The A-chain of Human Relaxin Family Peptides Has Distinct Roles in the Binding and Activation of the Different Relaxin Family Peptide Receptors

Mohammed Akhter Hossain,1 K. Johan Rosengren,2,† Linda M. Haugaard-Jönsson,‡ Soude Zhang,§ Sharon Layfield,† Tania Ferraro,† Norelle L. Daly,† Geoffrey W. Tregear,¶ John D. Wade,** and Ross A. D. Bathgate†

From the †Howard Florey Institute and ‡Department of Biochemistry and Molecular Biology and the **School of Chemistry, University of Melbourne, Victoria 3010, Australia, the ††School of Pure and Applied Natural Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden, and the †§Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia

The relaxin peptides are a family of hormones that share a structural fold characterized by two chains, A and B, that are cross-braced by three disulfide bonds. Relaxins signal through two different classes of G-protein-coupled receptors (GPCRs), leucine-rich repeat-containing GPCRs LGR7 and LGR8 together with GPCR135 and GPCR142, now referred to as the relaxin family peptide (RXFP) receptors 1–4, respectively. Although key binding residues have been identified in the B-chain of the relaxin peptides, the role of the A-chain in their activity is currently unknown. A recent study showed that INSL3 can be truncated at the N terminus of its A-chain by up to 9 residues without affecting the binding affinity to its receptor RXFP2 while becoming a high affinity antagonist. This suggests that the N terminus of the INSL3 A-chain contains residues essential for RXFP2 activation. In this study, we have synthesized A-chain truncated human relaxin-2 and -3 (H2 and H3) relaxin peptides, characterized their structure by both CD and NMR spectroscopy, and tested their binding and cAMP activities on RXFP1, RXFP2, and RXFP3. In stark contrast to INSL3, A-chain-truncated H2 relaxin peptides lost RXFP1 and RXFP2 binding affinity and concurrently cAMP-stimulatory activity. H3 relaxin A-chain-truncated peptides displayed similar properties on RXFP1, highlighting a similar binding mechanism for H2 and H3 relaxin. In contrast, A-chain-truncated H3 relaxin peptides showed identical activity on RXFP3, highlighting that the B-chain is the sole determinant of the H3 relaxin-RXFP3 interaction. Our results provide new insights into the action of relaxins and demonstrate that the role of the A-chain for relaxin activity is both peptide- and receptor-dependent.

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1 To whom correspondence should be addressed: Howard Florey Institute, University of Melbourne, Victoria 3010, Australia. Tel.: 61-3-8344-5648; Fax: 61-3-9348-1707; E-mail: bathgate@florey.edu.au.

Relaxin was first identified more than 90 years ago and subsequently shown to be a peptide hormone having a two-chain structure similar to insulin (Fig. 1) (1). It has since been established that relaxin is a member of the relaxin peptide family, comprising a total of seven members in the human (2). These are the H1, H2, and H3 relaxin peptides that are encoded by the three relaxin genes RLN1 to -3 and the insulin-like peptides INSL3 to -6 (insulin-like peptides 3–6). Phylogenetic analyses indicate that all of these relaxin family peptides evolved from a relaxin-3 (H3 relaxin equivalent) ancestral gene prior to the emergence of fish (3). In most mammals other than humans and higher primates, there are only two relaxin genes that encode relaxin and relaxin-3. The RLN1 gene in these species is equivalent to the RLN2 gene in humans (encoding H2 relaxin) and higher primates and encodes the relaxin peptide that is expressed by the corpus luteum and/or placenta (2). The function of the RLN1 gene in higher primates is unknown, and an H1 relaxin peptide has not been isolated.

In contrast to the receptors for insulin and insulin-like growth factors I and II, which are tyrosine kinases, the receptors for relaxin family peptides are members of two unrelated branches of the G-protein-coupled receptor (GPCR) family. LGR7 (leucine-rich repeat-containing G-protein-coupled receptor) is the receptor for relaxin and is characterized by an unusually large ectodomain that terminates with a low density lipoprotein receptor class A module (4). Relaxin also has high affinity for the related receptor, LGR8, which is the receptor for INSL3 (5). The native receptor for H3 relaxin is the unrelated receptor GPCR135, also known as the somatostatin- and angiotensin-like peptide receptor (6). H3 relaxin also has high affinity for the related receptor GPCR142, which is the receptor for INSL5 (7). Both receptors are classic peptide ligand GPCRs and lack a large ectodomain. Importantly, H3 relaxin has a high affinity for LGR7 and will also interact with LGR8, albeit with a significantly lower affinity (8) and hence will interact with all of

The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

The abbreviations used are: H1, human relaxin-1; H2, human relaxin-2; H3, human relaxin-3; GPCR, G-protein-coupled receptor; LRR, leucine-rich repeat; RXFP, relaxin family peptide; MALDI-TOF, matrix-assisted laser desorption time-of-flight; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HEK, human embryonic kidney.
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the relaxin family receptors. Neither INSL4 (9) nor INSL6 (10) can bind to the relaxin family receptors, and their native receptors are unknown. Based on IUPHAR–NC nomenclature, the receptors were recently named relaxin family peptide (RXFP) receptors: LGR7-RXFP1, LGR8-RXFP2, GPCR135-RXFP3, and GPCR142-RXFP4 (11).

The determinants for H3 relaxin activity on RXFP3 and RXFP4 are probably located in the B-chain alone, since synthetic S-reduced H3 relaxin B-chain is an RXFP3 and RXFP4 agonist (12, 13). It has been recently demonstrated that key residues in the H3 relaxin B-chain are responsible for both binding affinity and cAMP-inhibitory activity (14). Further, a series of chimeric peptides that consist of the B-chain of H3 relaxin in combination with A-chains from other members of the relaxin family demonstrated that the A-chain from H1 relaxin, H2 relaxin, INSL3, and INSL6 does not change the pharmacological properties of the H3 relaxin B-chain significantly. However, substitution of the relaxin-3 A-chain with the A-chain from INSL5 results in a chimeric peptide that selectively activates RXFP3 over RXFP1 (13). Hence, although the A-chain of H3 relaxin is not necessary for RXFP3/4 activity, it is essential for RXFP1 activity.

