Identification of a Glycosylated Relaxin-like Molecule from the Male Atlantic Stingray, *Dasyatis sabina*†

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ABSTRACT: The alkaline gland fluid of the Atlantic stingray (*Dasyatis sabina*) contains a molecule that cross-reacts weakly to anti-porcine relaxin antibodies. This material was isolated and purified to homogeneity by reversed-phase high-performance liquid chromatography. In SDS gel electrophoresis, the molecule showed an apparent molecular mass of 13 kDa which upon reduction formed two polypeptide chains of 4 and 9 kDa, respectively. Sequence analyses revealed a 27-amino acid residue A chain and a 54-amino acid residue B chain which contained an N-glycosylation site in position B37. The distribution of the six cysteines and possibly the disulfide bonding is identical to that found in insulins and most relaxins. Although the stingray relaxin-like molecule contains the structurally relevant glycine residues within the A chain, in the midregion of the B chain it has only one of the two requisite binding site arginines, which explains the lack of relaxin bioactivity in standard mammalian assays. Stingray relaxin is the first member of the relaxin family identified in a nonhomeotherm male. Carbohydrate analysis of relaxin revealed an N-linked asialo, agalacto, bisected biantennary, and a core-fucosylated oligosaccharide in the position of Asn B37 which makes it the first reported glycosylated relaxin-like molecule.

Relaxin is a peptide hormone which is found in several species in both the vertebrate and invertebrate phyla (*1*). Relaxin has been isolated and sequenced from the ovaries of several species of mammals as well as from two groups of elasmobranch fishes, i.e., sharks and skates (*2*−*5*). However, its biological actions have been defined only in mammals where it induces the widening of the pubic symphysis ligament and softens the cervix, among other actions, in preparation for parturition (*6*). Although generally regarded as a female hormone, relaxin has also been identified in the prostate and seminal fluid of some mammals where it elicits increased sperm motility (*7*). Given the presence of relaxin in this mammalian male accessory reproductive gland and the fluid which it produces, we investigated the possibility that relaxin exists in the alkaline gland of stingrays which is a homologue of the mammalian prostate. In the present study, we describe the isolation of a relaxin-like molecule from the alkaline gland fluid of the stingray, its protein sequence, and the oligosaccharide sequence of the single N-glycosylation site.

MATERIALS AND METHODS

Adult male Atlantic stingrays, *Dasyatis sabina*, were captured off the coast of South Carolina. Animals were anesthetized with 1% 3-aminobenzoic acid ethyl ester (MS 222 Sigma, St. Louis, MO). Fluid from the two alkaline glands in each animal was harvested by aspiration into a 10 mL syringe after piercing the cloacal wall with a 27 gauge needle. Alkaline gland fluid (AGF) was used fresh or kept frozen at −80 °C for as long as 6−8 months.

Isolation

Pooled samples from 40 stingrays were thawed, and the resultant 100 mL of AGF was acidified to pH 5.5 with the addition of 600 μL of 20% trifluoroacetic acid. The sample was then centrifuged for 5 min at 13000*g*.

Samples were separated on a Waters dual-pump HPLC system (model 510, Waters Millipore, Milford, MA) with gradient programing and data collection controlled by a Digital computer (Professional 380, Digital Equipment Corp., Maynard, MA). Samples (2 mL) were injected through a manually operated Rheodyne valve, and the effluent was monitored by UV absorbance at 280 or 220 nm.

For the initial purification, the AGF supernatant (19 mL at a time) was fractionated on a Synchropak RP-P column (10 mm × 250 mm) at a flow rate of 3 mL/min with 0.1% trifluoroacetic acid in water as solvent A and 0.1% trifluoroacetic acid in 80% acetonitrile as solvent B. During the initial 10 min of loading, the gradient was kept at 20% B; thereafter, the concentration of B was raised linearly to 50% over 30 min. The column was washed with 100% B for about 2 min and then changed back to the initial condition. The total run time was 44 min followed by a 15 min equilibration period. The absorbance of the effluent was monitored at 280 nm, and 1 min fractions were collected.

Fractions showing RIA activity were dried *in vacuo*, dissolved in 0.1% trifluoroacetic acid, and rechromatographed using a 30 min linear gradient from 30 to 50% at a flow rate of 3 mL/min. The effluent was monitored at 220 nm, and fractions were collected manually.

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Analyses

Radioimmunoassays (RIAs) were performed in 50 mM phosphate buffer (pH 7.4) and 150 mM NaCl supplemented with 1% BSA and 0.01% NaN_3. Aliquots of the HPLC fractions were lyophilized and redissolved in 100 μL of RIA buffer. Samples were assayed with sheep anti-relaxin antibody (S540), rabbit anti-relaxin antibody (R6), and [^{125}I]porcine relaxin as a tracer (*8*).

For sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), precast Tricine gels were purchased from Novex (San Diego, CA). AGF fraction 33, porcine insulin, and human relaxin, 1 μg each, were dissolved in 25 μL of SDS containing sample buffer (Novex). The same set of proteins (2 μg) were reduced with 25 μL of sample buffer containing 1% dithiothreitol for 5 min at 90 °C. Electrophoresis was performed in an Xcell Mini-Gel apparatus (Novex) for 90 min at 125 V, and the mobility was compared to that of a wide-range protein standard (Mark 12, Novex). Gels were stained with Coomasie blue.

Reduction, Carboxymethylation, and Chain Separation. Fraction 33 from the second preparative HPLC run was dissolved in water [100 μg/(20 μL of H_2O)] and reduced with 100 μL of 50 mM dithiothreitol in 0.2 M Tris/HCl (pH 8.6) containing 6 M guanidinium chloride. After 60 min of reduction at 37 °C, the SH groups were alkylated with 100 μL of a 100 mM iodoacetic acid solution in water. After 30 min, the reaction was quenched with 20 μL of the 50 mM dithiothreitol solution and the chains were separated by analytical HPLC with a 30 min gradient of 20 to 80% B on a Synchropak RP-P 4.1 × 250 mm column. The UV absorbance was detected at 230 nm, and fractions were collected manually.

Enzymatic Digestion of the Chains

Fragments were isolated by chromatography on an ABI chromatograph, model 130A (Applied Biosystems-Perkin Ellmer), with an Aquapore 300 column (C8, 2.1 × 30 mm column). In all experiments, the flow rate was 100 μL/min and UV absorbance was detected at 215 nm.

Trypsin. Reduced and S-alkylated A or B chains (5 μg in 20 μL of water) were digested with 0.2 μg of trypsin in 20 μL of 0.05 M NH_4HCO_3 for 1 h at 37 °C, and then the reactions were quenched with 10 μL of 0.1% TFA in water and the mixtures injected onto an Aquapore 300 column. B chain fragments were separated by a linear gradient of 5 to 50% B and A chain fragments by a linear gradient of 0 to 30% B, both in 40 min. Fractions were collected manually, concentrated *in vacuo*, and lyophilized.

Carboxypeptidase A Digest. The A chain (2.5 μg in 10 μL of 0.1 M NH_4HCO_3) was incubated with 0.1 μg of carboxypeptidase A (EC 3.4.17.1) for 30 min at 37 °C. The reaction was quenched by freeze-drying and the sample treated with 20 μL of 2:1:2 ethanol/triethylamine/water (v/v/v) for 2 min followed by amino acid determination by Waters Pico·Tag method.

Cyanogenbromide Cleavage. The B chain (7.5 μg) was exposed to 500 μg of CNBr in 50 μL of 70% formic acid for 18 h at room temperature. Thereafter, the acid was evaporated *in vacuo*, and the remaining peptides were dissolved in 55 μL of HPLC solvent A and separated by HPLC on an Aquapore 300 column using a 40 min linear gradient of 5 to 50% B. Fractions were collected manually.

Amino Acid Analysis. With the exception of carboxypeptidase digests, all samples for amino acid analysis (1−10 nmol of peptide) were hydrolyzed in HCl (6 M, 1% phenol) vapor at 150 °C for 1 h. The amino acids were derivatized with phenyl isothiocyanate, followed by HPLC separation using the Waters Pico·Tag system.

Sequence Analysis. The primary sequences of the A and B chains were obtained by running the reduced HPLC-purified materials (200 pmol) in an ABI 477 pulsed liquid protein sequencer with an in-line ABI 120A phenylthiohydantoin analyzer. The cysteine residues gave rise to blanks under these conditions. Positive identification of the cysteine residues was achieved by sequencing the *S*-carboxymethylated chains. The corresponding phenylthiohydantoin-*S*-(carboxymethyl)cysteine eluted as a distinct peak between the phenylthiohydantoins of serine and glutamine.

The sequence of the A chain was verified after tryptic digestion and sequence analyses of the HPLC-purified fragments. In addition the C terminus of the A chain was confirmed by carboxypeptidase A (EC 3.4.17.1) digestion followed by analysis of the released amino acids.

The B chain sequence was secured by the same procedure. Overlapping sequences were established using CNBr fragments.

Mass Spectrometry. Mass spectra were recorded on a Jeol HX110/HX110 4 sector tandem mass spectrometer (Jeol, Tokyo, Japan). Samples were dissolved in 1:1 methanol/water (v/v) containing 6% acetic acid.

Carbohydrate Analysis

Determination of the N-Linked Oligosaccharide. One hundred ten micrograms of HPLC-purified fraction 33 was commercially analyzed (Glyko, Novato, CA) for the presence of carbohydrate residues. The nature of the N-linked glycosylation was determined by fluorochrome-assisted carbohydrate electrophoresis (FACE). The four major analytical steps involved were release and isolation of sugars from the glycoconjugate, quantitative labeling of the sugar with fluorophase, separation of the labeled sugars by polyacrylamide gel electrophoresis (PAGE), and analysis by densitometry (*9*).

