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Mouse Relaxin: Synthesis and Biological Activity of the First Relaxin With an Unusual Crosslinking Pattern

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SUMMARY: According to a recently published cDNA sequence, mouse relaxin has an extra amino acid in the C-terminal end of the A chain and thus an interchain loop consisting of 25 amino acids instead of the usual 24-membered ring. Because of the restrictive disulfide link arrangement the extra residue can be expected to cause a loop out in the C-terminal α-helix. We have chemically synthesized authentic mouse relaxin as well as an analog without the additional A chain residue and found that the native hormone, although active, was inferior to its insulin-like analog. This result is in harmony with our previous study which suggests that the surface of relaxin represented by the C-terminal helix of the A chain is positioned opposite to the surface that contains the receptor interaction site and therefore is less sensitive to modifications.

The major physiological activity of relaxin in mammals is to provide an adequate passage for the young at parturition (1, 2). The hormone which is produced in the ovaries of most mammals appeared to be an obligatory disulfide homologue of insulin. The discovery of a mouse cDNA sequence with a different disposition of the cysteines (3) challenges this concept and suggests that relaxin can function in a different disulfide configuration. The abnormal relaxin gene appeared to be the only gene of its kind in the mouse that was transcribed (3). This observation raised the question whether or not the gene product would be biologically active and if so how it would compare to a hypothetical revertant, i.e., des-Tyr (A24) mouse relaxin with a normal disulfide pattern. In the present paper we describe the synthesis of mouse relaxin and des-Tyr (A24) mouse relaxin and compare their chemical and biological properties.

Materials and Methods

Chemicals and solvents used in this study were the best analytical grade available. Solvents for HPLC and peptide synthesis were purchased from

0006-291X/93 $4.00

Burdick & Jackson (Muskegon, MI), reagents for peptide synthesis were from Applied Biosystems (Foster City, CA), and amino acid derivatives were from Bachem Inc. (Torrance, CA).

All the peptides were synthesized on an ABI 430 A synthesizer with either the Fast-moc (4) or the t-Boc methodology (5). In general the syntheses were performed as described for human relaxin II (6). The B chains were synthesized on PAM resin by Boc chemistry with HF-labile protecting groups for all side chains except for cysteines which were protected by the acetamidomethyl group (Acm, in position B11) and the S-2-(3-nitropyridinesulfenyl) group (Npys, in position B23), tryptophans were formylated (N^{in}-formyl), and methionines oxidized (sulfoxide). Following HF-treatment the partially protected B chain was purified by gel filtration on Sephadex G50 in 6 M guanidinium chloride at pH 4.5, dialyzed against water (Spectrapor 3 dialysis tubing), lyophilized, and further purified by HPLC.

The A chains were synthesized by Fast-moc chemistry on p-alkoxybenzyl alcohol resin with trifluoroacetic acid (TFA) labile protecting groups on all side chains except the C-terminal cysteine which carried the HF-labile 4-methybenzyl group, and Cys A11 which contained the acid-stable acetamidomethyl group. The intra-chain disulfide A10-A15 was formed by titration with iodine in 50% acetic acid and the A chains were purified by preparative HPLC.

Interchain disulfide bond formation was initiated by release of the 4-methylbenzyl group of the C-terminal cysteine of the A chain with HF. The monothiol A chain (25 mg) and 30 mg of relaxin B chain were incubated in 4 ml of 6M guanidinium chloride at pH 4.5 at 37°C for 24 h. Separation was achieved by gel filtration on Sephadex G50 sf, equilibrated in 20% acetic acid (2.5 cm x 40 cm), and followed by preparative HPLC (yield 21.4 mg). For the synthesis of the third disulfide bond 21.4 mg of the peptide was dissolved in 4 ml of 50% acetic acid and added dropwise to a stirred solution of 8.5 ml of glacial acetic acid, 5.5 ml of water, 44.7 µl of 6 M HCl, and 7.1 ml of 50 mM iodine in glacial acetic acid. Stirring was continued for another 10 min at room temperature, whereafter the excess iodine was reduced with 1 M ascorbic acid in water. The reaction mixture was concentrated to about 5 ml *in vacuo*, diluted with water, and separated by preparative HPLC. (yield 15.9 mg).

The side chains of tryptophan (N^{in}-formyl) were liberated by treatment with 3.2 ml of a chilled solution of isopropanol/water/2N NaOH (7/7/2 v/v/v) for 45 sec on ice, acidified with acetic acid, diluted with water, and separated by preparative HPLC as before (yield: 4.56 mg).