Similarly, H2 relaxin and INSL3 require both A- and B-chains for RXFP1 and RXFP2 activation. Ligand-mediated activation of RXFP1 and RXFP2 involves a three-stage process (15). Primary ligand binding occurs in the leucine-rich repeats (LRRs) of the receptor ectodomain, and there is a lower affinity secondary binding site in the transmembrane exoloops (16). Receptor signaling through cAMP then requires the unique low density lipoprotein receptor class A module at the N terminus of the receptors (15). RXFP1 and RXFP2 receptors without this domain bind ligand normally but do not signal. A recent study has shown that the B-chain of relaxin binds to specific residues in the RXFP1 receptor LRRs (17). This interaction is determined by the arginine residues at B13 and B17 and an isoleucine or valine at position B20 within the B-chain forming a “relaxin binding cassette” (Arg-X-X-X-Arg-X-X-Ile/Val) (18). Similarly, we have recently demonstrated that the INSL3 B-chain interacts with specific residues in the RXFP2 receptor LRRs (19). Primary ligand binding to the LRRs is directed by the B-chain-specific residues HisB12, ArgB16, ValB19, ArgB20, and TrpB27 (20). Importantly, B-chain only INSL3 peptides can bind to the primary binding sites in the LRRs but do not activate the receptor (21). These peptides are low affinity antagonists and highlight that the A-chain is required for receptor activation.

Very little is known about the residues involved in secondary binding to the receptors, although it is known that this site has a lower affinity for the ligand and is necessary for receptor activation (22). Recently, it was demonstrated that INSL3 can be truncated at the N terminus of its A-chain by up to 9 residues without affecting RXFP2 binding affinity (23). However, this truncated peptide does not stimulate cAMP signaling and in fact acts as a high affinity antagonist. It is therefore possible that the N-terminal region of the INSL3 A-chain is somehow involved in the secondary interactions with the RXFP2 receptor, which are necessary for receptor activation.

Similarly, it has previously been shown that the N terminus of the A-chain of porcine relaxin is important for its activity on the mouse pubic symphysis (24). Deletion of more than 3 amino acids resulted in the loss of the ability of porcine relaxin to relax the pubic symphysis. However, the role of the N-terminal A-chain residues in H2 relaxin upon RXFP1 and RXFP2 receptor binding and activation as well as the role of these residues in H3 relaxin upon RXFP1 and RXFP3 binding and activation is unknown. In this study, we have synthesized H2 and H3 relaxin peptides with A-chain truncations to examine whether the N terminus of the A-chain of the relaxin peptides is important for peptide structure, receptor binding, and/or activation. H2 relaxin A-chain-shortened analogs were tested for binding and activation on RXFP1 and RXFP2 only, since H2 relaxin does not bind to RXFP3 (6). H3 relaxin A-chain shortened analogs were tested for binding and activation on RXFP1 and RXFP3 only, since H3 relaxin has a poor affinity for, and does not activate, RXFP2 (22). Interestingly, we found that there are significant differences in the mechanisms by which the various relaxin family peptides activate their receptors. These data provide important new biochemical insights into the action of relaxin peptides.

EXPERIMENTAL PROCEDURES

Solid Phase Peptide Synthesis—Appropriately regioselectively S-protected individual A- and B-chains of H2, H3, INSL3, and their analogs were prepared as their C-terminal amide forms using either continuous flow or microwave-assisted solid phase methodology on an automated PerSeptive Biosystems peptide synthesizer and a CEM Liberty peptide synthesizer, respectively. After simultaneous cleavage, side chain deprotection, and purification of the individual chains, use of a previously reported sequential disulfide bond formation strategy (8) led to the production of the following A-chain-truncated relaxin analogs: A-(5–24) H3, A-(7–24) H3, A-(8–24) H3, A-(9–24) H3, A-(10–24) H3, Ala-A-(9–24) H3, Ala-A-(9–24) H3, A-(9–24) H3, A-(5–24) H2, A-(7–24) H2, A-(9–24) H2, Ala-A-(9–24) H2, Ala-A-(9–24) H2 (Fig. 1). Additionally, the A-chain-truncated INSL3 analogs A-(9–26) INSL3 and A-(10–24) INSL3 were also prepared. The native B-chains of H2, H3, and INSL3 were used in each case. The overall yield was ~5% for H3 analogs, 6% for the INSL3 analogs, and ~10–15% for H2 analogs relative to the starting B-chain peptide.

Peptide Characterization—The purity of each synthetic peptide was assessed by analytical reverse phase HPLC and MALDI-TOF mass spectrometry using a Bruker Autoflex II instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptides were quantitated by amino acid analysis of a 24-h acid hydrolysate using a GBC instrument (Melbourne, Australia).

Circular Dichroism Spectroscopy—CD spectra were recorded on a JASCO (J-185; Tokyo, Japan) spectrophotometer at 25 °C using a 1-mm path length cell. The peptides were dissolved in 10 mM phosphate buffer (pH 7.5) at a concentration of 0.01 or 0.1 mg/ml.