Bioassays. The potential biological activity of the stingray relaxin-like material was determined by two assays, i.e., the mouse symphysis pubis assay and the stingray sperm motility assay.

Immature Charles River CD mice (*10*) were primed on day 0 with a subcutaneous injection of 5 μg of estradiol cyclopentylpropionate in 0.1 mL of sesame oil. Five days later, each mouse was injected subcutaneously with 0.1 mL of 1% benzopurpurin 4-B containing either a placebo, a standard [porcine relaxin at 1 μg/mouse (0.17 nmol)], or AGF fraction 33 [25 μg/mouse (2.3 nmol)]. Eighteen to twenty-four hours later, the mice were killed in an atmosphere of CO_2, the interpubic joint was freed from surrounding connective tissue, and the distance between the pubic bones was measured under a dissecting microscope (*10*).

The second bioassay consisted of incubating stingray (*D. sabina*) sperm with aliquots of AGF fraction 33. Anesthetized mature stingrays as described above had the abdominopelvic contents exposed by an anterior−posterior midline incision. The terminal portion of the vas deferens was punctured with a 16 gauge needle and sperm aspirated into

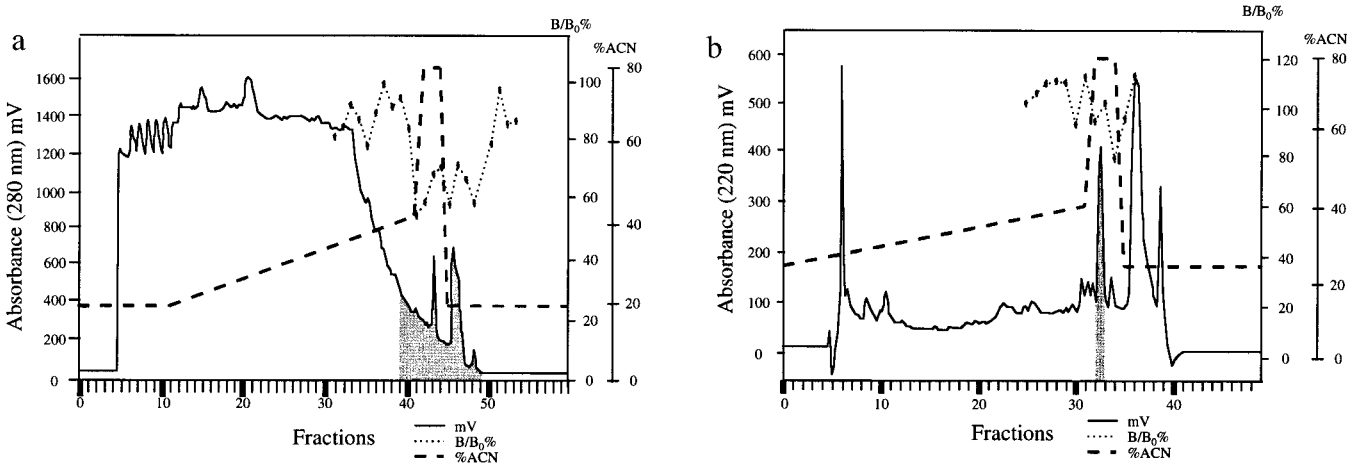

FIGURE 1: (a) High-performance liquid chromatography of alkaline gland fluid. Solid lines indicate absorbance, dashed lines the acetonitrile (ACN) gradient, and dotted lines the percent bound/free (B/B_0) labeled relaxin antibody in the radioimmunoassay (RIA). The shaded areas are fractions taken for rechromatography in part b. (b) Rechromatography of fractions 39−49 (part a) incorporating relaxin RIA. Lines and labels are as in part a. The shaded area is fraction 33 taken for further analyses.

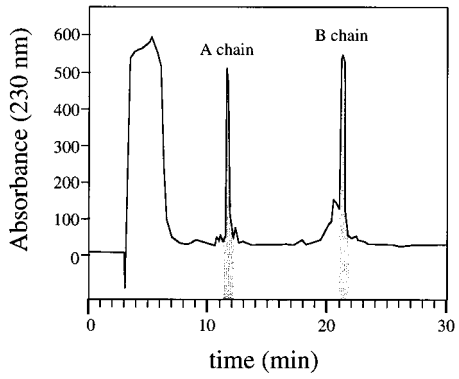

FIGURE 2: Analytical HPLC of fraction 33 (Figure 1b), reduction, and chain separation. Shaded areas indicate fractions taken for further analyses.

