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Conformationally constrained single-chain peptide mimics of relaxin B-chain secondary structure

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Abstract: Relaxin is a member of the insulin superfamily and has many biological actions including angiogenesis and collagen degradation. It is a 6 kDa peptide hormone consisting of two peptide chains (A and B) tethered by two disulphide bonds. Past structure–function relationship studies have shown the key receptor binding site of relaxin to be principally situated within the B-chain α-helix. Molecular dynamic simulations were performed to aid the design of conformationally constrained relaxin B-chain analogues that possess α-helical structure and relaxin-like activity. Restraints included disulphide bonds, both single and double, and lactam bonds. Each peptide was prepared by solid phase synthesis and, following purification, subjected to detailed conformational analysis by circular dichroism spectroscopy. Of 15 prepared relaxin B-chain mimetics, one was able to mimic the secondary structure of the native ligand as indicated by biomolecular recognition/interaction analysis using surface enhanced laser desorption ionization mass spectroscopy together with a relaxin antibody. However, none of the mimetics possess characteristic relaxin-like biological activity which strongly indicates that the pharmacophore comprises additional structural elements other than the relaxin B-chain α-helix. These findings will assist in the design and preparation of novel relaxin agonists and antagonists.

Keywords: α-helix; biomolecular interaction; molecular dynamics; peptidomimetics; relaxin

INTRODUCTION

Relaxin has traditionally been considered a hormone of pregnancy, regulating the growth and remodelling of reproductive tissues such as the cervix [1]. In rats, relaxin has been shown to inhibit both spontaneous as well as oxytocin-induced uterine contraction [2]. In addition to the known functions of relaxin in reproductive tissues [1], relaxin is secreted as a paracrine factor by several non-reproductive tissues in both sexes [3]. Further new insights into the physiological role of relaxin were recently derived from the analysis of relaxin knockout mice [4,5]. It was established that such animals exhibit abnormal collagen remodelling resulting in age-related lung fibrosis [6], and male-specific ventricular diastolic heart dysfunction [7]. Additionally, it has been shown that mutant males develop age-related abnormalities of the testis, epididymis and prostate [8]. It has also been demonstrated both in cell culture experiments and *in vivo* models that relaxin regulates proliferation, angiogenesis and connective tissue remodelling through activation of molecular targets such as vascular endothelial growth factor and matrix metallo-proteinases [9].

Relaxin is a structurally related member of the insulin family of proteins, consisting of two peptide chains (A and B) tethered by two disulphide bonds. In the human, there are three genes which each code for a relaxin [1]. Human relaxin-2 ('relaxin') is the principal expression product *in vivo* and forms the subject of this work. Structure–function relationship studies of relaxin suggest that three amino acids that reside within the B-chain α-helix (Arg B13, Arg B17 and Ile B20) are essential for bioactivity (Figure 1). It also seems from these studies that the A-chain acts at least in part as a scaffold for the B-chain, to help present key residues to the receptor in the correct conformation [10,11]. In support of this hypothesis, the relaxin B-chain has been shown previously to have no secondary structure once the A-chain is removed [12]. Structure–function studies have also been carried out on the A-chain within relaxin [10], but a complete analysis of all residues required for receptor binding and bioactivity has yet to be performed.

Recently it has been shown that a leucine-rich repeat containing G-protein coupled orphan receptor, LGR7, binds relaxin and subsequently leads to an accumulation of cAMP when expressed in cells [13]. LGR7 consists of a *C*-terminal tail that binds to a Gs protein, seven transmembrane domains and a large ectodomain comprising ten repeating leucine-rich regions. Ligands acting on G-protein coupled receptors

Abbreviations: ivDde, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl; Dmab, {N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}; LGR7, leucine-rich G-protein coupled receptor 7; RMSD, root mean square deviation; SELDI-TOF, surface-enhanced laser desorption ionization time-of-flight, mass spectrometry.

