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# Replacement of Disulfides by Amide Bonds in the Relaxin-like Factor (RLF/INSL3) Reveals a Role for the A11−B10 Link in Transmembrane Signaling

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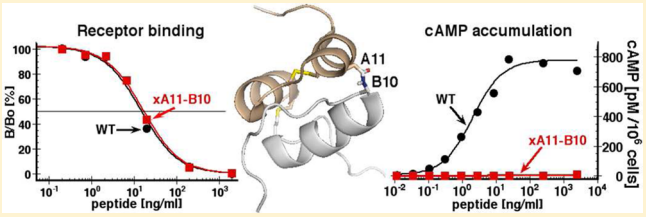
Supporting Information

**ABSTRACT:** The relaxin-like factor (RLF) also named insulin-like 3 (INSL3) consists of two polypeptide chains linked by two interchain and one intrachain disulfide bond. RLF binds to its receptor (LGR8 also named RXFP2) through the B chain and initiates transmembrane communication by activating the adenylate cyclase through the N-terminal region of both chains. Cystine A11−B10 occupies a unique position on the molecular surface just outside the binding region and between the two signaling ports. We have synthesized an RLF analogue in which the disulfide A11−B10 was replaced by a peptide bond and found that cAMP production ceased while receptor binding was not affected. In contrast, replacing the disulfide A24−B22 by a peptide bond reduced potency proportional to the binding affinity and lowered efficacy to 65%, while replacing disulfide A10−A15 by a peptide bond reduced binding affinity to 32% and lowered potency to 7% but maintained 100% efficacy. The exceptional properties of the derivative bearing an A11−B10 isopeptide cross-link suggests that the disulfide has a special role in signal transduction. We propose that disulfide A11−B10 serves as an insulator between the two ports, whereas the amide functionality disturbs the signal transmission complex likely due to changes in polarity. The clear separation between receptor binding and signal activation sites within this small protein permits one to study how the relaxin-like factor initiates the signal on the receptor that induces intracellular cAMP production.




The relaxin-like factor (RLF) is a member of the relaxin−insulin superfamily and is composed of a 26-residue A chain and a 31-residue B chain.[1] Like in relaxin and insulin the two chains are connected by two interchain and one intra-A chain disulfide bond. RLF interacts with the leucine-rich repeat G-protein coupled receptor-8, LGR8 also named RXFP2, and triggers the accumulation of the second messenger cAMP. Synthetic human RLF[2] shows sub-nanomolar receptor affinity and efficacy in cloned LGR8 expressing cell lines.[3]

G-protein coupled receptors are the most abundant and important class of cell-membrane receptors that are involved in many physiological and pharmacological processes. In spite of their significance, little is known about the molecular mechanism and how the extracellular messenger initiates the signal and causes the passage of the signal through the plasma membrane to set off the intracellular response.[4,5]

As concerns the relaxin-like factor and its receptor, LGR8, signaling may be a function of the electronic structure of the hormone. We have identified interacting structures on the hormone that could lead to the relative long-distance induction of intracellular cAMP production. A number of RLF derivatives revealed that the transmembrane signal-initiation site is located within the N-terminal segments of both RLF chains. The signal-initiating site requires L-$\alpha$-amino acids at the purported contact site, but the action is insensitive to the side chain length or shape of the contact residues.[6−8] The fact that the functional structure of the RLF signaling region lies next to the N-terminal interchain disulfide link, A11−B10, and that the actual signal transmission is sensitive to the electronic configuration of the signaling residues suggests that the electronic configuration of the cross-link may be an important factor in signal initiation.

In this paper we report on the synthesis of RLF derivatives in which disulfide bonds, one at a time, are replaced by peptide bonds. We have determined, and are reporting here, that only the disulfide bond A11−B10 connecting the two signal-initiating sites in the N-terminal domains of both RLF chains has a supporting role for the transmembrane signal-initiation function of the relaxin-like factor.

## MATERIALS AND METHODS

**Materials.** Human RLF and desA(1−8)hRLF (= RLFi) were synthesized as described.[2,6] Resins and protected amino acids for Fmoc and Boc peptide chemistry were purchased from AdvancedChemTech (Louisville, KY) or NovaBiochem (Gibbstown, NJ). Reagents and solvents for peptide synthesis were obtained from AdvancedChemTech (Louisville, KY). All other chemicals were of high purity and used without further

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manipulations. LGR8 cloned into the pcDNA3.1.zeo plasmid was a gift from Dr. Hsueh, Department of Obstetrics and Gynecology, Stanford University School of Medicine.[3] The human embryo kidney cell line 293T/17 was obtained from the American Type Culture Collection (ATCC CRL-11286) and stably transfected for LGR8 expression.

**Peptide Synthesis.** Peptides were synthesized on solid support using an ABI-model 433 peptide synthesizer for standard Fmoc chemistry and a manual shaker for Boc chemistry.

*1. Synthesis of RLF-(A11−B10)-isopeptide Amide$^{A26}$ (xA11-B10).* RLF bearing a peptide cross-link between A11 and B10 was synthesized from three partially protected fragments: Asp$^{A11}$A(1−14), A(15−26)amide$^{A26}$, and Dpr$^{B10}$B(1−31).

*1.1. Asp$^{A11}$A(1−14)-S-CH$_2$CH$_2$CO-Leu-NH$_2$ (I).* The C-terminal amino acid thioester Boc-Gly-S-CH$_2$CH$_2$CO-Leu was assembled on 4-methylbenzhydrylamine resin using the procedure described by Hackeng et al.[9] Boc-amino acids were activated using dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt). All side chain protecting groups and the leucine−resin bond were cleaved with HF in the presence of 10% cresol. The HF was evaporated, and the peptide resin mixture was precipitated with ether and filtered off. The peptide was extracted with 1 M acetic acid and purified by HPLC (yield: 279 mg, 34.7%; *m/z*: found 1609.9; calcd 1610.8).

*1.2. A(15−26)(OAll$^{A19}$Acm$^{A24}$)-amide$^{A26}$ (II).* Peptide **II** was synthesized by Fmoc chemistry on Rink-amide MBHA-resin (NovaBiochem) using TBTU/HOBt/DIEA carboxyl activation. Boc-Cys(Trt) was introduced in position A15. All protecting groups except Cys$^{A24}$(Acm) and Asp$^{A19}$(OAll) were TFA labile. Standard TFA deprotection was performed with TFA/water/phenol/ethanedithiol (87.5:5:5:2.5 v/v/w/v) for 2 h at room temperature. Peptide **II** was collected by ether precipitation followed by HPLC purification (yield: 25.1%, *m/z*: found 1529.9; calcd MNa$^+$ 1530.8).

