The relaxin-like factor (RLF) is thought to be responsible for the intra-abdominal migration of the testis during mammalian development. Our latest studies of RLF and LGR8 have revealed that the N-terminal region of the A chain is not required for receptor binding but is indispensable for cyclic AMP generation. RLF derivatives with six residues deleted from the N terminus of the A chain are active, whereas further truncation, up to the first A chain cysteine (A-10), yields tightly binding ligands devoid of signaling activity. These derivatives are specific competitive inhibitors (RLFi) of RLF. Although receptor binding is dependent upon B chain residues, the N-terminal region of the A chain is a generic trigger of the trans-membrane signaling activity.

The relaxin-like factor (RLF),1 the product of the InsI3 gene (1), appears to be crucial for testicular descent in human neonates. InsI3−/− male mice retain the testes in the body cavity and are infertile (2, 3). The same phenotype was observed when the G-protein coupled receptor (GREAT) was deleted (4). These receptors (GREAT, LGR8) that affect the descent of testis in mice (4, 5) and in humans (LGR8) (6) are RLF-specific (7, 8). Thus, an experimental system is now available that invites detailed investigation of the ligand/receptor interaction.

RLF, isolated from bovine testes, consists of an A chain comprising 26 residues and a B chain of 40 or 45 residues. The chains are linked by three relaxin-like disulfide bonds (9). This native RLF confirms the structure that has been implied for all synthetic molecules. RLF binds its receptor in part through the N-terminal region of the A chain and B chain residue Trp(B-27) (10) and via additional, yet unidentified, residues that would account for the high receptor binding affinity. However, the molecule can be pared down without loss of binding avidity. Full binding intensity, for example, is observed with RLF ending in Trp(B-27)-amide (11).

Our studies suggest that the N-terminal region of the A chain initiates the trans-membrane signal. Sequential shortening of the N-terminal end of the A chain reduces cAMP production, which becomes undetectable when 7 or more residues are removed. A chain truncations did not reduce binding avidity so that Ades-(1–7) RLF is a specific competitive inhibitor of RLF function. In this paper we report that the receptor-binding region of RLF is physically separate from the trans-membrane signal initiation site in the N-terminal region of the A chain.

EXPERIMENTAL PROCEDURES

**Materials—** Fmoc amino acid derivatives were purchased from either Advanced ChemTech (Louisville, KY) or Novabiochem. Reagents and solvents for peptide synthesis were obtained from Advanced ChemTech. The LGR8 cloned into the pcDNA3.1 neo 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.

**Peptide Synthesis—** All RLF chains were synthesized by Fmoc chemistry, using an automated peptide synthesizer (Applied Biosystems, Foster City, CA, model 433A). The C-terminal amino acids were linked to Wang resin. Side chain-protecting groups of all trifunctional amino acids were trifluoroacetic acid-labile, except for methionine (sulfoxide) in position B-5, acetamidomethyl protected cysteines in positions A-11 and B-11, and tertiary butyl protected cysteine in position A-24. O-Benzotriazol-N,N,N,N′,N′-tetramethyluronium-tetrafluoroborate (HBTU) (12) was used for carboxyl activation. Each polypeptide chain was deprotected and removed from the solid support by treatment with a freshly prepared mixture of trifluoroacetic acid/water/ethanedithiol/thioanisole/phenol (100/5/2/5/7.5) (v/v/v/v/w) for 2 h at room temperature (13). The resin was filtered off, the peptide was precipitated with ice-cold diethyl ether, the pellet was collected by centrifugation, washed twice with diethyl ether, air-dried, resuspended in water, and lyophilized. Each RLF chain was purified by reversed-phase HPLC on a Rainin C18-column (41.4 mm × 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 light absorbance of the effluent. Fractions of 10 ml were collected, and 10 μl of each fraction was subjected to homogeneity by HPLC on a Bakerbond column (see below). Fractions containing the pure peptides were pooled and lyophilized.