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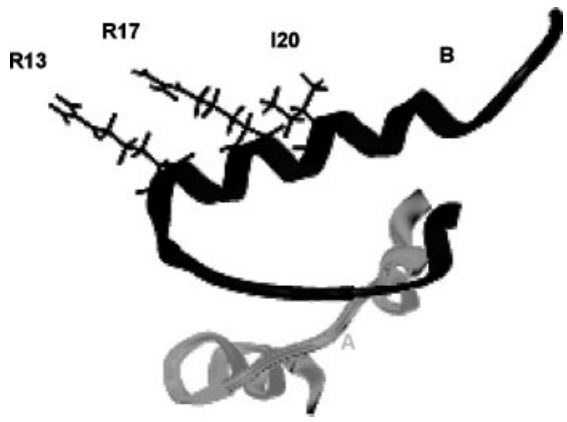

Figure 1 X-ray crystal structure of relaxin (**1**) showing the A-chain (grey), B-chain (black) and the relaxin binding motif (R13, R17 and I20) on the B-chain α-helix.

usually bind to an exoloop of the transmembrane region. In contrast, it has been shown that relaxin binds to the ectodomain alone [12], which would explain the ability of the ectodomain of LGR7 to antagonize relaxin *in vitro* and *in vivo* [12]. The expression of LGR7 throughout the body is also consistent with the wide distribution of relaxin binding sites found in various tissues.

To test the hypothesis that the B-chain is solely responsible for binding and activation of relaxin to LGR7, conformationally constrained analogues of the relaxin B-chain were designed. The likely stability of the designed compounds was assessed by molecular dynamics simulations. Subsequently selected analogues were synthesized, and their helical content determined using circular dichroism (CD) spectroscopy, and their ability to inhibit or mimic relaxin activity in cell culture was assessed.

EXPERIMENTAL PROCEDURES

Materials

Fmoc-amino acid derivatives for peptide synthesis were purchased from Auspep (West Melbourne, Australia). Reversed-phase high performance liquid chromatography (RP-HPLC) columns were obtained from Vydac (Hesperia, USA). Solvents and chemicals were all of peptide synthesis or analytical grade. Synthetic human relaxin-2 was obtained from stocks previously described [14] and goat anti-relaxin antiserum was a gift from Genentech (South San Francisco, USA).

Molecular Design of Relaxin Analogues

With the aid of SYBYL molecular modelling software (version 6.6, Tripos, St Louis, USA) on a Silicon Graphics O2 workstation, the x-ray crystal structure of human relaxin-2 B-chain [15] was used as a template to design analogues with theoretical increased α-helicity. The first analogues consisted of the key receptor binding domain situated within the B-chain (B13–20) and an i to i + 4 constraint. A suitable site in the sequence for a constraint was identified and the residues mutated either to cystine to form a disulphide constrained mimetic (peptide **3**), or Asp and Lys and to give a lactam (peptide **4**). The energies of the analogues were then minimized with respect to their geometries using the Sybyl forcefield and Gasteiger-Marsili charges. The backbones of the minimized analogues were then superimposed on the backbone of the native B13–20 peptide and the root mean square deviation (RMSD) of the backbone atoms was obtained, as a means of comparing the similarity of the analogues with native relaxin.

The next group of analogues designed consisted of the entire length of α-helix on the B-chain (B11–25). As described above, sites for an i to i + 4 constraint were determined. The constraints were then incorporated into the peptide and the resulting analogues were energy minimized. These peptides (**6–12**) were also examined for their similarity to the native sequence by comparing their backbones and obtaining an RMSD value.

A further set of analogues (**14–16**) was designed by incorporating a constraint between the α-helix and strand of the B-chain. Appropriate sites for this constraint were identified by generating a van der Waals dot surface for the relaxin B-chain, and examining the dot surface for juxtaposed/overlapping residues on the helix and strand. Three pairs of residues fell into this category: Trp^3 and Met^{25}, Met^4 and Ile^{22} and Ile^8 and Ala^{21} (residues within the binding motif were not considered). The three pairs of residues were modified to form a disulphide bonded-cystine residue and the compounds obtained subsequently minimized and their backbones compared with the native B-chain as described above.

