β-Scruin, a homologue of the actin crosslinking protein scruin, is localized to the acrosomal vesicle of *Limulus* sperm

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SUMMARY

Scruin (α-scruin) is an actin bundling protein found in the acrosomal process of *Limulus polyhemus* sperm. We have cloned and sequenced a second scruin isoform from *Limulus*, β-scruin, that is 67% identical to α-scruin. Northern and Southern analyses confirm that β-scruin and α-scruin are encoded by distinct genes. The sequence of β-scruin, like α-scruin, is organized into N- and C-terminal superbarrel domains that are characterized by a six-fold repeat of a 50 residue motif. Western analysis using rabbit polyclonal antisera specific for α- and β-scruin indicate that β-scruin, like α-scruin, is found in *Limulus* sperm but not blood or muscle. Both immunofluorescence microscopy and immunogold-EM localize β-scruin within the acrosomal vesicle at the anterior of sperm but not in the acrosomal process. The function of β-scruin in this membrane-bounded compartment that is devoid of actin is unknown. However, the location of β-scruin together with the fact that it contains two putative β-superbarrel structural folds, which are known to be catalytic domains in a number of proteins, suggests it may have a possible enzymatic role.

Key words: scruin-related sequence, duplicated superbarrel domain, sperm acrosomal vesicle

INTRODUCTION

The eggs of marine invertebrates are protected from the external environment by thick vitelline layers. This protective layer is also a barrier to sperm during the fertilization process. Several different strategies have evolved in the sperm of marine invertebrates to overcome this protective layer and achieve fertilization. For example, contact of abalone sperm with an egg releases a 16 kDa lytic protein, lysin, that rapidly creates a hole in the vitelline envelope for sperm entry (Messier and Stewart, 1994). In echinoderms and the horseshoe crab sperm, fertilization is achieved through the 'harpoon-like' action of an actin-based acrosomal process (Tilney, 1975, 1980; Tilney et al., 1973). At fertilization, the sperm cell extends an acrosomal process, a 60 μm membrane-covered bundle of actin filaments (DeRosier et al., 1980). Extension of the process is completed in a matter of seconds and occurs by two very different mechanisms. In unactivated *Thyone* sperm, G-actin located posterior to the acrosomal vesicle is kept in an unpolymerized state by profilin (Tilney, 1978). Contact with the egg elicits an acrosome reaction in which two processes take place simultaneously. The acrosomal vesicle fuses with the plasma membrane and releases its lytic enzymes. At the same time, G-actin rapidly assembles into filaments (Tilney and Inoue, 1982) which are crosslinked into a bundle by a 55 kDa actin binding protein, fascin (Maekawa et al., 1982). In contrast to the pool of G-actin in *Thyone* sperm, in unactivated *Limulus* sperm, the actin is preassembled into a bundle that is coiled around the base of the sperm nucleus (DeRosier et al., 1982; Tilney, 1975). During the acrosome reaction, the actin bundle uncoils and extends through a channel in the nucleus to form a 60 μm long acrosomal process (DeRosier et al., 1982; Tilney, 1975). In contrast to *Thyone*, the actin filaments in the *Limulus* acrosomal process are cross-linked by scruin, a 102 kDa protein present in a 1:1 molar ratio with actin (Schmid et al., 1991).

All available information suggests that scruin is organized into two domains. Helical reconstructions of scruin decorated actin filaments from the *Limulus* acrosomal process, at 13 Å resolution, reveal that scruin consists of two globular domains that bind adjacent actin subunits in the filament (Owen and DeRosier, 1993; Schmid et al., 1994). This two domain organization is also confirmed by limited proteolysis of purified acrosomal bundles (Way et al., 1995). Different proteases cleave in the middle of the scruin to yield an N-terminal ~47 kDa and a C-terminal ~56 kDa protease-resistant domain. In addition, the scruin sequence suggests that each protease-resistant domain is largely composed of a superbarrel structural fold that is derived from the tandem duplication of a six-fold 50 residue motif (Way et al., 1995).

Database searches show scruin is unrelated to any known actin binding protein, but is related to several sequences which contain between two and seven scruin-like repeat motifs (Way et al., 1995). These sequences include: kelch in *Drosophila* (Xue and Cooley, 1993), mouse IAP-promoted placental protein (MIPP) (Chang-Yeh et al., 1991), a MIPP

homolog in *C. elegans* (Wilson et al., 1994), expressed sequence tags (ESTs) for kelch and MIPP in humans (Adams et al., 1993a,b), galactose oxidase in fungi (Ito et al., 1994), and four ORFs in the genome of poxviruses (Massung et al., 1994; Senkevich et al., 1993). The atomic structure of galactose oxidase reveals that the repeated sequence corresponds to a four stranded anti-parallel β-sheet motif that forms the repeat unit in a superbarrel structural fold (Ito et al., 1994). The superbarrel fold is a common structural fold in a number of enzymes, including neuraminidase (Chothia and Murzin, 1993; Murzin, 1992; Bork and Doolittle, 1994; Crennell et al., 1993; Varghese et al., 1983). Although scruin is an actin-crosslinking protein, the functions of the scruin-related proteins are unknown, except in the cases of galactose oxidase, neuraminidase, and kelch. Galactose oxidase catalyzes the oxidation of the hydroxyl group at the C6 position in D-galactose (Ito et al., 1994) while neuraminidase hydrolyses sialic acid residues from glycoproteins (Varghese et al., 1983). Kelch may have a cytoskeletal function because it is localized to the actin-rich ring canals that connect the 15 nurse cells to the developing oocyte in *Drosophila* (Xue and Cooley, 1993). However, unlike scruin, kelch may interact indirectly with actin, to stabilize ring canals after they have formed, as *Drosophila* lacking kelch still contain ring canals, although they appear more disordered (Robinson et al., 1994; Xue and Cooley, 1993).