The distribution of the cysteine-protecting groups allowed for the stepwise synthesis of the three disulfide bonds. In the A chain, cysteine A-10 and A-15, liberated during the trifluoroacetic acid treatment, were oxidized by titration with iodine in 50% acetic acid (14). Excess iodine was immediately reduced with 1 M ascorbic acid in water. The reaction mixture was diluted to 10 ml with 10 ml of diethyl ether, and the pellet was collected by centrifugation, washed 3× with 10 ml of diethyl ether, and air-dried. The RLF A chain derivative was dissolved in 1M acetic acid and desalted on Sephadex G25 in 1 M acetic acid and lyophilized. To produce the first interchain disulfide bond, equimolar amounts of the thiol-activated A chain and the monothiol B chain were reacted in 3 ml of 8 M guanidinium chloride, 0.1 M acetic acid (adjusted to pH 4.5 with NaOH) for 24 h at 37 °C. The product was isolated by size separation on Sephadex G50 (2.5 cm × 55 cm) in 1 M acetic acid followed by separation on HPLC on a Jupiter C18-column (10 mm × 250 mm, Phenomenex). The last disulfide bond was formed by oxidative removal of

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1 The abbreviations used are: RLF, relaxin-like factor; CD, circular dichroism; HPLC, high performance liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; Fmoc, N-9-fluorenylmethoxycarbonyl; hRLF, human RLF.

2 S The on-line version of this article (available at http://www.jbc.org) contains supplemental material.

3 The abbreviations used are: RLF, relaxin-like factor; CD, circular dichroism; HPLC, high performance liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; Fmoc, N-9-fluorenylmethoxy carbonyl; hRLF, human RLF.
the acetamidomethyl groups in positions A-11/B-11 using iodoine in 95% acetic acid as solvent (10, 14). After 60 min at room temperature in the dark the reaction mixture was slowly added to a vigorously stirring solution of 0.1 M ascorbic acid. The peptide was isolated by size separation on Sephadex G25 in 1 M acetic acid and lyophilized. Lastly, the methionine sulfoxide (B-5) (1.25 mmol) was reduced with 800 μl of 50 mM ammonium iodide in 90% trifluoroacetic acid for 15 min on ice. The reaction was quenched by addition of 10 mM ascorbic acid in water (5 ml), and the peptide was isolated by gel filtration on Sephadex G25 in 1 M acetic acid, followed by HPLC purification on a Jupiter C18-column. The solvents were removed by lyophilization. Final yields and mass spectroscopy data are presented in Table I.

High Performance Liquid Chromatography—Peptides were analyzed in two HPLC systems. For both systems solvent A consisted of 0.1% trifluoroacetic acid in acetonitrile/water 4/1 v/v, and solvent B consisted of 0.1% trifluoroacetic acid in water, and 45% B at a flow rate of 100 μl/min. Peptides were hydrolyzed in vapor phase 6 l) was spotted on a sample plate. Peptides were analyzed in 24-well tissue culture plates at a density of 250,000 cells/well and grown without RLF. Assays of hRLF derivatives were compared with human RLF standards run in parallel. Assays were performed in duplicate, and 2–4 independent assays were averaged (± S.E.).

RESULTS

In the search for additional receptor-binding residues of human RLF (Fig. 1) we have synthesized RLF derivatives with truncations at the N termini. The yields of the corresponding derivatives and the MALDI-MS data are presented in Table I. All derivatives were homogeneous in two different HPLC systems. Upon reduction, two new peptides were generated, and the HPLC elution profiles were compared with a human RLF standard treated in parallel. As expected, altered retention times were observed only for the chain carrying the modification. Enzymatic digests and peptide mapping confirmed the parallel arrangement of the peptide chains. Treatment with Staphylococcus aureus V8 protease, followed by trypsin, liberated the N-terminal A-(9–19)-B-(9–16) and the C-terminal A-(20–26)-B-(21–26) disulfide peptides for all derivatives except for Ades-(1–9), which yielded the N-terminal A-(10–19)-B-(20–26) and AR8A hRLF, which yielded the N-terminal A-(1–
(19)-B-(9–16) disulfide peptide. Peptides were identified by MALDI-MS and HPLC. The fragmentation pattern is in agreement with the parallel arrangement of the two chains (see supplemental material) as observed in natural and synthetic bovine RLF (9).