NMR Structural Analysis—The truncated analogues A-(9–24) H2, A-(10–24) H3, and A-(10–24) INSL3 were each analyzed by solution NMR spectroscopy. For each of the peptides, 0.5-ml samples containing 1, 0.5, and ~1 mg, respectively, in the solvent system 90% H2O, 10% D2O at pH 7.5 were prepared. Two-dimensional homonuclear data, including TOCSY,
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NOESY, and DQF-COSY, were recorded at 600 and 900 MHz on Bruker Avance spectrometers. All two-dimensional spectra were generally recorded with 4000 data points in the direct dimension and 512 increments in the indirect dimension, which was zero-filled to 1000 data points prior to transformation. For A-(9–24) H2 relaxin, a series of TOCSY spectra were recorded at 288, 293, 298, 303, and 308 K in order to determine the amide temperature dependence. A temperature coefficient of $> -4.6$ ppb/K was considered indicative of a hydrogen bond (25). Based on this analysis, the following hydrogen bond donors were identified: A15, B5, B9, B10, B12, B19, B21, and B22. Additional data for structural restraints were also recorded on a lyophilized sample redissolved in 100% D$_2$O.

Structural restraints for structure calculations included interproton distances derived from a NOESY spectrum recorded at 900 MHz with a mixing time of 150 ms. Dihedral angle restraints of $-100 \pm 80^\circ$ were introduced for $\phi$ angles, where a positive angle could be excluded based on a stronger interresidual $\text{H}_i - \text{H}_i$ distance than intra-$\text{H}_i - \text{H}_i$ NOE. Hydrogen bond restraints were introduced where hydrogen bonds could be identified based on temperature coefficients in combination with preliminary structures. Preliminary structures were calculated using torsion angle dynamics within the program CYANA (26). The final structures were generated by torsion angle dynamics within CNS followed by Cartesian dynamics and refinement in explicit water as described in detail previously (27).

**Binding Assays**—Human embryonic kidney (HEK)-293T cells stably transfected with RXFP1 and RXFP2 (8) were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum, 100 $\mu$g/ml penicillin, 100 $\mu$g/ml streptomycin, and 2 mM l-glutamine and plated into 24-well poly-L-lysine-coated plates for whole cell binding assays. Competition binding experiments were performed as previously described with either 100 pm $^{33}$P-labeled H2 relaxin (8) or $^{125}$I-labeled INSL3 (28) in the absence or presence of increasing concentrations of unlabeled hormones. Nonspecific binding was determined with an excess of unlabeled peptides (500 nm H2 relaxin or INSL3).

CHO-K1 cells stably expressing RXFP3 (29) were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 5% (v/v) fetal calf serum, 2 mM l-glutamine, 100 $\mu$g/ml penicillin, and 100 $\mu$g/ml streptomycin. Crude membrane preparations were prepared for competition binding curves using 100 pm $^{125}$I-labeled H3 relaxin B-chain-INSL5 A-chain chimeric peptide (kindly labeled by Dr. Steve Sutton) as previously described (13). Nonspecific binding was determined by the addition of 500 nm H3 relaxin.

All data are presented as the mean ± S.E. of the percentage of the total specific binding of triplicate wells, repeated in at least three separate experiments, and curves were fitted using one-site binding curves in Graphpad Prism 4 (Graphpad Software). Statistical differences in pIC$_{50}$ values were analyzed using Student’s $t$ tests in Graphpad Prism 4.

**Inhibition of Forskolin-induced Intracellular cAMP Accumulation**—The influence of the various ligands on cAMP signaling in cells expressing RXFP receptors was assessed using a cAMP reporter gene assay as previously described (15). Briefly, HEK-293T cells in 96-well plates were co-transfected with either RXFP1 or RXFP2 and a pCRE-β-galactosidase reporter plasmid (30). 24 h later, co-transfected cells were treated with increasing concentrations of H2 relaxin, H3 relaxin, or INSL3 analogs in parallel with 10 nm H2 relaxin or INSL3 for RXFP1- or RXFP2-transfected cells, respectively. In addition, INSL3 analogs were tested for their ability to block 1 nM INSL3 stimulation over 6 h. CHO-K1 cells in 96-well plates were co-transfected with RXFP3 and the pCRE-β-galactosidase reporter plasmid, and 24 h later, they were treated with 5 $\mu$m forskolin together with increasing concentrations of H3 relaxin analogs. 10 nm H3 relaxin was used for maximal stimulation, whereas untreated cells were used as controls. After 6 h, the cell medium was aspirated, and the cells were frozen at $-80^\circ$C overnight. The amount of cAMP-driven β-galactosidase expression in each well was determined as previously described. Ligand-induced stimulation of cAMP was expressed as a percentage of the maximum H2 relaxin, INSL3, and H3 relaxin response for RXFP1, RXFP2, and RXFP3 cells, respectively. Data points were measured in triplicate, and each experiment was repeated at least three times.

**RESULTS**

**Synthesis of Relaxin Analogs**

A number of INSL3, H2, and H3 relaxin analogs as outlined in Fig. 1 were prepared for functional and structural studies. In all cases, a highly efficient solid phase peptide synthesis strategy using regioselectively S-protected relaxin or INSL3 A- and B-chains followed by sequential disulfide bond formation was utilized (8). This method assures a good overall yield and avoids a tedious and labor-intensive random oxidation method. Each peptide was comprehensively chemically characterized, either RXFP1 or RXFP2, and the pCRE-β-galactosidase reporter plasmid (30). 24 h later, co-transfected cells were treated with increasing concentrations of H2 relaxin, H3 relaxin, or INSL3 analogs in parallel with 10 nm H2 relaxin or INSL3 for RXFP1- or RXFP2-transfected cells, respectively. In addition, INSL3 analogs were tested for their ability to block 1 nM INSL3 stimulation over 6 h. CHO-K1 cells in 96-well plates were co-transfected with RXFP3 and the pCRE-β-galactosidase reporter plasmid, and 24 h later, they were treated with 5 $\mu$m forskolin together with increasing concentrations of H3 relaxin analogs. 10 nm H3 relaxin was used for maximal stimulation, whereas untreated cells were used as controls. After 6 h, the cell medium was aspirated, and the cells were frozen at $-80^\circ$C overnight. The amount of cAMP-driven β-galactosidase expression in each well was determined as previously described. Ligand-induced stimulation of cAMP was expressed as a percentage of the maximum H2 relaxin, INSL3, and H3 relaxin response for RXFP1, RXFP2, and RXFP3 cells, respectively. Data points were measured in triplicate, and each experiment was repeated at least three times.

