Molecular Cloning and Characterization of Spiggin

AN ANDROGEN-REGULATED EXTRAORGANISMAL ADHESIVE WITH STRUCTURAL SIMILARITIES TO Von Willebrand FACTOR-RELATED PROTEINS

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One of the most definitive examples of a vertebrate extraorganismal structural protein can be found in three-spined sticklebacks (Gasterosteus aculeatus). In the breeding male the kidney hypertrophies and synthesizes an adhesive protein called “spiggin,” which is secreted into the urinary bladder from where it is employed as a structural thread for nest building. This paper describes the first molecular characterization of spiggin and demonstrates that this adhesive is a protein complex assembled from a potential of three distinct subunits (α, β, and γ). These subunits arise by alternative splicing, and 11-ketoandrogens induce their expression in stickleback kidneys. Analysis of the predicted amino acid sequence of each subunit reveals a modular organization whose structural elements display a similarity to the multimerization domains found within von Willebrand Factor-related proteins. These results implicate that spiggin utilizes a conserved multimerization mechanism for the formation of a viscous agglutinate from its constituent subunits in the urinary bladders of male sticklebacks. This novel extraorganismal structural protein is therefore ideally suited to its function as an adhesive thread.

Extraneous proteins are common among invertebrates. Examples range from the fibroin threads forming spider webs and silkworm cocoons (1, 2) to the collagen-based matrixes of the byssus threads of Mytilus and the Drosophila sgs family (3, 4).

The production of extraorganismal structural proteins is not as common among vertebrates. However, a distinct example occurs in the teleostean stickleback family (Gasterosteidae) where the secondary proximal epithelium cells of the male kidney undergo hypertrophy during the breeding season (5). In male three-spined stickleback (Gasterosteus aculeatus) this structural reorganization is due to the synthesis of an adhesive protein called “spiggin,” which is under the regulation of 11-ketoandrogens (6). This agglutinate is subsequently secreted into the urinary bladder for storage and employed as a structural and highly elastic adhesive thread to assemble a nest from plant material in which the female lays her eggs (5, 6).

Previously we have characterized spiggin from the urinary bladder content of male sticklebacks as being a 203-kDa cysteine-rich glycoprotein (6). No similarities in amino acid composition could be observed between spiggin and murine kidney androgen-regulated protein, elastin, collagen, fibroin, or vitelline envelope proteins (7–9). These observations suggest that spiggin is a novel structural protein.

This paper describes the first molecular characterization of spiggin and demonstrates that this extraorganismal adhesive is a protein complex assembled from a potential of three distinct subunits (α, β, and γ). These subunits arise by alternative splicing from a single gene, and their expression is under the regulation of 11-ketoandrogens. Analysis of each subunit’s predicted amino acid sequence reveals that they exhibit a modular organization whose structural elements display a similarity to the multimerization domains found within von Willebrand Factor (vWF)-related proteins.

**EXPERIMENTAL PROCEDURES**

**Fish Maintenance**—Adult sticklebacks were routinely housed in a 200-liter aquarium containing brackish water (0.5% salinity) at 20 °C under a photoperiod of 16:8 h light:dark. The water was aerated and filtered. The fish were fed red midge larvae.

**Amino Acid Sequencing**—Urinary bladder contents from 10 mature male sticklebacks were pooled. Proteins were resolved by SDS-PAGE (10) and visualized by Coomassie staining (11). The 203-kDa spiggin bands were excised from the gel and subjected to in-gel digestion with porcine trypsin (Promega) or Lys-C protease (Wako Chemicals GmbH) (12). Individual peptides were isolated following acidification by microbore reversed phase liquid chromatography on a Kromasil C18 column operated in the SMART System (Amersham Pharmacia Biotech). The amino acid sequences of selected peptides were determined in an Automated Peptide Sequencer (model 494A, Applied Biosystems).

**Reverse Transcriptase-Polymerase Chain Reaction—**Total RNA was extracted from a pooled sample of five mature male kidneys using Tri Reagent™ (Sigma). cDNA was synthesized from 1 μg of total RNA using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). PCR reactions were performed in “Thermo Buffer” (Promega) and contained 500 ng of cDNA template, 0.2 mM dNTPs, 1 mM magnesium chloride, 1 unit of Taq DNA polymerase (Promega), and 25 pmol of spiggin NH2-terminal (5′-CARACIAARGATCARAC-3′) and spiggin peptide4 (5′-TTGTGIGAIATRTARTTYTCYTT-3′) oligonucleotides. Optimized reaction conditions (13) were as follows: 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min for 40 successive cycles using a

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF323732 (spiggin subunit-α), AF323733 (spiggin subunit-β), and AF323734 (spiggin subunit-γ). From whom correspondence should be addressed: Dept. of Cell and Molecular Biology, Unit of Physiology, Umeå University, SE-901 87 Umeå, Sweden. Tel.: 46-90-7869545; Fax: 46-90-7866991; E-mail: Per-Erik.Olsson@biology.umu.se.

