The Mode of Interaction of the Relaxin-like Factor (RLF) with the Leucine-rich Repeat G Protein-activated Receptor 8*

Erika E. Büllsbach and Christian Schwabe1

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

The relaxin-like factor (RLF, also named INSL3) is a critical component in the chain of events that lead to the normal positioning of the gonads in the male fetus. RLF and relaxin share features of the secondary structure to the extent that relaxin cross-reacts with the LGR8, the RLF receptor. Although both hormones interact with their receptors essentially via the B chain, the sharply defined binding cassette of relaxin is not present in RLF. Structure and function analysis of RLF derivatives with single amino acid replacements revealed that the most important binding residues are tryptophan B27, followed by arginine B16 and valine B19. Single alanine replacements for each individual position resulted in a relative receptor affinity of 4.0% (B16), 6.1% (B19), and 0.5% (B27). Tryptophan B27 is located on an extended structure, and arginine B16 and valine B19 are positioned on the exposed surface of the B chain helix. The 3 residues could be brought together to form a contiguous binding area if the C-terminal end of the B chain were free to fold back against the central portion of the B chain helix. Such a movement depends critically on the flexibility of the C-terminal end, which is controlled by positions B23–25. In as much as these major binding residues seem hardly sufficient to explain the strong binding of RLF to LGR8 we searched for and found an extended region where little contributions by individual residues added up to a strong receptor affinity. This mode of interaction could drive the binding energy sufficiently high to account for the picomolar binding constant of RLF and its receptor.

Targeted deletion of the relaxin-like factor (RLF) gene (named insl3) in mice by Adham et al. (1, 2) strongly suggests that RLF, a Leydig cell-derived hormone, is a vital factor in the physiological process of gonadal positioning. This work signaled a new approach to a long-standing problem of cryptorchidism in humans (3, 4) where the problem of testicular retention is exacerbated by a predisposition for testicular cancer (5, 6). The availability of synthetic RLF and RLF derivatives (7–12) has provided the opportunity to investigate several aspects of the role of RLF in gonadal migration. This research led to the potentially important discovery of a signal activation site in RLF that does not overlap the RLF binding site. The modified RLF is a potent, highly specific inhibitor of RLF action (12). Although it is clear from our investigations that modifications of the signaling site do not interfere with LGR8 binding, the site or region of RLF that is responsible for a nanomolar binding constant is not known.

Native RLF, isolated from bovine testes, is a polypeptide hormone in which a 26-residue A chain is linked by two interchain disulfide bonds to a 41-residue B chain and an intrachain cystine bond in the A chain (13). Previous studies indicated that RLF can be shortened at the C terminus of the B chain up to residue 27 without losing its potency if the C terminus is amidated (11). Receptor binding is retained even after additional removal of 6 amino acids from the N terminus of the A chain and 5 residues from the N terminus of the B chain (12). Consequently the binding site of RLF to LGR8 is located in the disulfide cross-linked 36-amino acid residue containing segment A10–26/B6–27-amide. In this study we are reporting experiments that provide evidence for a 5-amino acid region of strong receptor binding that is augmented by three more prominent interaction sites to produce the observed affinity constant for the LGR8 and its ligand.

EXPERIMENTAL PROCEDURES

Materials—The LGR8, cloned into the pcDNA3.1.zeo plasmid, was a gift from Dr. Hsueh, Department of Obstetrics and Gynecology, Stanford University School of Medicine. The human embryo kidney cell line 293T/17 was obtained from the American Type Culture Collection (ATCC CRL-11286) and used for LGR8 expression. Fmoc (N-(9-fluorenylmethoxycarbonyl) amino acid derivatives were purchased from either Advanced ChemTech, Louisville, KY or NovaBiochem, San Diego, CA. Reagents and solvents for peptide synthesis were obtained from Advanced ChemTech.