In this report, we have identified a second scruin isoform, β-scruin, which is 67% identical to the scruin (α-scruin) found in the acrosomal process of *Limulus*. Unlike α-scruin, β-scruin is localized to the acrosomal vesicle, a membrane-bounded compartment that contains hydrolytic enzymes and lacks actin. This finding suggests that α- and β-scruin are functionally different and that β-scruin is associated with the hydrolytic pathway and not the 'harpoon' action of the acrosome reaction.

MATERIALS AND METHODS

cDNA isolation and sequence analysis

The β-scruin cDNA was identified and isolated from a *Limulus* testes cDNA library using standard hybridization methods (Way et al., 1995). Two clones encoding β-scruin (L3 and L4) were excised from Lambda ZAP II into Bluescript in vivo according to the manufacturer's instructions (Stratagene, La Jolla, CA). The cDNA sequence of the larger clone, L3 was derived from double stranded sequencing of random clones generated by sonication (Bankier et al., 1987), using Sequenase II (US Biochemical Corp., Cleveland, OH) and the Bluescript SK/KS primers. Assembly and analysis of the L3 sequence was achieved using the DNASTAR software package (DNASTAR Inc, Madison, WI). Database sequence searches were performed using BLAST (Altschul et al., 1990).

Phylogenetic relationships between the repeats in α- and β-scruin were analyzed using the CLUSTAL W program (Higgins et al., 1992; Thompson et al., 1994). To establish statistical significance, 1,000 rounds of bootstrapping were performed.

Northern and Southern analyses

Briefly, 5 µg *Limulus* testes poly(A)$^+$ RNA was separated on a 1% formaldehyde agarose gel, blotted overnight onto Hybond N (Amersham, Arlington Hts, IL), and then UV crosslinked in a Stratalinker (Stratagene, La Jolla, CA). The blot was prehybridized in 50% formamide, 50 mM NaPO$_4$, pH 6.8, 4× SET, 5× Denhardt's and 100 µg/ml herring sperm DNA at 42°C for 2 hours. Hybridization was overnight in the same solution with either the α-scruin L1 clone or the β-scruin L3 clone insert labeled with [α-^{32}P]dCTP using the Prime-It labeling kit (Stratagene, La Jolla, CA). (20× SET = 3 M NaCl, 20 mM EDTA and 200 mM Tris-HCl, pH 7.5). Subsequently, hybridized filters were washed in 50% formamide, 5× SET and 0.5% SDS at 42°C for 1 hour and then sequentially at 65°C for 1 hour in 2× SET and 0.5% SDS; 0.5× SET and 0.5% SDS and 0.1× SET and 0.5% SDS. For low stringency conditions, blots were prehybridized in 20% formamide, 50 mM NaPO$_4$, pH 6.8, 6× SET, 5× Denhardt's and 100 µg/ml herring sperm DNA at 42°C for 2 hours, then hybridized with equal counts of the α- and β-scruin probes in the same solution. The blot was sequentially washed down to final stringency of 1× SET and 0.5% SDS at 65°C. For reprobing, the filters were stripped by washing with several changes of 50% formamide and 50 mM NaPO$_4$, pH 6.8, at 65°C for 2 hours followed by 2× SSC and 0.1% SDS for 20 minutes at room temperature.

For Southern analysis, 4 µg of digested genomic *Limulus* DNA was separated on a 1% agarose gel in 1× Loening buffer (10× Loening buffer = 0.4 M Tris-base, pH 7.6, 0.36 M KH$_2$PO$_4$ and 0.01 M EDTA). Gels were pretreated using standard methods prior to capillary transfer overnight onto Hybond N (Amersham). Blotted filters were UV crosslinked, prehybridized in 6× SSC, 5× Denhardt's, 1% SDS and 100 µg/ml herring sperm DNA at 65°C and hybridized overnight in the same solution with α-^{32}P labeled full length probes for α- or β-scruin. Subsequently filters were washed in 2× SSC and 0.5% SDS at 65°C for low stringency and 0.2× SSC and 0.5% SDS at 65°C for high stringency. Filters were stripped for reprobing by treatment at 65°C for 1 hour with 0.4 M NaOH followed by 0.1× SSC, 0.1% SDS and 0.2 M Tris-HCl, pH 7.5.

Generation of α- and β-scruin specific antibodies

Two peptides, M34 (CKAKPQPGSKPTSVK) and M35 (CTTRS-GSRKTQKTLK) corresponding to residues 407-420 and 403-416 of α- and β-scruin, respectively, were synthesized with an additional cysteine at the N terminus of each peptide (Fig. 1). Briefly, 2 mg of each peptide was coupled to Keyhole Limpet hemocyanin using the Imject activated immunogen conjugation kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Both conjugated peptides were separated from uncoupled peptide using a Presto desalting column (Pierce) and the pooled conjugate fractions adjusted to a final concentration of 1 mg/ml in PBS prior to injection into New Zealand white rabbits (Hazelton Research Products, Denver, PA). The M34 pre-immune (R213.0) and test bleed sera R213.1 to R213.5 for α-scruin were titred by western blot using crude or purified acrosome preparations using standard methods. The M35 pre-immune (R214.0) and test bleed sera R214.1 to R214.5 for β-scruin sera were titred by western blot against a fusion protein tagged with residues 390-585 of β-scruin expressed in *Escherichia coli*. We used PCR to insert an in-frame *Eco*RI site adjacent to residue 390 and a TAA-TAG double stop *Hin*dIII site after residue 585 of β-scruin. The resulting PCR product was cloned into the *Eco*RI- *Hin*dIII sites of the *E. coli* expression vector pMAL C2 (New England Biolabs, Beverly, MA). The fidelity of the construct was confirmed by sequencing prior to expression in DH5α. M35 β-scruin sera were titred on total DH5α protein samples that had been induced to express the pMAL-β-scruin fusion construct.

