Characterization of the Actin Cross-linking Properties of the Scravin-Calmodulin Complex from the Acrosomal Process of Limulus Sperm*

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During activation of the Limulus sperm acrosomal process, actin filaments undergo a change in twist that is linked with the conversion from a coiled to a straight scravin-actin bundle. Since scravin had not been purified, its identity as an actin-binding protein has not been demonstrated. Using HECAMEG (methyl-6-(N-heptylcarbamoyl)-D-glucopyranoside) detergent extraction in concert with high calcium, we purified native scravin and identified it as an equimolar complex with calmodulin. 125I-Calmodulin overlays and calmodulin-Sepharose indicate that scravin binds calmodulin in calcium but not in EGTA. Overlay experiments also map the calmodulin binding site between the putative N- and C-terminal β-propeller domains within residues 425-446. Immunofluorescence microscopy reveals that calmodulin colocalizes with scravin and actin in the coiled bundle. Although scravin binds calmodulin, pelleting assays and electron microscopy show that the scravin cross-links F-actin into bundles independently of calcium. Based on our biochemical and structural studies, we suggest a model to explain how scravin controls a conformational change in the actin filament and acrosomal process may be caused by the dynamic conformational changes in the acrosome reaction. We predict that calcium subtly alters scravin conformation through its calmodulin subunit and the conformation change in scravin causes a shift in the relative positions of the scravin-bound actin subunits.

In many examples of cell motility including: cytokinesis, phagocytosis, exocytosis, chemotaxis, and extension of the lamella, movement or force is generated by either actin-myosin interactions or the reversible assembly of actin filaments (1). Contrary to these examples, extension of the acrosomal process in Limulus sperm may be a movement of an actin spring in which potential energy, stored as a coiled bundle at the base of sperm body, is unleashed at fertilization to uncoil and extrude the bundle through a channel in the nucleus (2, 3). During the uncoiling process, the actin bundle untwists by an impressive 0.23° per 700 nm. This action is accompanied by slippage and a modest (−0.23° per subunit) untwisting of the actin filaments (4, 5). As a result of these events, the bundle forms a 60-μm-long membrane extension, which bridges the egg jelly coat to fuse with the egg plasma membrane.

The factors that maintain the coiled state of the bundle or signal its rotation and slippage are unknown, but the target of their action must be scravin, an actin-associated protein in the acrosomal process. Previous studies show that the acrosomal process consists of a 1:1 complex of actin and scravin (M, 102,000) (6). In EM2 reconstructions, scravin decorates the outside of an actin filament, with each scravin molecule bound to a pair of actin subunits along the actin one-start helix (7). Presumably, actin cross-links are maintained by interactions between scravin proteins on neighboring filaments.

Based on sequence analysis, limited proteolysis, and EM image reconstructions, scravin is organized into two 40-kDa domains connected by a highly helical protease-sensitive neck (7–9). Each domain consists of a six-fold repeat of ~50 amino acids that based on the work of Bork and Doolittle (10), is typifies a protein superfamily, which includes galactose oxidase (10), several open reading frames in the genome of pox viruses (11), a mouse placental transcript, MIPP (12), and Kelch, the Drosophila gene that is important for nutrient transport during oogenesis (13). Although there is some understanding of the structural organization of scravin, its regulation and biochemical properties are not understood because the protein has not been purified in a native, soluble state.

To identify the mechanism that causes the dynamic conformational changes in the acrosomal process during the acrosome reaction, we must first purify scravin and characterize its actin binding properties. In this report, we describe the isolation of scravin and report its association with calmodulin. Furthermore, we show that the scravin-calmodulin complex cross-links F-actin into bundles but, surprisingly, the cross-linking activity is independent of calcium. Our results suggest that scravin is always bound to actin filaments, and we hypothesize that during the acrosome reaction the conformational changes in the actin filament and acrosomal process may be caused by a subtle conformation change in scravin.