†The abbreviations used are: vWF, von Willebrand Factor; 11-KA, 11-ketoandrostenedione; PAGE, polyacrylamide gel electrophoresis; kb, kilobase(s); ORF, open reading frame.
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PTC-200 Thermal Cycler (MJ Research). Amplified products were ligated into pGEM-T (Promega), and recombinant plasmids were isolated using the Wizard® Plus SV Miniprep System (Promega). Cycle sequencing was performed using the Thermo Sequenase (version 2.0) Sequencing Kit (Amersham Pharmacia Biotech). The reactions were resolved on an ABI Prism™ 377 DNA Sequencer (PerkinElmer Life Sciences), and the data obtained were analyzed using EditView (version 1.0.1) (PerkinElmer Life Sciences).

Slot Blot Analysis—Total RNA was extracted from mature male tissues, from three individual fish, using Tri Reagent™ (Sigma). Aliquots of 5 μg of total RNA were mixed with denaturing solution (6 × SSC, 7% [v/v] formaldehyde) and transferred onto a nylon membrane (Amersham Pharmacia Biotech) using a Minifold II Slot Blot Apparatus (Schleicher and Schuell). Membranes were probed using a randomly primed [α-32P]dCTP radiolabeled spiggin cDNA fragment (636 base pairs) that was isolated by reverse transcriptase-polymerase chain reaction and sequenced as above. Hybridizations were performed at 65 °C overnight (6 × SSC, 0.1% [w/v] SDS, 100 μg ml⁻¹ tRNA, and 5 × Denhardt’s solution). The membranes were washed for 2 × 30 min periods at 42 and 65 °C in 0.1 × SSC, 0.1% (w/v) SDS and exposed to Hyperfilm™-MP film (Amersham Pharmacia Biotech) at −70 °C. The films were visualized using a Curix 60 Film Developer (AGFA).

Northern Blot Analysis—Total RNA was extracted from mature kidney of Tri Reagent™ (Sigma). Northern blots containing 5 μg of total RNA were performed as described previously (11). Hybridization to an [α-32P]dCTP spiggin cDNA probe and washing were performed as described above.

cDNA Library Construction and Screening—Total RNA was extracted from a pooled sample of 20 mature male kidneys using Tri Reagent™ (Sigma). The mRNA fraction was isolated using the poly(A) Quick® mRNA Purification Kit (Stratagene), and an unidirectional cDNA library was constructed in Lambda ZAP Express® (Stratagene). A total of 2 × 10⁹ plaques were screened (11). Hybridization to an [α-32P]dCTP spiggin cDNA probe and washing were performed as described above. Positive plaques were purified through four successive hybridization rounds, and individual clones were isolated by phagemid excision. These clones were sequenced by Cybergene AB (Huddinge, Sweden).

Southern Blot Analysis—Genomic DNA was isolated from a whole mature stickleback using the GenElute™ Mammalian Genomic DNA Kit (Sigma). Southern blots containing 20-μg aliquots of DNA digested to completion with BamHI, EcoRI, HindIII, KpnI, Ncol, and XhoI were performed as described previously (11). Hybridization to an [α-32P]dCTP spiggin cDNA probe and washing were performed as described above.

Effects of Steroid Treatments—Steroid treatments were performed using adult females, as their kidneys do not undergo hypertrophy under natural conditions (6). Nonbreeding females housed at 9 °C and a photoperiod of 8:16 h light:dark were anesthetized with 0.1% (v/v) 2-phenoxyethanol (Sigma) and implanted with cocoa butter containing steroids dissolved in cocoa butter. The steroids were 1,11-KA and 2,11-KA only. Tubes containing cocoa butter were used as controls. Following implantation the fish were maintained in a 50-liter aquarium containing brackish water (0.5% salinity) at 17 °C for 16 days. Nontreated females served as controls. Following implantation the fish were maintained in a 50-liter aquarium containing brackish water (0.5% salinity) at 17 °C for 16 days. Nontreated females served as controls.

