The Trap-like Relaxin-binding Site of the Leucine-rich G-protein-coupled Receptor 7* 

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The pleated sheet region of the leucine-rich G-protein-coupled receptor 7 supports a relaxin-binding group of amino acids that perfectly matches the binding cassette of relaxin. Arginines B13 and B17 are each chelated by an aspartic acid/glutamic acid pair and by isoleucine B20, which, offset by a one-quarter helix turn from a straight line connecting the arginines, interacts with a cluster of hydrophobic amino acids. The binding cassette of relaxin cuts at an angle of ~45° across five parallel leucine-rich repeats. The arginine residues 13 and 17, which evolve parallel from the B-chain α-helix of relaxin, neutralize the charge repulsion of the juxtaposed acidic groups on the receptor and thereby trigger closure of a hydrogen bonding network around the guanidinium groups. Thus, relaxin is bound by synchronized chelation of two arginines and stabilized by hydrophobic interaction of isoleucine B20 with tryptophan, isoleucine, and leucine in neighboring leucine-rich repeats of the receptor. Deletion of any one of the three features diminishes interaction to the level of non-specific binding. This model explains the exquisite sensitivity of relaxin binding avidity to minute changes in the disposition of the guanidinium and the size dependence of the hydrophobic binding residue in position B20.

The peptide hormone relaxin supports vital processes such as heart function, coronary and peripheral blood flow, and physiological adaptation to the stresses of pregnancy (1, 2). It acts on the capillary bed as a vasodilator, which, in part, explains its anti-fibrotic action. The positive effect of this hormone on peripheral and cardiac blood flow makes relaxin, along with its receptor, a prime target for research on chronic debilitating diseases.

The discovery that the orphan receptor LGR7 binds relaxin specifically (3–5) makes it possible to investigate ligand-mediated activation of a member of the group of leucine-rich repeat (LRR) receptors. This genre of receptors, including those for thyrotropin, luteotropin, and human chorionic gonadotropin, is known to have leucine-rich repeats in their ectodomains and to be anchored to the cell surface by seven helical transmembrane segments. The binding sites are thought to be large and to cover much of the inner aspects of the concave pleated sheet structures (6, 7). In contrast, our earlier work on relaxin has revealed sharply defined binding structures (8) that would require an equally well defined binding region on the receptor surface.

LRR proteins, such as the RNase inhibitor for example, can bind proteins with extraordinarily high affinity (9, 10). Binding constants reach into the femtomolar range when as many as 26 residues make surface contact (11). In contrast, our structure function studies with synthetic relaxin showed clearly that the contact with LGR7 is essentially limited to three residues located in the center of the B-chain helix. Thus, the binding energy sufficient to yield an affinity constant of ~10−10 M had to be derived from an unusual receptor/ligand interaction. A binding cassette of the general formula RXXXXRX(RIV) was discovered that appears in every species-specific relaxin regardless of major primary structure variations in the molecule (8). In addition, steric effects are exerted by glycine A14, which, by its smallness, controls the position of the A-chain loop (12). In this paper we are presenting evidence for the identity of a contact residue on LGR7 for each of the members of the relaxin-binding cassette.

MATERIALS AND METHODS

Strains, Plasmids, and Oligonucleotides—Escherichia coli strain XL1Blue MRF’ was used for plasmid amplification and the human embryo kidney cell strain 293T/17 (American Type Culture Collection number CRL-11286) for protein expression. Plasmid pUC18 served as cloning vector, and plasmid pcDNA3.1.zeo (Invitrogen) was utilized as expression vector. Mutagenic primers used in this study were custom synthesized and purified by polycrylamide gel electrophoresis (Integrated DNA Technologies). Sequences are available upon request.

