

# The Relaxin Receptor-binding Site Geometry Suggests a Novel Gripping Mode of Interaction\*

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**Relaxin has a unique, clearly identifiable, mixed function receptor-binding region comprising amino acid residues that evolve sequentially from the central portion of the B chain  $\alpha$ -helix. Two arginine residues in positions B13 and B17 that project like forefinger and middle finger from the helix provide the electrostatic element opposed by the hydrophobic (thumb) element isoleucine (B20), offset from the arginines by about 40°. The binding intensity of relaxin to its receptor decreases by 3 orders of magnitude if alanine is substituted for the newly discovered binding component isoleucine in position B20. The arginine residues cannot be replaced by other positive charges, nor can the guanidinium group be presented on a longer or shorter hydrocarbon chain. In contrast, the hydrophobic interaction is incremental in nature, and the contribution to the total binding energy is roughly proportional to the number of hydrocarbon units in the side chain. It appears that a hydrophobic surface exists on the receptor that offers optimal van der Waals' interaction with  $\beta$ -branched hydrophobic amino acids. The binding energy increases roughly 10-fold with each methylene group whereby  $\beta$ -branching is more effective per surface unit than chain elongation. Aromatic side chains appear to demarcate the extent of the binding region in so far as residues larger than phenylalanine decrease receptor binding. The exceptional clarity of binding site geometry in relaxin makes for an excellent opportunity to design peptido-mimetics.**

Relaxin, a small, two-chain protein, the physiological mediator of parturition in most mammals (1), has recently been shown to influence significantly the symptoms of scleroderma (2, 3). Since its discovery (4) relaxin has provided a share of unusual features including an insulin-like structure (5–9) and a receptor-binding site composed of two charged residues, *i.e.* arginine in position B13 and B17 (10, 11). The binding residues are positioned one turn apart on the major B chain helix and are projecting parallel into the surrounding water (12). This observation led to the suggestion that relaxin would bind to the receptor by a dual prong mechanism involving the interaction of the guanidinium groups with two negative charges at the bottom of a binding pocket in the receptor (11). Although both

arginines are indispensable, the fact that arginine-containing peptides would not interfere with binding suggested that other binding site members might exist. In this paper, we are reporting that the receptor-binding site of relaxin includes isoleucine in position B20, which is located three-quarter of one turn further toward the C-terminal end of the same helix so that the hydrophobic side chain opposes the two arginines forming a quasi-prehensile unit that points to a novel binding mechanism. Evidence presented in this paper supports the conclusion that Ile-B20 is as important for receptor-binding as either of the critical arginines and that the relaxin/receptor interaction is trivalent.

## EXPERIMENTAL PROCEDURES

### Materials

Amino acid derivatives were purchased from either Advanced ChemTech (Louisville, KY), Bachem Bioscience (Torrance CA), or Nova Biochem (San Diego, CA). Solvents for peptide synthesis and HPLC<sup>1</sup> were Burdick and Jackson high quality grade. Reagents for peptide synthesis were purchased from PerkinElmer Life Sciences. Other chemicals and reagents were of analytical grade.

### Methods

#### Peptide Syntheses

Human relaxin B29 and B33, Gln-B14, Asp-B14, and GRER-dpp insulin were synthesized as described (13, 14). All other human relaxin derivatives and GRERI-dpp-insulin B chain were synthesized by Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry using trifluoroacetic acid labile protecting groups (15, 16) for all side chains except unprotected tryptophan, methionine sulfoxides, and *S*-acetamidomethyl cysteine (B11). The synthesis was performed on an ABI model 433A protein synthesizer starting with 0.25 mmol of peptide on the resin up to residue B21. Thereafter the resin was split into three equal portions, each of which was used to produce one B chain analog using the standard 0.1-mmol chemistry protocol. The peptidyl resin was deprotected with trifluoroacetic acid/ethanedithiol/thioanisole/phenol/water (10:0.25:0.5:0.75:0.5 v/v/v/v/v) (17) for 2 h at room temperature, the resin was filtered off, and the peptide was collected by ether precipitation and dried and purified by preparative HPLC (yield, 15–25 mg). The B chain was dissolved in 5 ml of 1 M acetic acid, and 20 mg of 2,2'-dipyridyldisulfide in 2 ml of methanol added, and the solution was stirred for 30 min at room temperature. The mixture was separated by gel filtration on Sephadex G25sf in 1 M acetic acid and lyophilized (yield, 90–100%). The partially protected B chain carried an acetamidomethyl group in cysteine B11, a 2-pyridinesulfonyl group in cysteine B23, and sulfoxides in the methionine side chains.