Peptide Synthesis—RLF chains were synthesized by a combination of automated peptide synthesis (model 433A; Applied Biosystems, Foster City, CA) and site-directed sequential disulfide bond formation. Syntheses were performed using the Wang resin (14) with trifluoroacetic acid-labile side chain-protecting groups for all trifunctional amino acids but methionine in position B5, which was used as sulfoxide, cysteines in positions A11 and B10, which were acetamidomethyl protected, and cysteine A24, which carried a tertiary butyl-protecting...
group. O-Benzotriazol-N,N,N′,N′-tetramethyluronium-tetrafluoroborate served as carboxyl group-activating agent (15). Polypeptide chains were deprotected and released from the resin with a freshly prepared mixture of trifluoroacetic acid/water/ethanedithiol/thioanisole/phenol (100/5/2.5/5/7.5) (v/v/v/v/v) for 2 h at room temperature (16). The resin was filtered off and the peptide precipitated with ice-cold diethyl ether. The pellet collected by centrifugation was washed two times with diethyl ether, air dried, and lyophilized as an aqueous suspension. Each RLF chain was purified by reversed phase high performance liquid chromatography (HPLC) on a Rainin C18 column (41.4 × 250 mm), using 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 83% acetonitrile (solvent B) at a flow rate of 40 ml/min. The peptide was eluted using a 30-min linear gradient and detected by UV absorbance of the effluent. Fractions containing the pure peptides were pooled and lyophilized.

The strategic distribution of the cysteine-protecting groups was essential for the stepwise synthesis of the three disulfide bonds. In the A chain, cysteines A10 and A15, after deprotection by trifluoroacetic acid treatment, were oxidized by titration with iodine in 50% acetic acid (peptide concentration, 10 mg/ml). Excess iodine was immediately reduced with 1 M ascorbic acid in water. The reaction mixture was diluted with water to reduce the acetic acid concentration to <20% and purified by preparative HPLC. Cysteine (tert-butyl) (A24) was converted to the 2-pyridylsulfenyl derivative (17) by dissolving the protected A chain (17 μmol) and 2,2′ dipirylyl disulfide (Aldrich) (240 μmol) in 900 μl of trifluoroacetic acid. The solution was chilled on ice, and thioanisole (100 μl) and 1 ml of trifluoromethanesulfonic acid in trifluoroacetic acid (1:4 v/v) were added. After 30 min at 0 °C the A chain was precipitated with chilled diethylether and the pellet collected by centrifugation, washed three times with 10 ml of diethylether, and air dried. The RLF A chain derivative was dissolved in 1 M acetic acid and desalted on Sephadex G25 sf in 1 M acetic acid and lyophilized.

The interchain disulfide bonds were established first by reacting the thiol-activated A chain with the B22 monothiol B chain, followed by oxidative removal of the acetalidomethyl-protecting groups in cysteines A11 and B10 (8) and by methionine sulfoxide reduction as reported before (18). The various RLF analogs were characterized by several HPLC systems as follows.

System 1: ~10–20 μg of peptide was loaded onto a Bakerbond wide pore C18 column (4.1 × 250 mm). The peptide was eluted with a 30-min linear gradient from 20 to 60% B at a flow rate of 1 ml/min. The UV absorbance of the effluent was recorded at 220 nm.

System 2: An Aquapore 300 column (C8, 1.2 × 30 mm) was used in combination with an ABI HPLC model 130A. ~1 μg of peptide was loaded and eluted with a 60-min linear gradient from 20 to 45% B at a flow rate of 100 μl/min. The UV absorbance of the effluent was recorded at 230 nm. In a parallel experiment the peptide was reduced prior to separation with 25 mM dithiothreitol in 3 mM guanidinium chloride in 50 mM Tris buffer, pH 8.6, for 30 min at 37 °C.

Amino Acid Composition—Peptides were hydrolyzed in vapor phase 6 M HCl containing 1% phenol. Amino acids were modified with phenylisothiocyanate and separated by HPLC, using the Waters Pico-Tag system.

Enzymatic Digest—RLF analogs (5 μg) were dissolved in 10 μl of 50 mM phosphate buffer, pH 7.5, and digested with staphylococcal protease V8 (E:S 1:10) for 16 h at 25 °C (19) followed by trypsin (E:S 1:20) for 30 min at 37 °C. The reaction was quenched by addition of 50 μl of 0.1% trifluoroacetic acid in water.

Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)—MALDI-MS was performed at the mass spectrometry facility at the Medical University of South Carolina, Charleston. The RLF analog (1 μg), dissolved in 1 ml of 0.1% trifluoroacetic acid, or 1 μl of the enzymatic digest was mixed with 3 μl of α-cyano-4-hydroxycinnamic acid (50 mg in acetonitrile/water 4:1 v/v), and 1 μl was spotted on a sample plate. Mass spectra were acquired on an Applied Biosystems (ABI) Voyager DE-STR instrument in the linear mode.