Cloning and Site-directed Mutagenesis of the LGR7 Receptor—The full-length cDNA of the human relaxin receptor LGR7 in the pUC-18 cloning vector encoded the prolactin signal peptide and the LGR7 mature protein (courtesy Dr. Hsu, Department of Gynecology and Obstetrics at Stanford University). This cDNA was further modified to contain the coding sequence for the FLAG epitope at the N terminus of the mature LGR7 protein, a unique BamHI site at the 5’-end and a unique EcoRI site at the 3’-end of the coding sequence. Site-directed mutagenesis was carried out using pairs of custom oligonucleotides in combination with a polymerase chain reaction. The reaction was performed starting with 50 ng of the DNA template, 10 pmol of each primer, 250 μM each deoxynucleotide triphosphate, and 1 unit of Vent polymerase (New England Biolabs) in 50 μl of the reaction buffer. The reaction mixture was heated to 94 °C for 2 min followed by 18 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 6 min, with a final extension of 7 min at 72 °C. Wild-type DNA was digested with 10 units of the restriction enzyme DpnI for 1 h at 37 °C, and the PCR reaction mixture was used to transform competent XL1-Blue-MRF’ cells. Individual colonies of the transformed cells were grown, and the plasmid was isolated and subjected to DNA sequencing using the primers LGR7–14F (5’-GGCATACAGCTTGTTCTC-3’), LGR7–24R (5’-GAAATTCATCAG-TGTCTG-3’), or LGR7–4F (5’-GAATGTTTGGTCGGTTCTG-3’). The cDNA of the mutant was subcloned into the BamHI/EcoRI site of the suitable pcDNA3.1.zeo vector. Individual clones were cultured in Luri-a-Bertani broth containing 50 μg/ml ampicillin and purified using a plasmid isolation kit (Qiagen). The primers used to sequence the DNA

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1 The abbreviations used are: LGR7, leucine-rich G-protein-coupled receptor 7; LRR, leucine-rich repeat; 293T/17, human embryonic kidney 293T clone 17.
were T7 (vector), BGH, LGR7–4f (the coding cDNA), LGR7–4r (5′-CGAGACTGTTATGCC-3′), LGR7–14f, LGR7–14r (5′-GAGAACAGCTGTTATGCTG-3′), LGR7–24r, and LGR7–24f (5′-CAGAACCAGCTGTTATGCTG-3′).

Cell Culture—Cells (293T/17) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, catalog number 11995-065) in 5% CO2 at 37 °C in a water-saturated atmosphere, detached with trypsin/EDTA (Invitrogen), and transferred to 6-well plates (106 cells/well). The 293T/17 cells were grown for 24 h before transfection with either the pcDNA3.1.neo vector alone (negative control), the vector bearing the LGR7 coding sequence (positive control), or the vector containing the mutant sequences. For transfections, the Polyfect™ reagent (Qiagen) and protocols provided by the manufacturer were used, and the transfected 293T/17 cells were assayed after a 48-h culture period.

Relaxin and Antibody Binding Assays—The transfected 293T/17 cells were dislodged by the addition of 1 ml of 0.5% EDTA (pH 7.5) per 10 ml of conditioned medium for 10 min at 37 °C. The cells were collected by centrifugation at 2,000 × g for 10 min, suspended twice in 1 ml of ice cold binding buffer (20 mM Hepes, pH 7.5, 1% bovine serum albumin, 0.1 mg/ml lysisine, 1.5 mM CaCl2, 50 mM NaCl, and 0.01% NaN3), and centrifuged at 4 °C for 10 min at 2,000 × g. The pellet, collected and reconstituted to 25 × 106 cells/ml, was transferred to ice-cold binding buffer. For binding assays, 0.25 pmol of 125I-Tyr(B30)-Phe(A3) human relaxin-II (based on 80% counting efficiency, the molar activity is 3900 cpm/fmol) (8) of human relaxin II, and 40 ng was incubated at room temperature for 60 min. Thereafter, the cells were washed with 1 ml of the same buffer and centrifuged for 10 min at 5,000 × g at 4 °C. The cells containing the pellets were placed in tubes and transferred to a γ-counter.

Determination of the FLAG Antigen—A radioactive, anti-FLAG antibody (Sigma) was prepared by the chloramine T method (13). Free 125I was removed from the antibody by size separation on Sephadex G25 in 50 mM phosphate buffer saline at pH 7.4 (ratio of labeled per unlabeled antibody was ~1:10). Antibody binding assays were performed in parallel with receptor binding assays. About 106 cells were used in a total volume of 100 μl containing the labeled antibody (100,000 cpm/assay) in the presence or absence of 0.2 μM (0.2 nmol) of the synthetic FLAG peptide and incubated for 1 h at room temperature. The cells were recovered, washed as described above, and the pellet was analyzed in a γ-counter.