The A chain with the intact intra-chain disulfide bond, an acetamidomethyl group in position A11, and a sulfhydryl group in position A24 was prepared according to the literature (13, 14). The A chain was dissolved in 0.1 M acetic acid, pH 4.5, in 8 M guanidinium chloride (5 mg/ml) and added to the dry B chain (1:1 molar ratio), which dissolved instantaneously. The reaction was stirred for 24 h at 37 °C, and the products were separated on Sephadex G50sf in 1 M acetic acid, followed

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<sup>1</sup> The abbreviations used are: HPLC, high pressure liquid chromatography; GRER-dpp, Gly (A10), Arg(B9), Glu(B10), Arg(B13) des-panapeptide(B26–30)insulin amide.

by HPLC on Synchropak RP-P C18 (10 × 250 mm) (yield, 50% based on the chains). To remove the acetamidomethyl groups partially protected relaxin (9 mg) was dissolved in 0.9 ml of 0.1 M HCl, diluted with 6.1 ml of glacial acetic acid and 2 ml of 50 mM iodine in glacial acetic acid were added. After 15 min at room temperature excess iodine was reduced by pouring the reaction mixture slowly into a stirred solution of 40 ml of 0.1 M ascorbic acid. The relaxin was desalted on Sephadex G25sf in 1 M acetic acid, lyophilized, and further purified by reversed phase HPLC (yield, 1.55 mg; 17.2% for relaxin and 39.4% for GRERI-dpp insulin). The methionine sulfoxides were reduced with ammonium iodide in 90% trifluoroacetic acid (13), and the relaxin analog was HPLC purified (yield, 60–80%).

#### High Performance Liquid Chromatography

Semipreparative HPLC was performed on Synchropak RP-P (C<sub>18</sub>, 10 × 250 mm). The solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 83% acetonitrile (solvent B). These solvents were used unless stated otherwise. The flow rate was 3 ml/min, and a 30-min linear gradient from 30 to 50% B was employed for all separations. Peptides were detected by UV absorbance at 226 nm.

#### Chemical Analyses of the Relaxin Analogs

**Analytical HPLC**—Two different HPLC systems were used. System 1: a Bakerbond widebore C<sub>18</sub> column (4.1 × 250 mm) was used in combination with a Waters HPLC system. About 10–20 μg of the peptide was injected and separated using a 30-min linear gradient from 20 to 60% B at a flow rate of 1 ml/min. The effluent was monitored by UV absorbance at 220 nm.

System 2 consisted of an ABI model 130A chromatograph equipped with an Aquapore 300 (2.1 mm × 30 mm) C8 column. About 1–2 μg of the corresponding relaxin was applied via an automatic sample injector. Separation was achieved at a flow of 100 μl/min, and the eluate was detected by UV absorbance at 230 nm. Intact relaxins were separated using a 60-min linear gradient from 25–45% B.

For reduction 2 μg of the protein was dissolved in 30 μl water and 30 μl of 50 mM DTT in 0.2 M Tris/HCl at pH 8.6 in 6 M guanidinium chloride was added. After 60 min at 37 °C the solution was acidified with 10 μl of glacial acetic acid and the product separated by reversed phase HPLC (system 2) using a 60-min linear gradient from 25–60% B.

For tryptic digestion and peptide mapping 2 μg of the relaxin was dissolved in 20 μl of 25 mM Tris/HCl at pH 7.5. Tosylphenylalanyl chloromethyl ketone-treated trypsin (EC 3.4.21.4) (100 ng in 2 μl of 50 mM NH<sub>4</sub>HCO<sub>3</sub>, E:S 1:20) was added, and the digest was maintained at 37 °C for 1 h. Hydrolysis was stopped by the addition of 38 μl of 0.1% trifluoroacetic acid, and 50 μl were used for analysis in HPLC (system 2). Tryptic fragments were separated using a 40-min linear gradient from 0 to 40% B.

**Amino Acid Composition**—Peptides were hydrolyzed in vapor phase 6 M HCl containing 0.1% phenol for 1 h at 150 °C. The amino acids were modified with phenylisothiocyanate and separated by HPLC (Pico-Tag system, Waters).

**Sequence Analysis**—Phe-B20 or Ala-B20 relaxin was sequenced using a Procise protein sequencer (PerkinElmer Life Sciences) connected to an inline phenylthiohydantoin analyzer.