Molecular Dynamics Simulations of α-helix Melting

To assess whether the analogues designed were likely to adopt an α-helical structure, a theoretical assay was designed to determine α-helical stability. Native whole relaxin, the linear B-chain and shortened analogues were subjected to a series of 1ps gas phase molecular dynamic simulations at a range of temperatures from 50K to 1000K, using the Tripos forcefield. At the end of each molecular dynamics simulation, ϕ and φ backbone torsion angles for each residue of the resulting final conformation were measured and compared with the corresponding torsion angles of the minimized conformation prior to molecular dynamics simulation. If the torsion angles for a residue fell out of the ranges $-50^\circ < \phi < -30^\circ$ or $-60^\circ < \varphi < -40^\circ$, i.e. the observed values for α-helices [16], then that residue was deemed to be no longer α-helical. A minimum of three consecutive residues (i.e. one turn of an α-helix) with torsion angles corresponding to α-helical structure were required for that region to be classified as helical. From this, the percentage of α-helix remaining after the molecular dynamic simulations was determined, and plotted against temperature. From these graphs, the temperature at which 50% of the initially present α-helix had melted (MT_{50}) could be determined for each compound and which was used as a measure of helical stability.

Solid-phase Peptide Synthesis

Relaxin B-chain analogues were synthesized via continuous flow Fmoc-solid phase methodology as previously described [14] using Fmoc-PAL PEG-PS (PerSeptive Biosystems, USA) as the solid support and HBTU-activated Fmoc-amino acids. N^{α}-Fmoc deprotection was with 20% piperidine in DMF. Lactam bond formation was performed on resin as follows: the *N*-terminus of the peptides was acetylated by treatment with acetic anhydride for 30 min. Lys (ivDde) and Asp (ODmab) residues were then selectively deprotected by treatment with 3% hydrazine in DMF for 1 h. The free side chains of these residues were subsequently condensed by treating the partially deprotected resin-bound peptides with HATU/HOAt/DIEA for 24 h. Ninhydrin tests were used to confirm that the reaction had proceeded to completion. Cleavage of all the peptides from the solid support and simultaneous side chain deprotection was achieved by 3 h treatment with trifluoroacetic acid (TFA) in the presence of phenol/anisole/triisopropylsilane/water (82.5/5/5/2.5/5, v/v). The crude peptides were subjected to RP-HPLC on a Vydac C18 column using a 1%/min gradient of CH_3CN in 0.1% aqueous TFA. Peptides containing cysteine residues were then subjected to intramolecular oxidation by 1 mM 2, 2′-dipyridyldisulfide (DPDS) in a buffer containing H_2O/MeOH/TFA (89.9/10/0.1, v/v) [17] and the reaction progress monitored by both RP-HPLC and matrix-assisted laser desorption time of flight (MALDITOF) mass spectrometry.

Characterization

The purity of each synthetic peptide was assessed by analytical RP-HPLC, and MALDITOF mass spectrometry using a Bruker Biflex instrument (Bremen, Germany) in the linear mode at 19.5 kV. Peptide quantitation was by amino acid analysis of a 24 h acid hydrolysate using a GBC instrument (Melbourne, Australia).

Circular Dichroism (CD) Spectroscopy

CD spectra were taken on a Jasco J-720 instrument at room temperature in a 1 mm path-length cell using doubly distilled water, 10 mM sodium phosphate buffer containing 120 mM NaCl, pH 7.4, or spectroscopy grade trifluoroethanol (TFE) as solvents. Peptide solutions were made to a concentration of 0.01 μM, determined by quantitative RP-HPLC and amino acid analysis [14]. Curves were smoothed by the algorithm provided by Jasco. Mean residue ellipticity (θ_{MR}) is expressed in degrees $\times$ cm^2/dmole. CD spectra evaluations were based on comparison with known peptide conformations [18] and the amount of α-helicity was calculated according to the algorithm of Greenfield and Fasman [19].

Functional cAMP Assays

Human 293T cells derived from human embryonic kidney fibroblasts stably transfected with LGR7 were maintained as previously described [20]. Cells were preincubated in the presence of 0.25 mM 3-isobutyl-1-methyl xanthine (IBMX, Sigma, Australia) before various treatments in triplicate for 30 min. At the end of the incubation, cells were lysed for measurement of cAMP using a well-characterized cAMP ELISA [21]. All experiments were repeated three times using cells from independent transfections. The results are plotted as pmol cAMP/10^4 cells.