EXPERIMENTAL PROCEDURES

Materials—Pepstatin, leupeptin, aprtinin, benzamidine, and ATP were purchased from Sigma. HECAMEG and calciion ionophore A23187 were purchased from Calbiochem. 125I-Calmodulin was purchased from DuPont NEN. Artificial sea water was purchased from Tropic Marin. Protein assays were performed with the Bio-Rad assay reagent.

Purification of the True Discharge—The true discharge was isolated using a protocol modified from Schmid et al., (6). Twenty ml of sperm,
collected from 30–40 adult male horsehoe crabs, was divided into six aliquots, layered on 33 ml of a 1:1 mixture of artificial sea water and 50 mM calcium chloride, and then gently mixed. Sperm were activated by adding 100 μl of 19 mM A23187 (in dimethyl sulfoxide) to each tube and incubated in the dark for 30 min. To prevent proteolysis, a mixture of protease inhibitors were added to each step (final concentrations: 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, 0.1 mM pepstatin A, 1.0 KIU/ml aprotinin, and 0.2 μg/ml leupeptin). The activated sperm were sheared three times through a 21-gauge needle and centrifuged twice at 2420 × g for 10 min to remove the sperm heads. The supernatant was then centrifuged at 43,140 × g for 15 min to pellet the acrosomal bundles. Each pellet was resuspended in 5 ml of scrin buffer A (50 mM Tris, pH 8.0, 1 mM dithiothreitol, 1 mM CaCl₂, and 0.01% NaN₃) with 19 mM HECAMEG for 15 min on ice. The volume was diluted to 30 ml with scrin buffer A and centrifuged at 43,140 × g for 15 min. The pellets were pooled into 1 ml of buffer A and pelleted at 16,000 × g in a microcentrifuge for 15 min. To quantify the stoichiometry of scrin and calmodulin by HPLC, this washing step was repeated five times to reduce the protein contaminants. In a typical preparation, 5.2 mg of true discharge was obtained as determined by the Bio-Rad assay. The purity of the true discharge preparations was assessed by SDS-PAGE.

Purification of Soluble Scrin—Scrin was dissociated from actin by gently shaking the isolated bundles in 1 ml of 1 M calcium for 1 h at room temperature, and then centrifuging the extract for 15 min at a microcentrifuge (16,000 × g) to remove most of the actin filaments. The low speed supernatant containing primarily scrin, calmodulin, actin, and some minor contaminants (3.7 mg of total protein) was successively filtered through 0.8-μm filters. In the later stages of the project, nucleic acids in the extract were removed by filtration through a 0.8-μm filter and then a QIagen Tip 5. The filtrate was loaded onto a Superdex 200 HR FPLC gel filtration column pre-equilibrated and run in scrin buffer A supplemented with inhibitors. The fractions that contained scrin were pooled (1.1 mg of total protein), loaded onto a Q-HiTrap (Pharmacia Biotech Inc.) ion exchange column with scrin buffer A eluted with a 0–5 M NaCl gradient in buffer A. Scrin protein eluted at 0.4 M NaCl. The scrin-containing fractions (300 μg of total scrin) were dialyzed in a 10,000-Dalton cut-off membrane in liquid nitrogen and stored as a lyophylate or concentrated by Ultrafree-MC centrifugal membrane filters (Millipore) and used immediately.

Identification of Calmodulin—The stoichiometry of the scrin-actin-calmodulin complex was determined by size exclusion chromatography through a TSK 300SWXL column (Toso Soda) under denaturing conditions (6 M guanidine HCl). The absorbance was measured at 230 nm. The molar ratio was calculated from the integrated peak areas. The 17-kDa polypeptide was identified as calmodulin by protein sequence obtained by mass spectrometry or chemical sequencing. First, the scrin-calmodulin complex was dissociated in 6 M guanidine HCl and separated by size exclusion chromatography. The purified calmodulin was cleaved with cyanogen bromide in 70% formic acid for 12 h as described (Matsudaira, 1992). Following incubation the same calmodulin sample was separated by size exclusion chromatography. The purified calmodulin complex was determined by size exclusion chromatography through a TSK 300SWXL column (Toyo Soda) under denaturing conditions.

