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The Exocytosis Regulatory Proteins Syntaxin and VAMP Are Shed from Sea Urchin Sperm during the Acrosome Reaction

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Syntaxin is a cytoplasmically oriented plasma membrane protein and VAMP (vesicle-associated membrane protein; synaptobrevin) is a protein associated with the secretory vesicle membrane. These two proteins form part of a complex which is thought to mediate the fusion of plasma and vesicle membranes during exocytosis. This paper reports the identification of syntaxin and VAMP homologues in sea urchin sperm. During fertilization, sea urchin sperm release the contents of a single vesicle, the acrosomal vesicle, exposing the membrane destined to fuse with the egg. During acrosomal exocytosis, the plasma membrane over the acrosomal vesicle fuses at multiple points with the acrosomal membrane (vesiculation) and syntaxin and VAMP are shed with the resulting membrane vesicles. Sea urchin sperm syntaxin and VAMP are associated in a complex as detected by immunoprecipitation. Following acrosomal exocytosis, syntaxin and VAMP cosediment to denser fractions on sucrose gradients showing that they have undergone associative changes during or after the acrosome reaction. Syntaxin and VAMP localization and loss during acrosomal exocytosis support a role for these proteins in regulating the acrosome reaction. © 1997 Academic Press

INTRODUCTION

Sperm of many animals, including sea urchins and mammals, contain a single exocytotic vesicle, the acrosomal vesicle, in close association with the plasma membrane. When sea urchin sperm encounter the outer investments of the egg, a sulfated fucan (Alves *et al.*, 1997) in the egg jelly activates a receptor in the sperm plasma membrane (Moy *et al.*, 1996). Occupancy of the egg jelly receptor activates ion channels (Darszon *et al.*, 1996) which regulate exocytosis of the acrosomal vesicle (the acrosome reaction). The acrosome reaction is unique in that the plasma membrane and acrosomal vesicle membrane fuse at multiple points (Epel and Vacquier, 1978; Yanagimachi, 1988; Barros *et al.*, 1967). These membrane fusions result in the formation of acrosome reaction vesicles (ARVs)[2] which are shed from sperm. This event exposes the gamete recognition protein bindin (Vacquier *et al.*, 1995) from the acrosomal vesicle and is followed by elongation of the acrosomal process (Tilney and Inoue, 1985). Acrosomal exocytosis requires influx of Ca^{2+} and can be induced by ionophores (Podell and Vacquier, 1984) or by addition of Ca^{2+} to permeabilized sperm cells (Castellano *et al.*, 1995).

When treated with egg jelly or ionophore, an entire population of sea urchin sperm undergoes acrosomal exocytosis simultaneously, making these cells a useful model for studying Ca^{2+}-triggered exocytosis. The molecular details underlying the signaling events from sperm receptor for egg jelly to acrosomal exocytosis remain unknown (Darszon *et al.*, 1996; Moy *et al.*, 1996). Similarities between sea urchin and mammalian sperm, such as the presence of voltage-dependent Ca^{2+} channels with the same biophysical properties, suggest that conserved regulatory mechanisms underlie the acrosome reaction (Beltran *et al.*, 1994; Darszon *et al.*, 1996).

Isolation and characterization of proteins on the presynaptic and synaptic vesicle membranes of neurons have increased our understanding of the regulation of exocytosis. Two of these proteins are syntaxin, an intracellular protein integral to the plasma membrane (Bennett *et al.*, 1992,

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[2] Abbreviations used: VAMP, vesicle-associated membrane protein; ARVs, acrosome reaction vesicles; HMVs, homogenized sperm membrane vesicles.

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1993), and VAMP, also referred to as synaptobrevin, which is integral to the synaptic vesicle membrane (Baumert *et al.,* 1989; Trimble *et al.,* 1988). In the presence of the ATPase NSF and SNAPs (soluble NSF attachment proteins), syntaxin and VAMP form a 20S complex with SNAP-25 (synaptosome-associated protein, 25 kDa; Oyler *et al.,* 1989). This complex is disrupted by ATP hydrolysis and is believed to be an intermediate in the vesicle priming step leading to membrane fusion (Söllner *et al.,* 1993a; Wilson *et al.,* 1992). These findings implicate syntaxin and VAMP as being involved in the priming and fusion steps of synaptosomal exocytosis. Furthermore, syntaxin binds to (Martin *et al.,* 1996; Sheng *et al.,* 1994, 1996) and presumably attenuates voltage-dependent Ca^{2+} channels (Bezprozvanny *et al.,* 1995) in the presynaptic plasma membrane, linking the channel to the regulated influx of Ca^{2+} prior to membrane fusion. In the present study we have identified syntaxin and VAMP homologues in sea urchin sperm. Due to the unique nature of acrosomal exocytosis, which involves the fusion of the plasma and vesicle membranes at multiple points, these proteins are released from sperm with the shed membrane vesicles and undergo associative changes.