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A. Pooled binding affinities (pKᵢ) and cAMP activity (pEC₅₀) of INSL3 A-chain truncated analogs

| Ligand       | ¹²⁵I-INSL3 RXFP2 pKᵢ | RXFP2 cAMP pEC₅₀ |
|--------------|-----------------------|------------------|
| INSL3        | 9.34 ± 0.02 (4)       | 10.35 ± 0.12 (3) |
| A(9-26) INSL3| 9.14 ± 0.10 (4)       | antagonist        |
| A(10-24) INSL3| 8.67 ± 0.04 (3)       | antagonist        |

*p<0.001 vs INSL3 and A9-26 INSL3; *From (20)

B. ¹²⁵I-INSL3 competition binding curves

C. Inhibition of INSL3 stimulated cAMP activity

including by MALDI-TOF mass spectrometry, and high purity was confirmed.

A-chain-shortened INSL3 Analogs

Analogs of INSL3 were first prepared to confirm the reported effects of A-chain shortening on the INSL3 peptide in our RXFP2-expressing cell line. As previously reported (23), A-(9–26) INSL3 demonstrated equivalent binding affinity to native INSL3 (Fig. 2, A and B). Additionally, a further truncated analog A-(10–24) INSL3 also showed high binding affinity, although it was significantly lower (p < 0.001) than both native INSL3 and A-(9–26) INSL3 (Fig. 2, A and B). Neither peptide was able to stimulate cAMP in RXFP2-expressing cells (data not shown); however, they were both able to dose-dependently inhibit INSL3-mediated cAMP signaling (Fig. 2C).

A-chain-shortened H3 Relaxin Analogs

Analogs of H3 relaxin that had been sequentially shortened by 7, 8, or 9 amino acids at the N terminus of their A-chains were synthesized and first tested for their ability to bind to and activate RXFP3. Analogs A-(8–24) H3, A-(9–24) H3, and A-(10–24) H3 relaxin all demonstrated high affinity binding to RXFP3-expressing cells (Fig. 3A and Table 1). Additionally, they were all able to inhibit forskolin-stimulated cAMP production with a potency similar to that of native H3 relaxin (Fig. 3B). Hence, A-chain shortening had no effect on H3 relaxin activity on RXFP3.

These peptides were then tested for their ability to bind to and activate RXFP1. In stark contrast to the effects of A-chain shortening on the INSL3 peptide, the H3 relaxin analogs demonstrated a progressive loss of binding affinity with A-chain shortening by 7, 8, or 9 amino acids (Fig. 4 and Table 1). Most importantly, the peptides were able to stimulate cAMP activity in RXFP1-expressing cells with activities matching their binding affinities (Fig. 4C and Table 1). Hence, A-(8–24) H3, A-(9–24) H3, and A-(10–24) H3 all had a lower affinity and potency than H3 relaxin (p < 0.001; Table 1), and additionally the affinity of A-(10–24) H3 for RXFP1 was significantly lower than A-(8–24) H3 (p < 0.05; Table 1).

Two additional A-chain-shortened peptides, A-(5–24) H3 and A-(7–24) H3 relaxin, were synthesized to determine the
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TABLE 1
Pooled binding (pK<sub>i</sub>) and cAMP (pEC<sub>50</sub>) data from H2 and H3 relaxin A-chain-truncated peptides

| Ligand | RXFP1 | RXFP3 | RXFP2 |
|--------|--------|--------|--------|
|        | <sup>3</sup>P-relaxin binding pK<sub>i</sub> | cAMP activity | <sup>125</sup>I-H3/INSL3 binding pK<sub>i</sub> | cAMP activity | <sup>3</sup>P-Rx relaxin binding pK<sub>i</sub> | cAMP activity |
| H3     | 7.69 ± 0.04 (3) | 9.36 ± 0.21 (4) | 8.48 ± 0.04 (3) | 8.26 ± 0.09 (3) | NA<sup>a</sup> | NA |
| A-(5–24) H3 | 8.18 ± 0.11 (3)<sup>b</sup> | 8.34 ± 0.06 (3)<sup>b</sup> | ND<sup>c</sup> | ND<sup>c</sup> | NA | NA |
| A-(7–24) H3 | 6.87 ± 0.03 (3)<sup>c</sup> | 6.88 ± 0.13 (3)<sup>c</sup> | ND | ND | NA | NA |
| A-(8–24) H3 | 6.30 ± 0.02 (3)<sup>c</sup> | 6.58 ± 0.31 (3)<sup>c</sup> | 8.34 ± 0.08 (3) | 7.96 ± 0.07 (2) | NA | NA |
| A-(9–24) H3 | 5.84 ± 0.16 (3)<sup>c</sup> | 7.14 ± 0.01 (3)<sup>c</sup> | 8.02 ± 0.12 (3) | 8.75 ± 0.12 (3) | NA | NA |
| A-(10–24) H3 | 5.58 ± 0.16 (3)<sup>c</sup> | 6.45 ± 0.09 (3)<sup>c</sup> | 8.22 ± 0.08 (3) | 7.98 ± 0.11 (3) | NA | NA |
| Ala-4 A-(9–24) H3 | 7.86 ± 0.11 (3)<sup>c</sup> | 7.23 ± 0.13 (3)<sup>c</sup> | ND | ND | NA | NA |
| Ala-5 A-(9–24) H3 | 7.69 ± 0.16 (3)<sup>c</sup> | 6.99 ± 0.30 (4)<sup>c</sup> | ND | ND | NA | NA |
| H2     | 9.24 ± 0.16 (3) | 10.37 ± 0.04 (4) | NA | NA | 8.48 ± 0.22 (3) | 9.13 ± 0.06 (3) |
| A-(5–24) H2 | 8.85 ± 0.09 (3) | 10.37 ± 0.12 (5) | NA | NA | 7.78 ± 0.16 (3)<sup>f</sup> | 7.64 ± 0.30 (3)<sup>f</sup> |
| A-(7–24) H2 | 8.62 ± 0.06 (3) | 9.92 ± 0.24 (4) | NA | NA | 7.01 ± 0.09 (3)<sup>f</sup> | 7.06 ± 0.34 (3)<sup>f</sup> |
| A-(9–24) H2 | 6.55 ± 0.10 (3)<sup>f</sup> | 9.12 ± 0.20 (3)<sup>f</sup> | NA | NA | 6.77 ± 0.23 (3)<sup>f</sup> | <6 |
| Ala-4 A-(9–24) H2 | 8.43 ± 0.04 (3)<sup>j</sup> | 9.88 ± 0.16 (3)<sup>j</sup> | NA | NA | 7.34 ± 0.13 (3)<sup>f</sup> | 7.52 ± 0.43 (3)<sup>f</sup> |
| Ala-5 A-(9–24) H2 | 8.49 ± 0.06 (3)<sup>j</sup> | 9.79 ± 0.19 (4)<sup>j</sup> | NA | NA | 7.59 ± 0.12 (3)<sup>j</sup> | 7.19 ± 0.12 (3)<sup>j</sup> |
| INSL3   | NA | NA | 0.01 ± 0.08 (3)<sup>f</sup> | 10.35 ± 0.12 (3)<sup>f</sup> | NA | NA |