Relative relaxin binding of the mutants was calculated as B/B0 (%) = ([TBm − NBm]−[TBw − NBw]) ([TBw − NBw]−[TBm − NBm]) × 100, where B is total binding, NB is nonspecific binding, TB is total binding, and subscript M is mutant, V is vector, subscript W is wild type, and F is the correction factor to compensate for differences in protein expression as determined by the binding of 125I-labeled anti-FLAG antibody in the presence and absence of the FLAG-peptide where F = (TBW − NBW)/(TBm − NBm) (subscript A is antibody). Each data point was measured in duplicate. Data are presented as the mean (± S.E.) of 2–4 independent experiments.

Molecular Modeling—To model the leucine-rich repeat domain of LGR7 on the protein sequence of the porcine ribonuclease inhibitor, the algorithm described by Kajava et al. (14) was used in combination with the Sybyl software (version 6.91, Tripos, St Louis, MO). The 24-residue repeats of LGR7 were aligned with the 28/29-residue repeats 3–12 based on the x-ray structure of the porcine ribonuclease inhibitor (2BHN). The corresponding residues of LGR7 were mutated, and additional residues located in the helical region on the outer surface were excised. Thereafter, prolines, side chain amides, and other side chains were fixed, and the molecule was energy-minimized using MAXIMIN for 1,000 iterations at default setting. The resulting molecule was checked for the chirality, and differences were corrected. Thereafter, the LGR7 model was again energy-minimized.

RESULTS

The leucine-rich repeat domain of LGR7 was modeled on the coordinates of the porcine ribonuclease inhibitor using the approach of Kajava et al. (14). The reduction in length of the repeats, from the 28/29-amino acid repeat of ribonuclease inhibitor to the 24-residue repeat of LGR7, was achieved by removing the corresponding residues in the helical structure of each repeat. The sequences of the individual repeats are shown in Fig. 1. The energy-minimized molecule shows the expected parallel pleated sheets inside and helices at the outside of a horseshoe-shaped structure. The conserved asparagines appear on the edge of the sheet-turn-helix motif. There was no deviation of the bond lengths from the ideal, as all of the chiral centers were correct and >90% of the φ/ψ angles of the backbone structure are within the allowed values. Further analysis of the backbone structure showed a cis-peptide bond between Ser-201 and Leu-202 located in the β-turn between repeat 4 and 5 and a 164° bond angle of the peptide bond between Cys-248 and Ser-249. The pseudo-energy of this structure was assessed by the program MatchMaker, which revealed an overall average pseudo-energy of ~0.04 (15, 16). The pseudo-energy showed periodic changes to more negative values for the pleated sheet regions and positive values for the helical regions. Although the overall negative pseudo-energy values indicated a good match between the primary structure and the tertiary structure, the probability of a valid structure is higher for the internal surface than for the outer circumference. On this inner surface of the receptor we replaced single residues using site-directed mutagenesis.

In parallel experiments, 293T/17 cells were transiently transfected with the genes for the mutant receptor, the LGR7 wild type for positive control, and the vector for negative control. Binding assays were conducted with radioactively labeled human relaxin (8) in the presence and absence of unlabeled human relaxin. The tracer concentration was sufficient to sat-
LGR7-Relaxin Interaction

Table I

Representative receptor binding data of the mutants

| Mutant     | TB cpm | NB cpm | TB cpm | NB cpm |
|------------|--------|--------|--------|--------|
| W180A      | 37,273 | 7,475  | 7,996  | 5,693  |
| I182A      | 37,273 | 7,475  | 7,996  | 5,693  |
| L204A      | 31,490 | 8,773  | 12,594 | 6,968  |
| V206A      | 31,490 | 8,773  | 12,594 | 6,968  |
| D231N      | 33,180 | 6,769  | 11,393 | 6,284  |
| D231E      | 47,835 | 9,789  | 14,683 | 8,231  |
| E233Q      | 22,037 | 6,754  | 8,042  | 6,621  |
| V235A      | 46,914 | 12,537 | 12,501 | 11,541 |
| V255A      | 46,914 | 12,537 | 12,501 | 11,541 |
| D276N      | 27,568 | 7,229  | 7,925  | 6,600  |
| D277Q      | 34,840 | 13,229 | 13,269 | 11,541 |
| D279N      | 27,568 | 7,229  | 7,925  | 6,600  |

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Data points were determined in duplicate, and five curves of LGR7 and three curves of LGR7-D276N were averaged (bars are ± S.E.).

