

Specific, High Affinity Relaxin-like Factor Receptors*

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The relaxin-like factor (RLF) is a circulating hormone that binds to specific membrane-bound uterine receptors in the mouse. Mono-iodinated RLF tracers were produced and characterized specifically to study the properties of the RLF receptor. The tracers bound to the RLF receptor in uterine crude membrane preparations with high affinity (73 nM for ¹²⁵I-Tyr(A9) RLF and 90 nM for ¹²⁵I-Tyr(A26) RLF) as determined by Scatchard analysis. The specificity of binding was confirmed by chemical cross-linking experiments. Binding of ¹²⁵I-Tyr(A9) RLF to the putative receptor was inhibited in the presence of a 640-fold excess of unlabeled human RLF but not by the same excess of human relaxin. SDS-gel electrophoresis of the RLF-receptor complex revealed a molecular mass of >200 kDa, which remained unchanged upon reduction. The size and the lack of subunit structure of the receptor is similar to the features reported for the relaxin receptor. In this regard both, the RLF and the relaxin receptor are different from the insulin- and the insulin-like growth factor-type 1 receptors. This observation supports the relaxin-likeness of this new factor not only toward potential target tissues but also as regards receptor features.

The relaxin-like factor (RLF)¹ is synthesized in gonadal tissues of males and females. The mRNA was first detected in Leydig cells and named, accordingly, Leydig insulin-like peptide (LEY I-L) (1). Later distinct but low levels of RLF mRNA were detected in female tissues, *i.e.* human trophoblasts (2), human corpora lutea (2), and mouse ovaries (3). The location of RLF expression implied a potential physiological role in reproduction and thereby a functional relatedness to relaxin rather than to insulin. This impression was reinforced when the protein was synthesized so that it seemed appropriate to adopt the name relaxin-like factor (RLF) (4).

In humans RLF is a circulating hormone with higher levels in post-puberty males than in children and post-puberty females (5). This is in agreement with the relative expression levels in gonadal tissue during development in the mouse (3). The potential activity of RLF in reproduction is supported by the observation that transcription of the gene is mediated by the steroidogenic factor-1 (6) and by experiments with knock-out mice in which males without a functional RLF gene are sterile because of premeiotic arrest of the sperm maturation

process; female knock-out mice did not show defects (7). The role of RLF in the female remains to be elucidated. The fact that RLF expression is cycle-dependent in some animals and that the mRNA is present during pregnancy (3, 8, 9) as well as the presence of RLF-specific receptors in mouse uteri again hint at a potential function in reproduction (4).

The structure of human RLF as predicted from its cDNA has two chains and a disulfide bonding pattern characteristic of the relaxin/insulin family (1, 10). Human RLF, synthesized according to this prediction (4) served to elicit production of structure-specific polyclonal antibodies in rabbits that recognized RLF in human serum (5). Synthetic human RLF interacted with membrane-bound receptors in mouse uterus and brain (4), which means that synthetic human RLF projects the proper binding site from the native structure of the molecule. In fact, structure and function studies revealed that tryptophan (B27) is essential for RLF interaction with its receptor (11). In the present paper we describe the properties of a mouse uterine RLF receptor distinct from the relaxin receptor.

EXPERIMENTAL PROCEDURES

Materials

Human RLF and human relaxin II were synthesized as described (4, 12). Solvents for chromatography and synthesis were Burdick and Jackson high purity solvents; all other chemicals were of ACS grade and used without further purification. Enzymes and enzyme inhibitors were obtained from Sigma. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, precast gradient gels were purchased from Novex (San Diego, CA). Molecular mass standards (range 6.9 to 205 kDa) were Kaleidoscope prestained standards purchased from Bio-Rad.

The buffers used are as follows. Homogenizing buffer: 25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 8 mg/liter soybean trypsin inhibitor, and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.5. Binding buffer: 25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 25 μM MgCl₂, 1.5 mM MnCl₂, 1.6 mM CaCl₂, and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.5. Wash buffer: 25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 0.01% sodium azide, and 1% bovine serum albumin, pH 7.5.

Methods

Protein concentrations were determined using the modified Lowry method adopted for membrane proteins (13).