*1.3. Asp$^{A11}$A(1−26)(OAll$^{A19}$Acm$^{A24}$)-amide$^{A26}$ (III).* Native ligation was performed according to the method reported by Hackeng et al. at pH 7.0 for 2.5 h at 37 °C.[9] The reaction was acidified with glacial acetic acid, and thiols were removed by extraction with ethyl acetate followed by desalting on Sephadex G25 in 1 M acetic acid. The lyophilized peptide was dissolved in 50% acetic acid at a concentration of 10 mg/mL and the intrachain disulfide A10−A15 formed by titration with 50 mM iodine in acetic acid. Excess iodine was reduced with 0.1 M ascorbic acid in water. The reaction mixture was diluted with two volumes of water and purified by HPLC using a linear acetonitrile gradient (yield: 70.4%; *m/z*: found 2900.3; calcd MH$^+$ 2900.3).

*1.4. Asp$^{A11}$-A(1−26)(Aloc$^{A1}$OAll$^{A19}$Acm$^{A24}$)-amide$^{A26}$ (IV).* To 16 mg of **III** (5.52 μmol) dissolved in 1.6 mL of DMSO triethylamine (14.92 μL, 0.107 mmol) was added immediately followed by 320 μL of a 50 mM solution of allyloxycarbonyl-*N*-hydroxysuccinimide in DMSO (16 μmol). The reaction was complete within 5 min at room temperature and quenched by addition of 250 μL of acetic acid, diluted with water, and purified by HPLC (yield: 85.6%; *m/z*: found 2983.2; calcd MH$^+$ 2982.4).

*1.5. Dpr$^{B10}$-B(1−31)(bis-Aloc$^{\alpha B1,\varepsilon B8}$O$^{B5}$Acm$^{B22}$)-B Chain (V).* The B chain was synthesized by Fmoc chemistry, and all amino acids were incorporated under standard TBTU/HOBt/DIEA activating and coupling conditions. The three amino groups (B1, B8, and B10) were protected as follows: The TFA-labile Boc group was used for the β-amino group in Dpr$^{B10}$, whereas the allyloxycarbonyl group was used to mask the N-terminal amino group and the ε-amino group in Lys$^{B8}$. Aloc-Pro was synthesized according to Cruz et al.[10] In addition, methionine in position B5 was sulfoxide protected, and the single cysteine in position B22 was Acm-protected. The B chain resin was TFA-deprotected (as described for **II**), the resin was filtered off, and the peptide was precipitated with methyl *tert*-butyl ether and HPLC purified (yield 382 mg, 40.8%; *m/z:* found 3760.30; calcd MH$^+$ 3760.30).

*1.6. Formation of the Peptide Bond between Asp$^{A11}$ and Dpr$^{B10}$ (VI).* The A chain (**IV**) 15.6 mg (5.2 μmol) was dissolved in 500 μL of isopropanol/water 1:1, and 5.72 μL (52 μmol) of *N*-methylmorpholine was added to generate the carboxylate in Asp$^{A11}$ and to neutralize excess TFA. After lyophilization the peptide was dissolved in 125 μL of DMSO and diluted with 125 μL of DMF. The solution was chilled to −10 °C, and the carboxyl group in position A11 was activated by 5.2 μmol of isobutylchloroformate at −10 °C for 5 min. The activated A chain was added to the B chain (**V**) (20.3 mg, 5.4 μmol) dissolved in 500 μL of 0.2 M *N*-methylmorpholine/HCl in DMF/water 4/1 v/v "pH 7.4" and left to react for 12−18 h at room temperature. Thereafter, the reaction mixture was acidified with 50 μL of acetic acid and separated on Sephadex G50 sf in 1 M acetic acid. UV-light-absorbing fractions corresponding to proteins eluting with an apparent molecular mass of 5000−8000 were pooled and further purified by reversed-phase HPLC (yield: 4.2 mg, 12.0%; *m/z*: found 6722.0; MH$^+$ calcd 6723.7).

*1.7. Deprotection of Asp$^{A11}$ and Dpr$^{B10}$ Cross-Linked RLF and Disulfide Synthesis (VII).* First the allyl groups were removed using 0.7 μmol of peptide (= 2.8 μmol of allyl) dissolved in 280 μL of DMSO. A premixed solution of 0.56 μmol of tetrakis(triphenylphosphine)palladium(0) in 100 μL of methylene chloride and 2.8 μmol of sodium boranate in 28 μL of DMF was added, and the reaction was continued under argon for 1 h at room temperature. The reaction was stopped by addition of 1 mL of 20% acetic acid, and the peptide was purified by reversed-phase HPLC. Yield: 1.4 mg, 35.9%.

Next, the acetamidomethyl groups were removed by treatment with iodine in 95% acetic acid.[11] The peptide (5.1 mg, 0.78 μmol) was dissolved in 255 μL of 80 mM HCl, and glacial acetic acid (4.85 mL) was slowly added to reach a final concentration of 95% acetic acid. The reaction was initiated by adding iodine (1.79 mL, 50 mM in 95% acetic acid) and stirred for 1 h at room temperature in the dark. The reaction was quenched with 15.3 mL of 0.1 M ascorbic acid, desalted on Sephadex G25-sf in 1 M acetic acid, and lyophilized. Methionine sulfoxide was reduced as previously described.[12] Peptide at the concentration of 10 mg/mL was reduced with a 15-fold excess of ammonium iodide in TFA/water 9:1 (v/v) for 15 min at 0 °C. The reaction was quenched with 3 mL of 5 mM ascorbic acid and purified by reversed-phase HPLC (yield: 52%, *m/z*: found 6270.56; MH$^+$ calcd 6271.16).

*2. Synthesis of RLF-(A24−B22)-isopeptide Amide$^{A26}$ (xA24−B22).* RLF bearing a peptide cross-link between A24 and B22 was made from three peptides: (1) A1−14-thioester (Acm$^{A11}$); (2) Asp$^{A24}$A(15−26)(OAll$^{A19}$)-amide$^{A26}$ and Dpr$^{B22}$B(1−31)(bis-Aloc$^{B1,B8}$,O$^{B5}$,Acm$^{B10}$)-B chain. The fragments were assembled as described above in similar yields and with similar purities (final product *m/z*: found 6271.01; MH$^+$ calcd 6271.16).