The side chains of methionine (sulfoxide) were specifically reduced with 500 µl of chilled 0.5 M NH_4I in water/TFA 1:9 v/v for 15 min on ice. The reaction was quenched by dilution with 4 ml of water containing 50 µl of 0.5 M

ascorbic acid, and was immediately subjected to preparative HPLC purification (yield: 2.9 mg). Both derivatives were synthesized with equal efficiency.

For high performance liquid chromatography (HPLC) the solvent system consisted of 0.1% TFA in water (A) and 0.1% TFA in 80% acetonitrile (B). For preparative HPLC we used a Synchropak RP-P column (10 x 250 mm) and linear gradients as follows: B chain: 30-60% B in 30 min, A chains 25-45% B in 25 min, relaxins and intermediates: 30-50% B in 30 min. The flow rates were 3 ml/min and the eluates were detected by UV absorbance at 226 nm.

Analytical HPLC was performed on an Aquapore 300 column (C8, 2.1 x 30 mm), using a linear gradient from 25% B to 45% B in 90 min at a flow rate of 100 µl/min.

The amino acid analyses were performed after vapor phase hydrolysis (6N HCl) at 110°C for 24 h followed by derivatization with phenylisothiocyanate (PicoTag system, Waters) and analyzed using a Waters 840 HPLC system.

Biological potencies were determined by their effect on the target tissue, the symphesis pubis of the mouse (7). Young female ovariectomized IRC mice (18-20 g) were injected s.c. with 5 µg of estradiol cyprionate in 100 µl sesame oil 5 days prior to injection of relaxin in 0.1% benzopurpurin 4B. Controls received benzopurpurin only. The length of the symphyseal ligament was measured under a dissecting scope.

Receptor-binding assays were performed on mouse brain membrane preparations as described before (8). Freshly prepared crude brain membranes were incubated with a constant amount of tracer and varying amounts of relaxin for 1 hour at room temperature. Total binding was measured in the absence of cold relaxin and nonspecific binding in the presence of 2 µg/ml of relaxin. The amount of relaxin that was needed to inhibit 50% of total binding was used to estimate biological potencies.

CD Spectroscopy was performed on an Aviv-Cary 60DS spectropolarometer in 0.05 M Tris-HCl buffer at pH 7.8. Data were collected in 0.5 nm intervals, and 5 runs were averaged for each curve. Protein concentrations were estimated by specific absorbance at 280 nm and verified by quantitative amino acid analysis.

The molecular weight of both relaxins were determined on a Kratos Maldi-3 time-of-flight mass spectrometer.

Results and Discussion

In Figure 1 the primary sequences of mouse relaxin, the des Tyr (A24) mouse relaxin, rat relaxin, and human relaxin are compared. Human relaxin is representative of all 24 known relaxins as concerns the relative position of the cysteine residues.

A chains

Mouse	E S G G L M S Q Q **C C** H V G **C** S R R S I A K L Y **C**
Mouse (des-Tyr)	E S G G L M S Q Q **C C** H V G **C** S R R S I A K L **C**
Rat	Q S G A L L S E Q **C C** H I G **C** T R R S I A K L **C**
Human	Q L Y S A L A N K **C C** H V G **C** T K R S L A R F **C**

B chains

Mouse	R V S E E W M D G F I R M **C** G R E Y A R E L I K I **C** G A S V G R L A L
Rat	R V S E E W M D Q V I Q V **C** G R G Y A R A W I E V **C** G A S V G R L A L
Human	D S W M E E V I K L **C** G R E L V R A Q I A I **C** G M S T W S K R S

Fig. 1. The primary sequences of A and B chains of mouse relaxin, des-Tyr mouse relaxin and, for comparison, rat and human relaxin II. The B chains of mouse relaxin and des-Tyr mouse relaxin are identical.