**Protein Determination**—Protein concentrations were measured by UV spectroscopy using an Olis Cary-15 spectrophotometer conversion (On-Line Instrument Systems, Inc.). Relaxin analogs (0.2–0.5 mg/ml) were dissolved in water. The specific absorption coefficient was calculated with 1.95 cm<sup>-2</sup> mg<sup>-1</sup> for B33 relaxin and relaxin analogs and 2.19 cm<sup>-2</sup> mg<sup>-1</sup> for B29 relaxin analogs.

**CD Spectroscopy**—CD spectra were measured on a Jasco J710 spectropolarimeter at a resolution of 0.2 nm, with a bandwidth of 2 nm. Ten spectra were averaged for each derivative. For far UV spectroscopy (250–190 nm), the relaxin analogs were dissolved in 25 mM Tris/HCl, pH 7.5, at a concentration of 0.0833 mg/ml using a cell of 0.1 cm pathlength. Mean residue ellipticity was calculated according to the literature (18). Protein concentrations were derived from UV spectroscopy and confirmed by amino acid analysis after total acid hydrolysis.

**Matrix-assisted Laser Desorption/Ionization Mass Spectrometry**—Relaxin analogs (1 μg/μl) were dissolved in 0.1% trifluoroacetic acid and mixed with 50 mM α-cyano-4-hydroxycinnamic acid in 80% acetonitrile (1:3 v/v). 1 μl was placed on a sample probe and air dried. Mass spectra were acquired with a Voyager-DE Biospectrometry Workstation (Perseptive Biosystems). Analyses were performed at the MUSC Mass Spectrometry Facility.

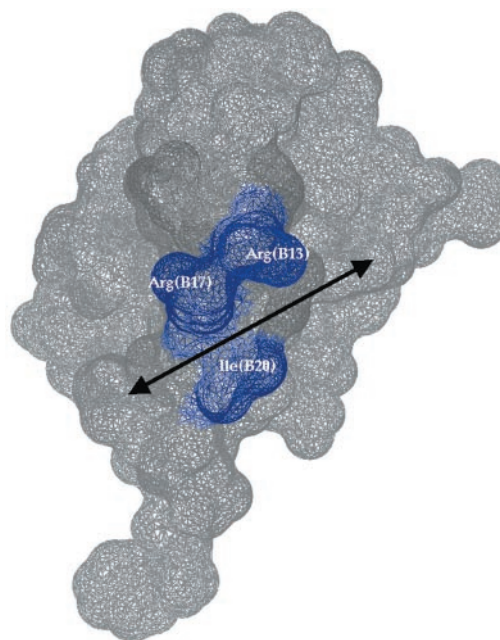


FIG. 1. Three-dimensional structure of human relaxin with view on the B chain helix emphasizing Arg-B13, Arg-B17, and Ile-B20 in blue. The arrow marks the purported path of the binding ridge on the receptor.

#### Biochemical Characterization

**Tracer Preparation**—Phe-A3,Tyr-B30 human relaxin protected at the tryptophan (formyl) and methionine side chains (sulfoxides) was synthesized as described for the human relaxin synthesis (13). Radioactive labeling of Tyr-B30 with <sup>125</sup>I<sup>-</sup> was performed by the chloramine T method, followed by the removal of the indole protecting groups (19). Phe-A3-<sup>125</sup>I-Tyr-B30 relaxin di-sulfoxide was isolated by HPLC on an Aquapore 300 column using a 60-min linear gradient from 25 to 40% B. The eluate was collected into 100 μl of a 1% bovine serum albumin solution in water. For receptor-binding assays this tracer was remade every 2 weeks.

