Structure of the R3/I5 Chimeric Relaxin Peptide, a Selective GPCR135 and GPCR142 Agonist*

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Linda M. Haugaard-Jönsson1, Mohammed Akhter Hossain5, Norelle L. Daly5, Ross A. D. Bathgate5, John D. Wade5, David J. Craik1, and K. Johan Rosengren1,2

From the 1School of Pure and Applied Natural Sciences, University of Kalmar, SE-391 82 Kalmar, Sweden, the 5Howard Florey Institute, The University of Melbourne, Victoria 3010, Australia, and the 4Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia

The human relaxin family comprises seven peptide hormones with various biological functions mediated through interactions with G-protein-coupled receptors. Interestingly, among the hitherto characterized receptors there is no absolute selectivity toward their primary ligand. The most striking example of this is the relaxin family ancestor, relaxin-3, which is an agonist for three of the four currently known relaxin receptors: GPCR135, GPCR142, and LGR7. Relaxin-3 and its endogenous receptor GPCR135 are both expressed predominantly in the brain and have been linked to regulation of stress and feeding. However, to fully understand the role of relaxin-3 in neurological signaling, the development of selective GPCR135 agonists and antagonists for in vivo studies is crucial. Recent reports have demonstrated that such selective ligands can be achieved by making chimeric peptides comprising the relaxin-3 B-chain combined with the INSL5 A-chain. To obtain structural insights into the consequences of combining A- and B-chains from different relaxins we have determined the NMR solution structure of a human relaxin-3/INSL5 chimeric peptide. The structure reveals that the INSL5 A-chain adopts a conformation similar to the relaxin-3 A-chain, and thus has the ability to structurally support a native-like conformation of the relaxin-3 B-chain. These findings suggest that the decrease in activity at the LGR7 receptor seen for this peptide is a result of the removal of a secondary LGR7 binding site present in the relaxin-3 A-chain, rather than conformational changes in the primary B-chain receptor binding site.

The human relaxin family of peptide hormones comprises seven members in humans, including relaxins 1–3 (1–3), and the insulin-like peptides INSLs 3–6 (4–7). Relaxin-1 is the result of a gene duplication only present in higher primates and is involved in stress responses (17) and in regulation of feeding (18), suggest a physiological role in neuroendocrine and sensory processing. An interesting feature of relaxin-3 is its ability to activate both GPCR142 (19) and LGR7 (20) in addition to its own receptor, GPCR135 (11). This is particularly puzzling as these receptors represent two different subfamilies, and raises questions about what structural characteristics enables the cross-interactions and activations to occur. Although the relaxin/LGR7 system is primarily involved in reproduction, LGR7 is also expressed in the brain, and thus the development of selective GPCR135/142 ligands for pharmacological studies to more precisely determine the physiological role of relaxin-3 in the brain has been a priority. Recently, it was shown that indeed

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The atomic coordinates and structure factors (code 2k1v) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 Australian Research Council Professorial Fellow.

2 Supported by the Faculty of Natural Science and Technology, University of Kalmar and Åke Wiberg’s Foundation. To whom correspondence should be addressed. Tel.: 46-480-446152; Fax: 46-480-446262; E-mail: johan.rosengren@hik.se.

3 The abbreviations used are: GPCR, G-protein-coupled receptor; H3 relaxin, human relaxin-3; INSL5, insulin like peptide-5; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; DQF-COSY, double quantum filtered correlation spectroscopy; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; RXFP, relaxin family peptide receptor.
such ligands can be achieved by creating chimeric relaxin-3 peptides. In particular an analogue in which the relaxin-3 B-chain is combined with the INSL5 A-chain (R3/I5) selectively activates GPCR135 and GPCR142 over LGR7 (12). Furthermore, if the relaxin-3 B-chain in R3/I5 is truncated at the C termini, R3(B23–27)/I5, a high affinity selective GPCR135/142 antagonist is obtained (21).

We recently determined the NMR solution structures of human relaxin-3 (22) and INSL3 (23). From these structures together with the crystal structure of relaxin-2 and extensive structure-activity studies, a picture of the complex events involved in the recognition of a relaxin by its receptor is starting to emerge. A conserved binding motif Arg-X-X-X-Arg-X-Ile has been identified as a crucial interaction point for relaxin binding to LGR7 (24), and a similar motif (His-X-X-X-Arg-X-X-Val) is used by INSL3 for binding to its receptor (23). However, it is clear that several additional interactions are needed both for strong binding and subsequent activation of the receptors. For example, the C-terminal Trp(B27) in relaxin-3 is involved in activation of GPCR135 but not the primary binding based on the potent antagonistic activity of the R3(B23–27)/I5 chimeric peptide. In contrast, this Trp is not needed for the activation event but is required for high affinity binding of INSL3 to LGR8 (25).

