Synthetic Cross-Links Arrest the C-Terminal Region of the Relaxin-like Factor in an Active Conformation†

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ABSTRACT: All kinetic indicators suggest that the receptor-binding site of the relaxin-like factor (RLF) is located in the flexible C-terminal region of the B chain and is centered about the tryptophan in position B27. Conformational restraints of varying lengths were used to confine Trp (B27) to a narrow range of positions relative to the C terminus of the A chain. Total synthesis of variants involving residues proximate to Trp (B27) assured us that none had a role in receptor binding. Even arginine B26, next to the important tryptophan, can be replaced without deleterious effects. To fix the distance between the C-terminal end of the A chain and Trp (B27) at predetermined lengths, we synthesized RLF with covalent cross-links between a lysine, which was placed in position B26, and the α-carboxyl group at the C terminus of the A chain (A26). The affinity of the cross-linked ligands for the RLF receptor varied as a parabolic function of length whereby the range of 10.0−11.1 Å provided the closest approach to the binding conformation. Wild-type transmembrane signaling activity, as determined by cAMP accumulation, was reached only with a glycine cross-linker.

Male mice lacking a functional RLF gene retain the testes in the body cavity and are infertile (*1*, *2*). The relaxin-like factor (RLF)[1], also named Leydig insulin-like peptide (Ley I-L) or insulin-like peptide 3 (Insl3), which mediates testicular migration, is a circulating proteohormone (*3*) produced by testicular Leydig cells (*4*) and, in smaller amounts, by ovarian theca cells (*5*). According to the prevailing view, RLF acts on the gubernaculum through RLF receptors and causes remodeling of the tissue to facilitate the intra-abdominal migration of the testes (*6*). However, RLF receptors were also identified on cells of the uterus (*7*), gubernaculum (*8*), and brain (*7*). Expression of the purported RLF receptor gene in the mouse (GREAT) (*9*, *10*) and in humans (LGR8) (*11*, *12*) has been verified in these target tissues.

Structurally and functionally RLF is related to relaxin. Our investigations with synthetic hormones provided the first evidence that relaxin and RLF cross-react at the receptor level (*7*). The corresponding receptors are similar to each other and distinct from insulin/IGF-type-1 receptors (*13*−*15*). The recent cloning of relaxin receptors LGR7 and LGR8 (*11*) and the high affinity of LGR8 for RLF (*10*, *12*) support these earlier findings and thus justify the name relaxin-like factor (*7*).

Analogy modeling based on the X-ray coordinates of relaxin (*16*) suggests that RLF can assume a relaxin-like conformation in particular in the region confined by the cystine bonds. The conformations of the N- and C-terminal portions of RLF are less predictable (*17*). Located within the region of structural ambiguity, in the C-terminal region of the B chain of RLF, is tryptophan B27, which, according to our previous report, is an important residue for receptor binding (*17*). In the present paper we show that the restriction of Trp (B27) motion in the C-terminal region of RLF by cross-linkers of various lengths influences the activity of the hormone in a predictable way.

EXPERIMENTAL PROCEDURES

Materials

Fmoc- and Boc-amino acid derivatives were obtained through Advanced ChemTech (Louisville, KY), Novobiochem (San Diego, CA), or Bachem (King of Prussia, PA). Boc-Leu-S-3-(mercaptopropionic acid) ester was synthesized as described (*18*), and Boc-Cys(Trt) was produced from cysteine hydrochloride, introducing first the thiol protecting group (*19*) followed by amino protection (*20*). Azido-(ε-Fmoc)lysine was synthesized according to Lundquist (*21*). High-purity solvents (Burdick and Jackson) were used for synthesis and chromatography. Reagents for peptide synthesis were purchased through Applied Biosystems. All other chemicals were ACS grade.

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[1] Abbreviations: Ahx, ε-aminohexanoic acid; βAla, β-alanine; Boc, *tert*-butyloxycarbonyl; But, *tert*-butyl; CD, circular dichroism; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate; Fmoc, 9-fluorenylmethyloxycarbonyl; Gaba, γ-aminobutyric acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(butanesulfonic acid); HPLC, high-performance liquid chromatography; IGF, insulin-like growth factor; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; MES, 2-(morpholino)ethanesulfonic acid; NEV, microcanonical ensembly at constant energy and volume; Nle, norleucine; NMR, nuclear magnetic resonance spectroscopy; PyBop, benzotriazol-1-yloxytrispyrrolidinophosphonium hexafluorphosphate; RLF, relaxin-like factor; Tris, tris(hydroxymethyl)aminomethane; Trt, triphenylmethyl.

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Methods

Peptide Synthesis. The RLF derivatives were obtained by site-directed sequential synthesis of the three disulfide bonds as previously described (*7*, *17*). The synthesis of RLF derivatives, incorporating an additional interchain iso-peptide cross-link, was based on segment condensation by native ligation (*22*, *23*). Two segments, one branched peptide in which the RLF B chain is linked to the C terminus of the A chain and one A chain peptide A(1−23), were synthesized by solid-phase chemistry.

The branched peptide was assembled on Rink amide MBHA resin (Novobiochem (San Diego, CA)) by Fmoc chemistry. All side-chain-protecting groups were trifluoroacetic acid-labile. Tryptophan and methionine remained unprotected.