MATERIALS AND METHODS

Amplification and Sequencing of VAMP and Syntaxin Homologues

VAMP and syntaxin homologues were PCR amplified from a testis cDNA library following a standard PCR protocol (94°C, 30 sec; 52°C, 45 sec; and 72°C, 90 sec) for 35 cycles. This was followed by a termination cycle of 94°C, 30 sec; 52°C, 45 sec; and 72°C, 10 min. Degenerate primers were used to amplify VAMP (BRVNF, 5′-CARCARACNCARCNCARGT-3′; BRVNR, 5′-CATCATYTTRCARTTYTTCCACCA-3′) and a mixture of primers were used to amplify overlapping syntaxin PCR products in seminested reactions as well as across the entire fragment encoding the sequence presented in Fig. 1 (TAXAF, 5′-GCGGATCCGAGCTCAGAGATCGCTTAGGGTCG-3′; SYX1A, 5′-AGNAARTTYGTNGARGT-3′; SYX2A, 5′-CATYTCNCCYTGNBWYTC-3′; TXN4, 5′-CATDATYTTYTTNCKNCKNGC-3′) (N = A,C,G,T; R = A,G; Y = C,T; K = G,T; B = C,G,T; W = A,T). PCR products were sequenced using ABI Prism dye terminator cycle sequencing on an ABI Model 300 automated sequencer (Perkin–Elmer Corp., Foster City, CA).

Gametes and Preparation of Sperm Proteins

Sea urchins, *Strongylocentrotus purpuratus,* were spawned by intracoelomic injection of 0.5 *M* KCl. Isolation of sperm heads and flagella was as described (Vacquier, 1986) and cortical granule lawns were prepared as described (Vacquier, 1975). Isolated lawn proteins were precipitated with 10% trichloroacetic acid (TCA), washed with 95% ethanol, and solubilized in Laemmli sample buffer containing 5% (v/v) β-mercaptoethanol. Sperm were separated from phagocytic pigment cells and sedimented to remove seminal plasma proteins as described (Vacquier, 1986). The washed sperm cells were then used for the preparation of membrane proteins. Sperm membranes were prepared by homogenization of sperm in Ca^{2+}-free seawater buffered with 10 m*M* Hepes, pH 7.8 (Vacquier, 1986), removing sperm heads and flagella by centrifugation at 10,000*g* (Sorvall SS-34) and collecting membrane vesicles by centrifugation for 30 min at 40,000*g* (Sorvall SS-34, 4°C). The collected membrane vesicles were approximately 10-fold enriched for known plasma membrane markers (Podell *et al.,* 1984) and are referred to as homogenized sperm membrane vesicles (HMVs). To induce acrosomal exocytosis, sperm (from 15 ml of undiluted semen, 6×10^{11} cells) were acrosome reacted by treatment with 40 μM ionophore nigericin (Sigma Chemical Co., St. Louis, MO; Podell and Vacquier, 1984) in seawater buffered with 10 m*M* Hepes (pH 7.8) at a 1:100 dilution of semen. After 10 min the cells were pelleted at 10,000*g* (Sorvall SS-34) for 30 min. Sperm acrosome reacted with ionophore are morphologically indistinguishable from sperm acrosome reacted with egg jelly. Shed membrane vesicles, referred to throughout as ARVs, were collected from the 10,000*g* (Sorvall SS-34) supernatant by centrifugation for 1 hr at 180,000*g* in an airfuge or Ti 45 rotor (Beckman Instruments, Palo Alto, CA). ARVs are enriched for known plasma membrane proteins as determined by SDS–PAGE and silver staining and are devoid of large particulate matter and fractured flagella visible by phase-contrast microscopy. To obtain solubilized proteins, ARVs or sperm, washed in Ca^{2+}-free permeabilization buffer, pH 7.2 (Castellano *et al.,* 1995), were resuspended in solubilization buffer (SB; 150 m*M* NaCl, 50 m*M* Tris, pH 7.4, 1 m*M* EDTA, 0.4% Nonidet P-40), rotated for 4 hr at 4°C, and cleared by centrifugation at 180,000*g* (Beckman Ti 45) for 30 min. Syntaxin and VAMP are solubilized under these conditions while the abundant histone proteins are not. Proteins were quantified with the microtiter BCA protocol using bovine serum albumin (BSA) as a standard (Pierce Chemical Co., Rockford, IL).