With the exciting pharmacological profile of the R3/I5 chimera and the promising general concept of chimeric relaxins as selective ligands it is of great importance to understand the structural consequences of combining peptide chains from two structurally related but physiologically different hormones. In this study we determined the three-dimensional structure of the chimeric peptide R3/I5, consisting of the full B-chain from human relaxin-3 combined with residues 4–24 of the A-chain from human INSL5, using solution NMR techniques. The chimeric peptide structure retains a relaxin/insulin-like fold with relaxin-3 B-chain adopting a conformation that is nearly identical to that seen in the native relaxin-3 structure. These findings provide important new biochemical insights regarding the features that are responsible for the interaction between relaxin-3 and the receptors GPCR135, GPCR142, and LGR7.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—Each of the two chains was assembled by regioselective disulfide continuous flow solid phase peptide synthesis as previously described (26, 27). From 0.1 mmol scale synthesis for both chains, a total of 3.2 mg of synthetic chimeric peptide were obtained (overall yield 6.28% relative to starting B-chain). RP-HPLC on an analytical Vydac C18 column (pore size 300 Å, particle size 5 Å, 4.6 × 250 mm) using a gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid showed a single product that, on a MALDI-TOF mass spectrometer (Bruker Autoflex instrument, Bremen, Germany), gave MH + 5239.83 (theoretical MH + 5240.17).

**NMR Spectroscopy**—For NMR studies and structure determination samples of 0.5 ml of ~1 mM R3/I5 in either 90% H2O, 10% D2O (v/v) or 100% D2O at pH ~ 4 were prepared. All spectral data were recorded at 600 MHz on Bruker Avance spectrometers and processed using Topspin (Bruker). As only unlabeled synthetic peptide was available, exclusively homonuclear two-dimensional data were used. Generally the data were recorded with 4k data points in the f2 dimension and 512 increments in the f1 dimension, with the latter subsequently zero filled to 1k data points prior to transformation. Data for the structure determination were primarily recorded at 298 K but additional data for resolving assignment ambiguities and for determination of amide proton temperature coefficients, to identify hydrogen bonds, were recorded in the 288–308 K temperature range. The two-dimensional experiments used for assignments and collection of structural restraints included DQF-COSY, TOCSY, and NOESY (using a mixing time of 100, 150, or 200 ms). Slowly exchanging amides were detected by recording TOCSY spectra immediately after dissolving the lyophilized peptide in D2O. Most amides disappeared within minutes but a number of resonances were still visible after 1 h. These included A23, B15, B16, B18, B19, and B20. Hydrogen bonds were also identified by the analysis of amide temperature coefficients (ΔδHN/ΔT). Amide protons involved in hydrogen bonds are protected from the solvent and their chemical shifts are less affected by changes in temperature. Amide protons with a ΔδHN/ΔT > ~4.6 ppb/K are likely to be involved in hydrogen bonds (28). The spectral data were calibrated to internal 2,2-dimethyl-2-silapentanesulfonic acid at 0.0 ppm.

**Structure Determination**—The spectral data were used to derive a set of structural restraints including inter-proton distances, dihedral angles, and hydrogen bond restraints. Distance restraints were derived from 100 ms NOESY spectra recorded in either H2O or D2O. The cross-peaks were integrated using the program CARA (29) and translated into distances with appropriate pseudo atom corrections using the program CYANA (30). Backbone φ dihedral angles were derived from the analysis of the splitting of the anti-phase Hα-HN cross-peaks in DQF-COSY spectrum with residues showing a coupling of <6 Hz being restrained to −60 ± 30° and residues showing a coupling of >8 Hz being restrained to −120 ± 30°. Side chain χ1 dihedral angles and stereospecific assignments of β-methylene protons were achieved by a combination of
Hα-Hβ coupling constants and Hα-Hβ/HN-Hβ NOE peak intensities (31). The structural restraints were used for calculation of structural families using simulated annealing using the program package CNS (32). Preliminary structures were used to resolve ambiguities in cross-peak assignments as well as identification of hydrogen bonds. Once a suitable acceptor could unambiguously be identified for amide protons that were found to be slow exchanging or having temperature coefficients (\(\Delta \delta_{\text{HN}}/\Delta T\)) consistent with hydrogen bonds these were included as restraints in the calculations. Preliminary structures were subsequently refined by further molecular dynamics and energy minimization in a water shell, as described in detail previously (22). For the final round of calculation a set of 50 structures was calculated from which the 20 structures with the lowest energies that were in agreement with the experimental data were chosen to represent the solution structure of R3/I5. The coordinates and experimental restraints for this structure have been deposited to the Protein Data Bank and given the accession code 2k1v.