The C-terminal segment (0.25 mmol) [Boc-Cys(Trt)-Pro-Tyr(But)-X-]-N_3-Lys-Trp-amide resin (X = none, or an ω-amino acid) was synthesized on a shaker using dicyclohexylcarbodiimide/1-hydroxybenzotriazole in DMF for carboxyl group activation. A 4-fold excess of activated amino acid over resin-bound amine was used for condensation for 2 h at room temperature or until the ninhydrin test (*24*) was negative. Condensation to proline was routinely run overnight. The resin was washed with 2-propanol (2×), methylene chloride (2×), and DMF (2×), and the Fmoc group removed by treatment with 20% piperidine in DMF for 2 min, followed by a second reaction for 8 min. The resin was washed with DMF (3×), methylene chloride (3×), and DMF (3×) before the next condensation reaction was conducted.

The reduction of the *N*α-azido group in position B26 (0.55 g of resin, 0.25 mmol) was achieved with 6 equiv of trimethylphosphine (1 M in toluene, Aldrich) in 5 mL of dioxane/water (4:1 v/v) at room temperature. After about 8 h when N_2 development had ceased (*21*, *25*), the resin was washed with dioxane and methanol and dried in vacuo. The B chain was further elongated by three amino acids using PyBop/DIEA (*26*) for condensation and piperidine/DMF (1:1 v/v) containing 1% Triton X-100 (2 × 10 min) for Fmoc group deprotection in each cycle (*27*). Thereafter, the synthesis of the B chain (0.1 mmol) was completed on an automated peptide synthesizer (ABI-433A, Applied Biosystems) with standard Fmoc chemistry. The peptide was deprotected with trifluoroacetic acid, phenol, water, ethanedithiol, and thioanisole (10:0.25:0.5:0.75:0.5 v/v/v/w/v) (*28*) for 2 h at room temperature, the resin filtered off, the peptide precipitated with ice-cold ether and collected by centrifugation, and the pellet washed three times with ether. The crude peptide was air-dried and then purified by preparative HPLC (yield 30.4 mg).

The A chain segment A(1−23) was synthesized by Boc chemistry on an ABI 433A peptide synthesizer. The C-terminal amino acid Boc-Leu-S-CH_2CH_2COOH (*18*) was condensed to 4-methylbenzhydrylamine resin (Advanced ChemTech). The side chains were protected by HF-labile groups except for the cysteines, which were acetamidomethyl-protected. Dicyclohexylcarbodiimide/1-hydroxybenzotriazole was used for activation with cycles modified such as to omit the base addition at the end of the condensation reaction. The corresponding peptidyl thioester was released by treatment with HF (5% *m*-cresol, 60 min, 0 °C) and purified by preparative HPLC (yield 131 mg, 10.5%). Prior to use, the acetamidomethyl groups were removed by treatment with iodine in 50% acetic acid and the peptide was desalted by gel filtration on Sephadex G25 sf in 1 M acetic acid and lyophilized.

Native ligation was performed essentially as described in the literature (*23*). For example, 4.8 μmol of the A chain segment and 600 mg of guanidinium chloride were dissolved in 500 μL of 250 mM phosphate buffer at pH 7.4. This solution was added to 3.3 μmol of the branched B chain peptide, followed by addition of a freshly prepared mixture of 25 μmol of tris(2-carboxyethyl)phosphine hydrochloride, 100 μmol of 3-mercaptopropionic acid, and 200 μmol of *N*-methylmorpholine in 105 μL of water. The reaction was performed at room temperature for 48 h. The peptides were separated by reversed-phase HPLC (yield 8.7 mg), the product was dissolved in 5.6 mL of water and 2.2 mL of 200 mM MES buffer (Sigma) at pH 6.5, and 870 μL of DMSO was added (peptide concentration 1 mg/mL). After 2 days the final peptide was isolated by preparative HPLC on a Jupiter column (C18, 5 μm, Phenomenex, 10 mm × 250 mm) and the resulting product lyophilized (yield 1.0 mg).

Characterization of the RLF Analogues. (1) Analytical HPLC. The homogeneity of all peptides was confirmed by analytical HPLC in two systems. For both systems the mobile phase consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 83% acetonitrile (solvent B). In the first system samples of about 20 μg were separated on a Bakerbond wide-pore C_{18} column (4.1 × 250 mm), using a 30 min linear gradient from 20% to 60% B at a flow rate of 1 mL/min. UV absorbance of the effluent was monitored at 220 nm. In the second HPLC system samples of 1 μg were separated on an Aquapore 300 column (C8, 2.1 mm × 30 mm) with a 60 min linear gradient from 20% to 40% B at a flow rate of 100 μL/min. The UV absorbance of the effluent was measured at 230 nm and recorded.

(2) Amino Acid Composition. Peptides were hydrolyzed in vapor-phase 6 M HCl (containing 0.1% phenol) for 1 h at 150 °C. The amino acids were modified with phenyl isothiocyanate and separated by HPLC (Pico·Tag system, Waters).

(3) Mass spectrometry was performed at the mass spectrometry facility at the Medical University of South Carolina. For matrix-assisted laser desorption ionization mass spectrometry a Perceptive Voyager DE was used. The RLF analogue (1 μg/μL in 0.1% trifluoroacetic acid) was mixed with 3 μL of α-cyano-4-hydroxycinnamic acid (50 mM in 80% acetonitrile), and 1 μL was spotted on a sample plate.