*3. Synthesis of Asn$^{A19}$ RLF-(A10−A15)-isopeptide Amide$^{A26}$ (xA10−A15). 3.1. Aloc$^{A1}$Asp$^{A10}$ Dpr$^{A15}$Asn$^{A19}$ Amide$^{A26}$ (Ac-*


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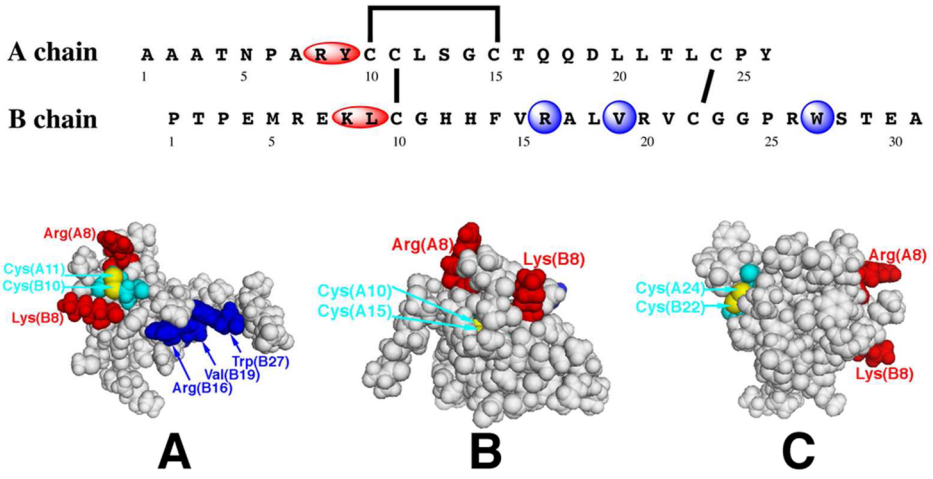


**Figure 1.** Upper panel: protein sequence of human RLF highlighting in red ovals the signal-initiation site. The peptide bond linking the pair of amino acids is the most important feature. The blue circles emphasize the major binding residues. Lower panel: structure pdb 2k6t showing (A) cystine A11−B10, (B) cystine A10−A15, and (C) cystine A24−B22. All structures are color-coded. Red, Arg(A8), Lys(B8) representing the signaling region; cyan one cystine, different in each structure, and yellow one disulfide bridge, different in each structure; blue the binding residues located in front (A) while invisible on the back site for (B) and (C).

*$m^{A11}But^{A24}$) A Chain (**VIII**).* The A chain was synthesized by Fmoc chemistry on Rink-amide MBHA-resin (NovaBiochem, Gibbstown, NJ) incorporating specially protecting amino acids, Aloc-$Ala^{A1}$, $Cys^{A11}$(Acm), and $Cys^{A24}$(*tert*-butyl). All other protecting groups were removed with TFA/scavengers (as described for **II**). The peptide was precipitated with methyl *tert*-butyl ether and purified by HPLC (yield: 143.7 mg, 65.2%, *m*/*z*: found 2983.12; $MH^+$ calcd 2982.39).

*3.2. Isopeptide Synthesis (**IX**).* The A chain (**VIII**) (143 mg, 48 μmol) was dissolved in 14.3 mL of DMSO, and the side chains of Asp in A10 and Dpr in A15 were condensed using benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBop)[13] (144.6 μmol), HOBt (144.6 μmol), and DIEA (434 μmol). The reaction was conducted for 2 h at ambient temperature, quenched with 1.5 mL of glacial acetic acid, diluted with water, and purified by HPLC (yield: 93.5 mg, 65.1%, *m*/*z*: found 2964.89; $MH^+$ calcd 2964.37).

*3.3. Chain Combination via Disulfide Synthesis (**XII**).* To convert cysteine (*tert*-butyl) to the 2-pyridylsulfenyl derivative, the protected A chain (42 μmol) and 2,2′-dipyridyl disulfide (Aldrich, Milwaukee, WI) (588 μmol) were dissolved in 1.8 mL of trifluoroacetic acid and chilled on ice. Thioanisole (200 μL) and 2 mL of triflic acid in trifluoroacetic acid (1:4 v/v) were added, and the reaction was continued for 30 min at 0 °C.[6,11] The A chain was precipitated with chilled diethyl ether, and the pellet was collected by centrifugation, washed 3 times with 10 mL of diethyl ether, 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 HPLC purified (yield: 13.7 mg, 31.1%; *m*/*z* found 2934.57, $MH^+$ calcd 2933.32 based on $Asp^{A10}Dpr^{A15}Asn^{A19}$-A chain amide$^{A26}$ ($Acm^{A11}$, $Spy^{A24}$ (**X**)).

RLF B chain (**XI**) was synthesized by standard Fmoc chemistry using TFA stable protecting groups $Cys(Acm)^{B10}$ and $Met(O)^{B5}$ as previously described.[6] The thiol-activated A chain (**X**) (4.5 μmol) and monothiol B chain (**XI**) (5.3 μmol) were dissolved in 900 μL of 0.1 M acetic acid/NaOH (pH 4.5) containing 8 M guanidinium chloride and stirred for 24 h at 37 °C. The reaction mixture was desalted on Sephadex G25 in 1 M acetic acid, and the chain-combination product was purified by HPLC (yield: 21.8 mg, 75.3%). Subsequent oxidative removal of the Acm groups and sulfoxide reduction was done as described above (**V**) (yield: 3.9 mg; 18.3%, *m*/*z*: found 6269.74; $MH^+$ calcd 6270.17).

**MALDI Time-of-Flight Mass Spectrometry.** RLF analogues (1 μg/μL) in water were diluted with 3 vol of 50 mM α-cyanocinnamic acid in 80% acetonitrile containing 0.1% TFA and 1 μL spotted onto a MALDI plate. MALDI-TOF MS was performed using a Bruker Autoflex III linear at the mass spectroscopy/proteomics facility at the Medical University of South Carolina.

**CD Spectroscopy.** Measurements were conducted using a Jasco J710 spectropolarimeter in combination with a 0.1 cm path length cell. Ten spectra were averaged. RLF derivatives were dissolved in water, and the protein concentration was determined by UV absorbance. Prior to CD measurements, samples were diluted into buffer pH 7.5 and 5.0.