RESULTS AND DISCUSSION

Peptide Synthesis—The relaxin-3 B-chain and INSL5 A-chain were prepared by continuous flow Fmoc (N-(9-fluorenyl)methoxycarbonyl)-solid phase peptide synthesis. Directed disulfide bond formation was achieved by selection and use of appropriate S-protection groups, which were subsequently removed in a stepwise fashion to individually form each of the three disulfide bonds, as previously described in detail (26). The oxidized chimeric two-chain peptide was purified using RP-HPLC and its identity and purity confirmed by RP-HPLC, MALDI-TOF MS, and NMR analysis.

NMR Spectroscopy, Resonance Assignments, and Structure Determination—The interesting pharmacological profile of the R3/I5 peptide warranted a detailed study to gain insights into the structural consequences of combining the A- and B-chains of two different relaxins. Thus the R3/I5 peptide was subjected to extensive two-dimensional NMR analysis at 600 MHz. All NMR data were recorded at \(pH = 4\) as the peptide was poorly soluble at higher pH. This pH is convenient for NMR studies, as the amide proton resonances are not broadened due to fast solvent exchange and we have previously used a similar pH for NMR studies of relaxin-3 and INSL3. For these peptides data were also recorded at higher pH and although the quality of the data was poorer there were no indications of structural changes as a result of the changed pH (23), and no such changes would be expected for the R3/I5 chimera either.

The spectral data for R3/I5 were of high quality in terms of signal dispersion but specific broadening was observed for some resonances, indicating internal dynamic processes (Fig. 2a). Resonance assignments were achieved using standard two-dimensional sequential assignment strategies and near complete assignments were possible for both backbone and side chain resonances. Resonances that could not be identified due to broadening included the amide protons of A12 and A16 and the H- protons of A15. Interestingly similar broadening of peaks from the region around the Cys(A10)–Cys(A15) disulfide bond has previously been observed for both native relaxin-3 (22) and INSL3 (23). In addition, a small set of additional spin systems could be identified as a minor conformation of residues B2–B5. These were confirmed to arise from a cis/trans isomerization of the only Pro residue in the sequence, Pro(B4). The conformation of the peptide bond preceding a Pro can be confirmed by the analysis of sequential NOE contacts. A sequential Hα,1-Hα, Hα, is consistent with a cis conformation, whereas a Hα,1-Hδ, is consistent with a trans conformation. Such NOE patterns
were observed for the two conformers and confirmed that the major conformer (>70%) is in the trans configuration. A similar observation was made for native relaxin-3, which also displays this second conformation (22), but in the light of recent structure-activity data, which show that the B-chain N terminus can be truncated with up to seven residues without losing activity (21), this is unlikely to have any significance for receptor binding.

Secondary Hα chemical shifts are particularly useful for providing an overview of the secondary structure in a protein (33). Fig. 2b presents a comparison of the secondary shifts of the R3/I5 chimera and native relaxin-3. From this analysis it is clear that the shifts are very similar, indicating that the overall fold of relaxin-3 is retained and that the typical relaxin secondary structure with three helical regions and a small extended region (A13–16, B7–11), with β-sheet like interactions between the chains are all present. It is also obvious that the B-chain terminal residues do not differ significantly from random coil, suggesting that they may be less structured in solution.

Amide temperature coefficients are very useful for providing a detailed picture of the hydrogen bond network in a protein. The Δδ_{H2O}/ΔT values determined for the R3/I5 chimera are presented in Table 1. Although a number of amides could not be followed throughout the temperature series as a result of resonance overlap or broadening, 16 amide protons were found to have a Δδ_{H2O}/ΔT consistent with a hydrogen bond and an additional 3 were found to have a slow exchange with the solvent. For all these 19 amides possible hydrogen bond acceptors were identified. The hydrogen bonding pattern in the B-chain is conserved in comparison to relaxin-3, albeit with a possible weakening at the end of the B-chain helix as suggested by the Δδ_{H2O}/ΔT of Thr(B21) and Cys(B22). For the A-chain the picture is less clear with a number of Δδ_{H2O}/ΔT values not being able to be determined. Nonetheless, for the amides that were found to be hydrogen bonded a pattern similar to native relaxin-3 is seen, but with the slightly poorer definition of the helices in the A-chain, in several cases it was not possible to unambiguously conclude a α-helical or 3_10-helical hydrogen bonding pattern.