The relaxin chains were synthesized by the solid-phase methodology and the three disulfide links were formed sequentially as outlined in Fig. 2. The results of our analytical work reinforce our confidence that the biological effects

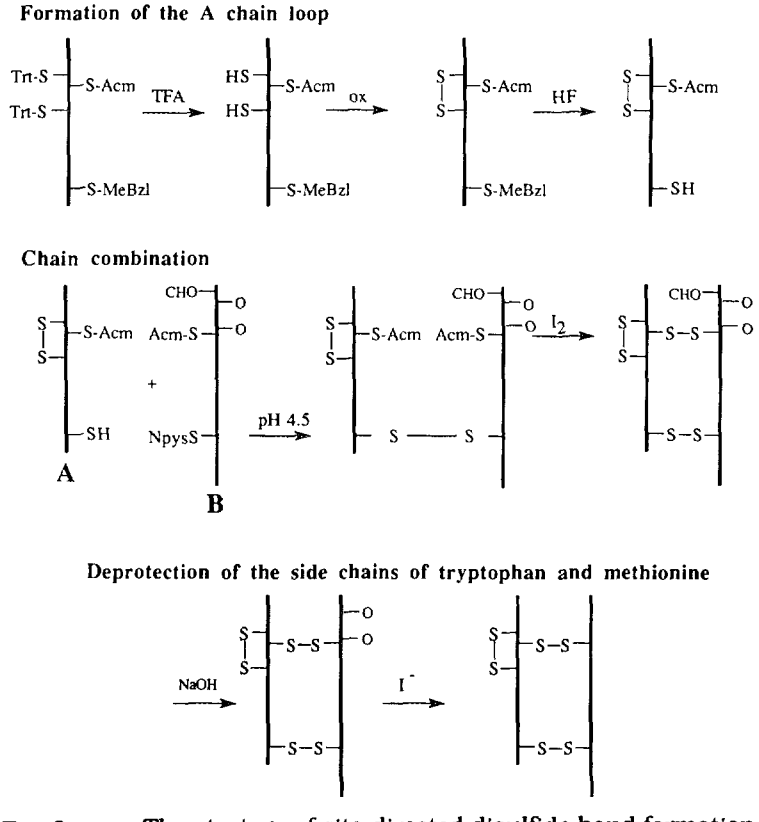

Fig. 2. The strategy of site-directed disulfide bond formation.

are indeed due to the proper molecules. HPLC data confirm the assumption that mouse relaxin is more hydrophobic (elutes later) than des-Tyr (A24) mouse relaxin. Both molecules were eluting as a single peak, indicating that there are no major contaminants (Fig. 3). Reduction of the relaxins, followed by HPLC analysis, verified the two-chain character of the molecules, and peptide mapping of tryptic digests by HPLC, followed by hydrolysis and amino acid analysis of the peaks, indicated that the two chains had the proper orientation with respect to each other.

The amino acid analysis (Table I) confirms again that mouse relaxin has an extra tyrosine residue which is absent in the insulin-like des-Tyr (A24) mouse relaxin. This result is reinforced by a mass spectrometry study of both molecules. The mass of mouse relaxin differs from des-Tyr (A24) mouse relaxin by 163.2 mass units, exactly the mass of tyrosine. Time-of-flight (TOF) mass spectrometry gives an average value for the mass ion of mouse relaxin of 6724.9 and for the des-Tyr derivative 6579.7.

The extra residue in the A chain was expected to cause crowding and subsequently loop-out of the C-terminal A chain helix (9). According to the CD spectra of both molecules, as compared to human relaxin, this appears not to be the case (Fig. 4). The spectra of mouse and des Tyr (A24) mouse relaxin do match within instrumental error. It seems possible that twisting of the S-C bond in the B chain cysteine could allow a disulfide bond to form with an angle approximately 90° to the original S-S bond and thus accommodate the extra A

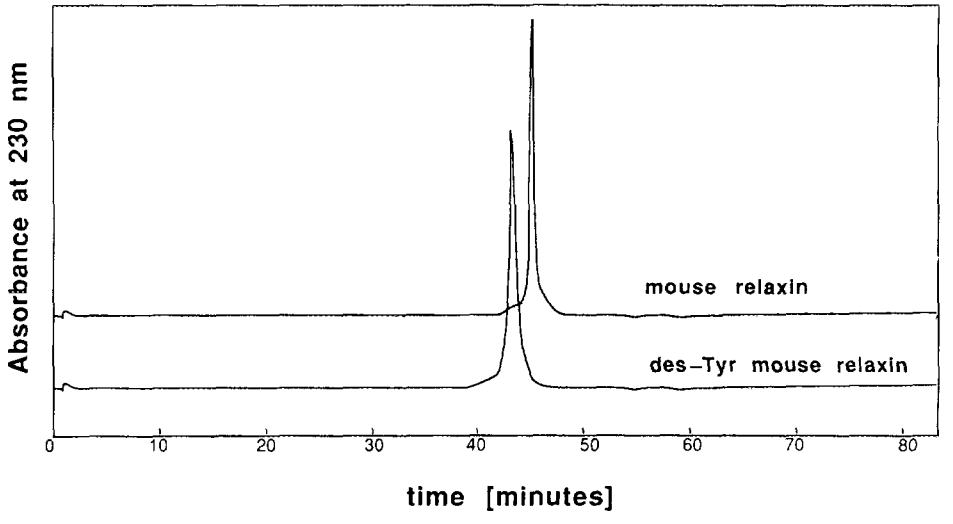

Fig. 3. The elution profile of synthetic mouse relaxin and the des-Tyr derivative of mouse relaxin. The additional tyrosine residue makes mouse relaxin more hydrophobic and increased retention on the C8 column. See methods for conditions.