Sperm immunofluorescence

Sperm were fixed for 10 minutes in 4.0% paraformaldehyde in artificial sea water. After fixation, the sperm preparations were absorbed onto polylysine-coated coverslips for 5 minutes, rinsed in sea water, permeabilized for 10 minutes with either −20°C ethanol or 0.1% Triton X-100, and non-specific binding sites were blocked by rinsing

3 times with 1.0% BSA in artificial sea water. The actin bundles were stained with bodipy-phalloidin as described previously (Sanders and Wang, 1990; Schliwa and van Blerkom, 1981), with the exception that artificial sea water was substituted for the Pipes-Hepes-EGTA-Mg buffer in order to maintain the integrity of the sperm. A number of anti-actin antibodies were used to test for the presence of G-actin in the acrosomal vesicle including the BTI anti-actin antibody (Bio-medical Technologies Inc., Stoughton, MA), the C4 anti-actin antibody (Sigma, St Louis, MO) and an anti-sea urchin actin antibody kindly provided by Dr Ed Bonder (Bonder et al., 1989). Coverslips were examined in either a Bio-Rad MRC600 Confocal microscope or a Zeiss Axioskop using DIC optics and a 100×/NA 1.3 Plan Neofluor Objective.

Immuno-electron microscopy

Sperm were fixed in 4.0% paraformaldehyde in sea water at 4°C overnight. After washing with sea water, the samples were dehydrated with increasing concentrations of dimethyl formamide and embedded in Lowicryl K4M embedding resin. Thin sections were incubated with either primary antibodies or the preimmune serum, and then treated with the goat anti-rabbit IgG-gold (10 nm) secondary antibody. The sections were examined in a Philips 410 (Philips Technologies, Cheshire, CT).

RESULTS

Isolation of β-scruin cDNA

During the screening for the scruin gene with the S42 PCR-derived scruin probe (Way et al., 1995), we identified two weak positives, L3 and L4. High stringency Southern analysis of rescued L3 and L4 clones suggested that, unlike the α-scruin-encoding clones L1 and L2, they were not identical to the original scruin probe, S42 (data not shown). Sequencing the larger clone, L3, confirmed that we had identified a scruin homologue. Fig. 1 shows the cDNA-derived protein sequence of L3 which we named β-scruin. The DNA sequence of β-scruin is 86.3% identical over 137 bp with the 144 bp S42 probe, which explains why β-scruin clones were identified during our screen for scruin.

Northern analysis using a full-length β-scruin probe detects a ~3 kb mRNA, a size in agreement with the 2.9 kb L3 clone (Fig. 2A). The β-scruin transcript is distinct from α-scruin and is not the product of differential splicing since full length probes for β-scruin do not hybridize with the 3.3 kb α-scruin message on the same northern blot (Fig. 2A). However, the β-
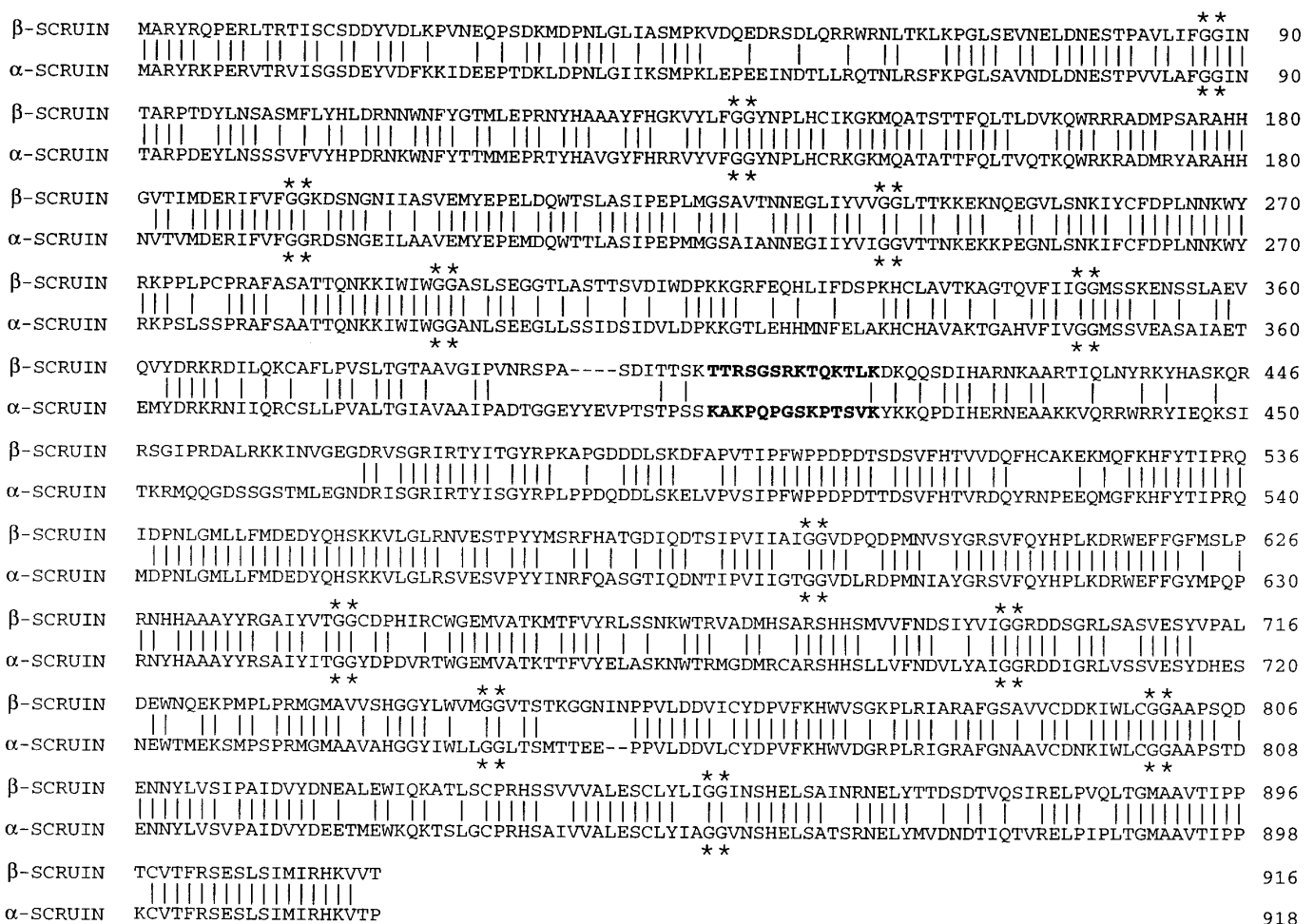
Fig. 1. An alignment between the predicted amino acid sequence of β-scruin (top) and α-scruin (bottom) generated by ALIGN using the default settings. The cDNA sequence has been submitted to the EMBL Sequence Database, accession number Z47541. Exact identities between the two sequences are indicated by the vertical bars and residue positions are indicated at the end of each line. The double asterisk above and below the aligned sequences indicates the double glycine motif found in all 12 repeats of each isoform (see Fig. 3). The positions of the peptide sequences M34 and M35 used to raise isoform specific antisera are indicated in bold type face.