Structural restraints in the form of inter-proton distances, dihedral angles, and hydrogen bonds were deduced from the NMR data and used to generate structures by simulated annealing followed by refinement and energy minimization in explicit solvent. The distance restraints were primarily derived from NOE cross-peak intensities in a NOESY spectrum recorded at 298 K with a mixing time of 100 ms. As expected for a peptide of around 50 amino acids, signal overlap was a problem in certain regions of the spectra, and degenerate frequencies for a number of resonances prevented the immediate assignments of certain cross-peaks to a specific proton pair. These problems were resolved by an iterative strategy in which preliminary structures were calculated and used to resolve ambiguities. In some cases ambiguous restraints were used in the calculations (22). Dihedral angle restraints were derived for both backbone and side chains based on coupling constants and NOE patterns. Hydrogen bonds were introduced for amide protons confirmed to be part of hydrogen bonds by either slow solvent exchange or temperature coefficients once their accepting partner could be identified in preliminary structures. In the final round 50 energy structures consistent with the experimental data were calculated and used to resolve ambiguities. From these the 20 lowest structures were calculated and used to resolve ambiguities. In some cases ambiguous restraints were used in the calculations (22). Dihedral angle restraints were derived for both backbone and side chains based on coupling constants and NOE patterns. Hydrogen bonds were introduced for amide protons confirmed to be part of hydrogen bonds by either slow solvent exchange or temperature coefficients once their accepting partner could be identified in preliminary structures. In the final round 50 structures were calculated and refined and from these the 20 lowest energy structures consistent with the experimental data were chosen to represent the solution structure of R3/I5. An overlay

### Table 1: Temperature coefficients and amide exchange for R3/I5

| Residue | Tc ppb/K | H/D exchange | H-bond acceptor a | Residue | Tc ppb/K | H/D exchange | H-bond acceptor a |
|---------|----------|--------------|-------------------|---------|----------|--------------|-------------------|
| PCA(A4) | — b      |              |                   | Arg(B1) | NA c      |              |                   |
| Asp(A5) | −6.0     |              |                   | Ala(B2) | −7.1     |              |                   |
| Leu(A6) | −5.9     |              |                   | Ala(B3) | −9.4     |              |                   |
| Gln(A7) | −6.0     |              |                   | Pro(B4) | NA        |              |                   |
| Thr(A8) | −3.9     |              |                   | Tyr(B5) | −9.9     |              |                   |
| Leu(A9) | −3.0     |              |                   | Gly(B6) | −5.5     |              |                   |
| Cys(A10)| −6.0     |              |                   | Val(B7) | −5.5     |              |                   |
| Cys(A11)| −6.0     |              |                   | Arg(B8) | −10.3    |              |                   |
| Thr(A12)| −6.0     |              |                   | Leu(B9) | −3.9     |              |                   |
| Asp(A13)| −4.0     |              |                   | Cys(B10)| −9.2     |              |                   |
| Gly(A14)| −2.6     |              |                   | Gly(B11)| −10.1    |              |                   |
| Cys(A15)| −4.3     |              |                   | Arg(B12)| −8.9     |              |                   |
| Ser(A16)| −6.0     |              |                   | Glu(B13)| −1.8     |              |                   |
| Met(A17)| −6.0     |              |                   | Phe(B14)| −2.7     |              |                   |
| Thr(A18)| −4.2     |              |                   | Ile(B15)| 0.7      | S             | Gly(B11) O d    |
| Asp(A19)| −3.0     |              |                   | Arg(B16)| −6 b     | S             | Arg(B12) O d    |
| Leu(A20)| −2.3     |              |                   | Ala(B17)| −6 c     |              |                   |
| Ser(A21)| −6.0     |              |                   | Val(B18)| −3.7     | S             | Phe(B14) O d    |
| Ala(A22)| −3.0     |              |                   | Asp(A19)| −6 d     | S             | Ile(B15) O d    |
| Leu(A23)| −3.2     | S             | Leu(A20) O e      | Thr(B21)| −9.1     |              |                   |
| Cys(A24)| 0.8      |              |                  | Cys(B22)| −7.0     |              |                   |
| Arg(B23)| −6 e     |              |                  | Gly(B24)| −4.6     |              |                   |
| Gln(B24)| −6.0     |              |                  | Gly(B25)| −6.7     |              |                   |
| Arg(B26)| −7.2     |              |                  | Trp(B27)| −5.0     |              |                   |