Truncation at the N terminus of the A chain up to the first cysteine in position A-10 resulted in derivatives with full receptor binding properties (Fig. 2A). CD spectra (Fig. 2B) showed signal intensities similar to full-length RLF at the absolute minimum but showed a slight reduction at 222 nm. In addition, a small blue-shift of the absolute minimum and the crossover point were observed amounting to ~0.5 nm for the Ades-(1–6) and Ades-(1–7) RLF derivatives and ~1 nm for Ades-(1–8) and Ades-(1–9) RLF derivatives. Measurements of the cAMP-response yielded a complete dose-response curve for Ades-(1–6) RLF albeit with a reduced activity of ~10% relative to the full-length RLF. In contrast, the activity of derivatives truncated by 7, 8, and 9 residues from the N terminus of the A chain showed significantly reduced cAMP production; none of them reached full response even at the highest concentration. This effect is more pronounced for Ades-(1–8) and Ades-(1–9) RLF than for Ades-(1–7) RLF (Fig. 2C).

RLF shortened at the N terminus of the B chain by 5 residues retains full receptor binding affinity (Fig. 3A). Its CD spectrum indicates a slight increase of signal intensity and a very small red-shift (~0.5 nm) when compared with the CD spectrum of the full-length RLF (Fig. 3B). Bdes-(1–5) RLF retains the wild-type trans-membrane signal activity (Fig. 3C). The additional deletion of 6 residues from the N terminus of the A chain still does not affect receptor binding (Fig. 3A), or the secondary structure, as evidenced by the CD spectrum (Fig. 3B). However, the dose-dependent cAMP production is reduced to 10% of the RLF response (Fig. 3C).

Derivatives in which either arginine in position A-8 or tyrosine in position A-9 are replaced by alanine have 100% binding avidity, an intact secondary structure, and 100% cAMP response (Fig. 4). Ades-(1–9) RLF supplemented with the missing A chain nonapeptide in equimolar amounts could not restore adenylate cyclase activity. For the Ades-(1–8) RLF derivative the ED₅₀ is attained at a 1:1 ratio of RLF to inhibitor.

FIG. 2. RLF truncated at the N terminus of the A chain. A, competitive binding of RLF derivatives to LG8 using A-¹²⁵I-labeled Tyr-9 hRLF (17) as tracer. Each data point was measured in duplicate, three independent experiments were performed, and the mean (±S.E.) of the pooled data is presented. B, far-ultraviolet CD spectra of the RLF derivatives were acquired. The protein concentration of each derivative was 83 µg/ml in 25 mM Tris/HCl buffer at pH 7.5. Ten spectra were averaged. C, cAMP assays on LG8 stably transfected 293T/17 cells. Each data point was collected in duplicate, and the data of two assays were pooled, and the mean (±S.E.) were reported.

**DISCUSSION**

Results of previous studies suggest that tryptophan B-27 (10) and arginine B-16² contribute to the binding energy of RLF/receptor interaction. However, the reduction in affinity upon

² E. E. Büllschach and C. Schwabe, unpublished results.
replacement of either amino acid by alanine is only ~50-fold, suggesting that additional binding sites exist that contribute to the relatively high energy of the RLF/LGR8 interaction (19). While searching for these binding regions by measuring both receptor-binding and cAMP production, we discovered a signal initiation region of RLF apart from the receptor-binding contacts.