Discussion

The arginines B13 and B17 in relaxin, located one helix turn apart, are pointing parallel to each other into the water, whereas a three-quarter helix turn toward the C terminus Ile, the third binding residue, introduces asymmetry into the binding site (9). Structure/function studies showed that arginines B13 and B17 in relaxin could neither be extended (homarginine) (17) nor replaced by lysine without a severe reduction in receptor binding avidity (18). Isoleucine B20 in relaxin could be exchanged for other hydrophobic residues, whereby amino acids smaller than Ile or Val reduced binding as a function of reduced overlap (8). To investigate which structural features would lead to these unusually stringent binding requirements, we expressed native and mutated LGR7 in 293T cells.

Initial direction for experimentation was derived from the coordinates of the intracellular porcine ribonuclease inhibitor (9, 10, 14). The surface produced by the LRR segments in these proteins is connected at the periphery by short α-helices that run normal to the plane of the pleated sheets. Alignment of the leucine-rich repeats 1–10 by the conserved asparagines provided a starting position for discovering configurations that would satisfy the requirements of the binding cassette of relaxin. The distance between the arginine residues was fixed by one helix turn to ~5.4 Å so that any of the negative charges, not falling into this range, could be ignored. In addition, a hydrophobic surface had to exist ~4 Å away on one side of a line connecting the negative charges. Based upon the precise determination of the three binding residues in relaxin, initial selection of complementary sites on the receptor was 3-fold strained. Statistically, such a configuration could be expected to occur.

Fig. 3. Relative binding of 125I-relaxin to mutant LGR7. All transient expression experiments were done in 293T/17 cells using side-by-side transfections of two or three mutants, the wild type (positive control), and vector only (negative control). Relative binding was calculated by comparison of the specific binding of 125I-relaxin to the receptor or receptor mutant corrected according to the number of receptors expressed on the cell surface determined by the specific binding of 125I-labeled anti-FLAG antibody.

Fig. 4. Comparison of dose-response curves of relaxin binding to LGR7 and LGR7 mutant D276N, a residue that is not part of the receptor-binding site. Data points were determined in duplicate, and five curves of LGR7 and three curves of LGR7-D276N were averaged (bars are ± S.E.).
chains of the binding residues are shown. A and B, “open sandwich” display of relaxin (A) and its receptor (B). Data support the proposals that relaxin Arg(B13) (blue) is chelated by Glu-277/Asp-279 (red) on LGR7, residue Arg(B17) (purple) is chelated by Asp-231/Glu-233 (orange) on LGR7, and Ile(B20) (cyan) interacts with Trp-180/Ile-182/Leu-204 (cyan). C and D, the two molecules relaxin (C) and LGR7 (D) are turned toward each other. This side view shows the locations of the binding sites of relaxin on the exposed amphiphilic helix surface and the binding residues on LGR7 on the parallel pleated sheets forming the concave surface of the horseshoe-shaped molecule.

only once in a relatively small molecule like the LGR7 ectodomain.

The topography of our model suggested that, on the pleated sheet, glutamic acid 277 and aspartic acid 279 on leucine-rich repeat number 8 and aspartic acid 231 and glutamic acid 233 on repeat number 6 would match the distribution of arginines in the binding cassette of relaxin (Figs. 1 and 5). Note that each pair of these residues is on the same repeat, i.e. they are fixed by primary sequence relative to each other. This configuration was so convincing that Glu-277 became the starting point for our mutational analysis. Glutamic acid 277 was exchanged for glutamine by site-directed mutagenesis. As a control, aspartic acid 276, which points below the plane of the pleated sheet, would not be able to bind the two arginines of relaxin each with an aspartic and glutamic acid pair. The arginines would neutralize the charge density in the binding pocket of relaxin. Aspartic acid 279 (Fig. 1), a potential partner in capturing the guanidinium group, was subsequently replaced by asparagine. This change also eliminated specific ligand/receptor interaction (Fig. 3). A chelating interaction with the guanidinium group, involving two acidic functions, would indeed explain the rejection of the primary amine of lysine in favor of a guanidinium group. Structure/function work on the binding cassette of relaxin, in combination with analogy modeling based on the porcine RNase inhibitor, has provided persuasive evidence for a chelating interaction between either arginine B13 or B17 in relaxin and Glu-277 and Asp-279 on LGR7.