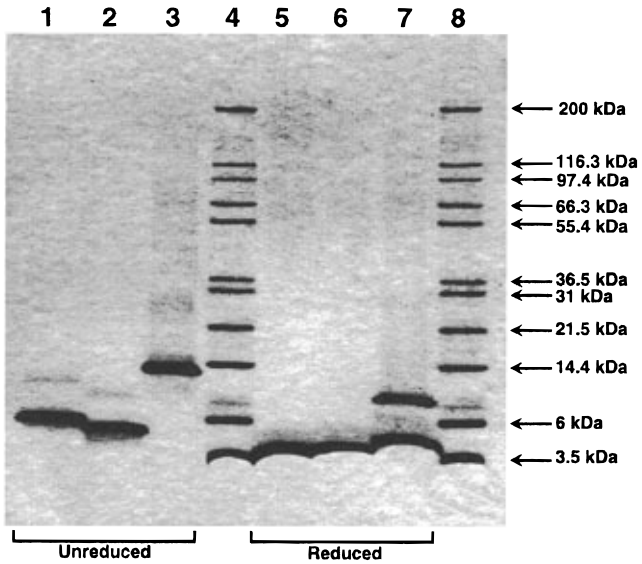

FIGURE 3: SDS gel electrophoresis of stingray relaxin-like material: lanes 1−3, unreduced samples; lanes 5−7, reduced samples; lanes 4 and 8, molecular weight standards; lanes 1 and 5, human relaxin; lane 2 and 6, human insulin; and lanes 3 and 7, fraction 33 from Figure 1b.

a 3 mL syringe. Sperm was diluted with elasmobranch Ringer (*11*) until the concentration was low enough to microscopically analyze sperm movement. Aliquots from this stock sperm/Ringer solution were added to yield concentrations of fraction 33 found in the native AGF (37 μg/mL) or diluted with Ringer to yield 1:8 and 1:16 concentrations. The relative motility was judged by placing equal volumes (10 μL) in a standard slide (Conception Technology, La Jolla, CA) and judging the sperm movement on a scale of 0 (no movement), 1 (twitching but no locomotion), 2 (slow forward), or 3 (fast forward).

Table 1: Amino Acid Compositions of Alkaline Gland Fluid Fraction 33 and Its Polypeptide Chains[a]

	A chain[b]	B chain[b]	fraction 33
Asp	2.03 (2)	3.47 (4)	5.25 (5)
Thr	0.99 (1)	1.68 (2)	2.22 (3)
Ser	1.70 (2)	2.35 (2)	3.84 (4)
Glu	1.16 (1)	2.74 (3)	3.30 (4)
Pro	2.32 (2)	8.12 (8)	8.77 (10)
Gly	2.16 (2)	6.75 (6)	8.99 (8)
Ala	1.08 (1)	3.13 (3)	3.55 (4)
Cys[c]	3.86 (4)	1.96 (2)	− (6)
Val	0 (0)	3.11 (3)	2.81 (3)
Met	0 (0)	4.89 (5)	4.41 (5)
Ile	0 (0)	2.93 (3)	2.97 (3)
Leu	1.85 (2)	2.16 (2)	4.41 (4)
Tyr	3.02 (3)	3.50 (3)	4.92 (6)
Phe	0.99 (1)	1.09 (1)	1.96 (2)
His	0 (0)	0 (0)	0 (0)
Lys	2.95 (3)	0 (0)	3.04 (3)
Trp	0 (0)	0 (1)	0 (1)
Arg	3.06 (3)	6.03 (6)	8.21 (9)

[a] The numbers of residues found in the final sequence are given in parentheses. [b] Average of three independent samples. [c] Determined as *S*-(carboxymethyl)cysteine.

RESULTS

Alkaline gland fluid (AGF) of the Atlantic stingray competed for porcine relaxin binding to anti-porcine relaxin antibodies. Using a radioimmunoassay, 26 μL of AGF was equivalent to 2 ng of porcine relaxin using the polyclonal sheep anti-porcine relaxin antibody (S540). Using the polyclonal rabbit anti-porcine relaxin antibody (R6), 350 μl of AGF was equivalent to 2 ng of porcine relaxin.

In order to localize the potential relaxin-like molecule, AGF was acidified and purified by reversed-phase HPLC. The cross-reactivity to the antibody was found at the end of the acetonitrile gradient (Figure 1a). Although the activity