SELDI MS-based Relaxin Antibody Binding

Peptides were assessed for their ability to bind to a relaxin polyclonal antibody using surface-enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF-MS). To do this, 2 μl of goat anti-relaxin antiserum was applied to the spots on a protein chip array PG20 chip (Ciphergen Biosystems, Fremont, CA), and the protein chip incubated overnight at 4 °C in a humidity chamber. The remaining solution was aspirated, and the spots washed three times with 5 μl of wash buffer (0.5% Triton X-100 in PBS) and a further three times with 5 μl of PBS. After the remaining buffer was aspirated, 2 μl of a solution of insulin-like peptide 3 (INSL3), relaxin or peptides **13–16** was added to individual spots. All peptide solutions were made up to 100 fmol/μl in PBS. The chip was then incubated for 1 h at 24 °C in a humidity chamber. The solutions were aspirated and washed three times with wash buffer and PBS as before. The chip was then rinsed with 5 μl of 1 mM HEPES and allowed to air-dry for 10 min. After drying, 1 μl of saturated sinapinic acid was added to each of the spots, and the chip air-dried again. The chip was then placed in a protein chip reader TBS-IIC (Ciphergen Biosystems, Inc., Fremont, CA) and subjected to SELDI-TOF-MS. Results are obtained using the software provided by Ciphergen.

RESULTS

Design, Dynamics Simulations and Synthesis of i to i + 4 Constrained Mimetics of the Relaxin B-chain

The sequences of all peptides examined were derived from the corresponding sequence of the B-chain of human relaxin, replacing Cys^{11} and Cys^{23} with Ser to prevent unwanted reactions between free thiol groups and incorporating any constraints as described. To assess the similarity of analogues to the native relaxin conformation, the conformation obtained following energy minimization for each analogue was overlaid on the corresponding relaxin B-chain sequence from the original x-ray crystal structure of relaxin by superimposing backbone atoms. The conformation of the unconstrained analogues of relaxin (peptides **2, 5** and **13**) did not alter on minimization, and thus these compounds returned RMSD values of 0 Å (Table 1). Of the analogues containing i to i + 4 helix stabilizing constraints, the short relaxin receptor binding motif analogues **3** and **4** gave the smallest RMSD values, indicating the greatest conformational similarity to native relaxin. The longer i to i + 4 constrained analogues encompassing the entire α-helix of the B-chain (analogues **6–12**) all gave higher RMSD values than compounds **3** and **4**, while compounds with two constraints (**8, 11** and **12**) gave higher RMSD values than their singly constrained counterparts.

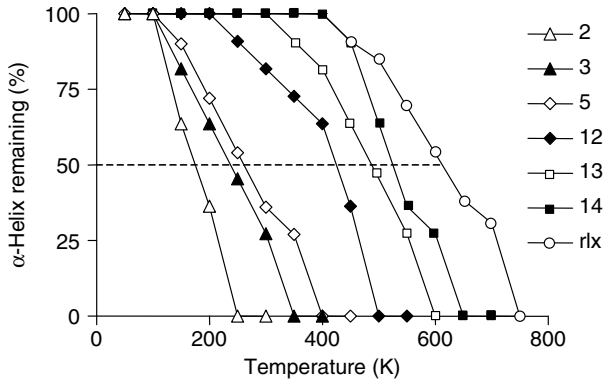

Figure 2 Graph of percentage of initial α-helix remaining after molecular dynamics simulations at different temperatures for a selection of relaxin B-chain peptide analogues examined in this study. Constrained analogues are shown as filled symbols, native unconstrained compounds are shown as open symbols. MT_{50} values were determined for each compound directly from the curves.

To determine the helical stability of the modelled compounds, molecular dynamic simulations were carried out at a series of temperatures, and the proportion of α-helix remaining at the end of each simulation was determined. This allowed helical stability curves to be plotted (Figure 2) and MT_{50} values to be determined (Table 1). Using this approach, native relaxin (**1**) gave the highest MT_{50} value (615K), suggesting that its helicity was the most stable of the peptides analysed. Four trends from the simulations of the analogues emerged: (a) The longer analogues were more stable (higher MT_{50}s) than shorter analogues; (b) peptides with an i to i + 4 constraint (e.g. **3** and **6**) were more stable than their unconstrained counterparts (e.g. **2** and **5**); (c) peptides with a cystine i to i + 4 constraint (e.g. **3** and **6**) were calculated to be more stable than the corresponding lactam derivatives (e.g. **4** and **9**); and (d) peptides with two i to i + 4 constraints (**8, 11** and **12**) were more stable than their singly constrained counterparts. Nevertheless, even the most stable i to i + 4 constrained analogue studied (**8**) appeared to be markedly less stable than relaxin itself, exhibiting an MT_{50} value 185K lower than native relaxin **1**.