Antibodies and Immunofluorescence

Antibodies to sea urchin egg syntaxin and VAMP were prepared, characterized and found to be specific for each protein (Conner *et al.,* 1997). Rabbit antisera were used at a dilution of 1:1000 for immunoblotting of 12.5% SDS–PAGE on Immobilon P membranes (Millipore, Bedford, MA); reactions were detected with Supersignal CL-HRP reagents (Pierce). Labeled goat antibodies (TRITC or peroxidase-conjugated) against rabbit IgG were obtained from Cappel (West Chester, PA).

For indirect immunofluorescence localization, acrosome-reacted or unreacted sperm cells were washed in Ca^{2+}-free permeabilization buffer (pH 7.2) lacking digitonin (Castellano *et al.,* 1995). Cells were fixed in 2% paraformaldehyde in the above buffer for 30 min at room temperature, permeabilized in 0.1% Triton X-100 (30 min, 4°C), and blocked with 1% BSA and 1% polyvinylpyrrolidone (PVP-40) overnight at 4°C. Cells were incubated on ice in permeabilization buffer with a 1/50 dilution of the primary antibody, followed by washing and a 1/200 dilution of the TRITC-conjugated secondary antibody. All antibody incubations and washes were in permeabilization buffer containing 1% BSA and 1% PVP-40.

For immunoprecipitations, 5 μl of each antiserum was added to 200 μg of solubilized sperm protein in 1 ml of SB and incubated at 0°C for 1 hr. Immunocomplexes were collected with prewashed protein A–Sepharose beads (Sigma Chem. Co.; 20 μl packed beads), washed five times in SB, and resuspended in Laemmli sample buffer. Samples were immunoblotted and primary antibodies were detected with HRP-conjugated protein A (Sigma).

Sucrose Density Gradients of Solubilized Sperm Proteins

Solubilized sperm proteins were prepared as described above. Aliquots of 200 μg were layered on 7.5 to 25% sucrose gradients

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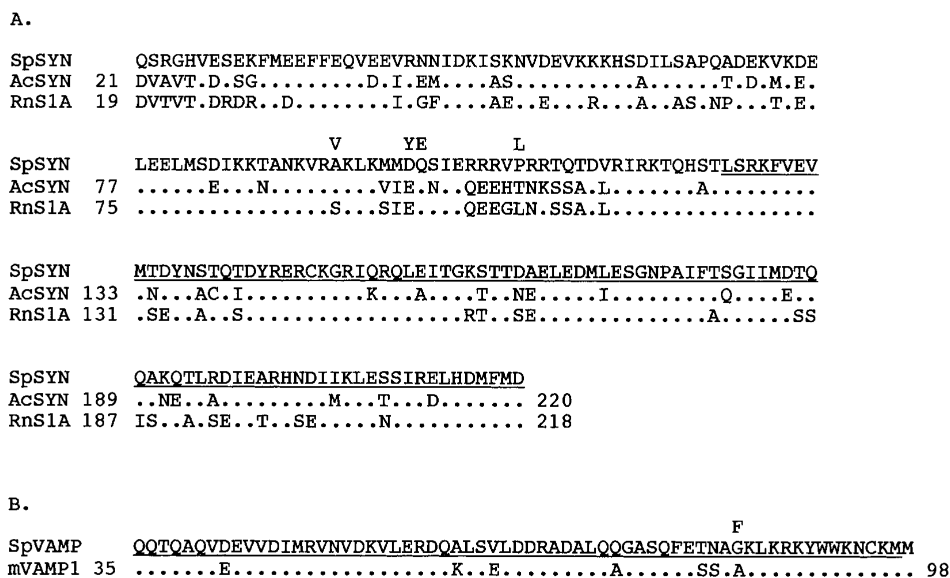