A

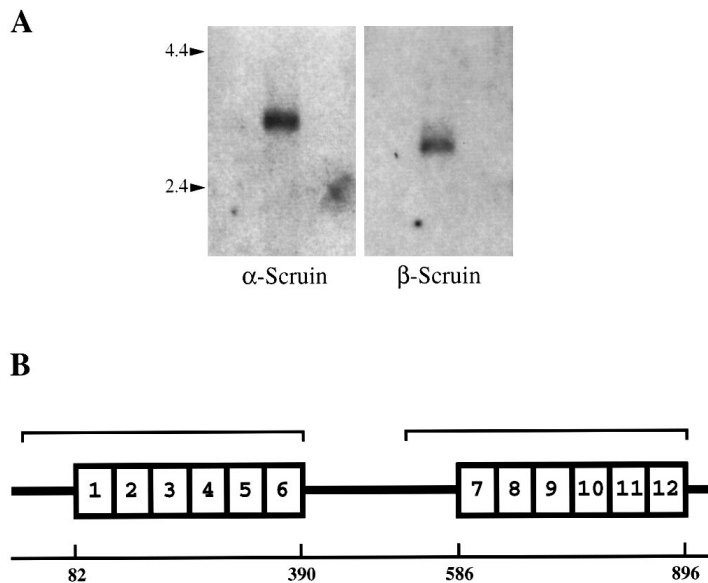α-Scruin   β-Scruin

B

82   390   586   896

Fig. 2. (A) Northern analysis of α-scruin and β-scruin on the same re-probed blot. Exposure time for β-scruin was twice that of α-scruin. RNA molecular mass standards are indicated on the left. (B) A schematic representation of the sequence organization of α- or β-scruin. The numbered boxes in two groups of six represent the ~50 amino acid residue repeat motif in both proteins. The two lines above the schematic indicate the tandem gene duplication within the molecule and the ruler below the schematic indicates the residue number positions for the beginning and end of the putative superbarrel domains in β-scruin.

scruin transcript is less abundant than α-scruin based on the double exposure time required to achieve the similar signal intensity (Fig. 2A). Southern analysis with full length α-scruin or β-scruin shows both probes hybridize with distinctly different sets of bands confirming that both cDNAs are products of separate genes (data not shown).

Sequence comparisons of α- and β-scruin

The β-scruin cDNA encodes a 917 amino acid residue protein, with a predicted molecular mass of 102 kDa, that is 67.4% identical to α-scruin (Fig. 1). The sequence of β-scruin contains no matches to peptide sequences obtained from scruin isolated from the acrosomal process. Like α-scruin, β-scruin is organized into a tandem pair of homologous domains (Fig. 2B). There is 34.5% identity between residues 16-389 of the N-terminal domain with residues 520-895 of the C-terminal domain. This duplicated region consists mainly of a six-fold tandem repeat of an imperfect 50 residue sequence motif (Fig. 2B).

β-scruin displays two major differences with α-scruin. The greatest sequence divergence, only 20.2% identity, between α-scruin and β-scruin occurs between residues 390-464 of β-scruin (Figs 1 and 2B). This region corresponds to the proteolytic sensitive region identified in α-scruin. Because β-scruin lacks the proteolytic sites detected in α-scruin, β-scruin may be more protease resistant than α-scruin. A second major difference between the two proteins is that β-scruin (pI 8.65) is considerably more basic than α-scruin (pI 7.22).

Phylogenetic analysis of repeats in α- and β-scruin

Our previous alignment of the 12 repeats in α-scruin was based on analysis of scruin-like repeat sequences in kelch and several viral open reading frames (ORFs), in relation to the crystal structure of galactose oxidase (Bork and Doolittle, 1994). However, with new information from β-scruin sequence, we re-analyzed the alignment of the sequence repeats. Dot plot analysis and visual inspection of all 24 repeats in α- and β-scruin indicates that our original alignment (Way et al., 1995) misplaced the start of the first repeat and consequently lacked

the last β-strand of each putative superbarrel domain. As a result, the phase of the alignment should be shifted toward the N terminus by 10 residues (Fig. 3). With the new alignment, the conserved features of the first and last β-strands in the repeat motif, originally seen in the α-scruin alignment are further enhanced (compare to Fig. 3 of Way et al., 1995).

Using the new phasing defined in Fig. 3 we performed a phylogenetic analysis of the repeat sequences in both proteins to examine their evolutionary relationship. The phylogenetic tree shows that the repeats at corresponding positions in both proteins have the greatest degree of similarity, i.e. repeat one in α-scruin is most similar to repeat one in β-scruin (Fig. 4A). Secondly, as expected from homology between the N- and C-terminal sequences in both molecules, the next level of similarity occurs between repeats from corresponding positions in the two halves of each molecule, i.e. repeats one and seven are most similar (Fig. 4A). Because the repeats at corresponding positions in both proteins are more similar than within their respective halves, we can conclude that α- and β-scruin diverged after the duplication event that generated the two domain organization. These observations suggest that both molecules evolved from an ancestral domain consisting of six repeats (Fig. 4B).