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RESULTS

Urinary bladder spiggin was purified to homogeneity, and six partial peptide sequences were determined by amino acid sequencing (Table 1). Degenerate oligonucleotides designed against the NH2-terminal sequence and peptide-4 were employed to amplify a partial cDNA sequence (636 base pairs) encoding for a spiggin subunit from male stickleback total kidney RNA by reverse transcriptase-polymerase chain reaction. The identity of this clone was established as the translated protein sequence also contained two peptides (SYYVR and IRDPQVLRK) predicted from amino acid sequencing (Table 1). This nucleotide sequence was subsequently employed for the generation of molecular probes.

Isolation of Spiggin Subunit-α, -β, and -γ—Slot blot analysis of spiggin spatial expression was performed on three separate animals. In all analyses the expression was restricted to the kidney of mature males (Fig. 1A) where a predominant message of 4.2 kb and lesser mRNAs of 2.2 and 1.6 kb were detected by Northern analysis. These transcripts were designated spiggin subunit-α (4.2 kb), subunit-β (2.2 kb), and subunit-γ (1.6 kb).

Table 1

| Peptide number | Peptide sequence | Location |
|----------------|------------------|----------|
| N-terminal     | KTKEIQTYY        | 25–32    |
| 2              | SYYVR            | 49–53    |
| 3              | IRDPQVLRK        | 190–197  |
| 4              | FENSQSI          | 230–236  |
| 5              | LIRYK            | 406–410  |
| 6              | RGTFSIR          | 842–848  |

Fig. 1A, slot blot analysis of spiggin expression in selected tissues of stickleback. All determinations were performed using 5 μg of total RNA from three separate animals. The same results were obtained for all animals. B, Northern blot analysis of spiggin expression in kidneys of mature male and female sticklebacks. Lane 1, 5 μg of total male kidney RNA; lane 2, 5 μg of total female kidney RNA. Positions of RNA molecular mass markers (kb) (Life Technologies, Inc.) are given in the left margin. The size (kb) of each spiggin subunit transcript is indicated in the right margin.
kb), respectively (Fig. 1B, lane 1). No hybridization was detected in mature female kidney even after prolonged exposure (Fig. 1B, lane 2). A total of 26 recombinant clones encoding for all transcripts were isolated from a mature male stickleback kidney cDNA library. Sequencing of these clones demonstrated that their untranslated regions were identical, and thus only two recombinants encoding for each subunit were sequenced entirely.

The deduced open reading frame (ORF) of subunit-α encoded for a 910-amino acid protein with a predicted molecular mass of 103 kDa. Both subunit-β and -γ encoded polypeptides of 613 and 472 residues with calculated molecular masses of 70 and 53 kDa, respectively (Fig. 2). The deduced NH2-terminal amino acid sequence of each spiggin subunit was located 25 amino acids upstream of the sequence obtained by NH2-terminal sequencing of urinary bladder content spiggin. The ORFs of each subunit were identical from the initiating Met1 through to Gln466. This was followed by six additional amino acid residues and a stop codon in subunit-γ, while the ORFs of both other peptides continued until Trp 606. At this point a further seven residues and a termination codon were observed in subunit-β.

The sequences of each subunit exhibited a conserved identity at both the nucleotide and protein levels that indicated that all subunits were derived from a single locus by alternative splicing. The hybridization patterns observed following Southern analysis of stickleback genomic DNA was compatible with the existence of a single Spiggin gene (Fig. 3).

Regulation of Spiggin Subunit-α, -β, and -γ Expression—Spiggin mRNA was induced by 11-KA (Fig. 4A) whose in vivo conversion into 11-ketotestosterone has been shown previously (17). A dose-response induction was first observed at an implant concentration of 1 μg ml−1 (18 ± 8%), and this was augmented 4- and 6-fold, respectively, with doses of 5 μg ml−1 (75 ± 16%) and 25 μg ml−1 (100 ± 10%). Kidney hypertrophy followed a similar pattern as implantation of 1 μg ml−1 11-KA induced intermediate hypertrophy, while treatment with 5 and 25 μg ml−1 produced clear hypertrophy. Implantation of other steroids did not induce spiggin expression or induce hypertrophy.