a H-bond acceptor suggested by final structure.
b H-bond acceptor unambiguously identified in structure and included as restraints in calculations.
c NA, not applicable.
d H-bond acceptor unambiguously identified in structure and included as restraints in calculations.
e Ambiguous hydrogen bond restraints with either A20 or A21 O as acceptors used in calculations.
of these 20 structures is shown in Fig. 3 and the structural statistics for this family is presented in Table 2.

**Description of the Structure and Comparison to Native Relaxin-3**—From Fig. 4, which shows a comparison of H3/INSL5 and relaxin-3, it is clear that the chimera has a distinct relaxin/insulin-like fold with three well defined helical segments comprising residues A5–12, A17–24, and B12–22. The helices enclose a significant hydrophobic core, which includes residues Leu(A6), Gln(A7), Cys(A10), Cys(A15), Leu(A20), Leu(A23), Cys(A24), Leu(B9), Phe(B14), Ile(B15), Val(B18), Ile(B19), Cys(B22), and Trp(B27). It is interesting to note that the key residues in the A-chain are generally highly conserved between INSL5 and H3 relaxin (Leu/Leu(A6), Gln/Ser(A7), Leu/Ile(A20), Leu/Leu(A23)). Thus a similar packing of the core is possible, which is likely the key for the chimeras retained overall fold. The Gln/Ser change is not conservative but as seen in Fig. 4 this residue is on the fringe of the core and its interactions with the Phe(B14) involve the Hø and Hβ protons, which show NOE cross-peaks to the aromatic protons and have unusual chemical shifts as a result of ring current effects. The interaction is particularly evident in Fig. 2b, which shows that the Hø chemical shift of residue A7 in both relaxin-3 and the chimera is shifted upfield by >1.5 ppm. The polar part of the side chain extends out in solution, which explains why different sized polar residues, *i.e.* Gln/Ser, can be accommodated at this position. In native relaxin-3 the A-chain is longer and thus the core is extended further and also includes Leu(A3), which in particular packs against Leu(A23). These interactions do not appear to be needed for stabilizing the fold.

Creating additional relaxin chimeras that can adopt a correct fold but that may have altered activity profiles offers a quick inroad to understanding the selectivity of the receptors, thus when discussing the hydrophobic core it is interesting to consider all relaxin sequences. The residues identified as key components in the core here are highlighted in Fig. 1, from which it is clear that generally hydrophobic amino acids are conserved in these positions. This suggests that a wide range of chimeric peptides are likely to produce a relaxin-like fold and thus are possible candidates for selective ligands. The notable exception is INSL4, which differs significantly, with Ser(A23), Gly(B9), and Gly(B15) all breaking the pattern. These changes could potentially considerably affect the fold of a chimera, including either the INSL4 A- or B-chain. It is also interesting to note the presence of the hydrophilic Gln at position B18 in relaxin-1 and -2. In the crystal structure of relaxin-2 this side chain forms hydrogen bonds to the hydroxyl of Tyr(A3), and thus chimeras in which only one of these partners has been removed may be destabilized.

Both the N and C termini of the relaxin-3 B-chain are fairly disordered in the chimera and appear to lack a well defined conformation in solution. For the first five residues in the B-chain no long-range NOE contacts are observed, consistent with a lack of interactions with other parts of the protein. This is in agreement with our previous studies of native relaxin-3 and INSL3, which both have disordered B-chain N termini. However, in native relaxin-3 a large number of NOEs were observed between Tyr(B5) and Lys(A17), which assisted in defining the conformation of the tail. No such NOEs were
observed between Tyr(B5) and the corresponding residue Met(A17) in R3/I5, and the NOE contacts across the strands in the short β-sheet are weaker, suggesting that the chimeric peptide may have become more flexible in this region. Recently it has been shown that the B-chain N terminus is not needed for activation of the GPCR135 or LGR7 receptors (21), and that in fact relaxin-3 can be truncated by up to seven amino acids without affecting biological activity. Thus it is not surprising to find that this region does not have a crucial structural role.

