ³[H]thymidine uptake in rat gubernaculum cocultured with testis of RLF^{+/-} mice, whereas testis of RLF^{-/-} mice did not show this effect. In the present study, the increase in BrdU incorporation was significantly higher in the presence of RLF than in controls which received medium only, medium with relaxin or medium with an inactive form of RLF that lacks the major binding residues (Büllesbach and Schwabe, 1999b). An additional point is that it now seems likely that the hormone affects the gubernaculum directly and that the process may be orchestrated by critical timing of Leydig cell activity.

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