(4) Protein determination was performed by UV spectroscopy using an Olis Cary-15 spectrophotometer conversion (On-Line Instrument Systems, Inc.). RLF analogues (0.2−0.5 mg/mL) were dissolved in water. The specific absorbance was calculated with molar absorbance coefficients of 1.34 M^{-1} cm^{-1} for tyrosines and 5.6 M^{-1} cm^{-1} for tryptophan.

(5) CD Spectroscopy: CD spectra were acquired on a Jasco J710 spectropolarimeter at a resolution of 0.2 nm, and 10 spectra were averaged. For far-UV/CD spectroscopy (250−190 nm) RLF analogues were dissolved in 25 mM Tris/HCl (pH 7.5) at a concentration of 0.083 mg/mL and transferred to a cell of 0.1 cm path length. Molar ellipticity was calculated according to the literature (*29*) considering the mean residual mass of each derivative.

(6) Fluorescence Spectroscopy. Measurements were made on a Hitachi F-2500 fluorescence spectrophotometer. Proteins were dissolved at a concentration of 0.1 mg/mL in 25 mM phosphate buffer at pH 7.4. Emission fluorescence spectra were recorded by scanning from 300 to 450 nm with fluorescence excitation at 295 or 280 nm. All experiments were performed at ambient temperature.

(7) Receptor-binding Assays on Uterine Crude Membranes of estrogen-primed mice were performed as previously described (*17*). The dose−response of competitive [^{125}I]Tyr (A26)-RLF binding was evaluated for each derivative and compared with that of human RLF measured in parallel.

(8) Assays of Receptor Binding to LGR8-Transfected COS-7 Cells. The LGR8 construct (*12*) was a gift from Dr. Hsueh (Department of Obstetrics and Gynecology, Stanford University School of Medicine). COS-7 cells were stably transfected using TransFast reagent (Promega) according to the manufacturer's recommendations. Stably transfected COS-7 cells, expressing the LGR8 receptor, were seeded at a density of 5×10^4 cells/well into 24-well tissue culture plates. Cells were grown to 90% confluence in Dulbecco's modified Eagle's medium (Gibco, 11995-065), supplemented with 10% fetal bovine serum, 100 international units/mL penicillin, and 100 μg/mL streptomycin. Cells were chilled on ice for 1 h, the conditioned medium was removed, and the cells were washed 3× with 0.5 mL of ice-cold binding buffer (20 mM Hepes, pH 7.5, 1% bovine serum albumin, 0.1 mg/mL lysine, 1.5 mM $CaCl_2$, 50 mM NaCl, 0.01% NaN_3). Increasing doses of RLF or RLF derivatives were added in 0.18 mL of binding buffer followed by 20 μL of [^{125}I]Tyr (A26)-RLF (100 000 cpm), and the assay was incubated for 1 h at room temperature. Thereafter, the medium was removed and the cells were washed three times with 0.5 mL of binding buffer. Attached cells were released by treatment with trypsin/EDTA (Gibco) (0.2 mL) for 10 min at 37 °C. Next 0.5 mL of phosphate-buffered saline was added and the suspension transferred to counting tubes for γ counting. Each data point was done in duplicate, and three independent experiments were averaged. The data presented are the mean ± standard error.

(9) Cyclic AMP Assays. Stably transfected LGR8 COS-7 cells were prepared as described above and seeded in 24-well tissue culture plates. When confluence reached about 90%, the conditioned medium was replaced by 100 μL of 3-isobutyl-1-methylxantine (2.5 mM) in complete culture medium and the cells were incubated for an additional 30 min at 37 °C. Thereafter, 100 μL of the hormone in complete medium, containing 2.5 mM 3-isobutyl-1-methylxantine, was added and the incubation continued for 16 h at 37 °C in a water-saturated atmosphere of 5% CO_2. The tissue culture plates were then transferred to a −80 °C freezer. For assays the plates were thawed and the cold conditioned medium transferred to 1.5 mL bullet tubes and heated to >90 °C for 15 min in a water bath. The concentration of the 2′-*O*-acetylated cyclic AMP was determined by RIA using [^{125}I]-succinyl-cAMP-tyrosyl methyl ester (*30*) and anti-cAMP antiserum (Chemicon, Temecula, CA) according to the manufacturer's recommendations. Assays were performed in triplicate, and three assays were averaged (±standard errors).

(10) Molecular Modeling. Sybyl software version 6.8 (Tripos, St. Louis, MO) was used for analogy modeling. From the X-ray structure of human relaxin (Protein Data Bank file 6rlx) the monomer that showed tryptophan B28 was selected, and the crystal water was deleted. Residues were exchanged one at a time to the corresponding residue of human RLF. For the dipeptide extension the coordinates of the corresponding residues of IGF1 were used (Protein Data Bank file 1HO2). All hydrogens were added in this starting structure, which was subsequently modified as follows.

(i) Control. The C-terminal carboxyl groups were capped as amides.

(ii) Directly Cross-Linked RLF. Arg (B26) was replaced by Nle, and the C termini were amidated. A cross-link was formed between the amide of Tyr (A26) and the methyl group of Nle in the trans conformation.