Amino Acid Analysis—Peptides were hydrolyzed in vapor phase 6 M HCl containing 0.1% phenol for 1 h at 150 °C. After modification with phenylisothiocyanate, the amino acids were separated by high performance liquid chromatography (HPLC) on a Waters Pico-Tag system.

HPLC—Small scale preparative HPLC was performed on an Applied Biosystems 130A chromatograph. The stationary phase was an Aquapore 300 column (C8; 2.1 × 30 mm) (Perkin-Elmer Applied Biosystems), and the mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 80% acetonitrile (solvent B). Peptides were loaded through a 50-μl sample loop and separated using a 60-min linear gradient (gradients used are given with the corresponding peptide). Unless stated otherwise, separations were performed at a flow rate of 100 μl/min, and the effluent was monitored at 230 nm. Fractions were collected manually.

RLF Tracer and Tracer Characterization

Tracer—The synthetic intermediate (N (in) formyl) (B27), S (oxide) (B5) human RLF (4) (10 μg) was dissolved in 10 μl of 250 mM phosphate

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¹ The abbreviations used are: RLF, relaxin-like factor; HPLC, high performance liquid chromatography.

buffer at pH 7.5 and chilled on ice. Na¹²⁵I (1.8 μ l, 0.9 mCi) was added followed by 5 μ l of chloramine T (2 mg/ml in 250 mM phosphate buffer at pH 7.5). The reaction was performed for 1 min on ice and quenched with sodium thiosulfate (5 H₂O, 50 mg/ml 250 mM phosphate buffer at pH 7.5). Thereafter the tryptophan side chain was liberated by addition of 5 μ l of piperidine. Two min later the reaction was quenched with 10 μ l of glacial acetic acid. After dilution with 20 μ l of water, the mixture was separated by HPLC, applying a 60-min linear gradient of 23 to 34% B. Proteins eluting after the excess unlabeled RLF analog were manually collected in an Eppendorf tube containing 100 μ l of a 1% aqueous bovine serum albumin solution, and an aliquot of 2 μ l was counted in a γ counter.

Location of the Radioactive Label—Two radioactive fractions (1 μ l, ~400,000 cpm) were dried and redissolved in 10 μ l of 50 mM phosphate buffer at pH 7.5 containing 1 μ g of protease from *Staphylococcus aureus* strain V8 (EC 3.4.21.19). Digest was performed for 16 h at 37 °C. Thereafter 10 μ l of L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (EC 3.4.21.4; 0.2 μ g in phosphate buffer at pH 7.5) was added, and the reaction continued at 37 °C for 1 h. The digest was quenched by the addition of 20 μ l of dithiothreitol (50 mM) in 0.2 M

Tris/HCl, pH 8.6, in 6 M guanidinium chloride, and disulfides were reduced for 1 h at 37 °C. The reaction mixture was spiked with a mixture of partially iodinated A chain peptide A9–19 and A20–26 and separated by HPLC using a linear gradient of 5 to 50% B. Two-min fractions were collected and counted on the γ counter.

Model Peptides to Aid Identification—Human RLF segments A9–19 and A20–26 were chemically synthesized using Fmoc (*N*-(9-fluorenyl)-methoxycarbonyl) chemistry and conventional side chain-protecting groups (14). The peptides were deprotected with trifluoroacetic acid containing ethanedithiol, phenol, and water as scavengers (15) and collected by ether precipitation. The HPLC-purified peptide (100 nmol) was dissolved in 100 μ l of 250 mM phosphate buffer at pH 7.5. KI (10 mM in water, 5 μ l), chloramine T (10 mM in 250 mM phosphate buffer at pH 7.5; 20 μ l for A(20–26) and 60 μ l for A(9–19)) were added, and the reaction was kept for 1 min at room temperature. The reaction was quenched with 100 μ l of dithiothreitol (100 mM in 0.2 M Tris/HCl, pH 8.6, containing 6 M guanidinium chloride), and the disulfide bonds were reduced for 1 h at 37 °C. Thereafter the reaction mixture was analyzed by HPLC on Aquapore 300 using a 60-min linear gradient from 5 to 50% B.