β-Scruin is localized to the sperm acrosomal vesicle

To investigate the localization of β-scruin we raised polyclonal antisera (R213 and R214) against two unique peptide sequences corresponding to a highly diverged region between α- and β-scruin (Fig. 1). Both sequences were predicted to be exposed at the protein surface because the region in α-scruin is immediately adjacent to a protease-sensitive region between the N- and C-terminal domains (Way et al., 1995). Immunoblots of pure acrosome preparations or pMAL β-scruin fusion protein indicated that the pre-immune sera, R213.0 and R214.0, respectively, were negative at 1:50 dilution (data not shown). The antisera, R213.5 and R214.5, for the two isoforms detect a single band of the correct size in positive controls at dilutions up to 1:5,000 dilution. However, while the M35-reactive sera R214.5 only detected the pMAL β-scruin fusion
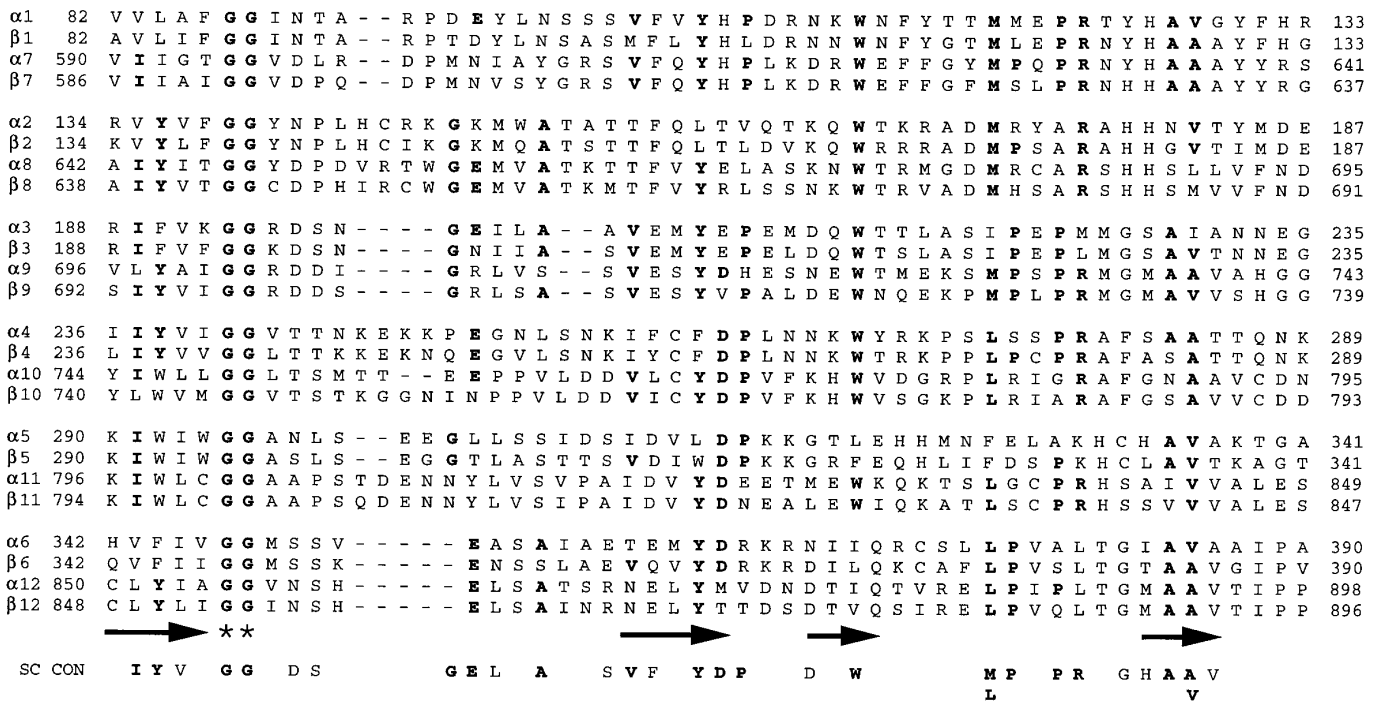
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α1    82   V V L A F G G I N T A - - R P D E Y L N S S S V F V Y H P D R N K W N F Y T T M M E P R T Y H A V G Y F H R   133
β1    82   A V L I F G G I N T A - - R P T D Y L N S A S M F L Y H L D R N N W N F Y G T M L E P R N Y H A A A Y F H G   133
α7    590  V I I G T G G V D L R - - D P M N I A Y G R S V F Q Y H P L K D R W E F F G Y M P Q P R N Y H A A A Y Y R S   641
β7    586  V I I A I G G V D P Q - - D P M N V S Y G R S V F Q Y H P L K D R W E F F G F M S L P R N H H A A A Y Y R G   637

α2    134  R V Y V F G G Y N P L H C R K G K M W A T A T T F Q L T V Q T K Q W T K R A D M R Y A R A H H N V T Y M D E   187
β2    134  K V Y L F G G Y N P L H C I K G K M Q A T S T T F Q L T L D V K Q W R R R A D M P S A R A H H G V T I M D E   187
α8    642  A I Y I T G G Y D P D V R T W G E M V A T K T T F V Y E L A S K N W T R M G D M R C A R S H H S L L V F N D   695
β8    638  A I Y V T G G C D P H I R C W G E M V A T K M T F V Y R L S S N K W T R V A D M H S A R S H H S M V V F N D   691

α3    188  R I F V K G G R D S N - - - - G E I L A - - A V E M Y E P E M D Q W T T L A S I P E P M M G S A I A N N E G   235
β3    188  R I F V F G G K D S N - - - - G N I I A - - S V E M Y E P E L D Q W T S L A S I P E P L M G S A V T N N E G   235
α9    696  V L Y A I G G R D D I - - - - G R L V S - - S V E S Y D H E S N E W T M E K S M P S P R M G M A A V A H G G   743
β9    692  S I Y V I G G R D D S - - - - G R L S A - - S V E S Y V P A L D E W N Q E K P M P L P R M G M A V V S H G G   739