(iii) RLF Cross-Linked through Gly. To the C terminus Tyr (A26) was attached a glycine in a reversed turn 1 conformation. The C termini Gly (A27) and Trp (B27) were amidated, the torsion angle Tyr (A26) C(O)−NH−C(α)−C(O) was adjusted to 120°, Arg (B26) was replaced by Nle, and a *trans*-amide bond was formed, replacing a hydrogen on Gly-amide and a hydrogen on the methyl group of Nle.

(iv) RLF Cross-Linked through β-Ala or Gaba. The C terminus of Trp (B27) was amidated, and Tyr (A26) was extended by either Asn or Gln in a reversed turn 1 conformation. The torsion angle Tyr (A26) C(O)−NH−C(α)−C(*β*) was adjusted to 120°, Arg (B26) was replaced by Nle, and a trans peptide bond was formed by replacing an H in the Asn (or Gln) side chain and a hydrogen of the methyl group of Nle. Thereafter, the α-carboxyl group was deleted.

(v) RLF Cross-Linked through Aminohexanoic Acid (Ahx). Both C termini were amidated. The methyl group of Nle-amide was joined to the amide of Tyr (A26) under formation of a trans peptide bond. The torsion angle Tyr (A26)-amide N(α)−C(α)−C(O)−N(*ϵ*) was adjusted to 42.6° and the torsion angle Tyr (A26)-amide C(O)−N(*ϵ*)−C(*ϵ*)−C(*δ*) was adjusted to 120°, matching the corresponding torsion angles of the Gly-cross-linked RLF. Arg (B26) was replaced by Nle and a new *trans*-amide bond formed by replacing a hydrogen of the methyl group of Nle (B26) and a hydrogen of the amide group in Nle-amide (positioned at A27). The α-amino group of Nle (A27) was removed last.

All RLF molecules were treated identically. First, in the presence of all the hydrogens the conformations of prolines, amide side chains, and all other side chains were fixed. Then all hydrogens were removed. Energy minimization was performed in two steps. First, the subset including Tyr (A26)-amide, Arg (B26), and Trp (B27)-amide and the cross-linking residue was minimized using the Tripos force field for 100 iterations including a 20 step simplexing. Finally, Kollman charges were loaded, and the Kollman united force field was used to minimize the molecule for 1000 steps. The peptide bonds in the resulting macrocyles were all in the trans conformation.

The dynamics of RLF and the cross-linked RLFs were studied in vacuo using an interval of 2000 fs at a temperature of 300 K in steps of 1 fs. Kollman charges were used in combination with the Kollman united force field. The initial velocity was set on "Boltzmann", and the microcanonical ensembly at constant energy and volume (NEV) was used.

Table 1: Human RLF Derivatives with Variations at the C Termini of the Two Chains and Single-Residue Replacement

derivative	B chain ends		A chain ends		receptor[a]	
	position	group	position	group	IC_{50} (SE) (%)	*m/z* (calcd)[b]
hRLF[c]	31	OH	26	OH	100.0 (±15.7)	6293.2 (6293.3)
hRLF	26	OH	26	OH	0.8 (±0.02)	5718.8 (5718.6)
hRLF	27	OH	26	OH	5.4 (±0.94)	5904.4 (5904.9)
hRLF (B27)-amide	27	NH_2	26	OH	84.1 (±8.6)	5904.5 (5903.9)
hRLF (A26)-amide	31	OH	26	NH_2	59.7 (±4.8)	6291.5 (6292.2)
desA(25−26)-hRLF	31	OH	24	OH	12.5 (±0.94)	6032.2 (6032.9)
Ala (B26)-hRLF	31	OH	26	OH	111.0 (±11.0)	6209.7 (6208.1)
Cit (B26)-hRLF	31	OH	26	OH	144.0 (±14.0)	6295.5 (6294.2)
Glu (B26)-hRLF	31	OH	26	OH	130.0 (±11.8)	6265.1 (6266.2)
Leu (B26)-hRLF	31	OH	26	OH	95.0 (±8.3)	6251.1 (6250.2)

[a] Receptor binding was determined using crude membranes of mouse uteri. [b] Mass/charge ratio determined by MALDI-TOF (the calculated value is given in parentheses). [c] For reference the IC_{50} of human RLF is 4.6 ng/mL = 0.73 nM.

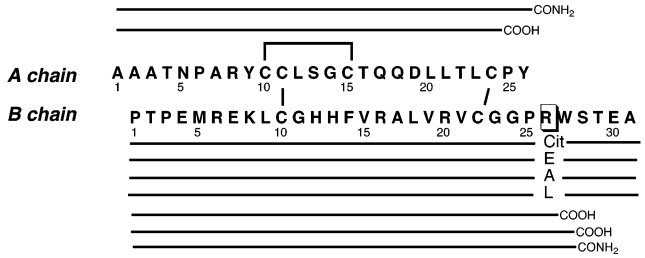

FIGURE 1: Primary structure of human RLF deduced from the cDNA sequence showing the single-point modifications performed in the present study.