Identification of the RLF Receptor

Membrane Preparation—Female ICR mice were primed with estrogen (5 μ g of β -estradiol 17-cypionate in 100 μ l of sesame oil). Five days later the mice were killed in an atmosphere of CO₂, and the uteri were collected. The uteri of five mice were dropped into 15 ml of chilled homogenizing buffer supplemented with 0.25 M sucrose. The tissue was homogenized with a Polytron homogenizer at setting 7 for 10 s. Thereafter the tissue preparation was centrifuged at 700 $\times g$ for 10 min at 4 °C, the supernatant was collected, the pellet was re-homogenized in 10 ml of homogenizing buffer supplemented with 0.25 M sucrose, and the suspension was treated as before. The combined supernatants were centrifuged at 20,000 $\times g$ for 60 min at 4 °C. The supernatant was discarded, and the pellet was suspended in 20 ml of homogenizing buffer without sucrose addition. The pellet was collected by centrifugation at 20,000 $\times g$ for 60 min at 4 °C. The crude membranes were suspended in 1 ml of binding buffer supplemented with 1% bovine serum albumin. The membranes were homogenized with a handheld homogenizer and used for assay.

Binding assays were performed in 1.5-ml Eppendorf vials. Dose-response curves were obtained using human RLF at concentrations from 0 to 3.17 μ M in binding buffer (40 μ l, tracer (20 μ l; 100,000 cpm, 275 pM in binding buffer), and 40 μ l of tissue. After gentle mixing the assay was incubated for 1 h at room temperature, quenched by the addition of 1 ml of chilled wash buffer, and centrifuged at 14,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was discarded, and the tip of the vial was cut and counted in a γ counter. Total binding was usually about 7 to 10% of the total counts added, and nonspecific binding was 50% \pm 10% of the total binding. Each assay was performed in duplicate, and three assays were averaged.

Scatchard analyses were done as described in the literature (16, 17). Nonspecific binding was determined using unlabeled human RLF at a concentration of 1.6 μ M (1 μ g/assay). Assays were run in duplicate or triplicate. Seven independent assays were performed with ¹²⁵I-

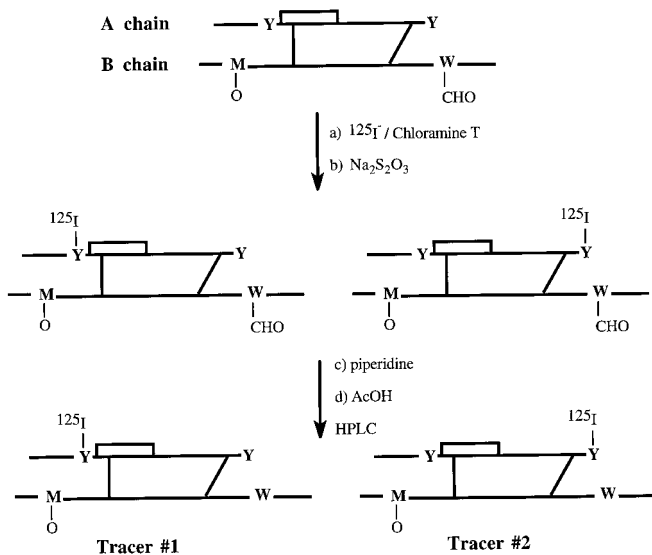


FIG. 1. Preparation of iodinated RLF starting with a partially protected synthetic precursor (CHO, N (in) formyl; O, sulfoxide). a, introduction of ¹²⁵I was performed with chloramine T as oxidant at pH 7.5. b, the reaction was quenched with thiosulfate. c) The N (in) formyl group was removed with piperidine (final concentration 20% in water). d, the reaction was acidified before loading onto the HPLC. Tracer #1 (¹²⁵I-Tyr(A9) human RLF) and tracer #2 (¹²⁵I-Tyr(A26) human RLF) refer to the position of the tracer in the HPLC chromatogram shown in Fig. 2.