The molar ratio was calculated from the integrated peak areas. The peptides and contaminants were separated by size exclusion chromatography. The purified calmodulin was cleaved with cyanogen bromide in 70% formic acid for 12 h as described (Matsudaira, 1992). Following incubation, the same calmodulin sample was separated by size exclusion chromatography. The purified calmodulin complex was determined by size exclusion chromatography through a TSK 300SWXL column under denaturing conditions.

Identification of the Calmodulin Binding Site—125I-Calmodulin gel overlays were performed as described previously (15) with minor modifications (16). 125I-Calmodulin nitrocellulose blot overlays were performed as described (17) using 0.05% Tween 20 and 30 mg/ml bovine serum albumin as the blocking agents. For scrin, we found that either methanol or acetone nitrocellulose overlays had a higher signal to noise ratio. In contrast, the myosin I positive control only worked with the gel overlay method. The percent binding was quantitated using NIH Image software and is represented as the ratio of the signal intensity and the total signal in calcium and EGTA × 100. A value of zero indicates the signal was not above the background levels. Competition assays with scrin and the peptide PSN1 (described below) were performed in calcium only.

Actin Cosedimentation Assays—Cosedimentation assays with purified scrin (0–4 μM), sperm calmodulin (15 μM), and rabbit skeletal actin (2 μM) were performed in the presence of 1 mM EGTA or 1 mM calcium as described previously (16). Briefly, samples were incubated at 4 °C overnight with 1% Triton X-100. The samples were negatively stained with 1.0% uranyl acetate and examined in a Siemens 101 electron microscope at an accelerating voltage of 80 kV. Some instances calmodulin was added to the samples at 0–5-fold molar excess of scrin.

Immunofluorescence Microscopy—A rabbit polyclonal antiserum (R2135) was generated against scrin; the specificity to crude sperm extracts has been described elsewhere (18). The mouse monoclonal anti-calmodulin antisera was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). To reduce the nonspecific background of the anti-scrin antibody for immunofluorescence, the antibody was affinity-purified against scrin blotted to nitrocellulose membrane (19). Sperm were fixed and stained as described elsewhere (18). Coverslips were examined in a Zeiss Axioskope microscope using differential interference contrast optics and a 100 μm filter. 3–3 Plan Neofluar objective configured with a B-2 Bauchard MC600 Confocal laser. The mean intensity in the bundle and the entire sperm was quantified using the standard confocal image analysis software. For reproduction, the images were transferred to Adobe Photoshop and Illustrator (Adobe Systems Inc., Mountain View, CA) for cropping, contrast/brightness adjustment, placement of each image panel, and annotation.

RESULTS

Treatment of true discharges, isolated in Triton X-100, with 1 M calcium dissociated scrin from actin. However, after a few hours, the soluble scrin precipitated from solution (not shown). Subsequent experiments determined that long term solubility depended on the removal of the Triton X-100 by dialysis at high ionic strength (0.45 M NaCl). Other treatments such as denaturing agents (8 M urea) and low pH (0.2 M glycine, pH 4.2) were found to also dissociate the scrin actin bundles, but less than half of the scrin remained active, as judged by high speed sedimentation with rabbit skeletal actin (100,000 × g for 30 min: not shown). Based on these findings, we replaced Triton X-100 with the non ionic detergent HECAMEG. HECAMEG is easier to remove by gel filtration or dialysis (critical micelle concentration 19.5 mM) and does not absorb at 280 nm. After demembranation of the actin bundle with HECAMEG and extraction of scrin with 1 M CaCl₂, one half of the actin pellets at low g-forces leaving scrin in the superna-
tant (Fig. 1A). The minor protein contaminants, DNA, and HECAMEG in the calcium extract were removed by gel filtration chromatography through Superdex HR 200 and ion exchange (Fig. 2). Based on relative molecular mass (Mr) standards, scruin fractionates as a monomer with an apparent molecular weight of 107,000.