The synthesis and purification of the i to i + 4 analogues proceeded without difficulty with each peptide being obtained in 5%–15% overall yield. All of the analogues were characterized by MALDITOF MS which showed principal products with the expected molecular weights. Analytical RP-HPLC was used to assess the purity of the analogues which, in each case, produced a single peak (results not shown).

CD Spectroscopy of i to i+4 Constrained Relaxin B-chain Analogues

The CD spectra obtained for native relaxin (**1**) in PBS (pH 7.4) showed a maximum at 190 nm, a minimum at 208 nm and another local minimum at 224 nm. These features are indicative of significant α-helical content, consistent with previous CD studies in PBS, water and 25% TFE [22]. In contrast, the i to i + 4 analogues **2–12** did not show any evidence of α-helical content in any of the solvents listed above, their CD spectra showing just one minimum at 215 nm (see Figure 3a).

Table 1 Primary Structure, RMS Deviation of Backbone Atoms Compared with Native Relaxin (RMSD) and Theoretical Melting Temperature of 50% of the Initially Present α-helix (MT_{50}) of Compounds Designed and Synthesized in this Study

Compound	Primary structure	RMSD (Å)	MT_{50} (K)
1	QLYSALANKCCHVGCTKRSLARFC DSWMEEVIKLCGRELVRAQIAICGMSTWS	0	625
2	RELVRAQI	0	195
3	RECVRACI	0.92	240
4	Ac-REDVRAKI	1.05	215
5	SGRELVRAQIAISGM	0	285
6	CGRECVRAQIAISGM	1.32	425
7	SGRELVRCQIACSGM	1.42	415
8	CGRECVRCQIACSGM	1.51	440
9	Ac-DGREKVRAQIAISGM	1.43	310
10	Ac-SGRELVRDQIAKSGM	1.48	325
11	CGRECVRDQIAKSGM	1.72	400
12	Ac-DGREKVRCQIACSGM	1.70	375
13	DSWMEEVIKLSGRELVRAQIAISGMSTWS	0	485
14	CMEEVIKLSGRELVRAQIAISGC	0.95	515
15	CEEVIKLSGRELVRAQIAC	0.92	475
16	CKLSGRELVRAQIC	1.13	450

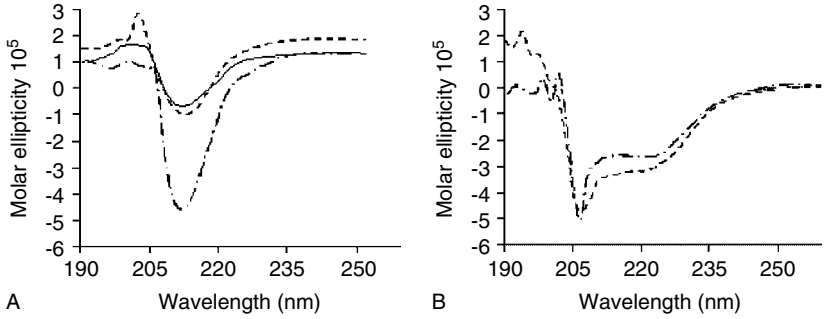

Figure 3 CD spectra in PBS of (A) peptides **2** (full line), **5** (dashed line) and **8** (dots and dashes) and (B) compounds **14** (dashed line) and **15** (dots and dashes).

Functional Characterization of i to i+4 Constrained Relaxin Analogues

Using native whole relaxin and HEK-293T cells expressing LGR7, i to i + 4 constrained peptide analogues **2–12** were tested for their ability to influence LGR7 mediated cAMP signalling. As previously reported [20], relaxin was able to produce a dose-dependant increase in cAMP. In contrast, none of the shortened relaxin B-chain peptides **2–12** were able to induce cAMP formation (for example, Figure 4) at doses up to 10 μM. The peptides were then tested for their ability to antagonize relaxin-stimulated cAMP accumulation. The relaxin B-chain peptide analogues **2–12** had no antagonistic effect on LGR7 at doses up to 10 μM (Figure 4).