FIG. 2. Isolation of RLF tracers. HPLC separation of the radioactive reaction mixture using an Aquapore 300 (2.1 \times 30 mm) column, 0.1% trifluoroacetic acid in water as solvent A, and 0.1% trifluoroacetic acid in 80% acetonitrile as solvent B. A linear gradient from 23 to 34% B was developed over 60 min. The flow rate was 100 μ l/min, and UV absorbance of the effluent was monitored at 230 nm. Fractions indicated as #1 and #2 were manually collected into 100 μ l of 1% bovine serum albumin in water.

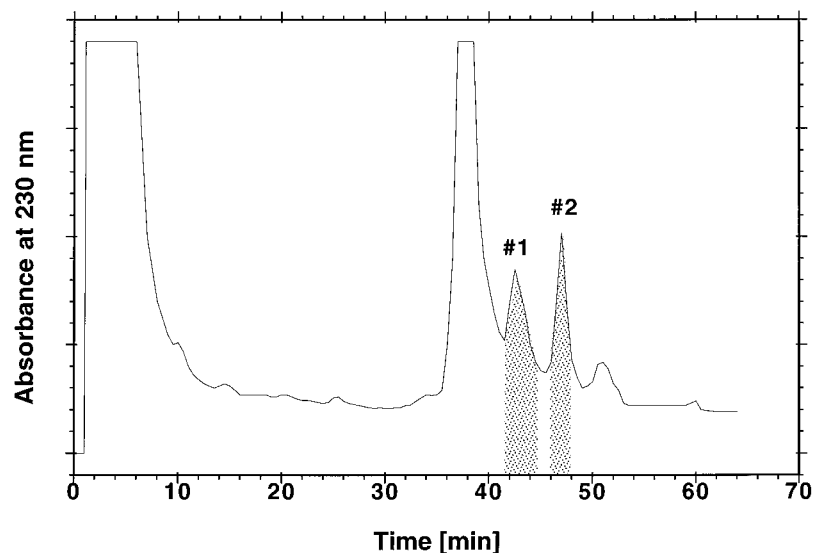


FIG. 3. Primary structure of synthetic human RLF including the cleaving sites for trypsin (*Tr*) and protease from *S. aureus* strain V8 are indicated.

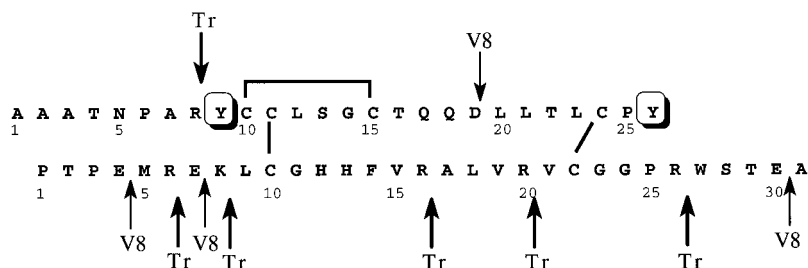


FIG. 4. HPLC separation of a tracer V8/trypsin digest followed by reduction. **A**, the reaction was spiked with partially iodinated reduced peptides of the sequence A(9–19) and A(20–26) on Aquapore 300 (2.1 × 30 mm). The mobile phase was 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in 80% acetonitrile. Separation was achieved by running a 1-h linear gradient from 5 to 50% B; the effluent was monitored by UV absorbance at 230 nm. **B**, digest of tracer #1. Fractions of 2 min were manually collected and counted in a γ counter. The majority of the counts were eluted in the position of mono-iodinated peptide A(9–19), indicating that tracer #1 is ^{125}I -Tyr(A9) human RLF. **C**, digest of tracer #2. Fractions of 2 min were

Tyr(A9)RLF, and two were performed with ^{125}I -Tyr(A26)RLF as tracer.