**Receptor Binding.** Assays were conducted using stably transfected LGR8−293T cells as previously described.[6] For receptor binding assays cells were suspended in binding buffer 20 mM Hepes at pH 7.5, 1% bovine serum albumin, 0.1 mg/mL lysine, 1.5 mM $CaCl_2$, 50 mM NaCl, and 0.1% $NaN_3$ and incubated with various concentrations of hRLF or cross-linked RLF-derivatives in the presence of $^{125}I$-$Tyr^{A9}$ hRLF (100 000 cpm, 25 fmol).[14] After 1 h at room temperature cells were diluted with 1 mL of ice-cold binding buffer and collected by centrifugation. The supernatant was discarded, and the tips of the vials containing the pellets were placed in counting tubes and transferred to a γ-counter for analysis. The total binding was determined in the absence and nonspecific binding in the presence of 0.4 μM unlabeled hRLF. In a regular assay, total binding was ∼70 000 cpm, and nonspecific binding amounted to less than 5% of the total binding. Assays of hRLF derivatives were compared with human RLF standards run in parallel. All data were collected in duplicates, and the data of at least three independent assays were pooled and presented as the mean (±SEM).

**cAMP Accumulation Assays.** For cAMP assays LGR8−293T cells were seeded in 24-well tissue culture plates with 250 000 cells/well and grown in 500 μL of DMEM, supplemented with fetal bovine serum (10%) and antibiotics. Twenty-four

hours later, the conditioned medium was replaced by 200 μL of 3-isobutyl-1-methylxanthine (IBMX) (0.5 mM) in DMEM containing 1% bovine serum albumin. The cells were incubated for 1 h at 37 °C in 5% $CO_2$ before 200 μL of hormone dilution, prepared in the same buffer, was added. The cultures were grown at 37 °C in 5% $CO_2$ in air for 16 h. Thereafter, the plates were moved into a −80 °C freezer and kept for 6 h. The plates were thawed within 15 min at 37 °C; the contents of the wells were transferred into 1.5 mL bullet tubes and heated to >90 °C for 10 min. Cell debris was removed by centrifugation at 14 000 rpm for 10 min at 4 °C. The supernatant was diluted into 50 mM acetate buffer (pH 6) and reacted with acetic anhydride/triethylamine. The 2-*O*-acyl-cAMP concentration was determined by radioimmunoassay using [$^{125}$I]-succinyl-cAMP-tyrosyl methyl ester[15] (20 000 cpm, 5 fmol) and rabbit anti-cAMP antiserum (Millipore, Billerica, MA). The total response was determined in the presence of 0.4 μM hRLF, and nonspecific response was determined in the absence of RLF. Assays of hRLF derivatives were compared with human RLF standards run in parallel. All data were collected in duplicates, and the data of at least three independent assays were pooled and presented as the mean (±SEM).

## RESULTS

The transmembrane signal generation site of RLF is located in the N-terminal segment of both chains of the hormone (Figure 1) and requires at least three L-amino acids in the A chain and two in the B chain immediately preceding the A11−B10 disulfide bond.[6,8] Replacement of amino acids by other L-α amino acids does not diminish transmembrane signaling as determined by cAMP production. Clearly signal initiation and transduction are not dependent upon the structure of the side chain but on the peptide backbone.[8] The A11−B10 cross-link is of prime interest because the disulfide is exposed on the molecular surface separating the signaling sites of the N-termini of the two chains. In contrast, the intra-A chain disulfide A10−A15 is buried in the hydrophobic core, and the C-terminal interchain cystine A24−B22 is located within the hormone receptor-binding region with the disulfide facing away from the receptor (Figure 1).[16,17]

We modeled and synthesized three RLF derivatives, each with a disulfide bond exchanged for an isopeptide cross-link. All were constructed from aspartic acid and 2,3-diaminopropionic acid (Dpr). The β-carboxyl group of aspartic acid was than activated and allowed to react with the β-amino group of Dpr. Models of these derivatives showed that the Asp/Dpr construct covered the distance of a cystine bond, and energy minimization suggested that the secondary structure remained intact.

The direction of the interchain peptide bond was dictated by the sequence of the B chain where both cysteines are followed by glycine. Replacing either B chain cysteine with Asp would, upon activation, lead exclusively to the succinimide[18] without forming the desired product. Therefore, both interchain links were synthesized between Asp in the A chain and Dpr in the B chain. For the intra-A chain disulfide replacement the Asp$^{A10}$/Dpr$^{A15}$ isomer was produced. To simplify the syntheses the C-termini of all A chains were amidated, a modification that does not affect the structure and function of RLF.[19]

The synthesis of both interchain isopeptide cross-linked RLFs was achieved using the same synthetic route as described for the Asp$^{A11}$−Dpr$^{B10}$ derivative (xA11−B10) (Figure 2). The synthesis is based on standard solid phase techniques in