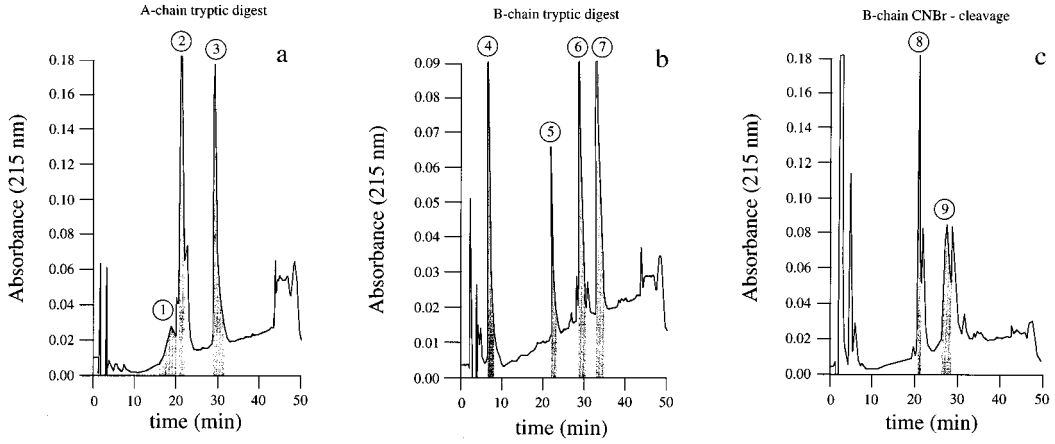

FIGURE 4: Tryptic digest of the two chains of fraction 33: (a) A chain, (b) B chain, and (c) CNBr digest of the B chain. Shaded areas indicate fractions collected for further analyses.

could not been attributed to a single fraction, the majority of the mostly colored impurities were removed. Rechromatography of pooled RIA-positive fractions, using a slightly different gradient, resulted in distinct UV-absorbing peaks with potential antibody cross-reactivity in fractions 28−35 (Figure 1b).

Fraction 33, the major UV-absorbing fraction, was further analyzed by analytical HPLC. Upon reduction, two peaks appeared, one more hydrophilic (A chain) and the other more hydrophobic (B chain) than the original component (Figure 2). SDS gel electrophoresis on Tricine gels showed that AGF fraction 33 had a molecular mass of about 13 kDa and was significantly larger than insulin or human relaxin (M_r = 6000). Reduction showed the appearance of two polypeptides with molecular masses of 4 and 9 kDa, respectively (Figure 3). Although AGF fraction 33 is a two-chain molecule, it is unusually large which is mostly attributed to the B chain.

The amino acid composition of the intact molecule, outlined in Table 1, showed the absence of histidine. The two chains are distinctly different in amino acid composition; for instance, lysine was only found in the A chain and valine, methionine, and isoleucine were unique residues of the B chain. After reduction and carboxymethylation, four cysteine residues were found in the A chain and two in the B chain, suggesting again that AGF fraction 33 is a relaxin-like molecule. This is further supported by the high concentration of basic amino acids in spite of increased levels of proline which are rarely seen in relaxins.

A Chain Sequence. The A chain was sequenced twice up to cysteine in position 25. Carboxypeptidase A treatment released only leucine and tyrosine. After tryptic digestion and HPLC purification, three peptides, A3−9, A11−22, and A23−27, were isolated and sequenced (Figure 4a). The small number of tryptic fragments found by HPLC was due to resistant Lys−Pro (A4−5) and Arg−Glu (A18−19) bonds. Sequences of the tryptic fragments confirmed the previous results and established the sequence of the C terminus. In addition, mass spectrometry of the *S*-carboxymethylated A chain suggested a molecular mass (m/z = 3392.6) identical to the calculated value, indicating the absence of posttranslational modifications.

B Chain Sequence. N-terminal sequencing of the B chain was repeated three times. An unambiguous sequence up to methionine (B30) was obtained twice. Tryptic digestion followed by HPLC separation gave rise to eight peaks (Figure 4b). Four of the fragments were sequenced. Fragments B1−3 and B12−20 verified the initial sequence, whereas fragment B21−34 extended the sequence toward the C terminus. Amino acid analysis of the most hydrophobic tryptic fragment indicated the absence of basic amino acids, identifying this peptide as the C-terminal fragment. The 18-amino acid sequences showed no overlap with peptide 1−34, and in addition, the first cycle showed no PTH amino acid. A clear sequence was observed, starting with glycine in cycle 2 (53 pmol) and ending with methionine in cycle 18 (1.1 pmol). The missing overlap was established using cyanogen bromide fragments (Figure 4c). At that time, the positions of the five methionines were known. A fragment of the C-terminal region starting with Arg-Pro-Arg followed by a blank led into the known sequence of the C-terminal tryptic fragment establishing a two residue (Met-Arg) overlap to segment B (21−34) (Figure 5).

Residue 37 remained unidentified in two independent experiments, indicating a possible posttranslational modification in this position (Figure 5). The sequence X-Gly-Ser strongly suggests N-glycosylation, a fact that was supported by the amino acid composition of the tryptic fragment. Comparison of amino acid analyses and sequence analyses revealed the difference of one aspartic acid residue. Mass spectrometry of the C-terminal tryptic peptide revealed a molecular mass *m/e* of 3512.0 as compared to the calculated value (*m/e* of 1864.1). The additional 1647.9 mass units account for the mass of the complex carbohydrate (see below).