CD Spectroscopy—RLF derivatives (~0.5 mg/ml) were dissolved in water and the protein content determined by UV spectroscopy, using calculated absorbance coefficients. An aliquot of 25 μg of RLF analog was diluted to 300 μl with a Tris/HCl buffer (pH 7.5; 25 mM final concentration) for spectroscopic analysis. A Jasco J710 spectropolarimeter was used to acquire far UV circular dichroism (CD) spectra at a resolution of 0.2 nm and a bandwidth of 2 nm. A cell of 0.1-cm pathlength was used, and 10 spectra were averaged. Molar ellipticity was calculated according to the literature (20).

Cell Culture—Stable cell cultures (293T/17), transfected with LGR8-pDNA3.1.zeo, were established using TransFast transfection reagent (Promega) according to the manufacturer’s protocol. The selected clone expressed 8600 receptors/cell and was used between passage 20 and 45. Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml zeocin (Invivogen, San Diego, CA) in a water-saturated atmosphere containing 5% CO2 at 37 °C. Cells were detached with trypsin/EDTA (Invitrogen) and 107 cells used to seed a 75-cm2 culture flask.

Receptor Binding Assay—Transfected 293T/17 cells were grown to 80% confluence and then dislodged with 0.5 mM EDTA, pH 7.5, (1 ml/10 ml of conditioned medium) for 10 min at 37 °C. Cells were collected by centrifugation at 2000 × g for 10 min, suspended two times in 1 ml of ice-cold binding buffer (20 mM Hepes, pH 7.5, 1% bovine serum albumin, 0.1 mg/ml lysis, 1.5 mM CaCl2, 50 mM NaCl, 0.01% NaN3), and recentrifuged at 4 °C for 10 min at 2000 × g. The pellet was reconstituted to 25 × 106 cells/ml of binding buffer. For binding assays 0.025 pmol (100,000 cpm) of A125I-Tyr-9 hRLF (21) in 20 μl of binding buffer was placed into a 1.5-ml Eppendorf vial together with 40 μl of binding buffer with or without human RLF or human RLF derivative and 40 μl (106 cells) of the cell suspension and incubated at room temperature for 60 min. Thereafter, the cells were diluted with 1 ml of ice-cold binding buffer and collected by centrifugation (10 min at 2000 × g at 4 °C), and the pellet was washed with 1 ml of the same buffer and centrifuged for 10 min at 5000 × g at 4 °C. The tips of the vials containing the pellets were placed in counting tubes and transferred to a γ counter for...
RLF-specific LGR8 Binding

Single replacements

A chain

| 1 | A | A | T | N | P | A | R | Y | C | 10 | L | E | G | C | T | Q | D | L | L | T | L | C | P | Y |

B chain

| 1 | P | T | P | E | M | R | E | K | L | C | G | H | F | V | R | A | L | V | R | V | C | G | P | R | W | S | T | E | A |

Single replacements

\[ \text{A A A A V A A A A A A A A A A A A A} \]

Segmental replacements

\[ \text{A A A A A} \]

![FIGURE 1. Primary structure of human RLF. RLF derivatives with a single amino acid replacement are depicted in bold below or above the native sequence. The syntheses of RLF derivatives reported by us previously are in italics and underlined. Replacements of B chain segments are in plain text. Panel A shows MALDI-MS data of B R20A RLF, for example. The two major peaks correspond to m/z for singly charged (6209.32) and doubly charged (3103.59) molecules. Panel B, MALDI-MS of endopeptidase Glu-C/trypsin digest of B R20A hRLF to identify the disulfide-containing peptides: A(9–19)/B (9–16) = 2186.5 (found 2185.5); A(9–19)/B (8–16) = 2314.9 (found 2313.6); A(9–19)/B (7–16) = 2443.8 (found 2442.7); A(20–26)/B (17–26) = 1763.9 (found 1763.2).} \]

analysis. Total binding was determined in the absence and nonspecific binding in the presence of 32 pmol unlabeled RLF. In a regular assay total binding was \( \approx 50,000 \) cpm and nonspecific binding amounted to <5% of the total binding. Data points were collected in duplicates, and the data of three independent binding assays were pooled and presented as mean \( \pm \) S.D.