**Receptor-binding Assays**—were performed on crude membrane preparations of mouse brain (20). Two freshly dissected mouse brains were dropped into 15 ml of chilled homogenizing buffer (25 mM Hepes, 0.14 M NaCl, 5.7 mM KCl, 8 mg/liter soybean trypsin inhibitor, supplemented with 0.25 M sucrose and 0.4 mM phenylmethylsulfonyl fluoride, pH 7.5) and homogenized using a Polytron homogenizer at position 7 for 10 s. After centrifugation for 10 min at 4 °C and 700 × g, the supernatant was collected. The pellet was again homogenized in 10 ml, the process was repeated, and the supernatants were pooled. The crude membranes were collected by centrifugation at 20,000 × g for 60 min at 4 °C, the supernatant was discarded, and the pellet was suspended in 25 ml of 25 mM Hepes buffer without sucrose. After a second centrifugation at 20,000 × g for 60 min at 4 °C, the supernatant was discarded, and the pellet of each vial was suspended in 1.5 ml of ice-cold binding buffer (25 mM Hepes, 0.14 M NaCl, 5.7 mM KCl, 2.8 mM glucose, 1.6 mM CaCl<sub>2</sub>, 25 μM MgCl<sub>2</sub>, and 1.5 mM MnCl<sub>2</sub>, supplemented with 1% bovine serum albumin and 0.2 mM phenylmethylsulfonyl fluoride). The pellets of six brains were pooled into a 6 ml polypropylene vial, chilled on ice, and homogenized with a hand-held Polytron for 20–30 s at maximum speed. Thereafter aliquots of 1.4 ml of the suspension were distributed into 1.5-ml Eppendorf vials and kept on ice. One vial was used for one dose-response curve. In general, six mouse brains were sufficient to generate four dose-response curves, each consisting of nine duplicate points.

Assays were performed in 1.5-ml Eppendorf vials. 40 μl of various concentrations of relaxin or analogs, 20 μl of tracer (60,000–80,000 cpm; final concentration, 125–165 pM), and 60 μl of crude membranes were added, gently mixed and incubated for 1 h at room temperature. Thereafter 1 ml of ice-cold wash buffer (25 mM Hepes, 0.14 M NaCl, 5.7 mM KCl, and 0.2% bovine serum albumin) was added, and the membranes were collected by centrifugation at 14,000 rpm for 10 min in an Eppendorf centrifuge. The supernatant was discarded, and the tip of the vial was cut and counted in a γ-counter. Nonspecific binding was determined in the presence of 2600 nM B33 human relaxin. In a typical

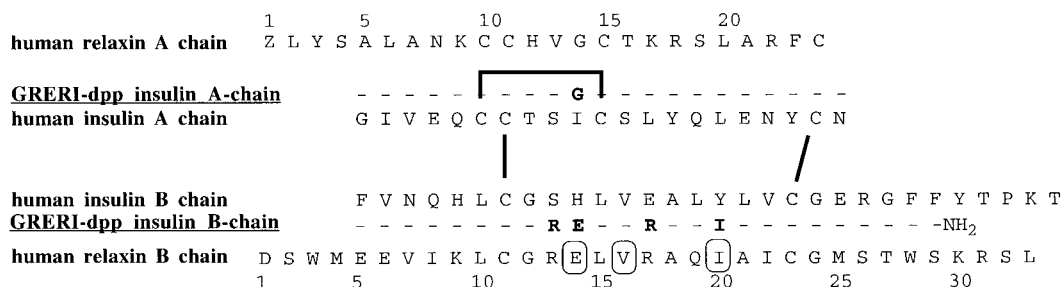


FIG. 2. Sequence of human relaxin II, human insulin and a insulin-relaxin hybrid (GRERI-dpp insulin) in which relaxin residues are substituted for the corresponding insulin residues. Circles indicate relaxin residues that were investigated during this study. Z, pyroglutamine).

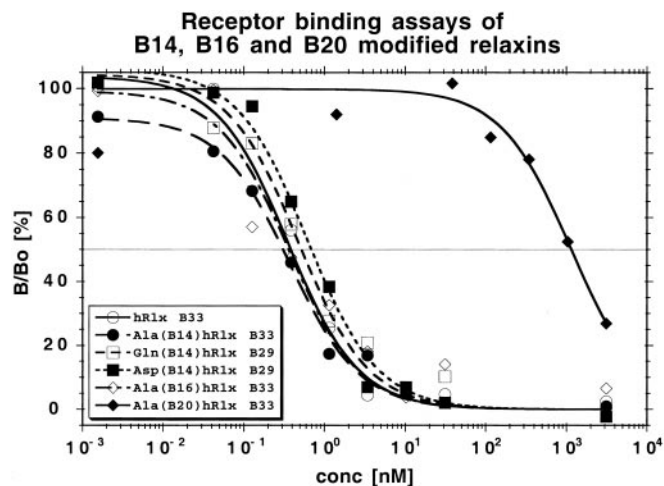


FIG. 3. Receptor binding assays of B14, B16, and B20 modified relaxins using crude membrane preparations of mouse brain and  $^{125}\text{I}$ -Phe-A<sub>3</sub>-Tyr-B<sub>30</sub> human relaxin for tracer. Three independent dose-response curves of each analog were averaged.

experiment total binding was 7–10% of the total radioactivity added, and specific binding was 35–50% of the total binding. Each point was measured in duplicate, and each analog was determined in at least three independent experiments. As a control each set of experiments contained a dose-response curve of human relaxin. Data were averaged and fitted as described by De Lean *et al.* (21).