The conformation of the B-chain C termini is considerably more important with the Trp(B27) of INSL3 being crucial for the binding to and activation of the INSL3 receptor LGR8. Furthermore, it was recently shown that Trp(B27) of relaxin-3 is needed for the activation of GPCR135 (21), as a chimeric peptide, with a truncated C-chain (R3(B23–27)/I5) despite maintaining full binding affinity, lacks the ability to activate the receptor. The chemical shift analysis suggests a random coil-like structure in this region and a distinct lack of NOEs was seen for residues B23–26. However, several NOEs were observed between the aromatic protons of Trp(B27) and the side chains of Ile(B19). This is consistent with what has previously been reported for relaxin-3 and INSL3. In the case of relaxin-3 a large number of NOE contacts were seen, placing the tail firmly packed against the peptide core, whereas in INSL3 the tail seemed more loosely associated with the rest of the peptide. In contrast in the crystal structure of human relaxin-2 the B-chain helix extends one turn further and the Trp side chain extends away from the peptide core (34). However, it is likely that this conformation is only adopted as a result of the possibility to interact with nearby molecules in the crystal. Although there may be differences in the degree of flexibility it seems likely that in all relaxins the C-terminal tail can move and adopt any conformation suitable for interacting with the receptor. This may well be a requirement for the activity of relaxin-3 and INSL3, which both rely on the C terminus for interactions with their receptors.

**Implications for Receptor Binding**—When using an NMR or x-ray structure of a “free” protein for analyzing structure-activity relationships, it is always important to consider the possibility that structural changes may occur in the protein upon binding to its receptor. However, as the insulin/relaxin fold is a conserved framework, which is cross-braced by three disulfide bonds, and which has been utilized by nature for the evolution of a whole family of hormones with various biological functions, we anticipate that such changes will be minor for the relaxin peptides.

The relaxin-3 B-chain alone has been shown to be an agonist for the GPCR135 and GPCR142 receptors, albeit with significantly less potency compared with the native peptide (11, 19). In contrast, the isolated B-chains of either relaxin-2 or -3 do not have the ability to interact with the relaxin-2 receptor LGR7. Based on these observations it has been suggested that activation of the smaller GPCR135 and GPCR142 receptors may rely on an interaction site located solely in the B-chain, whereas in contrast the more complex LGR7 and LGR8 receptors require additional interaction points, possibly involving also the A-chain. According to such a model it should be possible to create relaxin-3 chimeras, which could be specific GPCR135/CPCR142 ligands, and indeed this has been shown to be the case (21, 35). However, from these data it cannot be ruled out that the changes in activity are a result of the chimeras affecting the B-chain structure and that the various receptors differ in sensitivity to such changes. Indeed, we recently showed that a relaxin-3 with a truncated A-chain loses its well defined structure and also to a large extent the ability to interact with LGR7, but in contrast it retains full activity on GPCR135 (36). Thus the key question addressed in the current study was: how is the conformation of the relaxin-3 B-chain affected by the association with a new A-chain in such chimeras? The answer is that the native B-chain structure is fully retained, which allows for several conclusions to be drawn regarding the receptor interactions. It has been established that the primary binding site of relaxin for LGR7 is located within the R-X-X-R-X-X-I motif that is presented on one face of the B-chain helix (24), and recent mutational explorations of the relaxin-3 B-chain have shown that the same motif is crucial for the binding of relaxin-3 to both GPCR135 and LGR7 (21). The observation that the B-chain structure, including the receptor binding motif, is retained in the chimera thus provides a clear explanation for the high affinity binding to the GPCR135 and GPCR142 receptors, and is consistent with the idea of the B-chain alone carrying all structural features needed for activation of these receptors. However, the A-chain in relaxin-3 is crucial as a structural support for maintaining the correct structure and the high affinity for the receptors. Furthermore, based on the structure of the R3/I5 chimera, we can rule out the possibility that the additional features required for LGR7 binding are also located in the B-chain and that the altered pharmacological profile of the chi-
mera is only a result of a conformational change in any part of the B-chain, resulting from the replacement of the A-chain. Rather, it is clear that there is a second binding site needed for activation of the LGR7 receptor and it is present in the A-chains of both relaxin-2 and -3, but not INSL5, and thus future mutational studies aimed at characterizing the nature of this site should be focused on this region.