Cyclic AMP Assay—LGR8–293T/17 stably transfected with the LGR8 message were seeded in 24-well tissue culture plates at a density of 250,000 cells/well and grown in 500 \( \mu \)l of Dulbecco’s modified Eagle’s medium supplemented with fetal bovine serum (10%) and antibiotics. Twenty-four hours later the conditioned medium was replaced by 100 \( \mu \)l of 3-isobutyl-1-methylxanthine (5 mM) in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin. Following incubation of the cells for 1 h at 37 °C in 5% CO\(_2\), 100 \( \mu \)l of hormone dilution prepared in the same buffer was added. The cultures were maintained at 37 °C in 5% CO\(_2\)/air for 16 h. Thereafter the plates were moved into a –80 °C freezer and kept there for at least 6 h. Prior to the assay, 20 \( \mu \)l of 0.5 M EDTA was added to each well, the plate was incubated for 15 min at 37 °C, and the content transferred into 1.5-ml bullet tubes and heated to >90 °C for 10 min in a water bath. Cell debris was removed by centrifugation at 14,000 rpm for 10 min at 4 °C. The supernatant (100 \( \mu \)l) was treated with acetic anhydride and the 2'-O-

acyl-cAMP concentration determined by radioimmunoassay, using \([^{125}\text{I}]\)succinyl-cAMP-tyrosyl methyl ester (22) (20,000 cpm = 5 fmol) and anti-cAMP antiserum (Chemicon, Temecula, CA). Total response was determined in the presence of 80 pmol (2.5 \( \mu \)g/ml) human RLF, and nonspecific response was determined in the absence of RLF. Assays of hRLF derivatives were compared with human RLF standards run in parallel. Assays were performed in duplicate, and three independent assays were averaged \( \pm \) S.D.

RESULTS AND DISCUSSION

The structural similarity of the relaxin-like factor and relaxin and the limited cross-reactivity of both hormones with the RLF receptor LGR8 (23) suggested that some similarity could be expected in the binding sites. This train of thought was further supported by the fact that the RLF receptor LGR8 has all the binding residues that were positively identified on the leucine-rich repeat region of the relaxin receptor LGR7 (24). RLF has only 1 of the 2 critical arginines seen in relaxin, and an initial survey of potential receptor interaction sites on the surface of the relaxin-like factor revealed that this arginine B16 was an important binding residue although not nearly equivalent to the corresponding arginine in relaxin. The high affinity of RLF for its receptor mandated the involvement of other binding
sites. There was no choice but to survey the remaining B chain by substitution of alanine for potential binding residues. In the process we confirmed that tryptophan B27 is a primary binding site (8). The effect again was limited to two orders of magnitude, ~10-fold less than the effect observed after removal of a binding residue in relaxin (25, 26). The binding kinetics of RLF and LGR8 left no doubt that primary binding residues were not solely responsible for the overall receptor affinity but that multiple low affinity contact regions must exist that would bind LGR8 cooperatively. This working hypothesis could not be secured without extensive alanine scanning (27). Thus, we systematically replaced with alanine residues of the B chain and the surface residues of the A chain (Fig. 1). The RLF residues A1–9, B1–5, and B28–31 were excluded from this study in as much as evidence shown in our earlier report indicated these regions do not affect receptor binding (12).

All RLF derivatives were chemically synthesized and characterized by HPLC, MALDI-MS of the intact molecule (Table 1 and Fig. 1A), and an endopeptidase Glu-C *Staphylococcus aureus* V8/tryptic digest, showing the correct molecular masses and verifying the correct chain orientation in each derivative (Fig. 1B). To evaluate the integrity of the secondary structure each derivative was further characterized by a competitive receptor binding assay by its ability to trigger transmembrane signaling and by CD spectroscopy. Receptor binding and signaling were studied using a 293T cell line stably transfected with human LGR8. This allowed for direct comparison of the binding and signaling events on the same receptor.