**Mouse Symphysis Pubis Assay**—Mouse interpubic ligament assays were carried out as described by Steinetz *et al.* (22), using virgin female mice. Mice were primed with 5  $\mu\text{g}$  of estrogen cypionate in 100  $\mu\text{l}$  of sesame oil and 5 days later were injected subcutaneously with relaxin or relaxin analogs in 100  $\mu\text{l}$  of 1% benzopurpurin 4B or with 1% benzopurpurin 4B alone as control. After 16 h the mice were killed in an atmosphere of  $\text{CO}_2$ , the symphyses pubis were dissected free of adhering tissue, and the distance between the interpubic bones was measured with a dissecting microscope fitted with transilluminating fiber optics.

#### RESULTS AND DISCUSSION

The evidence that two arginine residues (B13 and B17) on the surface of the B chain helix are in the relaxin-receptor interaction site (11, 13) pointed to a novel binding mechanism. The x-ray structure of human relaxin shows that these arginines are located on the edge of the dimerization surface (12), which suggests that relaxin acts as a monomer and that the two side chains would project away from the molecular surface. Although it is clear that these two B chain arginines are indispensable, both in terms of charge and geometry, they are not sufficient for binding. For example, helical peptides with two arginines in positions  $i$  and  $i+4$  do not compete for the relaxin receptor-binding site regardless of concentration. Conversely, relaxins from different species that show more than 50% sequence differences still bind the test (mouse) receptor quite well. The display of the Connolly surface (23) derived from the

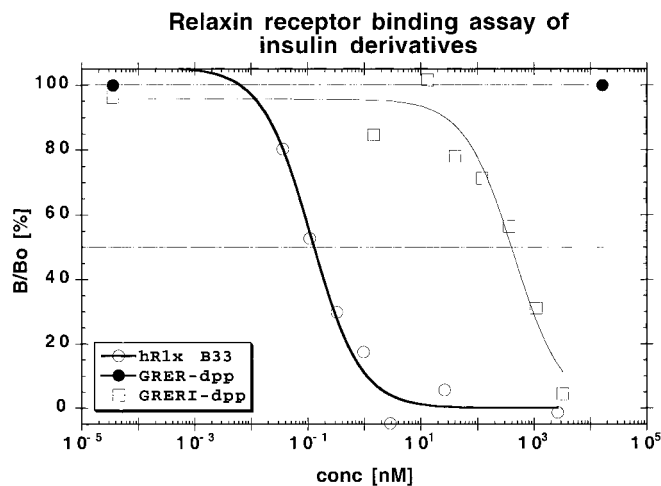


FIG. 4. Receptor binding assays of GRERI-dpp insulin on crude membrane preparations of mouse brain using  $^{125}\text{I}$ -Phe-A<sub>3</sub>-Tyr-B<sub>30</sub> human relaxin for tracer. The effect was compared with human relaxin and GRER-dpp insulin, which were run in parallel. Three independent dose-response curves were acquired. GRER-dpp insulin lacks the critical Ile-B20.

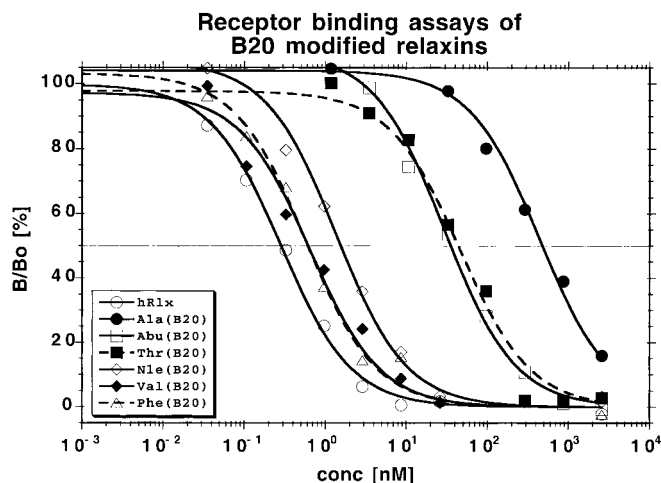


FIG. 5. Receptor binding assays of B20 modified relaxins using crude membrane preparations of mouse brain and  $^{125}\text{I}$ -Phe-A<sub>3</sub>-Tyr-B<sub>30</sub> human relaxin for tracer. Three independent dose-response curves of each analog were averaged.