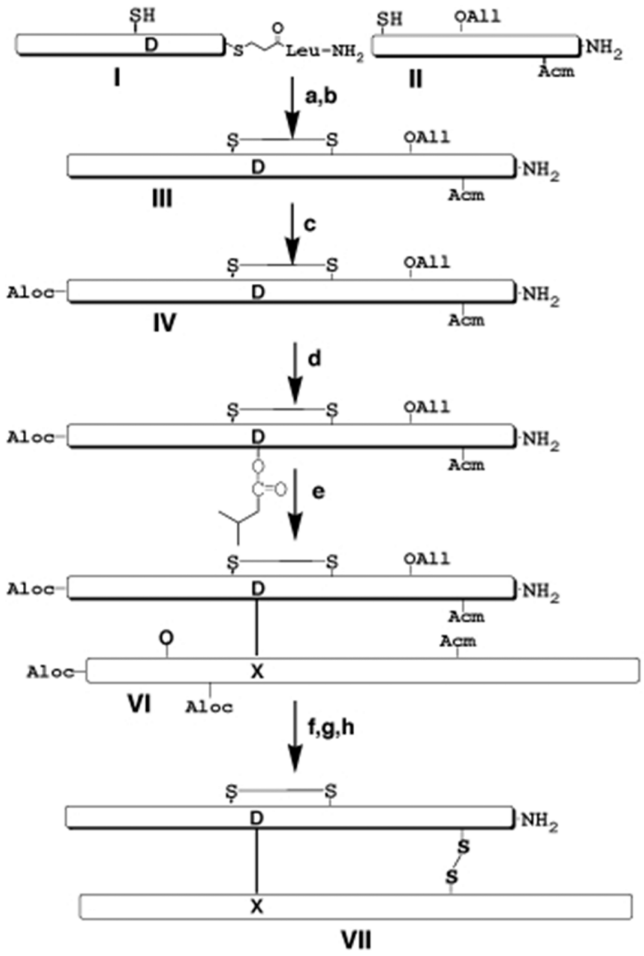


**Figure 2.** Synthesis of hRLF containing an isopeptide bond (xA11−B10) starting with three partially protected peptides, A1−14 (I), A15−26 (II), and B1−31 (V): (a) 0.1 M phosphate buffer, 6 M GuHCl, thiophenol, benzylmercaptan, 2.5 h, 37 °C; (b) titration with iodine in 50% acetic acid; (c) AlocONSu, DMSO, TEA; (d) NMM, isobutylchloroformate in DMSO/DMF 5 min, −10 °C; (e) B chain (**V**) in 0.2 M NMM 80% DMF "pH 7.4", 12 h, rt; (f) Pd(PPh$_3$)$_4$, NaBH$_4$, DMSO/DMF/CH$_2$Cl$_2$, 1 h, rt; (g) iodine, 95% acetic acid, 1 h, rt; (h) NH$_4$I in 90% TFA 15 min, 0 °C (X = Dpr).

combination with special protecting groups that permit the selective synthesis of the isopeptide bond as well as the two remaining disulfide links. Formation of the isopeptide bond required special TFA-stable protection of all amino, thiol, and carboxyl groups in the A chain except Asp$^{A11}$ and of the thiol and amino groups in the B chain with the exception of Dpr$^{B10}$. Allyl-based groups were used for amino group and Asp$^{A19}$ protection. Since the β-allyl ester in Asp$^{A19}$ has limited stability during the piperidine treatment used in each Fmoc-deprotection cycle, the A chain was generated from the two segments A1−14 and A15−26 by native chemical ligation. After ligation the intrachain disulfide bond was formed while Cys$^{A24}$ remained protected. The cross-link was established by preactivating the β-carboxyl group in Asp$^{A11}$ as mixed anhydride followed by reaction with Dpr$^{B10}$ of the B chain. Removal of the allyl-protecting groups, followed by formation of the A24−B22 disulfide bond by oxidative removal of the Acm groups, and reduction of methionine sulfoxide led to the final product.

For the synthesis of the intra-A chain isopeptide cross-linked RLF (xA10−A15) a different reaction scheme was developed


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(Figure 3). Since replacement of Asp$^{A19}$ does not affect structure and function,[7] the Asn$^{A19}$ derivative (xA10−A15) was

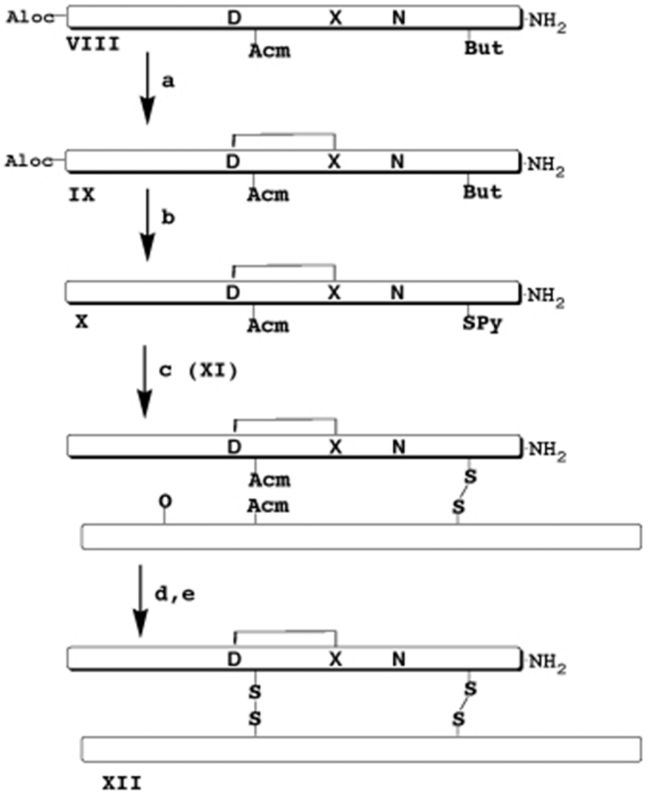


**Figure 3.** Scheme of the synthesis of hRLF containing an A10−A15 cyclic isopeptide bond. N = Asn replacing Asp in position A19. The A chain was synthesized by Fmoc chemistry on an amide resin. After TFA treatment the remaining protecting groups are allyloxycarbonyl (Aloc) at the N-terminus, acetamidomethyl Acm and tertiary butyl (But) at the cysteines and the C-terminus amidated; (a) PyBop, HOBt, DIEA, 2 h, rt; (b) Py-S-S-Py, thioanisole, 10% triflic acid, TFA, 30 min, 0 °C; (c) **XI** (B chain (Acm, O)), 8 M GuHCl pH 4.5, 24 h, 37 °C; (d) iodine in 95% acetic acid, 1 h, rt; (e) $NH_4I$ in 90% TFA, 15 min, 0 °C (X = Dpr).

produced instead. TFA-stable protecting groups at the N-terminus of the A chain and cysteines A11 and A24 are required for this approach. The cross-link between Asp$^{A10}$ and Dpr$^{A15}$ was formed in solution with PyBop as condensing reagent. Differential protection of the four cysteines enabled the directed synthesis of the two disulfide bonds.