The first target of our investigation was the N-terminal region of the A chain. The data suggest that binding to the LGR8 receptor expressed in 293T cells is not significantly perturbed by the removal of up to 9 residues from the N-terminal end of the RLF A chain. Small changes of the CD spectra imply changes in secondary structure, in particular, the blue-shift
and the reduction of the 222-nm minimum suggest presence of the less stable 3_{10}-helix (20) next to the α-helix (16). Increased changes are observed for derivatives truncated up to 8 residues. It is unclear how many of these changes can be contributed to the secondary structure of the removed N-terminal peptide. Residue 9 is tyrosine, which may make a structure-independent contribution to the spectrum.

RLF B chain truncation experiments further enhance the impression that the molecule can be pared down substantially without affecting the core of the binding region. Extensive deletion of amino acids from both ends of the B chain does not interfere with receptor binding. Even in combination with N-terminal shortening of the A chain by 6 residues, the B chain N-terminal deletion of residues 1–5 and elimination of C-terminal residues beyond residue 27 from the B chain does not attenuate receptor binding. It appears that RLF can be shortened to 42 residues without significant reduction in receptor affinity (Fig. 3A) and without disturbance of the CD spectra. Note that removal of the N-terminal pentapeptide from the B chain compensates for the differences in the CD spectrum observed after truncation of the A chain alone (Fig. 3D). This would speak against the idea that water exclusion from the hydrophobic core is important for either binding or signaling activity. The B chain truncation would admit water to the same region that is uncovered if the A chain N-terminal tail is removed so that one can assume that removal of both the N- and C-terminal fragments would certainly admit water to the core region.

Although deleting the N-terminal end of the A chain has no effect on receptor binding, it reduces cAMP production by RLF as a function of chain length. Shortening the B chain N terminus by 5 residues and converting the new C-terminal tryptophan B-27 to an amide have no effect on cAMP production. However, in combination with deletion of 6 amino acids from the A chain N terminus, the B chain deletions caused suppression of cAMP production similar to the effect of the A chain hexapeptide deletion alone.

The cAMP production begins to decrease notably when 6 residues are removed from the N terminus of the A chain and goes to very low levels with the deletion of residues 7, 8, and 9. It appears that at least 5 amino acids have to precede cysteine (A-10) in the A chain to elicit the full cAMP response. The sequences of RLF from various species show His/Arg or Arg/His in positions 8 and 9, respectively, with human Arg/Tyr providing the only known exception (Ref. 21 and references therein). To investigate the specificity requirements of the signaling initiation effect we synthesized RLF with alanine in position 8 and a second one with alanine in position 9; both derivatives were fully active. The fact that alanine is sufficient to restore signal initiation activity leaves one with the novel idea that, in this case, primary structure per se may be sufficient to illicit the most important communication function in an organism. The role of the A chain tail is merely to provide bulk to maintain a certain conformation of the receptor activation complex. Furthermore, it seems that RLF truncated beyond position A-6, while retaining some background activity, cannot stimulate full activity by a large excess of the ligand. This observation suggests that the A chain-truncated molecule is a specific competitive RLF inhibitor.

A potent inhibitor of RLF activity in vivo would provide an important research tool for investigating the ramifications and mechanisms of cryptorchidism. The idea was tested by measuring suppression of cAMP production by a uniform amount of truncated RLF added to each point of a human RLF dose-response curve as well as by adding a uniform amount of active RLF to a RLF inhibitor curve. The result, depicted in Fig. 5, confirms that the truncated molecule is a competitive inhibitor of cAMP production by RLF and that approximately equimolar concentrations of the inhibitor are needed to suppress the cAMP production by 50%.

These experiments have led to the most surprising observation that a segment of a primary structure (as opposed to specific amino acids) is responsible for signal initiation of RLF as it binds to the LGR8 receptor. It invites the suggestion that receptor side chains must be in place to make and break contact, in the mode of a vintage telegraph.

Acknowledgments—We thank Robert Bracey and George Fullbright for their technical assistance.

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