If our model is correct to this point, the other binding residues for relaxin must be within a circle of ~5.4 Å from a point centered on Glu-277 and Asp-279. Two acidic residues were observed that satisfied this requirement, i.e. aspartic acid 231 and glutamic acid 233, both in repeat number 6. Asp-231 was replaced by asparagine and Glu-233 by glutamine, and the two constructs were tested in transiently transfected 293T cells. Both mutants did not bind relaxin, suggesting that LGR7 bound the two arginines of relaxin each with an aspartic and glutamic acid pair. The arginines would neutralize the charge repulsion between residues 231/233 and 277/279 and thus induce a binding configuration comprising the guanidinium group sandwiched between two acidic functions. Receptor/ligand interaction is usually associated with a binding pocket, which is sort of a nesting feature on the receptor that is not conspicuous on the inner LGR7 surface. Despite the curvature of the inner aspect of LGR7 that seems to cradle the relaxin molecule, one is still faced with an end-to-end interaction between the arginines protruding from an α-helix in relaxin and acidic residues from a pleated sheet of the receptor surface. The inner aspect of the LGR7 receptor is slightly concave but without the varied landscape that a binding cleft in a globular protein provides.

At this point our results would allow relaxin to bind the receptor in two orientations, either with the C-terminal toward the cell or toward the cysteine-rich domain. This is very important information when one begins to search for the signal initiation site of relaxin.

The potential hydrophobic interaction region that would have to match Ile B20 in relaxin is located ~3.5–4 Å C-terminally to B17 arginine in relaxin, offset from the straight line connecting the arginine residues. This arrangement provides an element of asymmetry that would unambiguously orient the ligand on the receptor surface. Tryptophan 180, isoleucine 182, leucine 204, and valine 206 form such a hydrophobic cluster at the correct distance to engage isoleucine B20 in relaxin. This cluster of hydrophobic residues is located on LRRs 4 and 5 of LGR7 (Figs. 1 and 5).

Hydrophobic sites, by the nature of interaction, should show some tolerance to replacement by like functions of different size, which is exactly what we observed. Mutation of tryptophan (position 180 on repeat number 4) to alanine reduces binding to ~10% of control value. The exchange of isoleucine 182 on the same repeat again reduces binding to ~10%, and replacement of leucine 204 on repeat number 5 by alanine still binds relaxin to ~20% of the native receptor.

These observations are strongly supported by the structure function work that preceded this study (8). Isoleucine and valine in position B20 (the binding cassette of relaxin) would provide nearly the same overlap and, hence, essentially the same binding intensity. Substituting isoleucine for either norleucine or α-aminobutyric acid diminished binding progressively until it bottomed out with the introduction of alanine, leading to a ~1,000-fold reduction. A substantial decrease was
obtained when we converted members of the contact site in LGR7, i.e. tryptophan 180, isoleucine 182, or leucine 204 to alanine. Furthermore, the exchange of valine 206 on repeat number 5 for alanine caused no significant loss in binding avidity, thus providing a demarcation for the extent of the hydrophobic binding region. Based upon these experiments, we concluded that the hydrophobic cluster on repeats 4 and 5 of LGR7 is the interaction site for Ile B20 in relaxin. The discovery of the hydrophobic interaction site fixes the binding direction for relaxin on the LRR surface such that the C-terminal of the B chain must point toward the N-terminal end of the receptor.