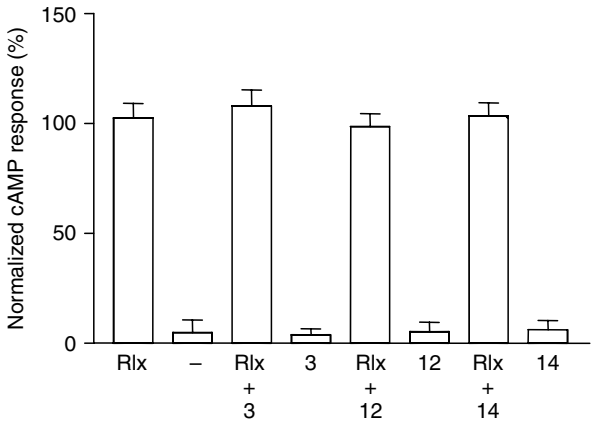

Figure 4 Graph of normalized cAMP production by HEK-293T cells transfected with LGR7 following treatment with 1 nM relaxin (Rlx), PBS (–) or 1 μM of relaxin analogue in the presence or absence of 1 nM relaxin.

Design, Dynamic Simulations and Synthesis of Terminally Constrained Cyclic Relaxin Analogues

Given the reduced theoretical helix stability, the absence of α-helical content by CD, and the lack of biological activity of any of the i to i + 4 constrained relaxin B-chain α-helix mimetics, terminally constrained cyclic mimetics of relaxin incorporating both the B-chain helix and strand (**14–16**) were designed and examined. These analogues gave relatively low RMSD values (Table 1), comparable to those of the short i to i + 4 constrained mimetics, suggesting that the terminal cystine constraint was well tolerated in these peptides. Indeed, peptides constrained by terminal lactam bridges were poor mimics of native relaxin with RMSD values above 2 Å (data not shown). Molecular dynamic simulations of the terminally constrained compounds **14–16** (Figure 2) showed higher MT_{50} values than any of the shorter relaxin B-chain analogues with a range of 450K–515K, with the most stable compound **14** having a higher MT_{50} value than the linear, unconstrained relaxin B-chain **13.**

The synthesis of the terminally constrained analogues proceeded without difficulty. However, their poor solubility contributed to substantial losses of peptide during purification, resulting in overall yields of 0.5%–1%. As before, the analogues were characterized by RP-HPLC and MALDI-TOF MS. An example of both is shown in Figure 5a and 5b.

Terminally Constrained Cyclic Relaxin Analogues Exhibit α-helical Structure

When measured in water, the CD spectra of the terminally constrained cyclic analogues **14** and **15** showed no evidence of α-helical structure. However, in contrast to the i to i + 4 constrained analogues and the unconstrained linear relaxin B-chain **13**, the CD spectra of the terminally constrained cyclic analogues **14** and **15** in either PBS and 25% aqueous TFE were similar to that of native relaxin indicating the presence of α-helix (see Figure 3b).

Terminally Constrained Cyclic Relaxin Analogues bind to a Relaxin Antibody

The ability of terminally constrained cyclic relaxin analogues to bind to an immobilized polyclonal relaxin antibody was determined using SELDI-TOF-MS. Using this approach, both relaxin and the terminally constrained cyclic analogue **14** were found to bind