<sup>a</sup> NA, no activity
<sup>b</sup> p < 0.001 versus H3 relaxin.
<sup>c</sup> ND, not determined.
<sup>d</sup> p < 0.01 versus H3 relaxin.
<sup>e</sup> p < 0.001 versus H3 A-(9–24).
<sup>f</sup> p < 0.01 versus H2 relaxin.
<sup>g</sup> p < 0.001 versus H2 A-(9–24).
<sup>h</sup> p < 0.05 versus H2 relaxin.
<sup>i</sup> p < 0.05 versus H2 A-(9–24).

FIGURE 4. Activity of the A-chain-truncated H3 relaxin analogs on the RXFP1 receptor. Shown are competition binding results of the A-chain-truncated analogs (A) and alanine-extended A-chain-truncated analogs (B) compared with H2 and H3 relaxin in HEK-293T cells stably expressing the RXFP1 receptor using <sup>3</sup>P-labeled H2 relaxin as the radioligand. Data are expressed as percentage of specific binding and were pooled data from at least three experiments performed in triplicate. Shown is cAMP activity of the A-chain-truncated analogs (C) and alanine-extended A-chain truncated analogs (D) compared with H2 and H3 relaxin in HEK-293T cells expressing the RXFP1 receptor using a pCRE-β-galactosidase reporter gene system. Data are expressed as percentage of maximum H2 relaxin-stimulated cAMP response and were pooled data from at least three experiments performed in triplicate.

minimum length of A-chain required for maximum binding affinity and potency. A-(7–24) H3 relaxin also demonstrated a significantly reduced affinity to RXFP1 compared with H3 relaxin, which was similarly reflected in a decreased potency (both p < 0.001; Table 1). In comparison with the shorter H3 truncates, A-(7–24) H3 relaxin was found to have a significantly higher affinity for RXFP1 (all p < 0.01 or p < 0.001), but interestingly, its potency in stimulating cAMP activity was not significantly different from these shorter truncated H3 relaxin peptides. The receptor binding of A-(5–24) H3 relaxin was fully restored, and in fact it even showed slightly higher affinity for RXFP1 than native H3 relaxin itself (Fig. 4A), but this was not statistically significant (p > 0.05; Table 1). Interestingly, in terms of potency A-(5–24), H3 relaxin was significantly more potent than the other A-chain truncated H3 relaxin peptides but, despite showing full affinity, remained significantly less potent than native H3 relaxin (all p < 0.001; Table 1). Truncations of the A-chain N terminus thus had a more pronounced effect on the potency than the affinity of H3 relaxin for RXFP1.

Alanine-extended A-chain-shortened H3 Analogs

To test the hypothesis that the decreased activity of the A-chain-shortened H3 relaxin peptides was due to a structural effect rather than specific side chain-driven influences, analogs of A-(9–24) H3 relaxin were synthesized with multiple alanine residue extensions. Hence, four or five alanine residues were added to the N terminus of the A-(9–24) H3 relaxin peptide by chemical peptide synthesis, resulting in the peptides Ala-4 A-(9–24) H3 and Ala-5 A-(9–24) H3 relaxin (Fig. 1). These
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peptides were tested for their ability to bind to and activate RXFP1 (Fig. 4, B and D). Remarkably, both peptides demonstrated significantly increased affinity for RXFP1 compared with A-(9–24) H3 relaxin (p < 0.001; Table 1), and the pKᵢ values were in fact not different from that of H3 relaxin. However, there was no change in the potency of the peptides compared with A-(9–24) H3 relaxin (Fig. 4D and Table 1). Hence, the addition of multiple alanine residues to the N terminus of the A-chain by 8 residues to influence both binding where there was a 500-fold lower affinity (Fig. 5B and Table 1). Hence, the H2 relaxin peptide needs to be shortened at the N terminus of the A-chain by 8 residues to influence both the binding and cAMP activity of the peptide, and in contrast to H3 relaxin, such a truncation has a bigger effect on the affinity than the potency.