Oligosaccharide Analysis. Analysis of HPLC-purified fractions showed the presence of N-linked oligosaccharides. Figure 6 shows the presence of one major and one minor band in the gel. The released oligosaccharides migrated into position 6.7 and 7.6 compared to the wheat starch digests characteristic of N-linked oligosaccharides. The absolute quantity of released oligosaccharides as determined by quantitative luminescence is presented in Table 2. The data showed that in 1.1 μg of fraction 33 (10% of the starting sample) there were 532.7 and 14.95 pmol of oligosaccharide present in the gel for bands at the 6.7 and 7.6 positions, respectively. This indicates that 97.3% of the oligosaccharides isolated from fraction 33 migrate to position 6.7 (Figure 6).

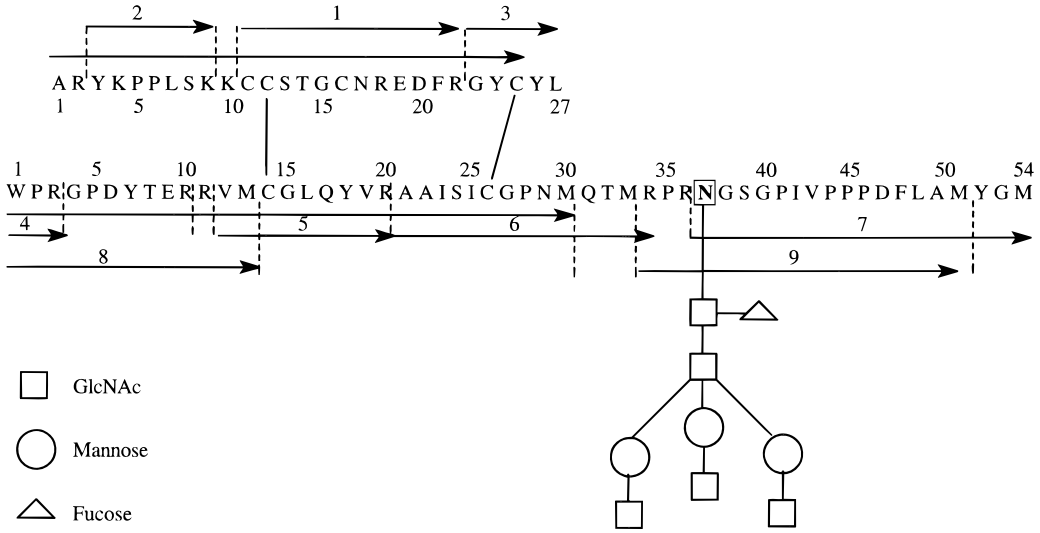

FIGURE 5: Primary structure and oligosaccharide structure of relaxin-like material in alkaline gland fluid. The cross-links are drawn so they are analogous to those of porcine relaxin. The vertical bars indicate peptide sequences from isolated chains and their tryptic and CNBr fragments. The N in the B chain at position 37 shows the site for the branched oligosaccharide binding. Fragments are numbered to correspond to numbers in Figure 4.

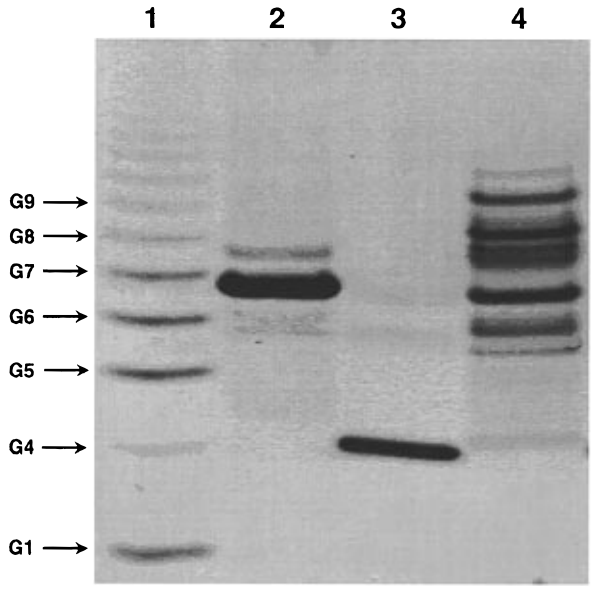

FIGURE 6: N-linked oligosaccharide profiling gel showing the position of standards of wheat starch digests (G1−9, other bands not labeled) in lane 1, the two bands of AGF fraction 33 digests in lane 2, the standard maltotetrase in lane 3, and the trypsin inhibitor control in lane 4. The fluorescence from each band is shown in Table 3.

Table 3 shows the enzymes used to sequentially cleave the specific monosaccharides from the glycoprotein and the number and identification of each monosaccharide. The results from the FACE gel showed the absence of mannose and galactose. However, HEXase III digestion indicated a core of two *N*-acetylglucosamines banded to a mannose residue. Further digestion with HEXase III and digestion with MANnose II indicated identical DP shifts with two monosaccharides released in each cleavage.