### Table 1: Chemical and biological data of RLF derivatives

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Yield&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MALDI-MS&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Binding to LGR8&lt;sup&gt;c&lt;/sup&gt;</th>
<th>cAMP&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Derivatives</th>
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<tr>
<td></td>
<td>Yield&lt;sup&gt;a&lt;/sup&gt;</td>
<td>m/z calc</td>
<td>m/z found</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; S.D.</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>A chain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A R8A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.2</td>
<td>6293.2</td>
<td>6293.2</td>
<td>1.37 ±0.70</td>
<td>100.0</td>
</tr>
<tr>
<td>A Y26A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.0</td>
<td>6201.1</td>
<td>6202.8</td>
<td>1.24 ±0.22</td>
<td>109.9</td>
</tr>
<tr>
<td>B chain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B R6A</td>
<td>13.5</td>
<td>6208.1</td>
<td>6209.2</td>
<td>1.63 ±0.28</td>
<td>84.0</td>
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<tr>
<td>B L9A</td>
<td>8.5</td>
<td>6251.1</td>
<td>6250.4</td>
<td>1.46 ±0.18</td>
<td>93.3</td>
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<tr>
<td>B G11V</td>
<td>10.1</td>
<td>6335.3</td>
<td>6334.4</td>
<td>4.35 ±0.74**</td>
<td>31.4</td>
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<tr>
<td>B H12A</td>
<td>25.4</td>
<td>6272.2</td>
<td>6275.5</td>
<td>1.86 ±0.24*</td>
<td>73.4</td>
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<tr>
<td>B H13A</td>
<td>17.9</td>
<td>6272.2</td>
<td>6282.1</td>
<td>1.12 ±0.52</td>
<td>121.9</td>
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<tr>
<td>B F14A</td>
<td>5.5</td>
<td>6217.1</td>
<td>6217.9</td>
<td>1.49 ±0.34</td>
<td>91.3</td>
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<tr>
<td>B V15A</td>
<td>10.5</td>
<td>6265.2</td>
<td>6266.6</td>
<td>3.23 ±0.91*</td>
<td>42.3</td>
</tr>
<tr>
<td>B R26A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.6</td>
<td>6267.2</td>
<td>6271.9</td>
<td>3.06 ±0.62**</td>
<td>104.0</td>
</tr>
<tr>
<td>B W27A&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.6</td>
<td>6267.2</td>
<td>6271.9</td>
<td>3.06 ±0.62**</td>
<td>104.0</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Yields are calculated on the initial equimolar reaction of partially protected A and B chains. Yields are optimized for hRLF; all other derivatives were synthesized only once.

<sup>b</sup> MALDI-MS, mass data of singly charged ions. Yields are calculated on the initial equimolar reaction of partially protected A and B chains. Yields are optimized for hRLF; all other derivatives were synthesized only once.

<sup>c</sup> Receptor binding assays of human RLF derivatives on LGR8 stable transfected 293T/17 cells. Dose-response curve of the derivative was run in parallel to human RLF standard.

<sup>d</sup> cAMP assays of human RLF derivatives using LGR8 stable transfected T293/17 cells. Dose-response curve of the derivative was run in parallel to human RLF standard.
It is remarkable as well that the binding of B W27A RLF to LGR8 is ~4 times weaker than we reported for the mouse uterine receptor (8).

In previous studies we showed that the orientation of the indole ring is crucial to binding. This can be demonstrated by changing the display of the indole ring as in D-Trp or by forcing it into a different space by inserting D-proline in position B25 (8). Lastly we modulated the affinity of RLF for the receptor by restricting the internal motion of the C-terminal area by cross-linkers of different lengths (11).

To account for the picomolar affinity constant of the hormone receptor complex, other, less conspicuous binding regions must support cooperatively the binding effects of tryptophan B27, arginine B16, and valine B19. Our work revealed that surface residues His B12, His B13, and Val B15 might induce ancillary binding of significant magnitude although each contributes little to the binding energy. Replacing the tetrapeptide B (12–15), HHFV, by tetra-alanine reduces receptor binding ~46-fold, whereas replacement of 1 additional residue B (11–15), GHHFV, by penta-alanine reduces LGR8 binding 1000-fold. This is by far the most significant attenuation of RLF binding observed as a consequence of amino acid exchanges. The binding affinity drops below an estimated additive affinity for these derivatives by a factor of 15 for the replacement of HHFV and a factor of 110 for the replacement of GHHFV.

Although we are exchanging helix-tolerant residues for typical helix formers, the drastic drop in binding propensity may be exacerbated by structural changes that affect more distant regions of RLF.