Fig. 5 shows a comparison of the surface characters of relaxin-3, R3/I5, and relaxin-2. It is possible that subtle changes in the shape rather than in the surface nature may play a role in the recognition and it is thus difficult to speculate too much into what features may be important based on the structures alone. Nonetheless, it is interesting to note the presence of positive charges at positions A12 and A17/18 in relaxin-2 and relaxin-3. These charges are not present in the INSL5 A-chain and thus are possible candidates for additional interaction points between relaxin-2/3 and LGR7 that could explain why a chimeric peptide carrying the INSL5 A-chain does not have the ability to interact with LGR7. The confirmation that the overall structure of the chimera is not disrupted presented here, will now allow further studies in which point mutated synthetic analogues are designed to examine these structure-activity questions to be performed.

In summary we have shown that the combination of a relaxin-3 B-chain with an INSL5 A-chain does not alter the native relaxin structure and thus changes in activity can be directly correlated to changes in the nature of the side chains rather than overall fold disruptions. In addition to providing proof that relaxin-3 relies on an additional binding site located in the A-chain for its interaction with LGR7 but not with GPCR135 or...
Structure of the H3 Relaxin/INSL5 Chimera

GPCR142, these findings emphasize the importance of chimeric peptides as a quick and powerful way of deducing structure–activity data for relaxins. A set of hydrophobic residues pack together in the core and are important for retaining the relaxin fold. These are well conserved between relaxins and thus most chimeras are likely to adopt similar structures, with the possible exception of INSL4, which displays considerable variation at these key positions.