RESULTS

We synthesized C-terminally truncated RLF to elucidate the minimal length required to exhibit full affinity to the mouse uterine receptor (Figure 1, Table 1). RLF, with a B chain shortened to 26 residues, shows less than 1% binding, the lowest level observed so far. RLF ending in tryptophan (B27) retained only 5.4% of the receptor binding, but amidation restored virtually full affinity (84.1%). Amidation of the C terminus of the A chain is fairly well tolerated, showing 59.7% affinity, whereas truncation to cysteine (A24) reduced the affinity to 12.5% (Table 1). On the basis of these experiments the minimal length of fully active RLF consists of a 26-residue A chain and a 27-residue, C-terminally amidated, B chain.

In parallel we investigated the role of residue B26 by replacing arginine (B26) by citrulline, glutamate, alanine, or leucine. The results outlined in Table 1 clearly suggest that the residue in position B26 can be freely replaced without affecting the affinity to the uterine receptor.

To visualize residues surrounding tryptophan (B27), RLF was modeled on the X-ray coordinates of human relaxin (Protein Data Bank file 6rlx). The distance between the two C termini was controlled by a link between position B26 and the C terminus of the A chain. Thus, arginine (B26) was replaced by lysine, and the ϵ-amino group was linked via various ω-amino acids to the C terminus of the A chain. Only when a seven-atom linker, ϵ-aminohexanoic acid, was incorporated was the distance between the two C termini similar to that of the average non-cross-linked structure. The space between the α-carbons of Tyr (A26) and Lys (B26) of the different derivatives varied by more than 4 Å (Figure 2, Table 2).

To determine which cross-linked RLF retained significant receptor-binding affinity, the various constructs were synthesized (Figure 3). The open reduced polypeptide consisted of a B chain with the C terminus of the A chain attached through a linker to the side chain of B26. Interestingly, folding of the final RLF derivative was independent of the length of the cross-linker. Product purity was confirmed by reversed-phase chromatography, and the molecular identity was established by mass spectrometry (MALDI-MS, Table 2).

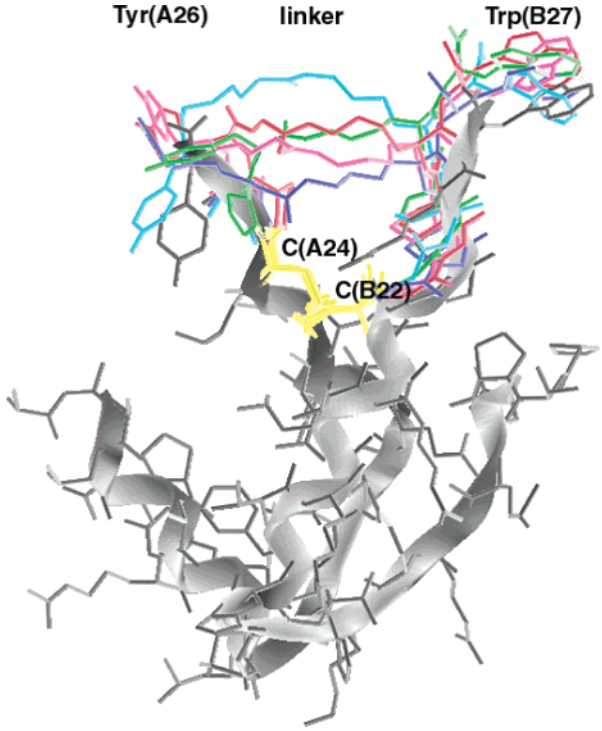

FIGURE 2: Human RLF (B27)-amide modeled on the X-ray structure of human relaxin. The C-terminal sequence forms a macrocycle of different ring size in cross-linked Lys (B26) human RLF. The aromatic rings of Tyr (A26) and Trp (B27) are indicated. Color code: black, RLF (B27)-NH_2; green, direct link (zero-atom linker); magenta, glycine link (three-atom linker); red, β-alanine link (four-atom linker); blue, γ-aminobutyric acid (five-atom linker); cyan, ϵ-aminohexanoic acid (seven-atom linker); yellow, cystine A24/B22.

The environment of tryptophan was explored by fluorescence emission spectroscopy (Figure 4). Upon excitation at 295 nm RLF (B27)-amide and all cross-linked derivatives, except for the directly linked derivative, showed a maximum emission at 349 nm indicative of a polar environment for the indole ring. The directly cross-linked derivative showed a slight blue shift of the maximum (emission maximum at

Table 2: Human RLF Derivatives Cross-Linked between the Side Chain of Lys (B26) and the C Terminus of the A Chain

derivative	linker[a]	distance[b] (Å)	*m/z* (calcd)[c]
hRLF (B27)-amide	none	12.9	5903.5 (5904.9)
direct	zero atoms	8.0	5858.5 (5858.9)
Gly	three atoms	10.1	5915.1 (5915.9)
β-Ala	four atoms	10.4	5928.1 (5929.9)
Gaba	five atoms	11.1	5944.3 (5944.0)
ϵ-Ahx	seven atoms	12.1	5971.7 (5972.0)

[a] The intervening atoms between the ϵ-amino group of lysine (B26) and the α-carboxyl group of Tyr (A26). [b] The distance measured between the α-carbons in positions A26 and B26 of the average structures shown in Figure 2. [c] Mass/charge ratio determined by MALDI-TOF (the calculated value is given in parentheses).