FIG. 1. Alignment of deduced partial amino acid sequences of sea urchin (*Strongylocentrotus purpuratus*) syntaxin and VAMP homologues (SpSYN and SpVAMP) with the highest scoring non-sea urchin sequences using BLASTP 1.4.9MP (Altschul *et al.*, 1990). Periods indicate amino acid identity between aligned sequences. Letters above the aligned Sp testis sequences indicate the four amino acid differences with the sea urchin (*S. purpuratus*) ovary forms (Conner *et al.*, 1997). Underlining of the sea urchin sequences indicates regions used to generate polyclonal antibodies against syntaxin and VAMP (Conner *et al.*, 1997). (A) Alignment of the sea urchin testis syntaxin sequence (SpSYN; GenBank Accession Number AF015486) to positions 21–220 of *Aplysia californica* syntaxin (AcSYN; GenBank Accession Number U03123). The aligned sequences are 72% identical. Also included in the alignment are positions 19–218 of *Rattus norvegicus* syntaxin 1A (RnS1A; Genbank Accession Number A38141) which is 71% identical to the sea urchin syntaxin sequence. The sea urchin testis partial sequence differs from the ovary sequence at four positions. (B) Alignment of the sea urchin testis VAMP (SpVAMP) sequence to positions 35–98 of mouse VAMP 1 (mVAMP1; GenBank Accession Number U61751). The aligned sequences are 89% identical. There is a single amino acid difference between the sea urchin testis and ovary sequences (F at position 50).

(4.4 ml), prepared in SB, and centrifuged at 300,000g for 20 hr at 4°C in a SW60 rotor (Beckman Instruments). Fractions of 190 μl were collected and 50 μl was analyzed by SDS–PAGE and immunoblotting.

RESULTS

Determination of Sequences Encoding Syntaxin and VAMP Homologues in Sea Urchin Testes

Using degenerate and exact match primers, multiple overlapping PCR products were amplified from a sea urchin testis cDNA library encoding a single form of syntaxin. The partial sea urchin sequence of 200 amino acids aligns with *Aplysia* syntaxin (AcSYN) positions 21–220 (full length = 289 residues); where the two sequences are aligned they are 72% identical (Fig. 1A), confirming that the two are homologues. For comparison, sea urchin syntaxin and rat syntaxin 1A (RnS1A) are 71% identical over this region (Fig. 1A), indicating a high degree of sequence conservation. The 200 residues of the sea urchin testis syntaxin are 98% identical to the syntaxin homologue expressed in the ovary of this species of sea urchin (Conner *et al.*, 1997). The differences between ovary and testis forms are localized to a region of high variability between AcSYN residues 93 and 115 (Fig. 1A). In addition to these amino acid differences, there are only two silent substitutions between the aligned sea urchin syntaxin testis and ovary DNA sequences, suggesting that these testis and ovary syntaxins are allelic and not different protein isoforms.

Using degenerate primers, a single PCR product was obtained from a testis cDNA library encoding 64 residues of the sea urchin VAMP homologue, which aligns to residues 35–98 (full length = 118 residues) of mouse VAMP 1 (Fig. 1B). Where they align the two VAMP sequences are 89% identical. The sea urchin testis VAMP homologue differs from the recently identified sea urchin ovary homologue (Conner *et al.*, 1997) at only one position (F at position 50), suggesting that the same VAMP isoform is expressed in both testis and ovary.

Identification of Syntaxin in Sea Urchin Sperm

Using antibodies generated against the underlined sequence shown in Fig. 1A (Conner *et al.*, 1997), syntaxin was identified in sperm which comigrated with syntaxin of sea urchin egg cortical granule lawns (Fig. 2, lanes 1 and 2). To determine if syntaxin is selectively localized, sperm were

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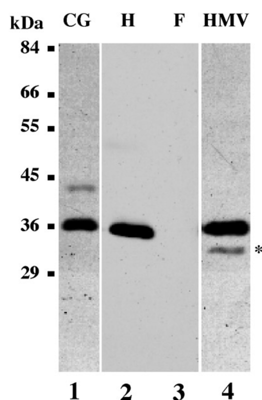

FIG. 2. Identification and localization of syntaxin in sea urchin sperm. Antibodies to sea urchin egg syntaxin recognize syntaxin (36 kDa) in sperm that comigrates with the egg form by immunoblotting egg cortical granule (CG) lawn protein (lane 1, 20 μg). Isolated sperm heads (lane 2) and flagella (lane 3) protein (10 μg) show that syntaxin is localized exclusively in sperm heads. Syntaxin is also present in homogenized sperm membrane vesicles (HMVs) (lane 4, 5 μg). Asterisk indicates an apparent syntaxin breakdown product of 34 kDa that appears in the HMVs (lane 4). A larger cross-reactive band is also present in the CG preparation which may be a result of crosslinking by ovoperoxidase released from disrupted cortical granules (Foerder and Shapiro, 1977; Hall, 1978; Heinecke and Shapiro, 1990).