α4    236  I I Y V I G G V T T N K E K K P E G N L S N K I F C F D P L N N K W Y R K P S L S S P R A F S A A T T Q N K   289
β4    236  L I Y V V G G L T T K K E K N Q E G V L S N K I Y C F D P L N N K W T R K P P L P C P R A F A S A T T Q N K   289
α10   744  Y I W L L G G L T S M T T - - E E P P V L D D V L C Y D P V F K H W V D G R P L R I G R A F G N A A V C D N   795
β10   740  Y L W V M G G V T S T K G G N I N P P V L D D V I C Y D P V F K H W V S G K P L R I A R A F G S A V V C D D   793

α5    290  K I W I W G G A N L S - - E E G L L S S I D S I D V L D P K K G T L E H H M N F E L A K H C H A V A K T G A   341
β5    290  K I W I W G G A S L S - - E G G T L A S T T S V D I W D P K K G R F E Q H L I F D S P K H C L A V T K A G T   341
α11   796  K I W L C G G A A P S T D E N N Y L V S V P A I D V Y D E E T M E W K Q K T S L G C P R H S A I V V A L E S   849
β11   794  K I W L C G G A A P S Q D E N N Y L V S I P A I D V Y D N E A L E W I Q K A T L S C P R H S S V V V A L E S   847

α6    342  H V F I V G G M S S V - - - - - E A S A I A E T E M Y D R K R N I I Q R C S L L P V A L T G I A V A K T G A   390
β6    342  Q V F I I G G M S S K - - - - - E N S S L A E V Q V Y D R K R D I L Q K C A F L P V S L T G T A A V G I P V   390
α12   850  C L Y I A G G V N S H - - - - - E L S A T S R N E L Y M V D N D T I Q T V R E L P I P L T G M A A V T I P P   898
β12   848  C L Y L I G G I N S H - - - - - E L S A I N R N E L Y T T D S D T V Q S I R E L P V Q L T G M A A V T I P P   896
                ────────►    * *                      ►            ►      ►           ►       ►       ────►

SC CON       I Y V   G G   D S              G E L     A       S V F   Y D P     D   W       M P   P R   G H A A V
                                                                                            L                 V
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Fig. 3. An alignment of the 12 repeat sequences in α- and β-scruin generated by the programme MEGALIGN. Repeats have been numbered according to the schematic in Fig. 2B with a prefix of α or β for α-scruin and β-scruin, respectively. The repeats have been grouped to emphasize the similarities between repeats at corresponding positions in both proteins as well as the gene duplication in both molecules. The residue positions at the beginning and end of each repeat are indicated. Residues shown in bold correspond to positions where at least 10 out of the 24 repeats have an identical residue. In addition, residues not shown in bold in the consensus correspond to positions where at least 8 out of the 24 repeats show identity. A 'scruin' consensus repeat is shown at the bottom of the alignment together with a double asterisk to identify the double-glycine residue motif indicated in Fig. 1. Bold arrows indicate the positions of β-strands in the putative structural fold of the repeat based on the analysis of related repeats by Bork and Doolittle (1994).

protein irrespective of dilution, the M34-reactive sera R213.5 showed a slight response to pMAL β-scruin fusion protein when used at low dilutions or when immunoblots were over-developed. This weak cross reactivity against β-scruin was

removed by blot affinity purification of the R213.5 sera on purified α-scruin.

Using the α- and β-scruin specific antisera at between 1:1,000-5,000 dilution we tested *Limulus* sperm, blood and muscle for the presence of α- and β-scruin by immunoblots. Neither scruin isoform was detected in blood or muscle samples (data not shown). However, we detected a single band of ~103 kDa in sperm preparations for both α- and β-scruin (Fig. 5). Although the large amount of DNA in total sperm samples was not conducive to producing good distinct bands, there was always a tendency for the β-scruin band to be broad and smeared compared to α-scruin suggesting this isoform may be post translationally modified.
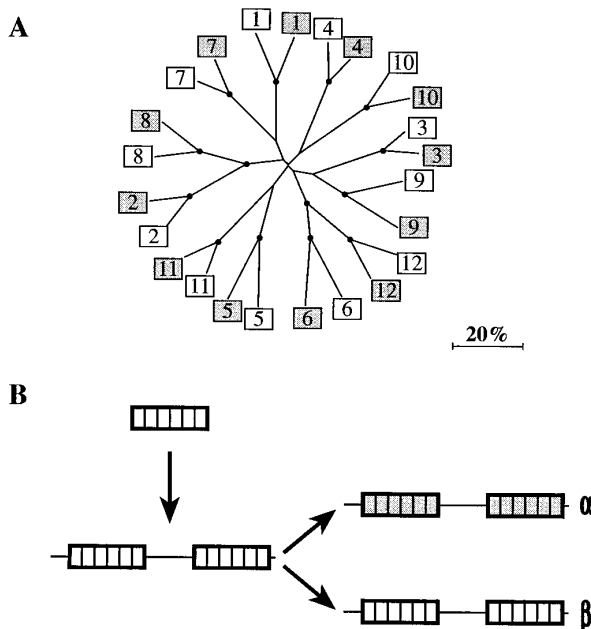
Fig. 4. (A) Phylogenetic analysis of the α- and β-scruin repeats defined by the alignment in Fig. 3. The filled numbered rectangles represent repeats 1-12 of α-scruin and the open rectangles the β-scruin repeats in the resulting tree. Black blobs at nodes indicate groupings which are statistically significant at the 95% confidence level and the scale bar represents a sequence divergence of 20%. The tree shows that each repeat groups with the corresponding repeat in the other isoform, suggesting that both isoforms arose by duplication of the same ancestral gene. Each pair of repeats then groups with the corresponding pair from the other block of repeats suggesting that the ancestral gene may have arisen by the duplication of a block of six repeats. The more distant relationships between the repeats are not clear. (B) A schematic representation for the evolution of α- and β-scruin, based on the phylogenetic analysis.
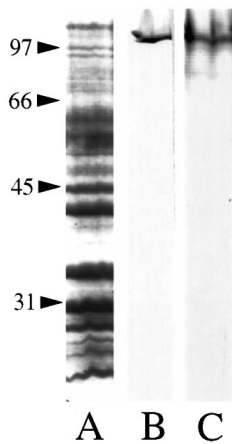
Fig. 5. Immunoblot analysis of α- and β-scruin. (A) A Coomassie stained SDS-PAGE gel of the crude sperm extract used in B and C. (B) Blot affinity purified anti-α-scruin R213.5 serum detects a single 103 kDa band. (C) Anti-β-scruin R214.5 serum recognizes a similar sized single but more diffuse band.