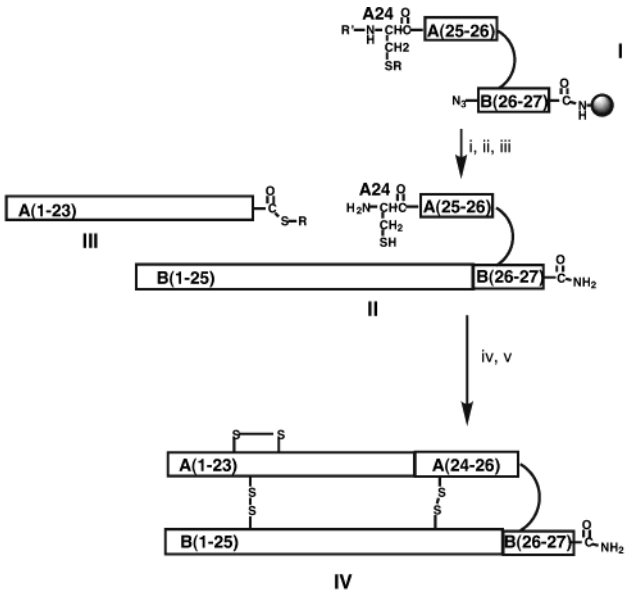

FIGURE 3: Chemical synthesis of C-terminally cross-linked RLF derivatives. **I** was synthesized on amide resin introducing azido-(ϵ-Fmoc)lysine into position B26. The peptide was elongated at the lysine side chain using Fmoc chemistry. First the linker (represented by the curved line) was introduced and extended by the C-terminal tripeptide of the A chain. (R and R′ are trifluoroacetic acid-labile protecting groups). (i) The azido group was reduced to the amine with trimethylphosphine. The B chain was (ii) elongated by Fmoc chemistry and (iii) deprotected with trifluoroacetic acid, yielding product **II**. The A chain peptide A(1−23) thioester (**III**) was synthesized by Boc chemistry on 4-methylbenzhydrylamine resin, and the peptide was deprotected with HF. (iv) Peptides **III** and **II** were reacted at pH 7.5 in the presence of reducing reagents. (v) The native ligation product was folded and oxidized at pH 6.5 in the presence of 10% DMSO, resulting in the final product **IV**.

347 nm) and a significantly quenched signal (54% when compared to that of the open structure). This effect was weaker for the RLF bearing a glycine spacer and disappeared as the distance increased. Fluorescence spectra of RLF containing β-Ala, Gaba, or Ahx were indistinguishable from the spectrum of the native hormone. Excitations at 280 nm resulted in emission spectra similar to the 295 nm spectra except that all derivatives showed identical emission maxima (data not shown).

The CD spectra (Figure 5) of the derivatives showed that none of the cross-links caused a significant structural disturbance when compared to the open structure. The largest difference, a red shift of 0.5 nm at the crossover point, was observed for the directly cross-linked derivative.

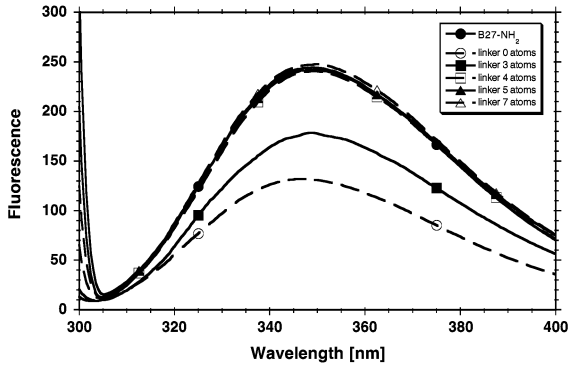

FIGURE 4: Fluorescence spectra at an excitation wavelength of 295 nm of the cross-linked RLF derivatives as compared to the open structure RLF (B27)-NH_2. The protein was dissolved in 25 mM phosphate buffer (pH 7.4) at a concentration of 0.1 mg/mL.

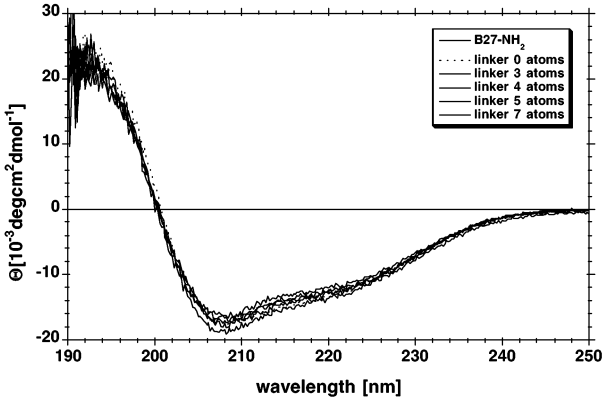

FIGURE 5: Far-UV/CD spectra of the cross-linked RLFs as compared to the open structure RLF (B27)-amide in 25 mM Tris/HCl (pH 7.5) at a concentration of 0.083 mg/mL using a cell of 0.1 cm path length. CD spectra were acquired at a resolution of 0.2 nm and a bandwidth of 2 nm, and 10 spectra were averaged.