fractionated into heads (acrosomal vesicle, nucleus, and midpiece) and flagella. Syntaxin localized exclusively to the sperm head based on reactivity on immunoblots (Fig. 2, lanes 2 and 3). Syntaxin was also detected in HMVs suggesting association with the plasma membrane (Fig. 2, lane 4). A proteolytic cleavage product was detected in HMV and ARV preparations at 34 kDa (Fig. 2, lane 4, asterisk). We believe this is a cleavage product of syntaxin and not a cross-reactive band because this product is not found in unreacted sperm heads (Fig. 2, lane 2). It only appears following preparation of HMVs or acrosomal exocytosis when ARVs are shed. Also, the abundance of this breakdown product increases, and full-length syntaxin decreases, with increasing length of incubation during sperm plasma membrane preparation (data not shown). It is not known whether this cleaved form of syntaxin results from increased protease sensitivity or from a reaction relevant to exocytosis. Although a trypsin-like protease has been identified in the sea urchin sperm acrosome (Green and Summers, 1980), inhibitors of trypsin and other eukaryotic proteases did not block the appearance of this syntaxin cleavage product.

Localization of Syntaxin to the Acrosomal Region of the Sperm and Release during Acrosomal Exocytosis

Indirect immunofluorescence of sea urchin sperm with antibodies to syntaxin showed that syntaxin is confined to the area of the acrosomal vesicle (Fig. 3A). This signal was absent in nonpermeabilized sperm which is consistent with an intracellular location of syntaxin (data not shown).

During acrosomal exocytosis, the plasma and acrosomal membranes covering the acrosomal vesicle are vesiculated and shed from sperm. Considering the localization of syntaxin to this area of the plasma membrane, we tested the fate of syntaxin following acrosomal exocytosis. When the sperm were acrosome reacted with ionophore, syntaxin was released with the ARVs shed to the supernatant during exocytosis (Fig. 4, lane 1). Syntaxin was not released from mock-reacted sperm (Fig. 4, lane 2). Instead, unreacted sperm retained syntaxin as detected on immunoblots (Fig. 4, lane 3), consistent with the acrosomal immunolocalization data (Fig. 3). Syntaxin was released from acrosome-reacted sperm (Fig. 4, lane 4) and immunolocalization showed no evidence of syntaxin present in acrosome-reacted sperm (Fig. 3B).

VAMP in Sea Urchin Sperm

Using antibodies specific for the sea urchin egg form of VAMP (Conner *et al.,* 1997), the sperm homologue was identified that comigrates with VAMP in egg cortical granule lawns on immunoblots (Fig. 5, lanes 1 and 2). As with syntaxin, sperm VAMP (17 kDa) was localized exclusively in sperm heads based on cellular fractionation of the sperm into heads and flagella (Fig. 5, lanes 2 and 3). Several unsuccessful attempts were made to localize VAMP on the sperm head by immunofluorescence of permeabilized sperm. It is possible that the epitopes for VAMP are masked in the fixed sperm. VAMP is present in mock-reacted sperm but absent from acrosome-reacted sperm (Fig. 5, lanes 4 and 5). As with syntaxin, VAMP is released with the ARVs during ionophore-induced acrosomal exocytosis but not from mock-reacted sperm (Fig. 5, lanes 6 and 7). Since in all other systems studied syntaxin is found in the plasma membrane and VAMP in the vesicle membrane, this supports the idea that the plasma and acrosomal membranes fuse at multiple points resulting in shed membrane vesicles, presumably containing components from both membranes. Neither VAMP nor syntaxin could be detected in the 180,000*g* supernatants of the pelleted shed ARVs (data not shown), indicating that these proteins do not become soluble following acrosomal exocytosis, but remain associated with the ARVs.