Immunofluorescence microscopy of unactivated *Limulus* sperm using the scruin isoform specific antisera at between 1:100 and 1:1,000 dilution confirmed β-scruin is in sperm but distinct from α-scruin (Fig. 6). While α-scruin was co-localized to the coiled actin bundle at the base of the nucleus (Fig. 6A and B), in contrast, β-scruin was found in the acrosomal vesicle at the anterior of the sperm head (Fig. 6C and D). The binding of R214 antisera was specific, as pre-incubation of the sera with 1 mg/ml M35 peptide completely abolished staining of the vesicle whereas control peptides did not (data not shown). The acrosomal vesicle lacks detectable actin filaments based on the absence of phalloidin staining (Fig. 6D). We also failed to detect G-actin in the acrosomal vesicle by immunofluorescence using an anti sea urchin actin antibody, although this antibody recognized a single band on westerns and stained other regions of the sperm, suggesting that β-scruin is not complexed to G-actin (data not shown).

To localize β-scruin in the acrosomal vesicle we examined the distribution of both scruin isoforms by immuno-EM (Fig.

7). For α-scruin we observed gold particles associated with the actin bundle, seen in cross-section, at the base of the sperm nucleus (Fig. 7A). In contrast, for β-scruin we always saw large numbers of gold particles dispersed evenly throughout the apical acrosomal vesicle. There was no clear association with the membrane or any discernible interior structures (Fig. 7B).

DISCUSSION

The acrosome reaction consists of two separate but linked events, rupturing of the acrosomal vesicle followed by immediate extension of the acrosomal process (Tilney, 1975; Tilney et al., 1979). Our results suggest that both scruin isoforms might be involved in these two processes but that they may perform different functions. Previously, we and others have shown that one scruin isoform, α-scruin, is a component of the acrosomal process and is certainly an actin cross-linking protein (Schmid et al., 1991; Way et al., 1995). Surprisingly, β-scruin, is a component of the acrosomal vesicle, a cellular compartment that does not contain actin. This finding was unexpected, given the sequence similarity between the two proteins and suggests that β-scruin must be functionally different from α-scruin.

In mammals the contents of the acrosomal vesicle are derived from the Golgi apparatus during spermatogenesis (Escalier et al., 1991; Peterson et al., 1992). Initially, in the so called Golgi phase, small dense carbohydrate rich granules appear within the Golgi apparatus. Subsequently, these proacrosomal granules bud off from the Golgi and begin to coalesce into a single large granule surrounded by an acrosomal vesicle. This acrosomal vesicle then becomes associated with the nuclear membrane at a region which will eventually be the anterior portion of the nucleus. In the second or capping phase the acrosomal vesicle spreads over the anterior of the nucleus and continues to enlarge by the fusion of further
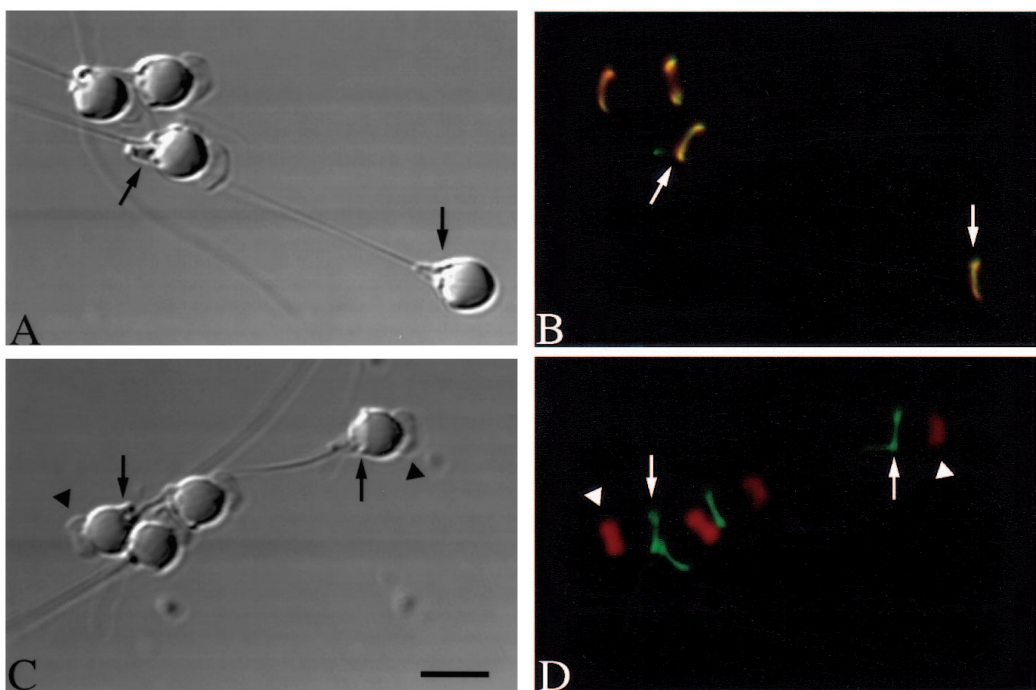