Induction of spiggin mRNA by 25 μg ml−1 11-KA over time was investigated (Fig. 4B). Expression was apparent 1 day after implantation (29 ± 14%) and increased following 3 days (43 ± 8%), 5 days (83 ± 8%), and 10 days (100 ± 23%) stimulation. The absence of kidney stimulation in normal females was confirmed, as no induction was detected in control groups (Figs. 4, A and B).
Analysis of Spiggin Subunit-α, -β, and -γ Proteins—The presence of spiggin within the kidney and urinary bladder content of male sticklebacks was investigated by immunodetection (Fig. 5). Antiserum raised against an NH₂-terminal motif common to both kidney subunits and urinary bladder content spiggin (KTKEIQTYTCRTFGS-C) recognized predominant bands of 130 and 51 kDa and a faint 90-kDa signal in the kidney (Fig. 5, lane 1). Conversely only a single distinct protein band of 203 kDa was detected in the urinary bladder content (Fig. 5, lane 2). This 203-kDa band was not detected in the kidney even after prolonged exposure.

Structural and Phylogenetic Analyses—Hydropathy analysis (18) demonstrated that subunit-α, -β, and -γ were overall hydrophobic (Fig. 6). All subunits contained a signal peptide with a predicted length of 19 residues at their amino terminus (Table II) (19) and up to four N-glycosylation sites and one O-glycosylation site (Table II) (20). Each subunit exhibited a modular organization and could be divided into different domains. Subunit-α was found to contain two full (D1 and D2) and one truncated (D3) nontandem motifs that displayed a high degree of sequence identity to the D domains found within vWF. Similarly subunit-β was determined to contain two full domains (D1 and D2), while subunit-γ contained one full (D1) and one truncated (D2) motif also arranged as nontandem repeats (Table II) (21). In addition, conserved vicinal cysteine motifs (CGLCG) were present in each D1 domain, while the D2 domains of subunit-α and -β contained an additional truncated version of this motif (GLCG) (Table II). A second characteristic of each subunit was that each D domain was bisected by regions exhibiting high cysteine content (10–12%) (Fig. 7).

Similarities between the predicted amino acid sequences of each subunit and vWF-related proteins were determined using the gapped BLAST (version 2.0) algorithm (22). Each subunit exhibited the highest similarity to *Xenopus* integumentary mucin B.1 (28%), rat MUC2 (27%), human MUC5AC (27%), human vWF (26%), and murine otogelin (25%), while lower similarities (<20%) were also exhibited to diverse vWF-related proteins such as *Xenopus* kielin, *Drosophila* hemolectin, and porcine zonadhesin. Phylogenetic analysis further confirmed that each subunit exhibited an ancestral relation to vertebrate mucins, vWF, and mouse otogelin (Fig. 8).

DISCUSSION

This study presents the first molecular characterization of spiggin, a novel extraorganismal adhesive protein synthesized by male three-spined sticklebacks. Spiggin was expressed in the kidney as three subunits (α, β, and γ). Alternative splicing from one locus generated each subunit. The expression of spiggin was only induced by 11-ketoandrogens. This is consistent with our previous findings that 11-oxygenated androgens were the most effective in stimulating stickleback kidney hypertrophy (23), and 11-ketotestosterone is regarded as the main an-
drogen in male teleosts (5). Spiggin is therefore the only currently known product being induced by 11-ketoandrogens in any species.

The amino acid sequence of subunit-α, -β, and -γ exhibited an overall hydrophobic character that is consistent with spiggin’s role as a water-insoluble adhesive (6) and contained signal peptides of a predicted 19 residues at their amino termini (19). Interestingly, amino acid sequencing of the NH₂ terminus of urinary bladder spiggin demonstrated that this translocation motif was absent and suggests that the subunits are processed into mature protein by signal peptide removal.

The ORFs of each subunit were predicted to encode for proteins with molecular masses of 103 kDa (subunit-α), 70 kDa (subunit-β), and 53 kDa (subunit-γ), respectively. However, immunodetection recognized predominant bands of 130 and 51 kDa and a faint 90-kDa signal in the kidney. The apparent differences between the predicted and observed molecular masses could be accounted for by post-translational modifications as up to four N-glycosylation sites and one O-glycosylation site were predicted (20) by their deduced amino acid sequences. This is consistent with our previous investigation where spiggin assayed positively for the presence of carbohydrate (6). Conversely, only a single distinct protein band of 203 kDa was detected within the urinary bladder of male sticklebacks that also concurred with our previous investigations (6). These observations suggest that formation of spiggin involves multimerization of the constituent kidney subunits in the urinary bladder. A structural analysis of each subunit was there-