Taken together, the sequence of the sugars (Figure 5) showed a core consisting of two *N*-acetylglucosamines (GlcNac) and a mannose, followed by an additional GlcNac. The initial GlcNac is attached to a fucose as a side chain. From the initial core mannose extend two additional mannose branches, each of which is attached to a GlcNac (Figure 5).

Table 2: Quantitative Analysis of Oligosaccharide Content from Figure 6 Based on the Percent Fluorescence from Each Lane and Band

	portion of lane luminescence (%)			
	lane 1	lane 2	lane 3	lane 4
	1.60			
	2.50			
	3.50			2.70
G9	5.00			12.70
G8	6.90			24.70
		2.70		12.20
G7	15.00			
		97.30		
				26.00
G6	20.10			
				13.10
				5.30
G5	22.10			
				3.20
G4	4.30		100.00	
G1	19.10			

Table 3: Release of Monosaccharides after Enzymatic Digestion

	released monosaccharide	
enzyme	name	number
NANase III	NeuAc	0
GALase I	Gal	0
HEXase III	bisecting GlcNAC	1
HEXase III	GlcNAc	2
MANose II	mannose	2

To investigate the biological potency of the potential stingray relaxin, fraction 33 was injected into estrogen-primed mice. Although the stingray relaxin-like material was used at a molar concentration 13.5-fold higher than that of porcine relaxin, it did not alter the interpubic width compared to a placebo injection. In addition, fraction 33 at any concentration did not alter stingray sperm motility or longevity.

DISCUSSION

AGF fraction 33 is structurally a member of the insulin/relaxin family. In some respects, the molecule appears to

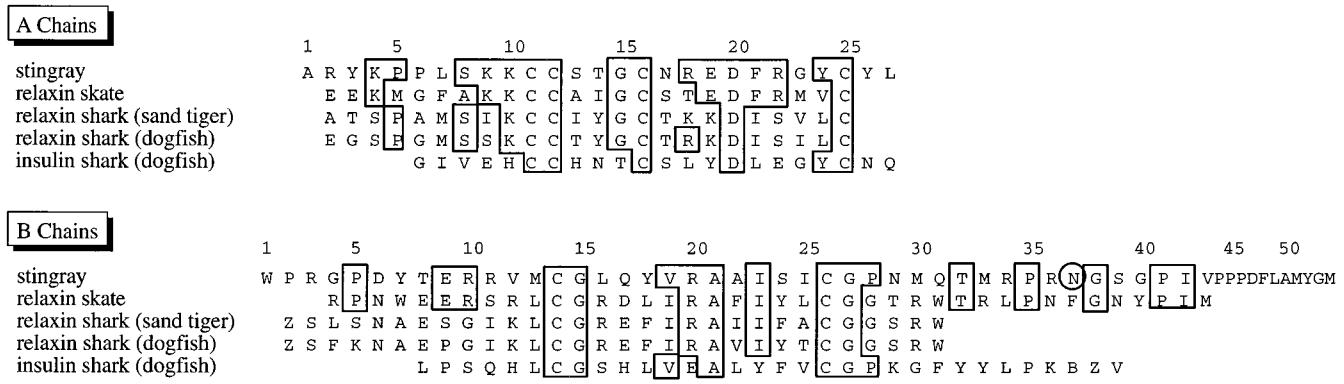

FIGURE 7: Comparison of stingray, shark, and skate relaxin and shark insulin amino acid sequences. Boxed areas are conserved with respect to the stingray sequence. The circle indicates the glycosylation site. Z indicates pyroglutamyl (N-terminal) or Glu/Gln. B indicates Asp/Asn.

be more like relaxin because all three conserved glycine residues of relaxin are present (A15, B15, and B27). Of the four structurally important glycine residues of insulin, two are identical to elasmobranch relaxin (B15 and B27) whereas the two others (GlyA1 and GlyB23, insulin numbering) are proline (A6) and methionine (B30), respectively, in stingray relaxin.

This molecule isolated from the stingray is the largest relaxin-like molecule yet described. The A chain of most relaxins has 22 or 24 residues except mouse (25 residues) (*12*), whereas the A chain of the stingray relaxin-like molecule has 27 residues, one extra amino acid at the N terminus and two residues at the C terminus. A two-amino acid extension at the C terminus of the A chain was observed also in dogfish shark insulin (*13*). The stingray relaxin-like molecule is the only member of the relaxin family with an A chain that does not terminate in cysteine. The B chain of this molecule is unusually long and exceeds the size of relaxin in the closely related skate and shark (*12*). The B chain of the stingray relaxin-like molecule is the longest known, exceeding that of the little skate relaxin by 13 amino acid residues, three at the N terminus and ten at the C terminus. The C-terminal extension of the stingray molecule is rich in prolyl and glycyl residues which indicates a flexible structure.