RLF Receptor Cross-linking—Tissue (640 μl) and tracer (700,000 cpm/20 μl ; final assay concentration 275 pM) in the presence or absence of hormone (40 μl , final assay concentration of 160 nM) were incubated for 1 h in binding buffer supplemented with 1% bovine serum albumin and aprotinin (2 $\mu\text{g}/\text{ml}$). The assay was diluted with 600 μl of binding buffer, and the membranes were collected by centrifugation at 14,000 rpm for 10 min in an Eppendorf centrifuge at 4 $^{\circ}\text{C}$. The supernatant was discarded, and the pellet was reconstituted in 200 μl of chilled binding buffer containing aprotinin. The suspension was kept on ice, and 5.5 μl of a freshly prepared solution of suberic acid (100 mM in dimethyl sulfoxide) was added. The reaction was performed for 30 min on ice, quenched by addition of 10 μl of a Tris/HCl solution (1 M, pH 7.5), diluted with 800 μl of binding buffer containing aprotinin, and centrifuged, and the pellet was collected. The receptor was extracted with 450 μl of 1% Triton X-100 in binding buffer containing aprotinin and shaken on an orbital shaker for 20 h at 4 $^{\circ}\text{C}$. After the addition of 500 μl of binding buffer, the extraction was centrifuged at 14,000 rpm at 4 $^{\circ}\text{C}$ for 10 min, and the supernatant was collected, diluted with 4 ml of binding buffer containing aprotinin, and purified on a wheat germ agglutinin column (1-ml column volume). The extract was passed through three times. Thereafter the column was washed with 10 ml of binding buffer containing aprotinin and 0.1% Triton X-100. The receptors were eluted with 5 × 1 ml of 0.3 M *N*-acetylglucosamine in binding buffer containing 0.1% Triton X-100. The eluate was collected, concentrated on Centricon-100, and washed with 3 ml of water. The receptor extract was concentrated to ~100 μl , lyophilized, and denatured in 50 μl of SDS sample buffer in the presence or absence of dithiothreitol and separated on a 4–12% SDS gradient gel. The gel was dried and exposed to Kodak X-AR film for 72 h.

RESULTS AND DISCUSSION

Tracer Preparation and Characterization—The synthesis of human RLF was achieved by a combination of solid phase chemistry and site-selective synthesis of the three disulfide bonds in solution (4). An intermediate of this synthesis carried a formyl-protecting group in the indole side chain of tryptophan (B27) and a sulfoxide in methionine (B5). Radioactive iodination of this partially protected human RLF by the chloramine T method (18) could be performed without oxidative degradation of the crucial tryptophan side chain (4, 19). After reduction of excess oxidant with thiosulfate, the indole group was liberated by treatment with aqueous piperidine (20) (Fig. 1). The resulting reaction mixture was separated into two radioactive peaks of equivalent amounts by HPLC (Fig. 2). Position B5 is methionine sulfoxide in all RLF tracers, and the peak broadening in the chromatogram is because of the partial separation of the two diastereomers.

Digest of human RLF (Fig. 3) with protease from *S. aureus* strain V8 (21) should cause cleavage between the two tyrosines at Asp (A19) and release, after reduction, two A chain peptides (A1–19 and A20–26). Unfortunately cleavage with protease V8 alone was incomplete, and only after the addition of trypsin was complete digestion observed. Upon reduction, peptides

manually collected and counted in a γ counter. The majority of the counts eluted in the position of mono-iodo A(20–26), indicating that tracer #2 is ^{125}I -Tyr(A26) human RLF.

FIG. 5. Scatchard plots of RLF receptor binding on crude uterine cell membranes using tracer #1 (^{125}I -Tyr(A9) human RLF) and tracer #2 (^{125}I -Tyr(A26) human RLF). Binding of tracer 1 was determined in seven independent assays, and tracer 2 was determined in two independent assays. The dissociation constant of the tracer #1-receptor complex was $78.3 \text{ pM} \pm 16.0 \text{ pM}$ (S.E.), whereas the dissociation constant for the tracer #2-receptor complex was 90.7 pM . Both experiments were performed simultaneously.