To assess whether the addition of alanine residues to the N terminus of the A-(9–24) H2 relaxin peptide could rescue binding of the peptide as with H3 relaxin, both Ala-4 A-(9–24) H2 and Ala-5 A-(9–24) H2 relaxin were synthesized and tested for their RXFP1 activity. Importantly, both peptides displayed significantly increased binding affinity compared with A-(9–24) H2 relaxin, although the binding affinity was still lower than H2 relaxin (Fig. 5A and Table 1). However, in contrast to the H3 relaxin peptides, there was also a significant increase in potency of the peptides compared with A-(9–24) H2 relaxin, although the potency matched the affinity and was still lower than H2 relaxin (Fig. 5B and Table 1).

The RXFP2 Receptor Activity of H2 Relaxin A-shortened Peptides

Finally, the activity of the H2 relaxin A-chain-shortened peptides was tested on RXFP2. H3 relaxin A-chain-shortened analogs were not tested, since H3 relaxin does not activate RXFP2 (16). As reported previously (22), H2 relaxin was able to bind to...
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RXFP2 with high affinity and activate cAMP signaling with high potency (Fig. 6, A and B, and Table 1). The A-chain-shortened H2 relaxin peptides displayed a progressive decrease in binding affinity compared with H2 relaxin (Fig. 6A and Table 1). In contrast to their binding affinity for RXFP1, both A-(5–24) H2 (p < 0.001) and A-(7–24) H2 relaxin (p < 0.001) showed significantly decreased binding affinity for RXFP2. The binding affinity of A-(9–24) H2 relaxin was not significantly different from A-(7–24) H2 relaxin (Table 1). The potency of A-(5–24) H2 and A-(7–24) H2 relaxin were both significantly lower than H2 relaxin (p < 0.001), matching the parallel decrease in their binding affinities (Fig. 6B and Table 1). A-(9–24) H2 relaxin, however, displayed extremely low potency (<1 μM; Fig. 6B and Table 1) on RXFP2. The low potency of this truncated peptide in conjunction with its relatively high affinity suggested that it may be a partial agonist of RXFP2. The peptide was therefore tested for its ability to antagonize INSL3-induced cAMP signaling. However, A-(9–24) H2 relaxin displayed no antagonist activity and in fact appeared to slightly enhance INSL3 activity (supplemental Fig. 1). Therefore, in stark contrast to A-chain truncated INSL3 analogs, A-chain truncation of H2 relaxin results in the loss of binding activity, which is associated with a progressive loss of potency. Importantly, the addition of alanine residues to the N terminus of the A-(9–24) H2 relaxin peptide improved RXFP2 binding affinity (Fig. 6A and Table 1) and more importantly cAMP activity (Fig. 6B and Table 1) in a similar fashion to the effects on RXFP1. Hence, both Ala-4 A-(9–24) H2 and Ala-5 A-(9–24) H2 relaxin demonstrated increased binding affinity, although in the case of the Ala-4 analog, this was not significantly different from A-(9–24) H2 relaxin. cAMP activity of the two peptides was significantly improved, and although this was still lower than native INSL3, it was not significantly different from A-(5–24) H2 relaxin.

**Structural Studies of H2, H3, and INSL3 Analogs**

**Circular Dichroism Spectroscopy**—As a first step to understanding the influence of A-chain truncation on the conformation of the H2/H3 relaxin peptides, they were studied by CD spectroscopy. Six peptides were selected for this study: native H3, A-(9–24) H3, Ala-5 A-(9–24) H3, native H2, A-(9–24) H2, and Ala-5 A-(9–24) H2 relaxin. The CD spectra of all six peptides show a typical α-helical pattern with double minima at 208 and 222 nm (Fig. 7) (31). The mean residual weight ellipticity at 222 nm, [θ]222, was used to calculate the helix content (32). The [θ]222 value for native H3 is −12,958.7, which corresponded to an α-helix content of 36%. Truncation of the N-terminal A-chain in A-(9–24) H3 relaxin caused a significant decrease in the [θ]222 value, −10,061.10, which corresponded to 29% of α-helix. Incorporation of five alanine residues into the analog Ala-5 A-(9–24) H3 relaxin, caused a partial recovery of the [θ]222 value, −11,866.6, which corresponded to 34% of α-helix.

Similarly, the [θ]222 value for native H2 is −17,303.5, which corresponded to α-helix content of 49%. Truncation of the N-terminal A-chain in A-(9–24) H2 relaxin caused a significant decrease in the [θ]222 value, −12,546.1, which corresponded to 36% of α-helix. Incorporation of five alanine residues, Ala-5 A-(9–24) H2 relaxin, caused a partial recovery of the [θ]222 value, −13,996.7, which corresponded to 39% of α-helix.