x-ray structure gives the impression that the two arginines form a contiguous surface feature in the binding region of relaxin together with Glu-B14, Val-B16, and Ile-B20 (Fig. 1). Ile-B20 was not an absolutely constant feature, but replacement was rare and only with large hydrophobic residues such as Leu in hamster (24) and Val in porcine relaxin (6). Positions B14 and B16 would show a glycine and alanine, respectively, in

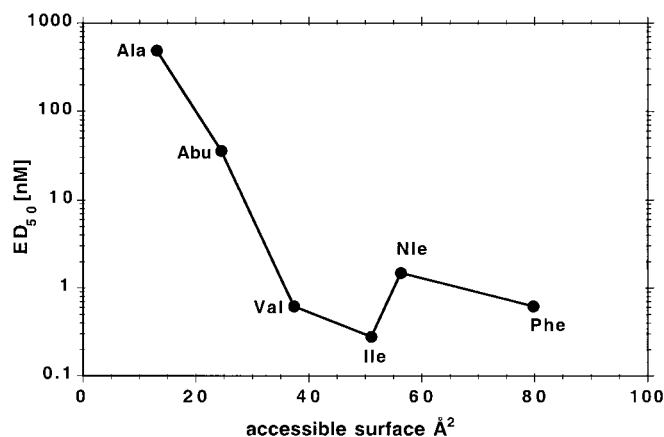


FIG. 6. Affinity of relaxin analogs for the relaxin-receptor *versus* the accessible area of hydrophobic amino acid residues in position B20. For each amino acid the Connolly surface was calculated using the Sybyl software (Tripos). The differences of the accessible surface areas of alkylamino acids and glycine are displayed. The binding affinity corresponds to the  $ED_{50}$  values measured in receptor binding assays.

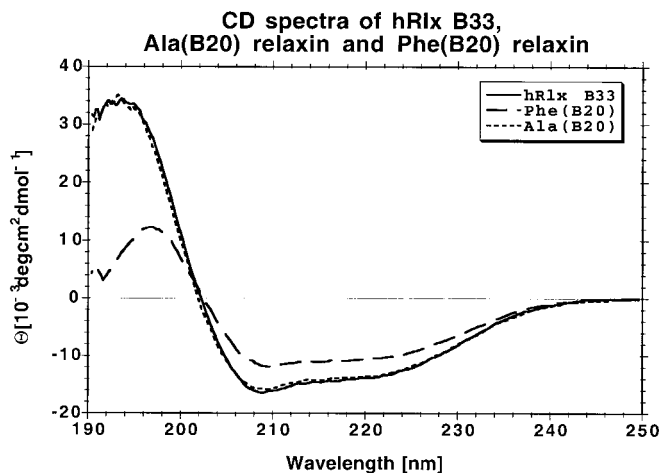


FIG. 8. CD of human relaxin in comparison with Ala-B20 relaxin and Phe-B20 relaxin. Relaxins were dissolved in 25 mM Tris/HCl buffer at pH 7.5 at a concentration of 13  $\mu$ M. Data were collected at a resolution of 0.2 nm and a bandwidth of 2 nm, and 10 spectra were averaged. The spectrum of Phe-B20 relaxin is severely perturbed when compared with other derivatives (Fig. 7).

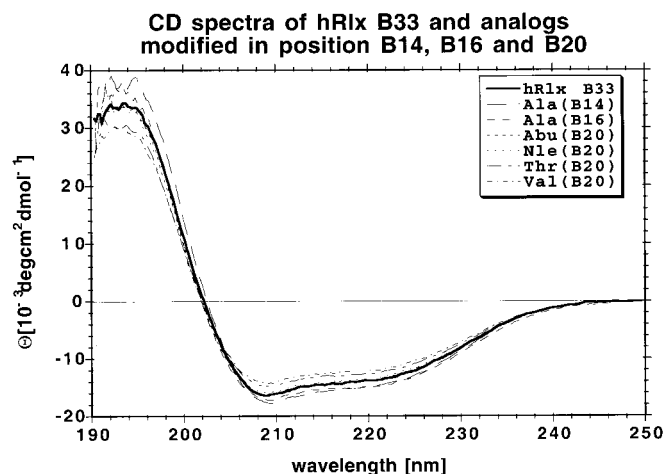


FIG. 7. CD of relaxin and relaxin analogs with modification in positions B14, B16, and B20. Relaxins were dissolved in 25 mM Tris/HCl buffer at pH 7.5 at a concentration of 13  $\mu$ M. Data were collected at a resolution of 0.2 nm and a bandwidth of 2 nm, and 10 spectra were averaged. Substitutions in positions B14, B16, and B20 do not induce major structural changes.

a few natural relaxins so that these positions seemed less critical (25).