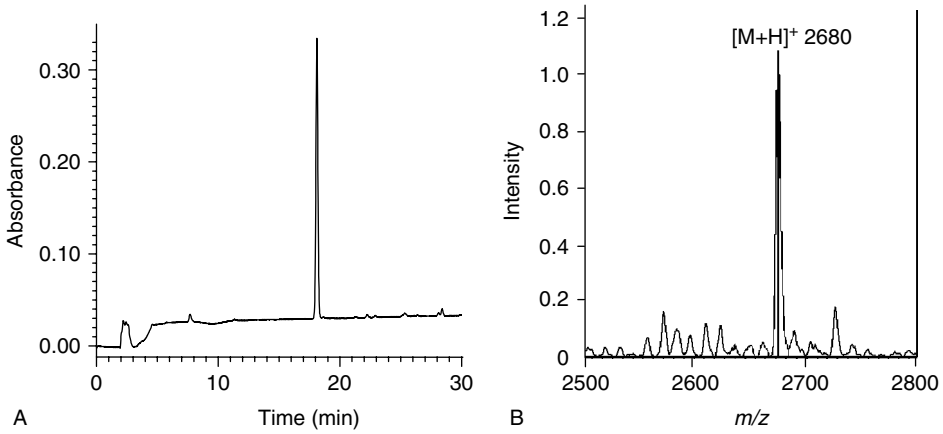

Figure 5 Analytical RP-HPLC trace (A) and MALDI-TOF-MS (B) of a sample of peptide **14**.

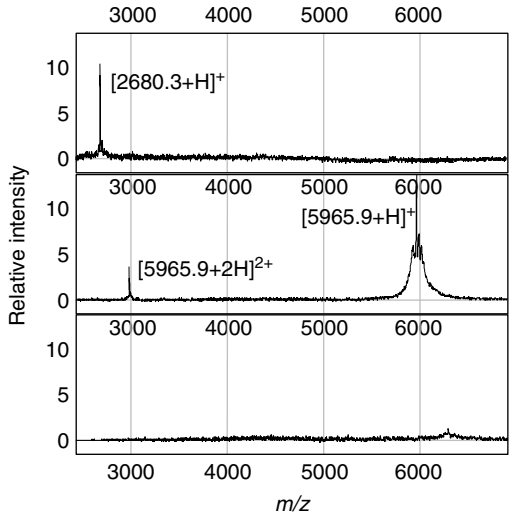

Figure 6 SELDI-TOF-MS traces for relaxin polyclonal antibody incubated with peptide **14** (top panel), relaxin (middle panel) and linear relaxin B-chain (bottom panel).

to the antibody (Figure 6). In contrast, neither the linear relaxin B-chain **13** (which was devoid of helical structure as determined by CD spectroscopy) nor the constrained cyclic analogues **15–16** (which did show helical structure) bound to the antibody.

Functional Characterization Terminally Constrained Cyclic Relaxin Analogues

The terminally constrained relaxin B-chain analogues **14–16** were tested for their ability to influence LGR7 directed signalling using the assay described above. Despite their α-helical structure, and the ability of peptide **14** to strongly bind to a relaxin antibody, all three compounds failed to cause either an increase in cAMP at doses up to 10 μM, or to antagonize relaxin stimulated cAMP accumulation (1 nM) (for example, Figure 4). The linear B-chain analogue **13** was also inactive in both these assays although this may be a consequence of its low solubility.

DISCUSSION

Many growth factors, cytokines and hormones have been shown, like relaxin, to have receptor binding sites that contain an α-helix. These include insulin [23], growth hormone [24] and interleukin 4 [25]. Using a range of biophysical approaches, many investigators have explored the possibility that small peptides based on these regions that retain helical structure would act as bioactive mimetics of the parent peptide or protein. One such approach based on molecular dynamics has been used to assist in the discovery of shortened mimetics of IL-4 grafted onto a helical scaffold derived from the yeast transcription factor GCN4 [26]. The IL-4 simulations were particularly computationally intensive, using explicitly solvated periodic boundary conditions over a long time period (at least 400 ps). This paper, describes an alternative approach to obtain such mimetics of the putative receptor binding site on the B-chain α-helix of relaxin. Molecular dynamics simulations were employed as an *in silico* assay of the helical stability of designed compounds. A series of short (1 ps) simulations were performed across a range of temperatures under non-solvated conditions. It was reasoned that by carrying out the simulations across a range of temperatures, it should be possible to use a simpler, computationally less demanding assay to gain a measure of thermal stability. To a large extent, this approach was vindicated: native relaxin was clearly the most thermally stable compound examined, all short i to i + 4 stabilized compounds denatured at low simulation temperatures, while longer terminally constrained compounds were of intermediate thermal stability This difference in helical stability was confirmed by CD studies. Only native relaxin showed significant helical content irrespective of the solvent examined while all shorter peptides failed to display helicity in any solvent. However, the longer terminally constrained peptides did show α-helicity, but only in PBS and the helix-promoting solvent, aqueous TFE. For this set of compounds at least, it was

possible to establish a minimum MT_{50} that translates to experimental helix stability.