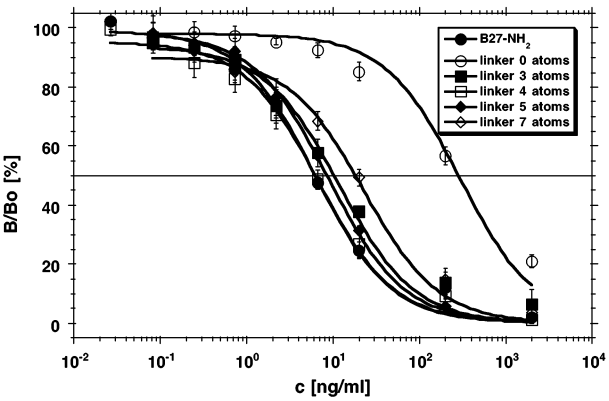

FIGURE 6: Receptor-binding assays on mouse uterine crude membrane preparations of cross-linked human RLF in comparison to the open structure consisting of RLF (B27)-NH_2 (for reference the IC_{50} of hRLF (B27)-amide is 5.9 ng/mL = 1 nM). Each data point was determined in duplicate. Three independent assays were averaged. The bars represent standard errors.

Receptor-binding assays of all RLF derivatives were performed using crude membranes from uteri of estrogen-primed mice (Figure 6). In addition, all cross-linked RLF analogues were tested in a whole cell assay using LGR8-transfected COS-7 cells (Figure 7). The RLF derivatives bearing either a three-atom linker (Gly) or a four-atom linker (β-Ala) retained full affinity in both assays. The RLF derivative bearing a five-atom spacer (Gaba) showed slightly

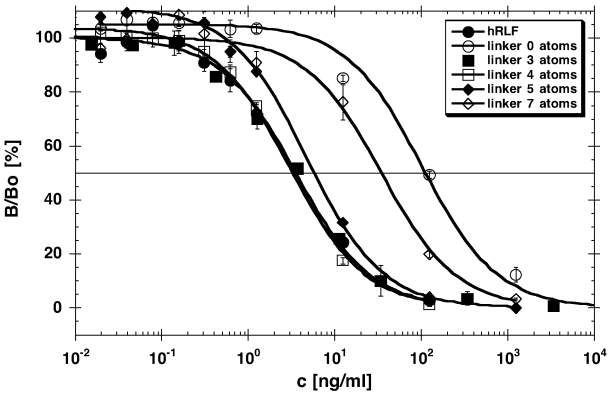

FIGURE 7: Receptor-binding assays on LGR8-bearing COS-7 cells. Cross-linked human RLF derivatives in comparison to human RLF (for reference the human RLF IC_{50} is 3.3 ng/mL = 0.5 nM). Each data point was determined in duplicate. Three independent assays were averaged. The bars represent standard errors.

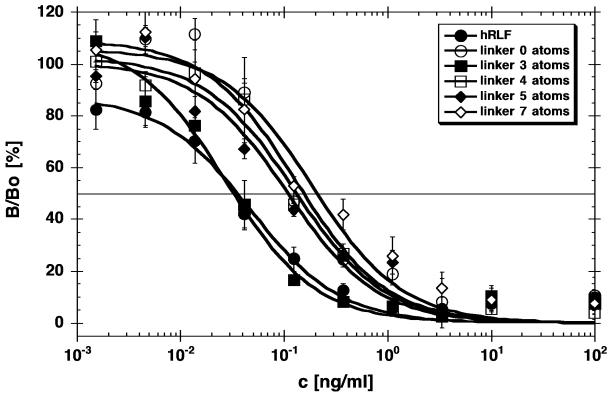

FIGURE 8: RLF-mediated cAMP accumulation in LGR8-bearing COS-7 cells, comparing cross-linked human RLF derivatives with human RLF. In parallel assays dose−response curves of the cross-linked RLF derivative and human RLF were compared (for reference the human RLF EC_{50} is 0.035 ng/mL = 5.6 pM). Assays were performed in triplicate, and three independent assays were averaged (SEM ± SE).

reduced affinities in both binding assays. In contrast, further elongation of the linker to seven atoms or, in the other extreme, directly linking the ϵ-amino group of Lys (B26) to the carboxylate of the A chain caused a drastic reduction in affinity in both RLF receptor-binding assays. While the longer cross-link reduced the affinity to 35% and 15% of that of the control, the shortest one exhibited an affinity of only 2.3% and 3.7%, respectively.

The pronounced differences in receptor binding do not parallel RLF-mediated cAMP production in LGR8-transfected COS-7 cells (Figure 8). While RLF with the glycine cross-link has standard potency, all others elicited a significantly weaker cAMP response (15−30% of that of RLF). Although the two weakest binding derivatives are still the least active, their ratio of activity to binding is reversed. Only the ϵ-aminohexanoic acid-cross-linked RLF shows binding proportional to the cAMP response.

DISCUSSION

Structure/function studies of RLF in our laboratory have led to the identification of Trp (B27) as a crucial residue for receptor binding (*17*). It is clear, however, that additional binding residues in RLF are required to achieve the subnanomolar affinity observed for the RLF−receptor complex (*7*, *12*, *15*). The search for these residues is hampered by the lack of the three-dimensional structure of RLF. While analogy modeling is a valuable aid, it remains difficult to position the active conformation of the indole side chain in relation to other residues. These limitations can be largely overcome by restricting the conformational freedom within the C-terminal end of the protein. The prerequisite was to define the minimum length at the C-terminal end of RLF and to find permissible positions to place a cross-linker. The minimum sequence was an RLF in which the B chain ended in Trp (B27)-amide. Modifications of arginine B26 and the C terminus of the A chain have little influence on receptor binding. Therefore, lysine was placed in position B26 and its side chain connected through a linker to the C terminus of the A chain (A26).