To test our ideas we have synthesized the several human relaxin analogs (Fig. 2) replacing Glu-B14 with either Ala, Gln, or Asp, replacing valine in position B16 with Ala, and replacing Ile in position B20 with Ala. The results of the receptor binding assays (Fig. 3) on crude membrane preparations of mouse brain suggested that positions B14 and B16 could be excluded as active site residues. In contrast, substitution of Ala for Ile-B20 reduced receptor binding by three orders of magnitude indicating that Ile-B20 is as important for receptor interaction as the arginines B13 and B17. This conjecture found impressive confirmation when we redesigned and synthesized our insulin-relaxin Zwitterhormon (GRER-dpp) (14) with Ile instead of Tyr in the position corresponding to B20 and found significant receptor-binding in the mouse brain receptor assay. In fact the binding curve for (GRER-dpp) runs parallel to that of relaxin (Fig. 4), whereas the Zwitterhormon without Ile-B20 recognized only the rat relaxin receptor as previously reported (14).

Probing for fine structural binding requirements as concerns position B20, we extended alanine by one methylene group

( $\alpha$ -aminobutyric acid) and by three methylene groups (norleucine) (Fig. 5). Remarkably, each  $CH_2$  group gives a 10-fold increase in binding energy. Reducing the chain length from norleucine and adding a  $\beta$ -branch (valine) resulted in a further 2.5-fold increase in binding intensity, suggesting that bulk at the base of the side chain is favorable. In line with this argument we noted that threonine, which is isosteric with valine, produced a significant improvement over alanine despite its polar character at the  $\beta$ -carbon. On the other hand, the larger surface of phenylalanine does not compensate for the missing  $\beta$ -branch (Fig. 5). Plotting the log of the binding affinity (nM) against the surface area shows a nearly linear relationship from alanine to isoleucine with norleucine and phenylalanine lying outside possibly because of size limitations (Fig. 6). We have synthesized a B20 *p*-benzoylphenylalanine relaxin derivative that, despite its hydrophobic character, does not bind, possibly because it exceeds the dimensions of the surface on the receptor and thus prohibits the proper alignment of Arg-B13 and Arg-B17 with the binding site.

These considerations invoke the rather unique but certainly plausible mode of prehensile binding action at the molecular level. Viewed normal to the axis of the B chain  $\alpha$ -helix, comparison with portions of a human hand suggests itself, with arginine fingers on one side opposed by a thumb that, if diminished in surface, will cause reduced holding power of the ligand to its receptor. The idea also invites the proposal that relaxin interacts with a ridge on the receptor as opposed to the more commonly found pocket. One is hard pressed to explain the drastic destabilization of the di-arginine-mediated relaxin/receptor interaction by removal of one member of the binding triad (Ile) if relaxin slides into a deep binding pocket on the receptor surface.

To confirm that our studies are based upon relaxin derivatives with the proper structure, the homogeneity of each analog was verified in two reversed phase HPLC systems. Upon reduction each analog yielded two chains; the A chain showed identical retention times for all analogs, whereas the retention times of B chains differed. Tryptic digest and peptide mapping by HPLC of the B (20) relaxin analogs showed the expected difference for the C-terminal cystinyl peptide A (23–24)/B (18–30), and all other fragments were identical. Mass spectrometry indicated the correct molecular mass of each analog. Amino acid analysis of the total acid hydrolysate resulted in the ex-