Table 1. Amino Acid Composition

Amino Acid	Mouse Relaxin	des-Y Mouse Relaxin
Asp	0.98 (1)	1.05 (1)
Ser	5.67 (6)	5.79 (6)
Glu	6.83 (7)	6.95 (7)
Gly	7.43 (7)	7.65 (7)
Ala	4.04 (4)	4.13 (4)
Cys**	3.95 (6)	3.90 (6)
Val	1.99 (2)	2.00 (2)
Met	2.95 (3)	2.94 (3)
Ile	4.93 (5)	4.96 (5)
Leu	5.17 (5)	5.36 (5)
Tyr	2.01 (2)	1.05 (1)
Phe	0.96 (1)	0.94 (1)
His	0.98 (1)	1.03 (1)
Lys	2.03 (2)	2.05 (2)
Arg	7.48 (7)	7.59 (7)
Trp*	1.03 (1)	1.06 (1)

*determined by UV spectroscopy.
**not quantified.

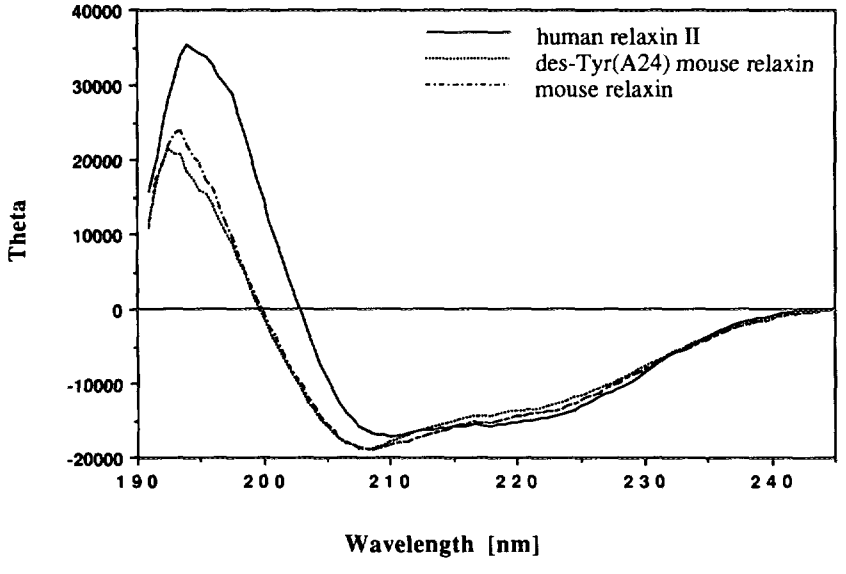

Fig. 4. The CD measurements were made on an Aviv-Cary 62DS. Samples were dissolved in 50 mM Tris at pH 7.8 and measured at room temperature at intervals of 0.5 nm. Five scans were averaged for each curve. The two murine relaxins are indistinguishable, but different from human relaxin II.

chain residue without forcing a helix loop-out. This problem will require additional studies.

The receptors in the symphysis pubis and brain show different affinity toward the mouse relaxin and its des-Tyr (A24) analogue. Surprisingly the hypothetical "revertant" is significantly more active than the mouse relaxin (Fig. 5) in the mouse pubic symphysis assay at the 1μg per mouse level. More interesting yet human relaxin II is more active in the mouse bioassay than the endogenous mouse relaxin or the des-Tyr (A24) mouse relaxin.. In the mouse brain receptor assay the des-Tyr (A24) mouse relaxin shows greater affinity for the receptor than the mouse relaxin (Fig. 6).

Although the CD spectrum does not show any structural disturbance the biological activity of mouse relaxin is diminished as compared to the molecule that contains the insulin-like 24-membered interchain loop. It may require x-ray crystallographic analysis to learn how precisely the additional amino acid is accommodated. Conversely it seems amazing that the insulin-like crosslink configuration can be changed to such an extent and still allow the hormone to retain even part of the original activity. Finally one wonders how the inferior molecule came to be adopted in mice in preference to the shorter more effective relaxin, from which it purportedly evolved.