Signal transduction as determined by the accumulation of cAMP parallels in general the binding avidity of the corresponding analog (Table 1 and Figs. 2B and 3, B and D). All A chain analogs and most B chain analogs are full agonists. Variations in the B chain are due to reduced binding avidity, i.e., B(11–15)Ala, RLF is de facto inactive because of its very low receptor affinity. Other weakly binding derivatives B(12–15)Ala, B R16A, and B W27A RLF achieve the maximum cAMP response at high concentrations (Fig. 3B). Apparent differences between binding and cAMP response for B L9A and B F14A RLFs are not statistically significant.

In general, all derivatives achieve a maximum cAMP response as determined for hRLF with the exception of B V19A RLF, which gives a maximum response of ~50%. Although valine B19 is thought to be a receptor binding residue, the inferior binding of B V19A RLF to LGR8 may cause subtle structural changes that reduce receptor activation, making it weakly antagonistic. Hall et al. (29) postulated that RLF by itself is only a partial agonist. Although it is tempting to attribute this antagonist effect to a conformational orientation of valine (B19), its role in signal transduction, if any, requires further investigation.

It seems justified to assume that the proposed binding residues B16, B19, and B27 are in contact with the large leucine-rich repeat ectodomain of LGR8 and that all residues promoting the transmembrane signal transmission are in the proper configuration. This would be consistent with a two-binding site receptor model with RLF binding to the high affinity site on the ectodomain of LGR8 such as to initiate signal transduction (29).

Thorough screening of the RLF surface has not revealed the identity of a single side chain as a cause of transmembrane signal transmission. LGR8 inhibitors require a deletion or replacement of larger domains, like the removal of the N-terminal octapeptide of the A chain (12).

The data presented here provide a first approximation to a binding configuration of RLF. Upon LGR8 binding the 3 strongly interacting residues B16 arginine, B19 valine, and B27 tryptophan could form either an extended structure or the 3 residues could join to form a contiguous area, particularly if the C-terminal end of the B chain can fold back onto its mid-region.

Such a binding structure is supported by our preliminary NMR studies, which imply that the side chains of B15, B19, and B27 are in contact with the large leucine-rich repeat ectodomain of LGR8 and that all residues promoting the transmembrane signal transmission are in the proper configuration. This would be consistent with a two-binding site receptor model with RLF binding to the high affinity site on the ectodomain of LGR8 such as to initiate signal transduction (29).

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The mobility of tryptophan B27 depends largely on a hinge region provided by the Gly-Gly-Pro (B23–B25) sequence. The role of proline B25 was first established by replacing t-proline by its D-enantiomer, which caused a significant loss of binding avidity, presumably by forcing the indole ring of tryptophan into a different space (8). Although alanine appeared to be tolerated in this position, as shown by binding studies using a...
The affinity of B P25A RLF to LGR8 is reduced to 29% (Fig. 3C) and cAMP accumulation to 45% (Fig. 3D). In 2001 Lim et al. (30) discovered a B P25S mutant in a patient with cryptorchidism and proposed that this point mutation caused testicular retention. Indeed, B P25S RLF reduced LGR8 binding to 3.3% of the native molecule (Fig. 3C) and signal transduction to 14% (Fig. 3D), probably by changing the flexibility parameters of the B chain, and the remaining function (31) may not be sufficient to support the physiological action of RLF. The relatively high incidence of cryptorchidism compared with the generally low frequency of mutations, however, suggests that this is not the major pathogenic event in our population.

Significant changes in affinity were by and large not accompanied by perturbation of the secondary structure. Thus, replacement of binding site residues arginine B16, valine B19, or tryptophan B27 by alanine did not change the secondary structure (Fig. 4A). On the other hand, analogs A G14I, B L9A, and B F14A RLF (Fig. 4B) gave rise to CD signals that were significantly reduced and the spectra blue shifted by 1 nm for B L9A, 2 nm for A G14I, and 2 nm for B F14A RLF, respectively. The three-dimensional structure implies that these residues are part of or in proximity to the A chain loop A(10–15) whereby B9 and B14 are also part of the hydrophobic core. Leucine B9 is in a “turn structure” N-terminal of the B chain helix (B12–22) and glycine A14 is in a turn structure N-terminal of the A chain helix (A17–24). Either one of the residues may destabilize the α-helix it precedes. Located within the area is glycine B11, which when replaced by valine, caused only a small change of the CD signal (Fig. 4C). Spectral changes not withstanding, B L9A and B F14A RLF show ~100% LGR8 binding and a slight but insignificant reduction in cAMP response, whereas A G14I RLF has an intermediate affinity and cAMP activity of ~50%. It is surprising that the core residue valine B21 can be replaced by alanine without loss of binding affinity, signal transmission, or changes in the CD spectrum, yet our observation that protein analogs in which the secondary structure is severely changed can retain significant bioactivity is in agreement with observations described for B F24G insulin (32, 33).