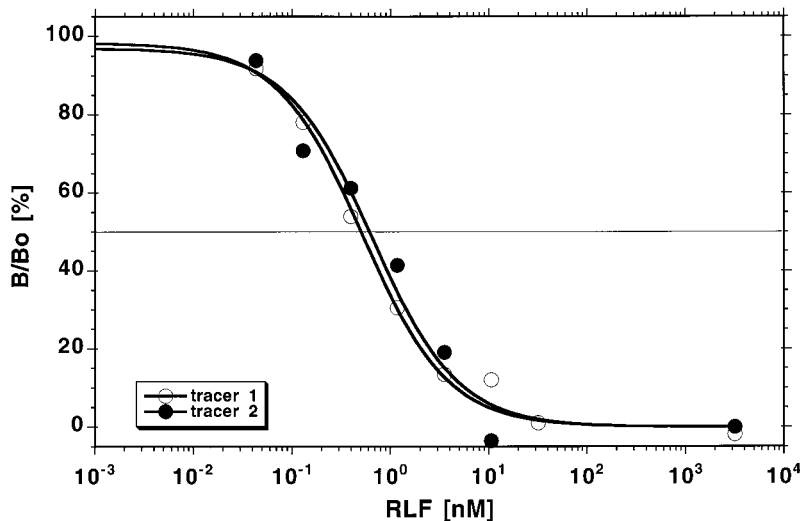
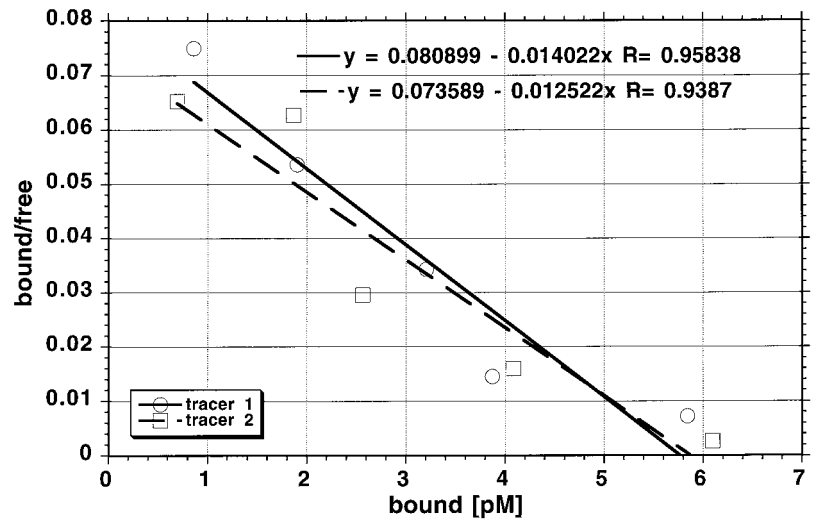


FIG. 6. Competitive binding of human RLF to mouse uterine receptors using two different tracers. Both tracers were tested in parallel. Each data point was collected in duplicate, and three assays were averaged. The ED_{50} of the two tracers are 0.51 nM for tracer #1 and 0.60 nM for tracer 2.

A9–19 and A20–26 could be separated by HPLC and subsequently identified by amino acid analysis. The result was verified with the aid of chemically synthesized peptides corresponding to the expected fragments in combination with HPLC analysis. The synthetic peptides were iodinated and served as standards to identify radioactive fragments derived from the digest of the corresponding RLF tracer. By this strategy it was possible to show clearly that both tracers are mono-iodinated; tracer #1 being labeled in position Tyr(A9), and tracer #2 labeled in position Tyr(A26) (Fig. 4, A–C).

Characterization of RLF Receptor Interaction—Both tracers have similar, if not identical, affinities to the RLF receptor on mouse uteri. Scatchard analyses revealed a single high affinity binding site with a dissociation constant of 78.3 pM ($\pm 16 \text{ pM}$ S.E.) for ^{125}I -Tyr(A9) human RLF and 90.7 pM for ^{125}I -Tyr(A26) human RLF (Fig. 5). The receptor concentration was 7 fmol/mg of membrane protein. Dose-response curves using 275 pM concentrations of the corresponding tracers resulted in ED_{50} of 0.55 nM for tracer #1 and 0.32 nM for tracer #2 (Fig. 6). Because ^{125}I -Tyr(A26) human RLF has a significantly lower specific binding on uterine tissue (27% versus 42% for the A9-labeled RLF in a parallel experiment), all subsequent studies were performed with ^{125}I -Tyr(A9) human RLF.