The identity and purity of the synthetic proteins were confirmed by reversed-phase HPLC (Figure 4) as well as by MALDI-TOF-MS analyses (Figure 5). CD spectra of the isopeptide cross-linked RLFs were compared with hRLF and with the inhibitor, RLFi, at pH 7.5 (Figure 6A). The helix content of hRLF and all derivatives was 35−42% (Table S1).[20,21] At pH 5.0 the CD signal of hRLF and xA24−B22 remains unchanged; however, the signal of xA11−B10 shows a blue shift of 2 nm. The spectra of RLFi and xA10−A15 changed to a great extent reducing the helix content to 14% and proportionally increasing the $\beta$-sheet content (Figure 6B and Table S2).[20,21] This pH sensitivity suggests that the structures of RLFi, xA10−A15, and, to a lesser extent, xA11−B10 are more flexible than wild-type RLF and xA24−B22. This structural relationship is also reflected in HPLC retention

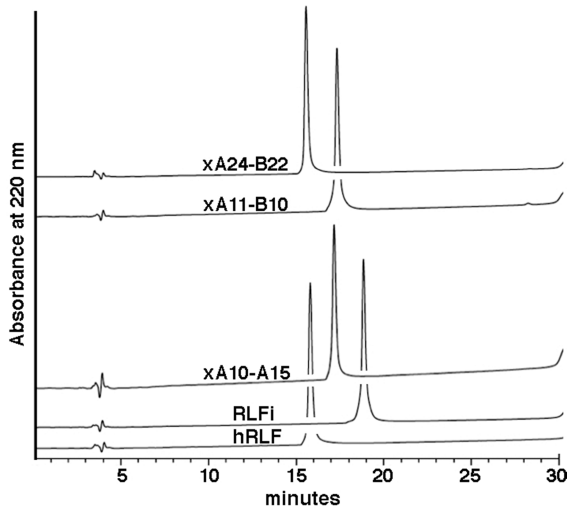


**Figure 4.** Reversed phase HPLC of isopeptide cross-linked RLF compared to human RLF and the RLF inhibitor (RLFi) on Jupiter $C_{18}$ (250 mm × 4.60 mm). The solvent system was 0.1% TFA in water (A) and 0.1% TFA in 83% acetonitrile (B). About 20 $\mu$g of protein was injected and eluted with a linear gradient from 20% B to 60% B over 30 min. The HPLC elution profiles were recorded by UV absorbance at 220 nm.

times where RLFi, xA10−A15, and xA11−B10 appear more hydrophobic than xA24−B22 and hRLF (Figure 4). For xA11−B10 the $^1$H NMR spectrum at pH 5.0 supports the CD data showing reduced chemical shift dispersion (Figure S1). The low solubility of hRLF at neutral pH renders structural investigation in solution difficult.

The human embryonic kidney cell line 293T stably transfected with LGR8 was used to evaluate receptor affinity (Figure 7) and the ability to initiate signaling through the plasma membrane to induce cAMP synthesis (Figure 8). By this measure the binding avidity of xA11−B10 (95% ± 7%) was identical to hRLF. In contrast, xA10−A15 and xA24−B22 showed receptor affinities reduced to 32 ± 2% and 7 ± 2% of normal, respectively. The diminished receptor affinity of xA24−B22 is in-line with previous observations that restrictions in the C-terminal region of RLF affect the proper exposure of the receptor-binding site.[17,19] The receptor affinity of xA10−A15 is lower than the one recently reported for the disulfide replacement by carbon-to-carbon double bonds.[22] The polar peptide bond is probably unable to accommodate the structure of the A10−A15 disulfide link, which is buried within the hydrophobic core. In terms of cAMP production xA24−B22 showed potency proportional to the binding affinity (12 ± 5% but lower efficacy (65 ± 8%), whereas xA10−A15 showed lower potency (7 ± 2%) and 100% efficacy (Figure 8).

## DISCUSSION

Isopeptide cross-linked derivative xA11−B10 interacted with the receptor as avidly as the disulfide-linked hormone but did not induce cAMP production. The effect was identical to that observed when the signal generating N-terminal octapeptide of the A chain had been removed as in RLFi,[6] when the cystine A10−A15 was deleted or the cysteines A10 and A15 were replaced by serines.[22] Truncation of the N-terminal region of the B chain by eight amino acids pointed to a second port of the signaling site albeit, the truncated derivative has reduced receptor affinity.[8]

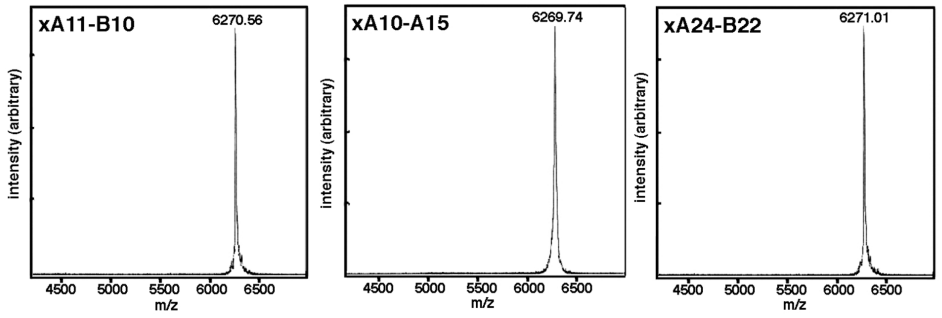


**Figure 5.** MALDI-TOF-MS of isopeptide cross-linked hRLF. Calculated $m/z$ = 6271.16 for xA11−B10 and xA24−B22 and 6270.17 for xA10−A15. The difference of about 1 mass unit for xA10−A15 is due to the replacement of Asp by Asn in position A19.

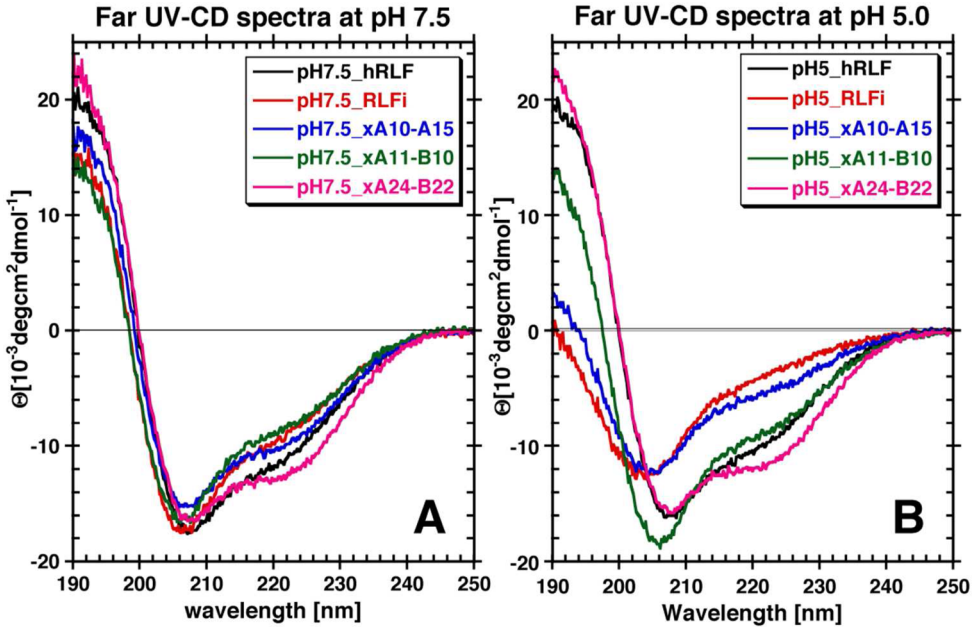


**Figure 6.** CD spectra of isopeptide cross-linked hRLFs compared to hRLF and the RLF inhibitor, RLFi at pH 7.5 (A) and pH 5 (B).