Fig. 6. Subcellular localization of α- and β-scruin in unactivated *Limulus* sperm. A and C show DIC images of unactivated sperm, clearly showing the acrosomal vesicle at the anterior (arrowhead) and the location of the actin bundle at the base of the nucleus at the posterior of the sperm cell body (arrow). B and D show merged images of bodipy phalloidin (green) and affinity purified anti-α-scruin R213.5 serum (B) or anti-β-scruin R214.5 serum (D) in red. In B α-scruin and F-actin co-localize at the base of the nucleus and appear yellow (arrow). In D β-scruin is localized to the apical vesicle (arrowhead) and is not associated with F-actin (arrow). Bar, 5.0 μm.
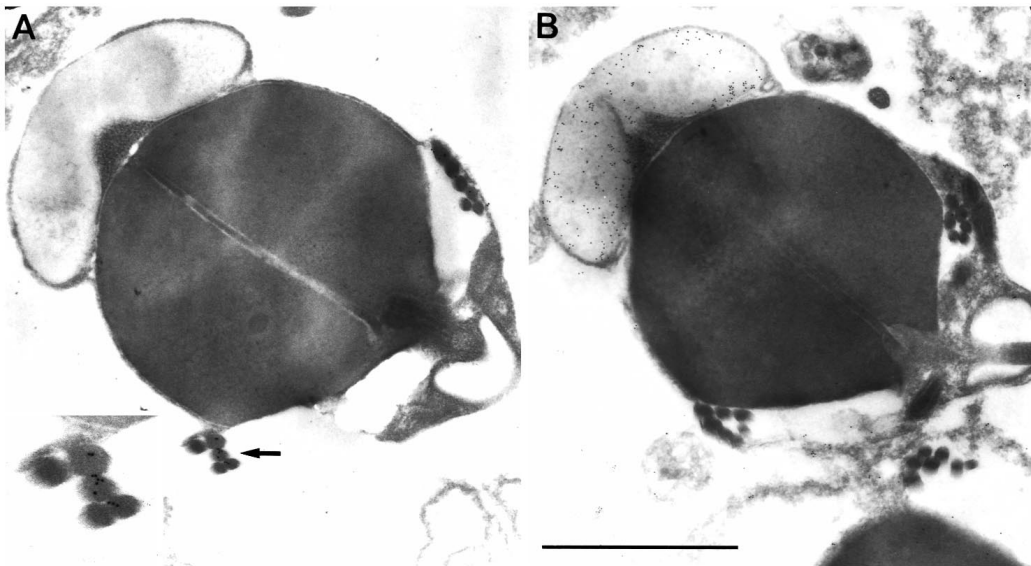
Fig. 7. Immuno-EM localization of α- and β-scruin in unactivated *Limulus* sperm. (A) Gold particles localize α-scruin to the actin bundle coiled around the base of the nucleus seen in cross-section (arrow). The insert shows the region indicated by the arrow at higher magnification. (B) The presence of numerous gold particles over the apical acrosomal vesicle suggests that β-scruin is present throughout the interior of this membrane bound compartment. Bar, 1.0 μm.

Golgi derived vesicles. Thus, the mammalian acrosome can be considered as a specialized secretory vesicle full of hydrolytic enzymes, that is 'stored' for long periods, until it is stimulated upon contact with the egg. It is not unreasonable to think that the acrosomal vesicle of *Limulus* is also derived from the Golgi apparatus by a similar process.

In light of the current information regarding acrosomal vesicle biogenesis, we would expect that any protein found in the lumen of this membrane-bound compartment would possess a hydrophobic signal peptide at its N terminus to direct translocation across the endoplasmic reticulum during its synthesis (Walter and Johnson, 1994). To date, all known examples of lumenal mammalian acrosomal vesicle proteins, including acrosin (Baba et al., 1989), acrogranin (Baba et al., 1993), calreticulin (Nakamura et al., 1993), apexin (Noland et al., 1994; Reid and Blobel, 1994) and the proacrosin binding protein sp32 (Baba et al., 1994) encode an N-terminal signal peptide in their sequence. However, the β-scruin sequence does not reveal such a signal peptide or any other obvious targeting or *trans*-membrane sequences, although the protein is clearly located in the lumen of the vesicle. Although we are currently unable to explain how β-scruin enters the lumen of the endoplasmic reticulum, there are a number of other proteins and peptides that are also transported into the endoplasmic reticulum or secreted from cells by unknown signal independent mechanisms (Muesch et al., 1990; Featherstone, 1990). In the future it may be possible through the transfection of α-and β-scruin hybrids, to localize both the sequence responsible for β-scruin targetting and the actin binding sites of α-scruin.

Given that β-scruin is in the acrosomal vesicle, what is its role? The sequence of β-scruin suggests it contains two putative β-superbarrel domains. To date all β-superbarrel domains, for which atomic structures are available, have only been identified in an extended family of enzymes, including quinoprotein alcohol dehydrogenases and sialidases (Bork and Doolittle, 1994). Given that the acrosomal reaction involves many different enzymes as well as specific glycoprotein mediated interactions with the zona pellucida of the egg, it is possible that β-scruin is a 'sialidase' and carries out an as yet unidentified function, such as modifying the egg or sperm membrane. Alternatively, it may be that the location of β-scruin is a remnant from an earlier role during the development of the sperm. Although localization of β-scruin during spermatogenesis might help to elucidate its possible function, in the absence of in vitro experiments we cannot rule out that it does not bind actin.

Are scruins present in other organisms?

Our phylogenetic analysis of α- and β-scruin suggests they are derived from an ancestral scruin that appears to have arisen from a duplication of a single superbarrel domain. We have also recently identified a partial 1.4 kb clone whose sequence encodes a scruin like protein (M. Way, unpublished results). The full sequence of the third scruin isoform, γ-scruin, may allow us to examine the more distant relationships of repeats in individual superbarrel domains. More importantly, given that *Limulus* diverged early during evolution and contains multiple scruin isoforms, we must ask the question whether scruin homologues exist in other organisms. Database searches using α- and β-scruin sequences have identified 21 human expressed sequence tags for unknown genes, which together contain a total of 36 scruin-like repeat sequences. If we assume that the maximum number of repeats in known sequences, such as kelch, is six, then it is clear that these human ESTs represent several genes, one of which may be a true scruin homologue. We are currently exploring this possibility by isolating full length clones using EST probes as well as using PCR based approaches to isolate a mammalian scruin in addition to isolating the full length γ-scruin gene from *Limulus*.

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