Adaptation of RLF binding site conformation to the receptor surface might be a critical criterion for protein interaction, and therefore flexibility could be an important factor. Based on B F14A RLF we succeeded in partially increasing the helix content by multiple alanine replacements in the B chain sequence 11–15. A comparison of the two analogs B(11–15)Ala₅ and B(12–15)Ala₄ RLF with the parent hormone and B F14A RLF is...
Our previous studies (8, 11), together with the current data, confirm that a major feature in RLF functionality is conformational adaptation. This is different for relaxin receptor binding, which depends upon the integrity of 3 residues RXRXRXI(V) on the B chain helix and the presence of a glycine (A14) within the A chain loop (26, 28). The replacement of one of the critical B chain helix arginines or the replacement of isoleucine by alanine caused the loss of receptor affinity by three orders of magnitude. This was originally reported using the mouse brain receptor (25, 26) but is now also observed for the human relaxin receptors LGR7 and LGR8. For relaxin-LGR7 binding we showed that the large contribution of the arginines to the binding energy is due to their chelation by two carboxyl groups (24). These relaxin binding residues as identified on LGR7 are also present on LGR8 but do not seem to play the important role in the cross-reactivity. Comparing the relaxin and RLF sequences it appears that the first arginine on the B chain helix is different. RLF and relaxin must interact differently with LGR8 because histidine B12 in RLF (corresponding to arginine in relaxin) has no effect on receptor binding and can be replaced either by asparagine, arginine, phenylalanine, or alanine while retaining full receptor avidity (data not shown). The replacement of the second arginine (B16) reduces binding but only by a factor of 25, not 1000-fold as observed for relaxin. The same holds true for the proposed hydrophobic interaction site where the valine-alanine exchange in position B19 reduces affinity for LGR8 by a factor of 20 in contrast to a factor of 1000 for relaxin receptor interaction.

One could consider that relaxin interacts with LGR8 using the RLF mode of binding because all 3 residues in the hotspot of the RLF receptor binding site are present in relaxin in the proper sequence. However, tryptophan at the C-terminal end of the B chain is unable to assume the receptor binding conformation in human relaxin-2 (34) and in human relaxin-3 (35) because in both cases there is a serine in a position corresponding to proline B25 in RLF. Although in human relaxin-3 the Gly-Gly-Ser sequence allows bending of the B chain back into the vicinity of valine B19, subtle differences of the interaction site may prohibit its LGR8 binding. As mentioned before, serine in position B25 reduces receptor affinity and may be one possible cause of cryptorchidism in man (30). In addition to the modest contribution of distinct B chain residues to LGR8 binding, receptor interaction is relatively insensitive to changes in the A chain loop. Exchange of glycine A14 for isoleucine, which inactivates relaxin (28), has little effect on the RLF-LGR8 interaction.

In summary, the data presented in this report indicate that RLF binds its receptor by multiple contacts in the central portion of the B chain, supported by tryptophan B27, which is moved into the proper binding coordinates by an induced bend in the C-terminal end of the B chain. This is quite different from the way LGR8 binds relaxin, and it seems intriguing that the two binding sites on LGR8 overlap to the point that RLF and relaxin can be displaced from LGR8 with an excess of either ligand. It is recognized that, despite a less concise defined RLF binding site on LGR8 the interaction is sufficiently precise to force the signal activation site at the N-terminal end of the molecule into its functional position.
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The Mode of Interaction of the Relaxin-like Factor (RLF) with the Leucine-rich Repeat G Protein-activated Receptor 8
Erika E. Büellesbach and Christian Schwabe

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