The lack of G-protein activation is associated with the introduction of a secondary amide for the natural disulfide. In this context it is important to consider that the peptide and the disulfide cross-link differ in chemical properties, such as bond length (1.33 Å vs 2.05 Å), geometry (planar vs tetrahedral), freedom of rotation (rigid vs flexible), and polarity (polar vs

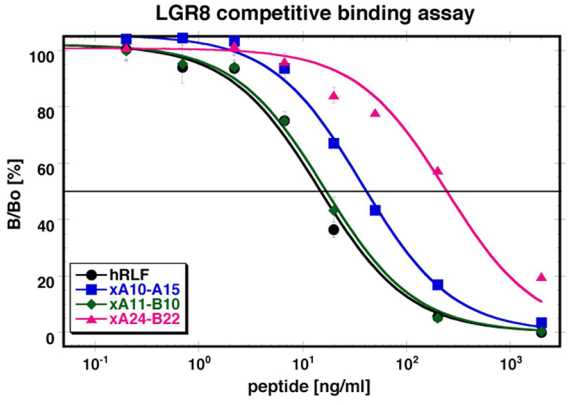


**Figure 7.** Receptor-binding assays: dose−response curves of isopeptide cross-linked RLF derivatives using stably transfected LGR8/293T cells and $^{125}$I-Tyr$^{A9}$ hRLF for tracer.[14] Data points were collected in duplicates, and the data of at least three independent binding assays were pooled and presented as the mean (±SEM).

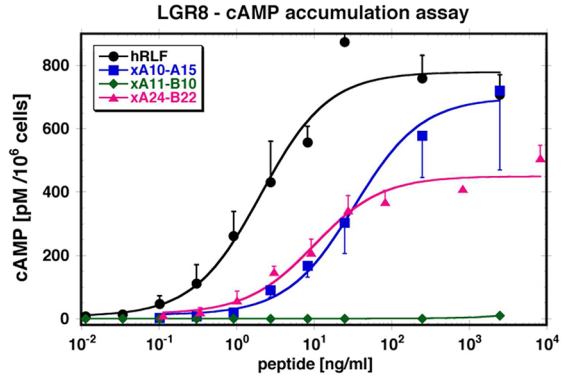


**Figure 8.** cAMP assays: hRLF and isopeptide cross-linked RLFs were assayed for their ability to stimulate cAMP accumulation using 293T cells stably expressing LGR8. Assays of the isopeptide cross-linked derivatives were compared with hRLF standards run in parallel. Assays were performed in duplicate, and 3−5 independent assays were averaged (±SEM). The total response was determined in the presence of 0.4 $\mu$M of hRLF, and the nonspecific response was determined in the absence of hRLF.


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nonpolar). Placed on the protein surface, the peptide bond can accept and donate hydrogen bonds, which may stabilize conformations within the hormone and interactions to the receptor. The impact on the structure is indicated by the pH dependence of the CD signal, implying a more dynamic structure for xA11−B10 and xA10−A15 than for wild-type RLF.

Dynamics as the cause of inhibition is difficult to assess since the active conformation of a protein−ligand represents only a small population within the large assembly of structural conformers. Extracting those transient structures is an active field of research[23] but currently out of reach for ligated membrane-bound receptors. The most sensitive measure of structural integrity available is the wild-type receptor binding affinity of xA11−B10, a clear indication that misalignment of the derivate on the receptor can be excluded. That implies that the isopeptide bond disturbs the local environment affecting signal transmission and thus the exposure of the G-protein binding site. The isopeptide bond could prevent such signaling in different ways, sterically it could cause a misalignment of the two signaling ports or electronically it could intercept the ligand-induced change of polarity on the transmembrane domain of the receptor.

Our postulated model of RLF-initiated transmembrane signaling requires the preservation of secondary amide bonds between L-amino acids linking residues A8 and A9 and residues B8 and B9 (Figure 1).[8] Disulfide bonds have always been viewed as the tie between peptide chains. Considering that the disulfide bond A11−B10 connects the two signaling ports of RLF one is led to the conclusion that this −S−S− bond may also be an insulator between the two signaling sites and that the peptide bond substitution creates a short circuit. One can envision that ligand binding provides the energy and the peptide bonds in the signaling site the polarity to induce signal transmission by reorienting bonds within the receptor. Once initiated this signal propagates through the membrane into the interior of the cell where cAMP synthesis is stimulated by exposure of a G-protein contact. To reach the G protein, this signal has to travel about 50 Å through the cell wall. The reaction reverses by reorienting the polarized bonds when the ligand dissociates.

Because the RLF receptor-binding site and signal-initiation site are located on different but distinct areas on the protein surface, it should be feasible to identify contacts of the different ligands to the receptor and thus prove or disprove the postulated mechanism of signal initiation.