Analyses of complex systems must be broken down into the various components that enter into the final equation. Stringent requirements for the interpretation of the data concerning the relaxin-binding site on the receptor have been derived from the precise description of the binding cassette of relaxin. It follows that the binding residues on the receptor have to be arranged like a mirror image of the binding cassette. Although finding one binding residue would be a questionable result, the discovery of three binding centers at exactly the distance from each other as dictated by the x-ray coordinates of relaxin represents very strong evidence. These conditions have been met by our results. We have shown that the two arginines, B13 and B17, in relaxin have charge and chain length requirements, and our experiments reveal that if any of these parameters is mismatched, binding will be reduced 1,000-fold. For example, amidation of only one of the acid functions in one of the two chelating pairs, either aspartate or glutamate, to the corresponding amide is sufficient to abolish binding. It was equally astounding to note that a change in length by one carbon, without changing the charge, of one of the chelating acids eliminated binding as well. The fact that mutating aspartic acid 231 to glutamic acid abolished binding not only speaks without changing the charge, of one of the chelating acids

| Mutant  | FLAG-LGR7 | FLAG-mutant |
|--------|-----------|-------------|
|        | TB cpm    | NR cpm      | TB cpm    | NR cpm      | F (correction) | B/Bo (corrected) |
| W180A  | 3,120     | 864         | 1,797     | 603         | 1.89          | -0.8           |
| I182A  | 3,120     | 864         | 2,462     | 801         | 1.36          | 13.5           |
| L204A  | 2,251     | 775         | 2,607     | 794         | 0.81          | 19.1           |
| V206A  | 2,251     | 775         | 2,345     | 741         | 0.92          | 6.91           |
| D231N  | 1,923     | 652         | 1,826     | 595         | 1.05          | -7.9           |
| D251E  | 3,313     | 1,121       | 3,218     | 1,083       | 1.03          | -14.7          |
| E235Q  | 1,582     | 636         | 1,762     | 690         | 0.88          | 21.3           |
| V253A  | 2,831     | 1,086       | 3,851     | 1,299       | 0.68          | 41.3           |
| V255A  | 2,831     | 1,086       | 4,071     | 1,189       | 0.61          | 24.8           |
| D276N  | 2,551     | 742         | 1,425     | 609         | 2.22          | 99.2           |
| E277Q  | 2,564     | 893         | 2,844     | 892         | 0.81          | 5.3            |
| D279N  | 2,551     | 742         | 2,548     | 647         | 0.94          | 8.7            |

FIG. 6. Mechanism of binding. Each arginine residue in the binding site of relaxin is chelated to two carboxylates. All atoms of the guanidinium group and the carboxyl groups are in the same plane. The spacing of the carboxyl groups allows for hydrogen bonds with an optimal orbital overlap. The second arginine in the binding site of relaxin interacts with a second set of two acidic residues. We propose that the resting position of the chelating acids is open and that approaching arginines trigger the formation of the hydrogen-bonding network.

Replacement of either of the valine residues (positions 253 or 255) in LRR segment number 7 by alanine caused a significant reduction of relaxin binding without a possibility of direct interaction with the ligand. The opposing potential contact residues Glu B14 and Val B16 in relaxin are located between the two binding arginines in relaxin and point away from the receptor interaction site. In addition, these residues can be replaced freely by smaller and larger residues without affecting binding affinity (8). It is likely that the valines in LGR7 orient the acidic residues on repeats number 6 and number 8 to permit the optimal overlap with the arginines on the B chain helix.

A construct of LGR7 without the cysteine-rich region will not bind relaxin. Our extensive structure function work with relaxin has revealed no residues that influence receptor binding other than the binding cassette and glycine A14 (8, 19). The fact that deletion of the cysteine-rich region eliminates binding completely suggests that the binding site has been disassembled by structural changes, because the cysteine-rich region

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2 E. E. Büllshbach and C. Schwabe, unpublished results.
may stabilize the relatively short pleated sheet segment of the receptor.

In summary, the LRR surface of proteins would, a priori, make this structure unsuited for the kind of binding pockets that come to mind when one thinks of very selective protein-protein interactions. LGR7/relaxin interaction is unique in that the specificity is established by pendulous arginine side chains, evolving in-line from an α-helix in relaxin, that are met by the protruding acidic side chains of the receptor, and all of this is supported by laterally displaced hydrophobic binding residues that act like the third leg of a tripod. To our knowledge, this is the first report of such a binding site that could well be typical for other protein binding leucine-rich repeat receptors. Our model explains the widely observed extensive cross-reactivity of relaxins that exists despite significant sequence variations as long as the binding cassette is present. Precise knowledge of the receptor-binding cassette in relaxin and the topology of its members has provided de facto confirmation of the analogy-derived model of LGR7, at least to the extent of the relaxin contact region.

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