**NMR Spectroscopy**—To further characterize the structural consequences of the A-chain shortening, we subjected the three analogues with the most significant truncations, A-(9–24) H2, A-(10–24) H3 relaxin, and A-(10–24) INSL3, to detailed analysis by two-dimensional NMR. For each of the peptides, TOCSY and NOESY spectra on 0.2–0.5 mM samples were acquired at 600 and 900 MHz. Strikingly, the quality of the data differed significantly between the three peptides, indicating a clear difference in the effects of the truncations on the peptide fold. A-(9–24) H2 relaxin showed relatively well dispersed signals with a significant number of both sequential and nonsequential NOEs, typical of a folded protein. A-(10–24) H3 relaxin similarly displayed resolved signals, but unlike for A-(9–24) H2 relaxin, the data contained only a very limited amount of NOEs, consistent with a less defined core structure. In contrast, A-(10–24) INSL3 showed very broad signals, clearly indicating either a degree of aggregation or the presence of significant dynamic processes, resulting in loss of signal through resonance frequency averaging and broadening. The quality of the data allowed for a nearly complete resonance assignment for the A-(9–24) H2 relaxin but only limited assignment for A-(10–24) H3 relaxin and virtually no confirmed assignments for A-(10–24) INSL3 using standard sequential assignment strategies (33). The deviations of the observed chemical shift for the Hα protons from random coil values generally provide good evidence for the presence of secondary structure (34). A comparison of these secondary shifts between the truncated and native H2 and H3 relaxins is presented in Fig. 8. From the analyses of both native H2 and H3 relaxin, the typical insulin/relaxin fold, comprising three helical segments and two extended regions, is evident. The latter segments form a small antiparallel β-sheet between the two peptide chains. Interestingly, the picture is clearly different in A-(9–24) H2 as a result of the removal of the
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First helical segment. A small degree of helical character may be retained in the A-chain, but the shifts suggest a close to random coil-like structure. In the region of the native B-chain helix (residues 13–22) a helical nature appears to be retained, although there is a clear general decrease in the size of the deviation from random coil consistent with a more flexible fold. Most strikingly, the β-sheet interaction is completely diminished, and in fact the majority of the B-chain appears to have a helical nature in contrast to the native peptide, where the first five residues are more disordered in solution. The observation of helical-like secondary shift were supported by the identification of typical medium range NOE contacts normally found in helices throughout most of the B-chain and the presence of helical-like hydrogen bonds identified by temperature coefficients once suitable acceptors were identified from the preliminary structures. The majority of these hydrogen bonds were found in the B-chain helical region, confirming the helical structure, but interestingly when the lyophilized protein was dissolved in deuterium oxide, no slow exchanging amide protons could be detected, consistent with the structure being flexible and undergoing continuous unfolding/refolding. Restraints and structural statistics for the family of 20 structures representing the solution structure of A-(9–24) H2 relaxin are summarized in Table 2. Fig. 9A shows the solution structure of A-(9–24) H2 relaxin, and from here it is clear that indeed most of the B-chain adopts a helical structure with the exception of a kink region around GlyB12. In contrast, most of the A-chain is highly flexible, and a lack of restraints and broad lines for the N-terminal region (residues A10–A15) results in an inability to define this region’s position in relation to the rest of the molecule.

DISCUSSION

In this study, we have demonstrated that the role of the A-chain of H2 and H3 relaxin in binding and activation of the different RXFP receptors is quite different from that of the related peptide INSL3 interacting with its receptor RXFP2. Truncation of the A-chain of INSL3 up to the CysA10 residue involved in the intrachain disulfide bond with CysA15 does not influence RXFP2 binding affinity. However, the truncation results in the complete loss of the ability of the peptide to stimulate cAMP signaling (23). A-(9–26) INSL3 and A-(10–24) INSL3 are thus high affinity RXFP2 antagonists and important research tools. In stark contrast, truncation of the A-chain of H2 or H3 relaxin up to residue 9 led to decreased RXFP1 binding affinity, which was directly related to lowered potency in stimulated cAMP signaling. Similar results were obtained for H2 relaxin A-chain-truncated peptides on RXFP2, and the effects on potency were more pronounced. Importantly, truncation did not result in an RXFP2 antagonist or partial agonist. A-chain truncation of the H3 relaxin peptide had no effect on hydrogen bonds that could be inferred from amide temperature coefficients once suitable acceptors were identified from the preliminary structures. The majority of these hydrogen bonds were found in the B-chain helical region, confirming the helical structure, but interestingly when the lyophilized protein was dissolved in deuterium oxide, no slow exchanging amide protons could be detected, consistent with the structure being flexible and undergoing continuous unfolding/refolding.

| Distance constraints | Values |
|-----------------------|--------|
| Total interresidue NOE | 280    |
| Sequential (|i - |j| = 1) | 152 |
| Medium range (|i - |j| = 4) | 26 |
| Long range (|i - |j| = 5) | 102 |
| Hydrogen bonds (for 6 hydrogen bonds) | 12 |

| Structure statistics | Values |
|----------------------|--------|
| Violations | 0.1/structure |
| Dihedral angle constraints (|>2 |°|) | 0 |
| Maximum dihedral angle constraints (degrees) | 0.34 |
| Deviation from idealized geometry | <2 |
| Bond length (Å) | 0.0033 ± 0.00022 |
| Bond angles (degrees) | 0.44 ± 0.024 |
| Improper (degrees) | 0.42 ± 0.027 |
| Average pairwise root mean square deviation* (Å) | 2.31 ± 0.55 |
| Backbone | 1.22 ± 0.43 |

* Pairwise root mean square deviation was calculated from 20 refined structures over amino acids B4–B23.
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binding or signaling on RXFP3. Hence, it would appear that the role of the A-chain is both peptide- and receptor-dependent.

Role of the A-chain for Peptide Structure—From the NMR solution structures of H3 relaxin (35) and INSL3 (20) and the crystal structure of H2 relaxin (36), it is clear that the peptides have a very similar insulin-like core structure, with the key differences being mainly around the chain termini. For both H2 and H3 relaxin, the A-chains are highly structured with two characteristic α-helical segments from A1 to A13 and from A17 to A24. Residues close to the A-chain N termini are involved in close contacts with other regions of the peptide; e.g., interactions are seen between LeuA2, TyrA3, LeuA6, and PheA23 in H2 relaxin and between LeuA2, LeuA5, LeuA6, LeuA23, and PheB14 in H3 relaxin, suggesting that the N-terminal region of the A-chain is of structural importance. In contrast, the INSL3 N-terminal residues are disordered, with the first structurally ordered residue being Asn at position 5. Consequently the N-terminal region has fewer long range contacts with the molecular core, with only the short side chains of ProA6 and AlaA7 forming some interactions with LeuA23 and PheB14. This is possibly the reason why truncation at the N terminus of the INSL3 A-chain did not affect the binding with its receptor RXFP2, since removal of residues A1–A8 would appear to be possible without major consequences for the overall fold. The NMR analysis of A-(10–24) INSL3 did not provide a clear answer as to how much of the structure was disrupted due to the severe signal broadening, but Bullesbach et al. (23) showed by CD studies that the overall structure was to a large extent retained after truncations up to A9. It should be noted that INSL3 is a rather dynamic peptide and that significant line broadening was observed in the region around the CysA10, CysA15 disulfide also in the native peptide (20). For H2 and H3 relaxin, we postulated that the loss of α-helix structure in the A-chain of H2 and H3 relaxin by removal of the N-terminal residues may have caused an overall loss of structure, resulting in the observed decrease in RXFP1 binding affinity and potency. CD and NMR studies confirmed that indeed the removal of the N-terminal A-chain helix in analogues A-(9–24) H2 relaxin and A-(10–24) H3 relaxin resulted in a significant decrease in A-chain shortening (37). The partial recovery of RXFP1 and RXFP2 binding affinity and potency in H2 relaxin, and RXFP1 binding affinity in H3 relaxin, by incorporating these α-helix-forming Ala residues suggests that the major role of this region of the peptide is structural. This is the same conclusion reached by the authors of the study on INSL3 N-terminal truncations (23). They suggested that “the role of the A-chain tail is merely to provide bulk to maintain a certain conformation of the receptor activation complex” (23). It therefore would appear that the alterations in activity seen as a result of the truncation of the A-chain N termini from the different peptides are having different roles; they similarly are driven by structural influences rather than specific side chain interactions.