Identification of Calmodulin—At each stage of the purification, a 17-kDa polypeptide consistently cofractionated with scruin (Figs. 1 and 2). Although the N terminus was blocked to chemical sequencing, we obtained sequence from two fragments generated by cyanogen bromide cleavage (MKDTD-SEEEI and MIREADIDGDQVNYEEFVTM). Data base searches with these two sequences showed an exact match with calmodulin from Drosophila. Additionally, this 17-kDa protein shows the characteristic calcium-dependent mobility shift on SDS-PAGE gels and is recognized by a monoclonal antibody to calmodulin (not shown).

The presence of calmodulin was also confirmed by immunofluorescence microscopy (Fig. 3). In unactivated sperm cells, calmodulin is localized to the base of the nucleus as a ring of fluorescence, which colocalizes with the rhodamine phalloidin staining pattern of F-actin or immunostaining of scruin. Quantitation of the fluorescence showed that 71% (S.D. = ± 7.6, n = 7) of the calmodulin was found to localize to the bundle at the base with a less apparent staining in the nucleus and the perimeter of the acrosomal vesicle. No calmodulin staining was observed within the flagellar region of the sperm or the interior of the acrosomal vesicle.

To determine the stoichiometry of the scruin, actin, and calmodulin in the acrosomal process, we quantified the peak areas of samples separated by gel exclusion chromatography in denaturing conditions. Based on the integrated peak areas and the known molecular masses for the proteins, the scruin:actin:calmodulin molar ratio was 1.00:1.15:0.97 (Fig. 1B). Although scruin and calmodulin were always seen to co-purify, the molar ratio of the two proteins was sometimes 1:0.5, depending on the individual preparation. This variability in the ratio of scruin to calmodulin after purification either is due to the extraction conditions or is merely a consequence of an equilibrium for binding and subsequent dissociation of calmodulin during purification.

Calmodulin Binding Site—To confirm that calmodulin is a scruin subunit, we tested binding of exogenous bovine calmodulin to scruin. By two criteria: 125I-calmodulin blot and gel overlays (Fig. 4) and calmodulin-Sepharose chromatography (not shown), calmodulin binds scruin in calcium but not in EGTA.

Although scruin is predicted to be mainly β-sheet, secondary structure analysis predicted that the neck region (Fig. 5a) between the N- and C-terminal domains of scruin is highly helical and amphipathic. A comparison of this region with the calmodulin binding IQ motif of MYO2 highlights the similar pattern of basic and hydrophobic residues. A helical wheel representation of a portion of the neck region clearly shows a basic and a hydrophobic face of the helix (Fig. 5b; Refs. 20 and 21). Because calmodulin binds to the basic face of an amphipathic helix, we examined calmodulin binding to various synthetic peptides, proteolytic fragments, and GST fusions that

**Fig. 1.** Isolation and calcium treatment of scruin-actin bundles and stoichiometry of scruin-actin-calmodulin complex. A, SDS-PAGE of true discharges extracted with HECAMEG (lane T) show the presence of scruin, actin, and a 17-kDa protein. The supernatant (lane S) of the calcium extract contained mostly scruin and a 17-kDa protein, whereas the pellet (lane P) was enriched in actin. B, HPLC traces of washed HECAMEG true discharges indicate that the ratio of scruin:actin:calmodulin is essentially 1:1:1.