Several protein design studies have shown that an α-helix mimic can be achieved by introducing a conformational constraint between side chains along the face of an α-helix either at the i to i + 3 [27], i to i + 4 [28,29] or positions i to i + 7 [30]. This study showed that the inclusion of an i to i + 4 constraint, either as the disulphide or as the lactam, gave compounds with improved theoretical thermal stability over their linear, unconstrained counterparts. However, the MT_{50} of even the most stable i to i + 4 constrained peptide **8** was well below that of native relaxin or the best of the terminally constrained mimetics. The low MT_{50}s of the i to i + 4 constrained compounds was reflected experimentally in that they did not show any detectable α-helical content as assessed by CD spectroscopy. In contrast, the terminally constrained peptides, which incorporated a significant portion of the B-chain β-strand, were found to be more thermally stable in the molecular dynamics assay. Furthermore, the increase in theoretical stability was sufficient to translate into a significant degree of α-helical content that was detected by CD spectroscopy although only in helix promoting solvents. Taken together, these data suggest that other features of native relaxin, including the B-chain β-strand (which makes multiple hydrophobic contacts with the B-chain helix), likely play a crucial role in stabilizing the B-chain α-helix.

Numerous studies have shown that key residues within the B-chain α-helix are directly involved in the binding not only of relaxin to its receptor, LGR7, but also of insulin to its tyrosine kinase receptor [25]. In this study, it was demonstrated that terminally constrained relaxin B-chain peptide analogues possessed a significant α-helical nature. Use of SELDI-TOF mass spectrometry-based biomolecular interaction analyses by measuring binding of the peptides to a monoclonal antibody that was specific for relaxin [31] demonstrated that the peptides either largely or wholly maintained their putative LGR7 binding motif. However, despite these observations, none of these conformationally constrained peptide analogues were found to possess either relaxin agonistic or antagonistic actions. These data indicate that relaxin-like actions through LGR7 require features other than the B-chain binding motif being presented in an α-helical conformation. It may be that the A-chain of relaxin, which has long been thought to be solely a conformational scaffold for the B-chain, is actually required for binding to the receptor. Another possibility is that although the terminal constraint holds the B-chain β-strand in an appropriate conformation sufficiently to stabilize the B-chain α-helix, the β-strand actually needs to undergo reorganization to allow receptor binding and that this event is hindered by the constraint. Indeed, there is evidence that the equivalent β-strand of the B-chain of insulin undergoes conformational adjustment upon binding to the insulin receptor [32]. It would be of interest to assess the ability of equivalently terminally constrained insulin B-chain mimetics to either mimic or inhibit insulin action. Indeed, our approach has recently been successfully employed to design cyclic B-chain analogues of the insulin-like peptide 3 (INSL3) which possess potent antagonistic activity (manuscript in preparation). Nevertheless, the terminally constrained, and significantly α-helical relaxin peptide analogue **14** was both recognized and bound by a relaxin-specific antibody. Again however, α-helical structure *per se* was obviously clearly not a sufficient criterion for antibody binding as shorter terminally constrained peptides did not bind to the immobilized antibody. Taken together, these observations suggest that the requirements for antibody binding differ to, and are likely less stringent than, those of binding to LGR7.

CONCLUSIONS

Increasing evidence that relaxin has a critical role in the breakdown of fibrotic tissue by collagen remodelling [7,33,34] along with the discovery of the relaxin receptor [13] has made relaxin an attractive therapeutic target. Previous studies have shown that i to i + 4 conformational constraints have aided in the promotion of α-helical structure for many peptides [28,35]. However, these constraints proved to be of little value in the promotion of α-helix in the B-chain. Terminal constraints, which have also been used to great effect [36], were able to stabilize α-helical structure to the point where one analogue was able to recognize the relaxin antibody. The inability of this analogue to have a relaxin-like biological effect suggests that the A-chain of relaxin is likely to offer more than just structural support to the B-chain, and might indeed be required for receptor binding.

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