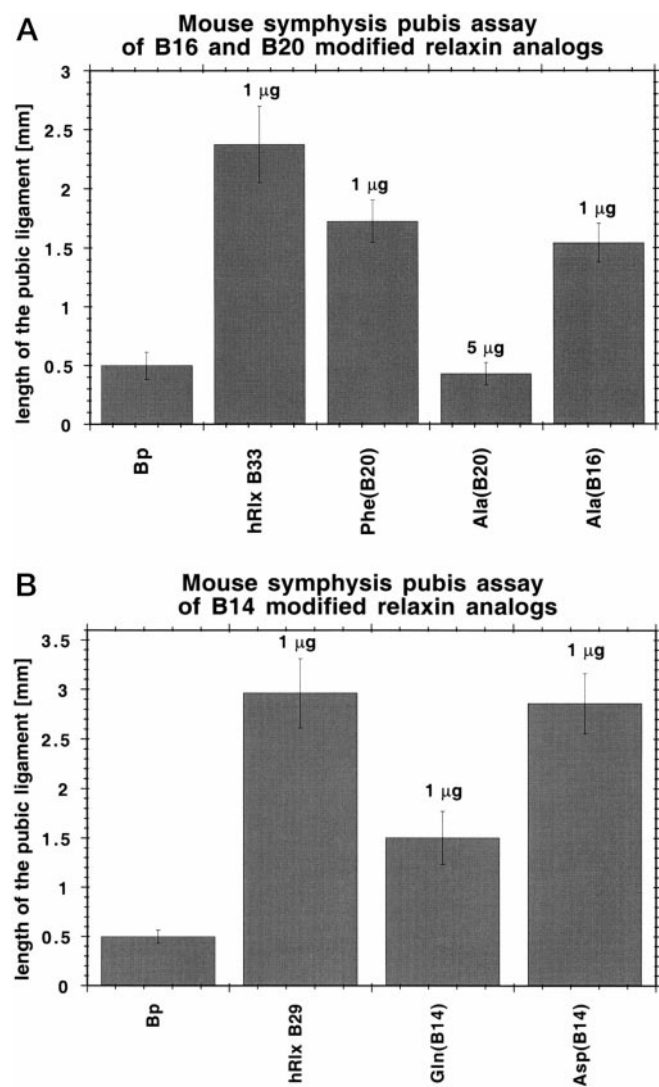


FIG. 9. **Mouse symphysis pubis assay.** Estrogen-primed mice were injected subcutaneously with relaxin or relaxin analog in 100  $\mu$ l of 1% aqueous benzopurpurin 4B. The mice were killed, the symphyses pubis were freed of adherent tissue, and the distances between the pubic bones were measured. *A*, benzopurpurin (*Bp*) (seven mice), 1  $\mu$ g of human relaxin B33 (eight mice), 1  $\mu$ g of Phe-B20 B33 (eight mice), 5  $\mu$ g of Ala-B20 B33 (seven mice), and 1  $\mu$ g of Ala-B16 B33 (seven mice). *B*, benzopurpurin (*Bp*) (15 mice), 1  $\mu$ g of human relaxin B29 (15 mice), 1  $\mu$ g of Gln-B14 B29 (14 mice), and 1  $\mu$ g of Asp-B14 B29 (14 mice).

pected amino acid composition, and sequence analysis of Ala-B20 and Phe-B20 relaxin confirmed the structure.

The results of CD spectroscopy shown in Fig. 7 support the idea that the reduced affinity is not due to a conformational change. In contrast, the dichroic intensity of Phe-B20 relaxin is reduced (Fig. 8), and a red shift of the maximum to 197 nm is observed as well as a reduced maximum to minimum ratio ( $\Theta_{197\text{ nm}}/\Theta_{209\text{ nm}} = 0.93$ ) when compared with human relaxin ( $\Theta_{195\text{ nm}}/\Theta_{209\text{ nm}} = 1.71$ ). The changes seem, however, mostly

quantitative and of such nature that this analog retained a relatively high potency.

To assure that binding would be an indicator of bioactivity, a selected number of analogs were tested in the mouse symphysis pubis assay. Relaxin derivatives with modification in positions B14 (Gln and Asp), B16 (Ala), and Phe(B20) were active at a dose of 1  $\mu$ g/mouse, whereas Ala-B20 relaxin was inactive at a dose of 5  $\mu$ g/mouse (Fig. 9). These results are in full agreement with the receptor binding assays and suggest that binding may be synonymous with bioactivity.

We conclude that the relaxin receptor-binding site comprises three crucial binding residues, Arg-B13, Arg-B17, and Ile-B20, which form a triangular contact region on the relaxin surface (Fig. 1). Remarkably, the components of this binding site are strongly hydrophilic on one side, opposed by a strongly hydrophobic component on the other, and all held in proper relation to each other by the geometry of the same  $\alpha$ -helix.

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