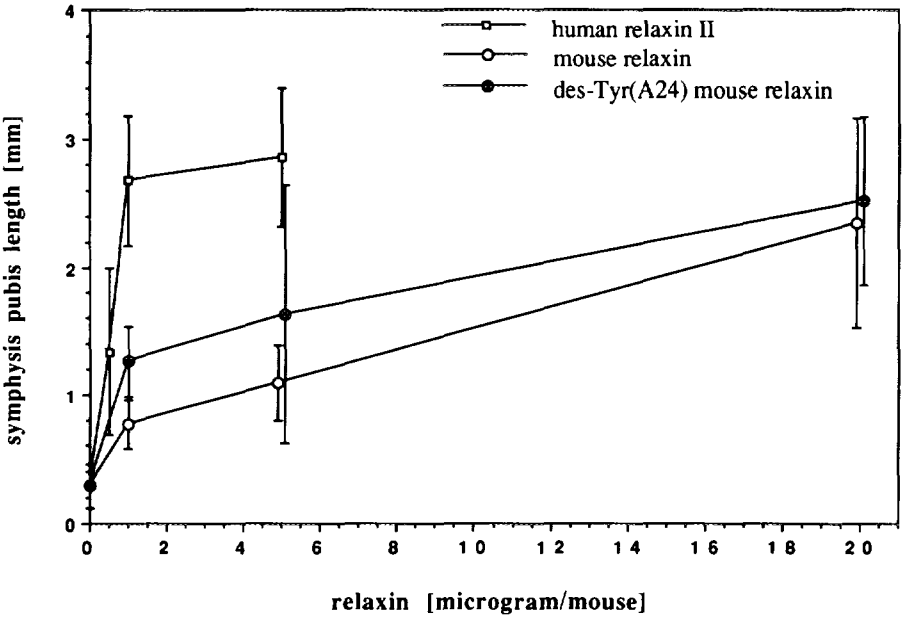

Fig. 5. Bioassay of mouse relaxin, des-Tyr mouse relaxin, and human relaxin II. The "revertant" shows significantly better activity than mouse relaxin at a dose of 1 μg/mouse. Human relaxin II is significantly more potent than mouse relaxin or the des-Try derivative.

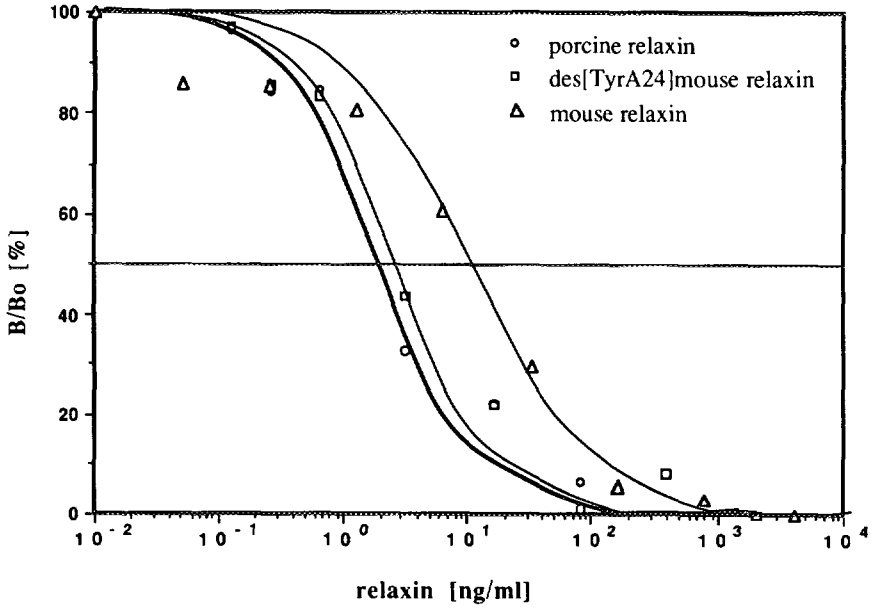

Fig. 6. Mouse brain relaxin receptor-binding assay. The des-Tyr derivative (ED_{50}=2.8 ng/ml) is about 5 times more effective than mouse relaxin (ED_{50}=13ng/ml) and similar to native porcine relaxin (ED_{50}=2ng/ml).

Acknowledgments

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