**Fig. 2.** Copurification of calmodulin and scruin by gel filtration and quaternary amine ion exchange chromatography. A, the low speed supernatant was chromatographed through Superdex 200 HR (top panel); SDS-PAGE (bottom panel) of the fractions shows scruin and a 17-kDa polypeptide co-fractionate. B, a pool of scruin-containing fractions was loaded onto a Q-HiTrap column. The chromatograph (top panel) indicates a single peak elutes at 40% buffer B. SDS-PAGE (bottom panel) shows that scruin co-purifies with a 17-kDa band that was determined by internal protein sequencing to be calmodulin.
spanned the neck region. Two GST fusion constructs, GST1 and GST3, contain sequences that border outside of the predicted calmodulin binding site. These fusion constructs did not bind the radiolabeled calmodulin (Table I). The third GST fusion (GST2) which contains this region did not express in E. coli; however, a synthetic peptide, PSN1, to the region 425–446 did bind calmodulin in EGTA and calcium. The peptide at a concentration of 50 nM also inhibited calmodulin binding to intact scruin by 61% (Fig. 4D). In other experiments, the C-terminal half of scruin (454C and 590C, produced by expression in E. coli; Ref. 9), a tryptic digestion of scruin, or natural breakdown products of scruin did not bind to the radiolabeled calmodulin in the presence or absence of calcium (not shown). These proteolytic sites have been previously mapped to the protease-sensitive neck region of scruin (9), suggesting that the calmodulin binding site is not in the C- or N-terminal halves of scruin.

Characterization of Actin Cross-linking Activity—Because scruin is the only actin-associated protein in the acrosomal process, we tested it for actin binding activity using a cosedimentation assay and electron microscopy. Scruin binds actin independently of calcium. In calcium and EGTA, the majority of scruin is found associated with actin in the pellet (Fig. 6). There is no difference between binding in EGTA and calcium. Furthermore, the amount of actin found in the pellet is invariant suggesting that scruin does not alter the F- to G-actin equilibrium. In the absence of actin, scruin remains in the supernatant in high g-force sedimentation assays (not shown). Since exogenously added calmodulin may affect the binding affinity of scruin for actin, cosedimentation assays were also performed with a 3.75-fold molar ratio of exogenously Limulus calmodulin (not shown). Examination of the samples by electron microscope before centrifugation shows that actin bundles form in the presence and absence of calcium (Fig. 7). The results from sedimentation assays and electron microscopy show that the presence or absence of excess calmodulin does not detectably affect actin binding activity of scruin.

DISCUSSION

The long term goal of our studies is to understand how the actin bundle uncoils during the acrosome reaction to form the 60-μm-long acrosomal process. We have made major strides toward this goal through the development of methods to separate and purify native scruin from actin. Although there are several three-dimensional views of the filaments in the acrosomal process (7, 8, 22), this study provides the first biochemical information about the protein components of the acrosomal process. Two tricks enabled us to purify scruin as a soluble and native protein and characterize it as an actin cross-linking protein. First, because calcium disrupts the acrosomal process (2), we were able to extract scruin as a native protein without using denaturants. Second, we modified the demembranation step by replacing the detergent Triton X-100 with HECAMEG. This eliminated the aggregation of the purified scruin and improved the overall yield of protein.
b.-sheet domains, which are separated by a highly helical region. The domain organization suggested by the sequence is in exact correspondence with the EM reconstructions; thus, we propose that the helical region of sequence 907–927 in scruin is the neck region. Within this helical region is the calmodulin binding site. In this position, changes in the conformation of calmodulin could alter the relative positions of the calmodulin binding domains (Fig. 8). Based on our studies, we propose a model that calmodulin acts as a wedge between the actin-binding domains. We had not detected calmodulin previously because of its low molecular weight and relatively poor dye-binding capacity (6, 8, 23). Because the previous EM reconstructions were of acrosomes that may have been partially depleted of calmodulin (prepared in the absence of calcium), we are checking the new preparations to see if the neck region is thicker from the presence of calmodulin.