Figure 7 shows a comparison of the primary structure of the stingray relaxin and the other elasmobranch relaxins and insulin. The secondary structure prediction of the stingray molecule indicates a potential to form an α-helix in the midregion of the B chain. The potential for helix formation in the N-terminal and C-terminal region of the A chain is reduced when compared to that in the X-ray structure of human relaxin (*14*). Prolines located in positions A5 and A6 as well as glycine in position A23 are likely to disrupt both the N-terminal and C-terminal helices of the A chain of relaxin. The helix-breaking glycine in position A23 is not observed in all other relaxin structures.

Based on a relaxin-like structure (*14*), side chains forming the hydrophobic core of relaxin are mostly hydrophobic in the stingray molecule; i.e., CysA11, CysA16, AlaB21, and IleB25 are identical residues in human relaxin, and PheA21 and TyrB18 are replacing leucines in human relaxin. Position B11 which is arginine in stingray relaxin replaces a relatively constant isoleucine in most other relaxins.

The physiologic role of relaxin remains uncertain in many species, including humans (*6*, *12*, *15*). The physiologic role of relaxin has been best characterized in rodents in which it acts to soften the interpubic bone ligaments and softens the cervix and vagina in preparation for parturition. It is likely that it plays this role in female elasmobranch fish such as sharks and skates, which have soft cartilaginous skeletons. Koob et al. (*16*) showed that shark as well as porcine relaxin was effective in increasing the natural and estradiol-primed thickness of the dogfish shark cervix.

We were unable to demonstrate biological activity of the relaxin-like molecule in the standard mouse pubic symphysis assay. This was not unexpected because the mammalian binding site, which is on the B chain between the disulfide bonds (*17*), is not complete in the stingray molecule. One of the conserved arginines of that site is replaced by leucine (B16), whereas the other arginine (B20) is present. This was confirmed in our radioimmunoassays in which a rabbit antibody directed against the binding site (*18*) was only weakly cross-reactive with the stingray relaxin-like molecule. On the other hand, a polyclonal sheep antibody directed against different sites on porcine relaxin showed highly specific binding to the stingray molecule.

Despite relaxin's existence and actions having been characterized almost exclusively in the female of several phylogenetic groups, including elasmobranch fish, there is clear evidence for its existence in the prostate, seminal fluid, and testes of various mammals (*19*−*24*). It was this latter observation, coupled with the previous identification of relaxin in elasmobranch fish, that led us to suspect that a relaxin-like molecule might be present in the accessory reproductive organs of male stingrays. Both skates and stingrays possess paired sacs which open into the cloaca in close proximity to that of the vas deferens. The fluid produced by these sacs is highly alkaline (pH 8.2−9.5) and led early workers to postulate that it was secreted over sperm to protect it from the acidic urine (*25*). The anatomical similarities between the openings of the alkaline gland and the sperm duct in the Atlantic stingray and the openings of the prostate into the urethra just distal to the ejaculatory ducts in mammals suggested a similar function for the fluid of both accessory reproductive organs. Although we were able to isolate a relaxin-like molecule from the stingray alkaline gland fluid, our bioassays on stingray sperm did not reveal any effect on the motility of those cells. We can only suspect that this molecule's actions are on parameters of sperm different from those that were tested here or may be in the female since fertilization is internal in these fish. Further-

Table 4: Amino Acid Sequence Homology with the Stingray Relaxin-like Molecule for A and B Chains of Relaxin and Relaxin-like Molecules from Various Species

species[a]	percent homology		
	A chain	B chain	total
little skate	40	33	36
sand tiger shark	33	25	29
dogfish shark	41	20	29
mouse	25	26	26
rat	33	20	25
guinea pig	33	18	24
dog	33	20	25
pig	46	21	31
human ii	29	34	32
human relaxin-like factor	19	29	24

[a] Original data taken from refs 4, 5, and 26.

more, we assume that female Atlantic stingrays, like the closely related skates and sharks, have relaxin in the ovaries. We are investigating the possibility that female elasmobranch relaxin has the same basic structure and is nonglycosylated. If this is indeed the case, then the Atlantic stingray would provide the first evidence for sex-specific relaxin-like molecules within the same species.

Schwabe and Büllesbach (*12*) have shown the lack of correlation between the amino acid sequence homology of relaxin and the classical phylogenetic hierarchy. Comparisons of the stingray relaxin-like sequence with other species showed that the stingray sequence has the greatest homology with the ovarian relaxin of the closely related skate (36%, Table 4). All other species, including shark and man, have sequence homology narrowly defined between 24 and 32%.

Clearly, the most unique aspect of the stingray relaxin-like molecule is the oligosaccharide residue which resides on the B chain in position 37. Typical of all N-linked oligosaccharides, it contains a common pentasaccharide core consisting of two *N*-acetylglucosamine residues and a mannose to which two additional mannose monosaccharides are attached. The stingray relaxin-like molecule follows the complex type of additional monosaccharide branching by having fucose and *N*-acetylglucosamines linked to the pentasaccharide core. We do not know the functional significance of this oligosaccharide chain.

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