Activation of RXFP3 by H3 Relaxin—The structure of RXFP3 is markedly different from RXFP1 and -2, being a classic peptide GPCR without a large ectodomain. Previous studies have shown that the H3 relaxin B-chain alone is an agonist of RXFP3 (6) although with a significantly lower affinity. More recent studies have demonstrated that the B-chain carries all of the features necessary for receptor binding and activation (14) but that presumably without the A-chain it is largely unstructured, and hence the interaction with the receptor would be expected to be less favorable. Here we have shown that in contrast to removal of the whole A-chain, truncation of the A-chain N terminus of H3 relaxin up to CysA10 has no effect on binding and potency on RXFP3. However, interestingly, the NMR data reveal a significant disruption of overall structure in the A-(10–24) H3 relaxin. Thus, although the shorter A-chain does not have the ability to fully stabilize the native fold, it appears to be able to provide enough structural support to retain native biological activity.

Activation of RXFP1 by H2 and H3 Relaxin—A number of studies have demonstrated that binding to RXFP1 and RXFP2 requires a more complex interaction involving several contact points, which at least to some extent relies on the peptide A-chain. Our findings confirm this idea but also provide new information about the mechanism. The general trend is that truncation to the A-chain N termini in both H2 and H3 relaxin of up to 4 amino acids does not have a significant affect on the
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binding or activation of the receptor. Further truncations reduce both the affinity and potency, highlighting that H2 and H3 relaxin are binding to and activating RXFP1 in a similar manner. Interestingly, the effect on affinity rather than potency is more pronounced in the case of H2 relaxin, whereas the opposite is true for truncated H3 relaxin. Given that it is well established that the primary relaxin binding motif is located in the B-chain and that the binding and to some extent activity is regained when the N-terminal residues are replaced by Ala, this change is most likely due to a disruption to the overall fold rather than removal of a key interaction site. The structural studies by both CD and NMR spectroscopy confirm this idea. Truncation of neither H2 nor H3 relaxin failed to produce an antagonistic analogue; thus, it seems likely that a second interaction site important for receptor activation is located in the C-terminal part of the A-chain, possibly in the region of the second helix rather than the region of the truncations.

Activation of RXFP2 by INSL3 and H2 Relaxin—The similarities in overall structure of H2 relaxin and INSL3 together with RXFP1 and RXFP2 would suggest an identical or at least similar way of binding and activation. This would indeed appear to be true for the primary binding mechanism, since we have previously shown that relaxin and INSL3 use similar residues in the RXFP2 LRRs for primary binding (19). However, as shown in this study, there is a distinct difference in the behavior of A-(10–24) INSL3 and A-(9–24) H2 relaxin. A-(10–24) INSL3 retains full binding affinity but lacks the ability to activate RXFP2. Thus, in the case of INSL3, binding and activation can be considered as two separate events. In contrast, our data on H2 relaxin suggest that binding and activation are intimately linked and that interactions that are responsible for the activation of the receptor also contribute to the overall binding affinity. Therefore, it is likely that there is a different mechanism for secondary binding to the TM exoloops and that this secondary binding site influences overall binding affinity. Further evidence for this concept is the lack of RXFP1 binding activity of B-chain-only analogs of H2 relaxin (38), whereas similar B-chain analogs of INSL3 bind to RXFP2 and act as antagonists (21). Interestingly, the behavior of the truncated H2 analogs is similar on RXFP1 and RXFP2, albeit with lower affinity and activity on RXFP2, suggesting that H2 relaxin activates RXFP2 in an “RXFP1-like” manner. Based on the data, it seems likely that the site mediating this interaction in INSL3 is located closer to the N terminus, whereas in H2 relaxin, it is probably closer to the C-terminal region.

Conclusions—In summary, we have presented an extensive structure function study on H2 and H3 relaxin. Our results provide a number of new biochemical insights into the mechanism of activation of RXFP1, RXFP2, and RXFP3 by the hormones H2 and H3 relaxin. It is clear that the A-chain is not needed for RXFP3 activation by H3 relaxin. Truncation of up to 9 residues leads to a significant disruption of the fold; nonetheless, the peptide retains almost full activity. In contrast, A-chain-truncated H2 and H3 relaxins show a decrease both in affinity and potency at RXFP1. Finally, the mechanism of activation of RXFP2 by H2 relaxin is distinctly different from that of INSL3. The fact that it is not possible to generate antagonistic analogs of H2 relaxin suggests that binding and activation are closely linked and that H2 relaxin interacts with RXFP2 in the same way as it interacts with its own receptor RXFP1. These data provide important new biochemical insights into the mechanisms of receptor activation of relaxin family peptides and will be essential for the future design of specific RXFP receptor agonists.

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