Our present evidence shows both calcium-dependent binding of calmodulin to intact scruin and calcium-independent binding to the PNS1 scruin neck peptide. We speculate that the flanking large domains in scruin partially inhibit the binding of calmodulin in the absence of calcium. In either case the immunofluorescence localization of calmodulin within the membrane-limited acrosomal process suggests the possibility that the local concentration of calmodulin could be high. A high calmodulin concentration would ensure a calmodulin-scruin complex is maintained in unactivated sperm.

The presence of a calmodulin subunit immediately suggests that actin binding by scruin is calcium regulated. Normally, in many enzymatic complexes, calmodulin is a regulatory subunit, which binds or dissociates from a target catalytic subunit and thus acts as a calcium-dependent switch to activate or inactivate the enzyme. However, our biochemical studies suggest a different type of regulatory mechanism, because scruin binds actin independently of calcium. We conclude that calmodulin does not regulate actin binding activity in an on-off fashion. This finding eliminates a simple on-off binding event as a mechanism for inducing the conformation changes in the actin filament. Instead, our results suggest that scruin is bound to actin before as well as after the acrosome reaction. Although the coiled bundle of unactivated sperm has not been studied, our speculation is supported by a related structure, the supercoiled false discharge, which has been shown to contain scruin (8). Based on our studies, we propose a model that calmodulin may instead control the twist of an actin filament or bundle by regulating the conformation of scruin. We envision a mechanism in which calmodulin acts as a wedge between the actin-binding domains (Fig. 8). In this position, changes in the conformation of calmodulin could alter the relative positions of the actin binding domains. The conformation change in scruin is then transmitted to the underlying actin subunits which allows the actin filaments to untwist by 0.23° between subunits (4, 5).
The local change in twist is multiplied along the length of the filament causing a large change in the filament. The change in filament twist breaks scruin-scruin cross-links between neighboring filaments which allows the filaments to slip as the bundle uncoils into the straight acrosomal process. The caveat to this model is that we do not yet appreciate how scruin-scruin interactions allow for the formation of bundled filaments, since under the present conditions purified scruin appears to be monomeric. It is possible that scruin interactions with itself are much weaker than actin-scruin or scruin-calmodulin interactions. Alternatively, the scruin-scruin binding site may not be exposed until scruin binds actin.

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**Fig. 6. Cosedimentation of scruin with actin in EGTA and calcium.** A, various concentrations of scruin were incubated with 2 μM actin in the presence of EGTA (■) and calcium (○). SDS-PAGE samples of supernatants and pellets show that in calcium and EGTA scruin binds avidly. B, quantitation of the pelleting assays shows the slightly higher affinity for actin in calcium than in EGTA. However, there is no apparent difference in actin binding in the presence of exogenous sperm calmodulin (data not shown). In the absence of actin, all scruin remained in the supernatant (data not shown).

**Fig. 7. Reconstitution of scruin actin bundles.** Based on electron microscopy, scruin is capable of forming bundles with rabbit skeletal F-actin in the presence of EGTA (a) and calcium (b). These bundles have a similar packing to the Limulus bundles (c) but are not crystalline. In the absence of scruin F-actin does not form bundles, and in the presence of scruin alone no filamentous structures are observed (data not shown). With the addition of exogenous sperm calmodulin at a 3.75-fold molar excess over scruin, there is no apparent difference in the formation of bundles (data not shown). Bar = 100 nm.

**Fig. 8. Model for extension of the bundle during sperm activation.** A cross-section diagram (viewed from the tip) of a scruin-bound actin filament in the acrosomal process before and after calcium binding. A pair of actin subunits is associated with a scruin calmodulin complex that is not drawn to scale, and the angular untwisting is exaggerated to show this subtle change in structure. Upon sperm activation, calcium ions bind to calmodulin, which initiates a series of conformation changes. First, in calcium, calmodulin binds scruin more tightly, which then induces a conformation change in scruin. Consequently, scruin binds more tightly to actin, which causes a subtle rearrangement in the pair of actin subunits. This rearrangement allows the unbending and extension of the actin bundle.
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