Crude membrane preparations of mouse uteri were incubated with tracer and cross-linked with the disuccinimide ester of suberic acid. The cross-linked hormone-receptor complex was

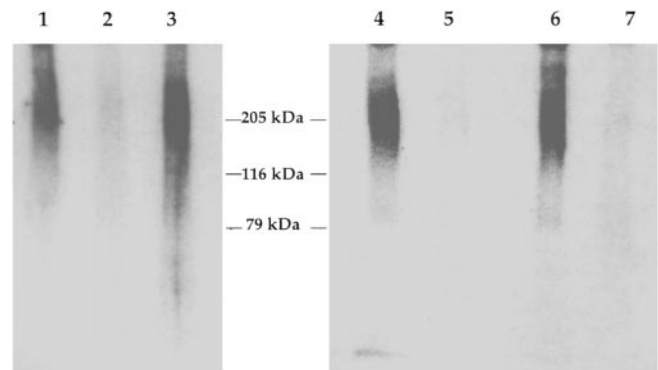


FIG. 7. RLF receptor of uterine membrane preparations cross-linked to ^{125}I -Tyr(A9)-RLF (275 nM) using DSS as cross-linker. Lane 1, receptor and tracer; lane 2, receptor + tracer + 640-fold excess of human RLF; lane 3, receptor + tracer + 640-fold excess of human relaxin II; lane 4, receptor reduced with dithiothreitol + tracer; lane 5, receptor reduced with dithiothreitol + tracer + 640-fold excess of human RLF; lane 6, receptor + tracer; lane 7, receptor + tracer + 640-fold excess of human RLF. SDS-polyacrylamide gel electrophoresis was performed on precast 4 to 12% gradient gels. The gels were dried and exposed to Kodak X-AR film for 72 h. The position of the bands were assigned using prestained molecular weight standards.

solubilized with 1% Triton X-100. The extract was diluted to 0.1% Triton X-100, and the RLF receptor was bound to a wheat germ agglutinin column and eluted with with *N*-acetylglucosamine. The binding to wheat germ agglutinin and the release of the receptor were monitored by radioactivity. The protein was collected and concentrated, and Triton X-100 was removed by centrifugation in a concentrator with a molecular weight membrane cut-off of 100,000. The retained high molecular weight biopolymers were denatured with SDS and separated on SDS gradient gels. As shown in Fig. 7, a broad radioactive band of the ¹²⁵I-Tyr(A9) human RLF receptor matches a molecular mass of about 220 kDa. The specificity of RLF binding to its receptor was demonstrated in parallel experiments performed in the presence of a 640-fold excess of either unlabeled human RLF or human relaxin. The radioactive band in Fig. 7 is absent in the presence of unlabeled human RLF (lane 2) but appears undisturbed when relaxin is added to the incubation mixture (lane 3). SDS-polyacrylamide gel electrophoresis in the presence of reducing agent indicates that the RLF receptor is a single-chain molecule.

Reduction of the cross-linked RLF-receptor complex will cleave the bound RLF into two chains. To detect the reduced hormone-receptor complex by radioactivity, the cross-link needs to be established between the only amino group of the RLF A chain (A1) and an unknown lysine side chain of the receptor. This implies that the N terminus of the A chain must be in contact with the receptor. The intensity of the radioactive signal of the reduced receptor is as strong as for the nonreduced receptor, implying that the majority of the cross-linked product is formed through this link with only small contributions of the two B chain amino groups.

Within the insulin family of receptors two groups have been identified. The multisubunit receptor of a molecular mass of >300 kDa includes the insulin receptor and the insulin-like growth factor receptor type 1 (22–24). They are biosynthesized as single-chain precursors and post-translationally modified by proteolytic conversion, dimerization, and subsequent disulfide bond formation. The single subunit receptors of a molecular mass of >200 kDa include the relaxin receptor (25, 26), the RLF receptor, and the insulin-like growth factor type 2 receptor (27, 28). Evidence presented in this paper suggests that the relaxin and the RLF receptor are cell membrane-bound glycoproteins, and both are present in uterine (4, 25, 29, 30) and brain tissue (4, 29–31). A weak cross-reactivity at the corresponding hormone/receptor level (4) has been reported. It will be interesting to see whether the cooperativity observed be-

tween RLF and relaxin is reflected in sequence homology of the two different hormone receptor systems.

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