## ■ ASSOCIATED CONTENT

### Supporting Information

Tables presenting the secondary structure estimation from CD spectra at pH 7.5 and 5.0 and a comparison of one-dimensional $^1$H NMR spectra of RLF and xA11−B10. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

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## ■ ABBREVIATIONS

Acm, acetamidomethyl; Aloc, allyloxycarbonyl; AlocONSu, allyloxycarbony-*N*-hydroxysuccinimide ester; Boc, *tert*-butyloxycarbonyl; But, *tert*-butyl; CD, circular dichroism; DIEA, diisopropylethylamine; DMF, *N,N*′-dimethylformamide; DMSO, dimethyl sulfoxide; Dpr, 2,3-diaminopropionic acid; Fmoc, 9-fluorenyloxycarbonyl; GuHCl, guanidinium chloride; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; hRLF, human RLF; LGR8, leucine-rich repeat G-protein coupled receptor 8; *m*/*z*, mass per charge; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; NMM, *N*-methylmorpholine; OAll, allyl ester; PyBop, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; PySSPy, 2,2′-dipyridyl disulfide; RLF, relaxin-like factor; RLFi, RLF inhibitor desA(1−8)human RLF; RXFP2, Relaxin/insulin-like family peptide receptor 2; rt, room temperature; SEM, standard error of the mean; SPy, 2-pyridine sulfenyl; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TOF, time-of-flight; Tris, tris(hydroxymethyl)-aminomethane; xA10−A15, $Asn^{A19}Asp^{A10}Dpr^{A15}$-cross-linked RLF; xA11−B10, $Asp^{A11}Dpr^{B10}$-cross-linked RLF; xA24−B22, $Asp^{A24}Dpr^{B22}$-cross-linked RLF.

## ■ REFERENCES

(1) Adham, I. M., Burkhardt, E., Benahmed, M., and Engel, W. (1993) Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. *J. Biol. Chem. 268*, 26668−26672.

(2) Büllesbach, E. E., and Schwabe, C. (1995) A novel Leydig cell cDNA-derived protein is a relaxin-like factor (RLF). *J. Biol. Chem. 270*, 16011−16015.

(3) Kumagai, J., Hsu, S. Y., Matsumi, H., Roh, J. S., Fu, P., Wade, J. D., Bathgate, R. A. D., and Hsueh, A. J. W. (2002) InsL3/Leydig insulin-like peptide activates the LGR8 receptor important in testis descent. *J. Biol. Chem. 277*, 31283−31286.

(4) Deupi, X., and Standfuss, J. (2011) Structural insights into agonist-induced activation of G-protein-coupled receptors. *Curr. Opin. Struct. Biol. 21*, 541−551.

(5) Liu, J. J., Horst, R., Katritch, V., Stevens, R. C., and Wuthrich, K. (2012) Biased signaling pathways in beta2-adrenergic receptor characterized by 19F-NMR. *Science (New York, N. Y.) 335*, 1106−1110.

(6) Büllesbach, E. E., and Schwabe, C. (2005) LGR8 signal activation by the relaxin-like factor. *J. Biol. Chem. 280*, 14586−14590.

(7) Büllesbach, E. E., and Schwabe, C. (2006) The mode of interaction of the relaxin-like factor (RLF) with the leucine-rich repeat G protein-activated receptor 8. *J. Biol. Chem. 281*, 26136−26143.

(8) Büllesbach, E. E., and Schwabe, C. (2007) Structure of the Transmembrane Signal Initiation Site of the Relaxin-Like Factor (RLF/INSL3). *Biochemistry 46*, 9722−9727.

(9) Hackeng, T. M., Griffin, J. H., and Dawson, P. E. (1999) Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. *Proc. Natl. Acad. Sci. U. S. A. 96*, 10068−10073.

(10) Cruz, L. J., Beteta, N. G., Ewenson, A., and Albericio, F. (2004) "One-Pot" preparation of N-carbamate protected amino acids via azide. *Org. Process Res. Dev. 8*, 920−924.

(11) Maruyama, K., Nagata, K., Tanaka, M., Nagasawa, H., Isogai, A., Ishizaki, H., and Suzuki, A. (1992) Synthesis of bombyxin-IV, an insulin superfamily peptide from the silkworm, bombyx mori, by

stepwise and selective formation of three disulfide bridges. *J. Protein Chem. 11*, 1−12.
(12) Büllesbach, E. E., and Schwabe, C. (1991) Total synthesis of human relaxin and human relaxin derivatives by solid phase peptide synthesis and site-directed chain combination. *J. Biol. Chem. 266*, 10754−10761.
(13) Coste, J., Le-Nguyen, D., and Castro, B. (1990) PyBop: A new peptide coupling reagent devoid of toxic by-products. *Tetrahedron Lett. 31*, 205−208.
(14) Büllesbach, E. E., and Schwabe, C. (1999) Specific, high-affinity relaxin-like factor receptors. *J. Biol. Chem. 274*, 22354−22358.
(15) Steiner, A. L. (1979) Cyclic AMP and cyclic GMP, in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M., and Behrman, H. R., Eds.) pp 3−17, Academic Press, New York.
(16) Rosengren, K. J., Zhang, S., Lin, F., Daly, N. L., Scott, D. J., Hughes, R. A., Bathgate, R. A., Craik, D. J., and Wade, J. D. (2006) Solution structure and characterization of the receptor binding surface of insulin-like peptide 3. *J. Biol. Chem. 281*, 28287−28295.
(17) Büllesbach, E. E., Hass, M. A. S., Jensen, M. R., Hansen, D. F., Kristensen, S., Schwabe, C., and Led, J. J. (2008) The solution structure of a conformationally restricted fully active derivative of the human relaxin-like factor (RLF). *Biochemistry 47*, 13308−13317.
(18) Bodanszky, M., and Martinez, J. (1981) Side reactions in peptide synthesis. *Synthesis*, 333−356.
(19) Büllesbach, E. E., and Schwabe, C. (2004) Synthetic cross-links arrest the C-terminal region of the relaxin-like factor (RLF) in an active conformation. *Biochemistry 43*, 8021−8028.
(20) Whitmore, L., and Wallace, B. A. (2008) Protein secondary structure analyses from circular dichroism spectroscopy: Methods and reference databases. *Biopolymers 89*, 392−400.
(21) Whitmore, L., and Wallace, B. A. (2004) DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res. 32*, W668−673.
(22) Zhang, S., Hughes, R. A., Bathgate, R. A., Shabanpoor, F., Hossain, M. A., Lin, F., van Lierop, B., Robinson, A. J., and Wade, J. D. (2010) Role of the intra-A-chain disulfide bond of insulin-like peptide 3 in binding and activation of its receptor, RXFP2. *Peptides 31*, 1730−1736.
(23) Bouvignies, G., Vallurupalli, P., Hansen, D. F., Correia, B. E., Lange, O., Bah, A., Vernon, R. M., Dahlquist, F. W., Baker, D., and Kay, L. E. (2011) Solution structure of a minor and transiently formed state of a T4 lysozyme mutant. *Nature 477*, 111−114.