REFERENCES

1. Hudson, P., Haley, J., John, M., Cronk, M., Crawford, R., Haralambidis, J., Treger, G., Shine, J., and Niall, H. (1983) Nature 301, 628–631
2. Hudson, P., John, M., Crawford, R., Haralambidis, J., Scanlon, D., Gorman, J., Treger, G., Shine, J., and Niall, H. (1984) EMBO J. 3, 2333–2339
3. Bathgate, R. A., Samuel, C. S., Burazin, T. C., Layfield, S., Claasz, A. A., Reytomas, I. G., Dawson, N. F., Zhao, C., Bond, C., Summers, R. J., Parry, L. J., Wade, J. D., and Treger, G. W. (2002) J. Biol. Chem. 277, 1148–1157
4. Burkhardt, E., Adham, I. M., Hobohm, U., Murphy, D., Sander, C., and Engel, W. (1994) Hum. Genet. 94, 91–94
5. Chassin, D., Laurent, A., Janneau, J. L., Berger, R., and Bellet, D. (1995) Genomics 29, 465–470
6. Conklin, D., Lofton-Day, C. E., Haldeman, B. A., Ching, A., Whitmore, T. E., Lok, S., and Jaspers, S. (1999) Genomics 60, 50–56
7. Lok, S., Johnston, D. S., Conklin, D., Lofton-Day, C. E., Adams, R. L., Jelmberg, A. C., Whitmore, T. E., Schrader, S., Griswold, M. D., and Jaspers, S. R. (2000) Bioul. Reprod. 62, 1593–1599
8. Schwabe, C., and McDonald, J. K. (1977) Science 197, 914–915
9. Hsu, S., Nakabayashi, K., Nishi, S., Kumagai, J., Kudo, M., Sherwood, O., and Hsueh, A. (2002) Science 295, 671–674
10. Kumagai, J., Hsu, S. Y., Matsumi, H., Roh, J. S., Fu, P., Wade, J. D., Bathgate, R. A., and Hsueh, A. J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 10501–10506
11. Liu, C., Kuei, C., Sutton, S., Chen, J., Roland, B., Kuei, C., Farmer, N., Jornvall, H., Sillard, R., and Lovenberg, T. W. (2003) J. Biol. Chem. 278, 50754–50760
12. Liu, C., Kuei, C., Sutton, S., Chen, J., Bonaventure, P., Wu, J., Nepomuceno, D., Kamme, F., Tran, D. T., Zhu, J., Wilkinson, T., Bathgate, R., Erste, E., Sillard, R., and Lovenberg, T. W. (2005) J. Biol. Chem. 280, 292–300
13. Wilkinson, T. N., Speed, T. P., Treger, G. W., and Bathgate, R. A. (2005) BMC Evol. Biol. 5, 14
14. Burazin, T. C., Bathgate, R. A., Macris, M., Layfield, S., Gundlach, A. L., and Treger, G. W. (2002) J. Neurochem. 82, 1553–1557
15. Sutton, S. W., Bonaventure, P., Kuei, C., Roland, B., Chen, J., Nepomuceno, D., Lovenberg, T. W., and Liu, C. (2004) Neuroendocrinology 80, 298–307
16. Ma, S., Bonaventure, P., Ferraro, T., Shen, P. J., Burazin, T. C., Bathgate, R. A., Liu, C., Treger, G. W., Sutton, S. W., and Gundlach, A. L. (2007) Neuroscience 144, 165–190
17. Tanaka, M., Iijima, N., Miyamoto, Y., Fukusumi, S., Itoh, Y., Ozawa, H., and Ibata, Y. (2005) Eur. J. Neurosci. 21, 1659–1670
18. McGowan, B. M., Stanley, S. A., Smith, K. L., White, N. E., Connolly, M. M., Thompson, E. L., Gardiner, J. V., Murphy, K. G., Ghatel, M. A., and Bloom, S. R. (2005) Endocrinology 146, 3295–3300
19. Liu, C., Chen, J., Sutton, S., Roland, B., Kuei, C., Farmer, N., Sillard, R., and Lovenberg, T. W. (2003) J. Biol. Chem. 278, 50765–50770
20. Sudo, S., Kumagai, J., Nishi, S., Layfield, S., Ferraro, T., Bathgate, R. A., and Hsueh, A. J. (2003) J. Biol. Chem. 278, 7855–7862
21. Kuei, C., Sutton, S., Bonaventure, P., Pudiak, C., Shelton, J., Zhu, J., Nepomuceno, D., Wu, J., Chen, J., Kamme, F., Seierstad, M., Hack, M. D., Bathgate, R. A., Hossain, M. A., Wade, J. D., Attack, J., Lovenberg, T. W., and Liu, C. (2007) J. Biol. Chem. 282, 25425–25435
22. Rosengren, K. J., Lin, F., Bathgate, R. A., Tregear, G. W., Daly, N. L., Wade, J. D., and Craik, D. J. (2006) J. Biol. Chem. 281, 5845–5851
23. Rosengren, K. J., Zhang, S., Lin, F., Daly, N. L., Scott, D. J., Hughes, R. A., Bathgate, R. A., Craik, D. J., and Wade, J. D. (2006) J. Biol. Chem. 281, 28287–28295
24. Büllesbach, E. E., and Schwabe, C. (2000) J. Biol. Chem. 275, 35276–35280
25. Büllesbach, E. E., and Schwabe, C. (1999) Biochemistry 38, 3073–3078
26. Bathgate, R. A., Lin, F., Hanson, N. F., Otvos, L. J., Guidolin, A., Giannakis, C., Bastiras, S., Layfield, S. L., Ferraro, T., Ma, S., Zhao, C., Gundlach, A. L., Samuel, C. S., Tregear, G. W., and Wade, J. D. (2006) Biochemistry 45, 1043–1053
27. Hossain, M. A., Lin, F., Zhang, S., Ferraro, T., Bathgate, R. A., Tregear, G. W., and Wade, J. D. (2006) Int. J. Pept. Res. Therap. 12, 211–215
28. Cierpicki, T., and Orleowski, J. (2001) J. Biomol. NMR 19, 249–261
29. Keller, R. (2004) The Computer Aided Resonance Assignment Tutorial, CATENA VERLAG, Reiskirchen, Germany
30. Guntert, P., Mummenhaler, C., and Wüthrich, K. (1997) J. Mol. Biol. 273, 283–298
31. Wagner, G. (1990) Prog. NMR Spectrom. 22, 101–129
32. Brungg, A. T., Adams, P. D., Clover, G. M., Delano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jang, J. S., Kszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. D. Bioul. Crystallogr. 54, 905–921
33. Wishart, D. S., Bigam, C. G., Holm, A., Hodges, R. S., and Sykes, B. D. (1995) J. Biomol. NMR 5, 67–81
34. Eigenbrot, C., Randal, M., Quan, C., Burnier, J., O’Connell, L., Rinderknecht, E., and Kossiakoff, A. A. (1991) J. Mol. Biol. 221, 15–21
35. Liu, C., Chen, J., Kuei, C., Sutton, S., Nepomuceno, D., Bonaventure, P., and Lovenberg, T. (2005) Mol. Pharmacol. 67, 231–240
36. Hossain, M. A., Rosengren, K. J., Haugaard-Jonsson, L. M., Zhang, S., Layfield, S., Ferraro, T., Daly, N. L., Tregear, G. W., Wade, J. D., and Bathgate, R. A. (2008) J. Biol. Chem. 283, 17287–17297