Restriction of the C-terminal chains caused side chain residues Lys (B26) and Tyr (A26) to occupy a formerly open space. It requires a seven-atom spacer to hold the two chains in the "open structure" position. Surprisingly, this configuration leads to 65−85% loss of the binding capacity. On the other hand, direct linking of the A chain C terminus and B26 lysine reduced the distance of the two ends by about 4 Å and led to about 97% loss of receptor-binding activity. Extension of the direct cross-link by insertion of a glycine residue restored nearly full binding intensity. The optimal binding conformation was observed when the distance between the α-carbons of Tyr (A26) and Lys (B26) was between 10.0 and 11.1 Å. Figure 2 shows an overlay of the differently cross-linked RLFs that reveals the average position of side chains as determined by the length of the connector; in particular the positions of the aromatic residues Tyr (A26) and Trp (B27) vary to a large extent.

The cross-linked RLF derivatives have a new 30−37-membered ring in the C-terminal region of the molecule (Figure 3). Tryptophanamide is located outside this macrocycle, thus reducing rotational restraints. The ring structure itself is flexible, and the various methylene groups of the spacers provide additional flexibility within the limits of the torsion angles. With the introduction of a defined linker, we selected a group of conformers, which would be seen only as transient structures in three-dimensional NMR without being recognized as the active conformation. The NMR or X-ray structure of the cross-linked RLF would provide a unique means of establishing an active conformation over and beyond what a three-dimensional structure determination of the native molecule could provide. Active conformation NMR could be used to select significant structures from an envelope of NMR conformations and thus to introduce a functional component (receptor binding) into 3D structure determination.

Molecular dynamic studies clearly indicate that different linkers affect RLF in different ways and that in an average structure the positions of the side chains of the derivatives vary significantly (Figure 2). The effect is confined to the C-terminal macrocycle while the N-terminal structure of the cystine A24/B22 remains unaffected. This prediction was experimentally confirmed by the high similarity of far-UV/CD spectra of all derivatives.

Fluorescence spectra indicated that the active site tryptophan is in a polar environment in the open structure of RLF (B27)-amide and in all cross-linked derivatives except the shortest one. Significant spectral differences imply a

transfer of the tryptophan side chain into a less polar surrounding and the presence of a fluorescence quencher. Fluorescence quenching was still significant for the tryptophan in the glycine-bearing cross-linked RLF and disappeared when the cross-linker was extended to four and more atoms. This energy transfer could be due to the closeness of tyrosine (A26) and tryptophan (B27) side chains. In our model the γ-carbons of the corresponding amino acids are within a distance of 15 and 17 Å in the directly cross-linked RLF and in the glycine-bearing cross-linked RLF, respectively. The distance between the donor tryptophan and acceptor tyrosine correlates well with the critical distance at which the energy-transfer rate equals the decay rate (15 Å) (*31*). Rotation around the C(β) and C(γ) bond is sufficient to appropriately align the aromatic rings for fluorescence quenching. The hydrophobic spacers, in particular ϵ-aminohexanoic acid, do not affect the tryptophan fluorescence.

The effect on receptor affinity changes as a function of the positioning of tryptophan B27 in a predictable way. Thus, without primary sequence changes, the receptor-binding activity of RLF can be modulated by 2 orders of magnitude by fixing in space the position of the binding-site tryptophan. The importance of the C-terminal conformation of the B chain for proper receptor recognition is also emphasized by the extremely low receptor affinity of D-Pro(B25)RLF, which turns the tryptophan (B27) away from the C terminus of the A chain (*17*).

While several conformers bind the receptor, the transduction of the signal as determined by the accumulation of cAMP shows full activity only for the derivative bearing a glycine cross-link. All other cross-linked derivatives are between 15% and 30% active. Clearly, binding is a prerequisite for activity, but conformational changes of the receptor must occur to transmit the signal through the membrane. For this class of receptors it has been implied that residues on exoloop 3 of the seven-membrane-spanning segments are required for signal transduction (*32*, *33*). The present work suggests that the complexed hormone itself might undergo structural changes and that the orientation of the C termini of RLF is an important factor.

RLF, in contrast to relaxin, appears to adjust its backbone conformation upon receptor contact. The dual affinity of the RLF receptor for relaxin and RLF (*7*, *12*) is probably due to the presence of two crucial binding residues such as Trp (B27) and Arg (B16) (*7*, *34*) in both hormones. The differences, however, are sufficient to account for the different affinity of the two hormones for LGR 8, the RLF receptor (*7*).

Structure fixation by covalent cross-linking provides information as to which of the many possible conformers of a flexible hormone actually interacts with the receptor. It has been argued that induced fit requires certain flexibility and that reduction of the degrees of freedom of motion may be detrimental for interaction. Here we show that freezing a binding residue in the correct position in space allows a hormone to bind the target as avidly as the flexible, native ligand.

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