| Protein Feature                  | Location                          |
|---------------------------------|-----------------------------------|
| Spiggin subunit-α               | 1–19 N-Glycosylation motif        |
|                                 | 105–108, 215–218, 249–252         |
|                                 | O-Glycosylation motif             |
|                                 | vWF type D domain                 |
|                                 | 27–169, 353–490, 796–883          |
|                                 | CGLCG motif                       |
|                                 | 162–166                           |
|                                 | GLGC motif                        |
|                                 | 487–490                           |
| Spiggin subunit-β               | 1–19 N-Glycosylation motif        |
|                                 | 105–108, 215–218, 249–252         |
|                                 | O-Glycosylation motif             |
|                                 | vWF type D domain                 |
|                                 | 27–169, 353–490                   |
|                                 | CGLCG motif                       |
|                                 | 162–166                           |
|                                 | GLGC motif                        |
|                                 | 487–490                           |
| Spiggin subunit-γ               | 1–19 N-Glycosylation motif        |
|                                 | 105–108, 215–218, 249–252         |
|                                 | O-Glycosylation motif             |
|                                 | vWF type D domain                 |
|                                 | 27–169, 353–470                   |
|                                 | CGLCG motif                       |
|                                 | 162–166                           |
|                                 | GLGC None present                 |

**Fig. 5.** Immunodetection of spiggin in the kidney and urinary bladder content of mature male sticklebacks. Lane 1, 1 μg of kidney-soluble protein fraction; lane 2, 1 μg of bladder content-soluble protein fraction. The positions of SDS-PAGE standards (kilodaltons) (Bio-Rad) are given in the left margin. The size (kilodaltons) of each immunodetected protein is indicated in the right margin.

**Fig. 6.** Hydropathy profiles of spiggin subunit-α, subunit-β, and subunit-γ. The hydropathy profiles were predicted using the Kyte-Doolittle algorithm using an 11-amino acid window. Plus (+) values indicate a hydrophilic character while minus (−) values represent a hydrophobic character.

**Fig. 7.** Structural domains identified in spiggin subunit-α, subunit-β, and subunit-γ. The location (amino acid numbers) of identified motifs is given below each polypeptide chain. The lengths of the polypeptides are not to scale.
fore conducted to identify motifs that could catalyze such a mechanism. Each subunit exhibited a modular structure and could be divided into distinct regions that displayed sequence similarity to the D domains found within vWF (21) and other related proteins such as vertebrate mucins, zonadhesin, and IgG Fc-binding proteins (24–27). However, the D domains within each spiggin subunit were organized into nontandem repeats bisected by several cysteine-rich regions (10–12%). This is unlike that of vWF-related proteins where D domains are arranged as repeating units with a disulfide-rich region located at their carboxyl terminus (24, 28). D domains and disulfide elements in vWF and porcine submaxillary mucin are required for their assembly into large multimeric proteins (28–31). The structural similarities between particularly vWF and subunit-α, β, and γ therefore suggest that the multimerization of spiggin is mediated by a conserved mechanism common to all vWF-related proteins.

Another interesting observation is that each subunit may have the ability to self-catalyze multimerization. In vWF this autocatalysis was dependent upon the presence of two sets of vicinal cysteine residues (CGLCG) present in its D1 and D2 domains (32–35). Each spiggin subunit possessed vicinal cysteine motifs in their D1 domains, while the truncated version (GLCG) present in the D2 domains of subunit-α and -β is also a characteristic of human MUC2 and porcine submaxillary mucin (24, 28). Mutation of these motifs in porcine submaxillary mucin resulted in proteins that were poorly secreted and suggested that the D domains interacted to induce correct protein folding (28). Such a process could therefore be envisioned for spiggin, as each subunit possesses the necessary catalytic motifs required to induce multimerization.

The conserved domain structure between each spiggin subunit and vWF-related proteins suggested an evolutionary relationship that was confirmed by phylogenetic analysis. Spiggin subunit-α, -β, and -γ were ancestrally related to vertebrate mucins, vWF, and mouse otogelin. These results clearly demonstrate that spiggin shares structural properties with vWF-related proteins. The presence of the D domains and the vicinal cysteine motifs suggest that spiggin utilizes a conserved multimerization mechanism for the formation of a viscous agglutinate from its constituent subunits in the urinary bladders of male sticklebacks. This novel extraorganismal structural pro-

![Phylogenetic relationships of spiggin subunit-α, subunit-β, and subunit-γ to von Willebrand Factor-related proteins.](image-url)
tein is therefore ideally suited to its function as an adhesive thread.

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