Association of Syntaxin and VAMP in Sperm

With the knowledge of the association of syntaxin and VAMP in neurons (Calakos *et al.,* 1994), we were interested in determining if the two proteins were associated in sperm. Immunoprecipitation of detergent-solubilized sperm proteins with syntaxin antibodies coprecipitated VAMP in unreacted sperm as shown by an immunoblot of the syntaxin immunoprecipitate with anti-VAMP serum (Fig. 6, lane 5). Similarly, syntaxin is present in VAMP immunoprecipitates as detected on anti-syntaxin immunoblots (Fig. 6, lane

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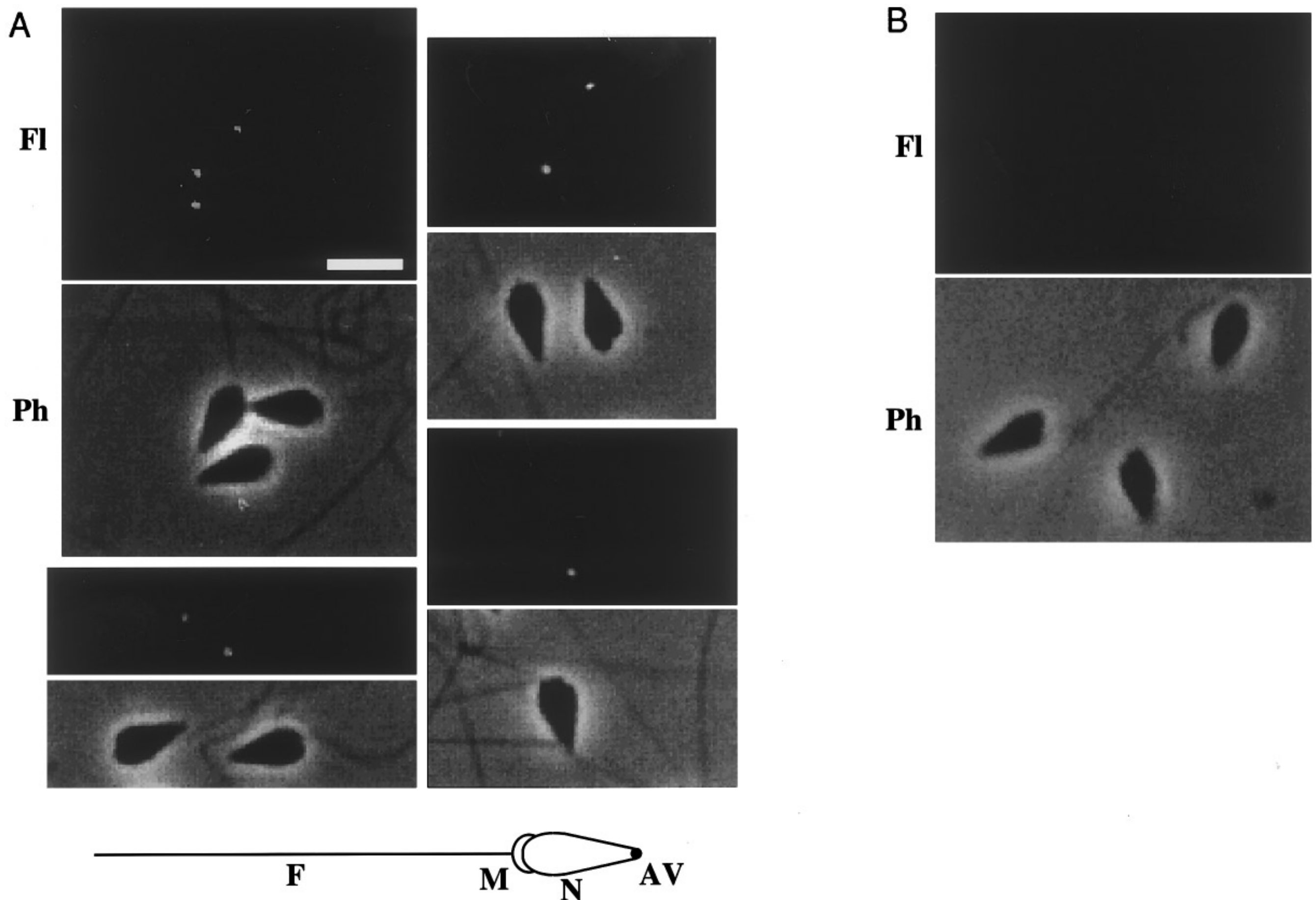

FIG. 3. Indirect immunofluorescence localizes syntaxin exclusively to the acrosomal region of the sperm (A). Micrographs at the top are rhodamine (TRITC) fluorescence of the secondary antibody (Fl). Micrographs at the bottom are the corresponding phase-contrast images (Ph). Schematic representation of a sea urchin sperm cell indicates the positions of the flagellum (F), midpiece (M), nucleus (N), and acrosomal vesicle (AV). Syntaxin localization is lost from acrosome reacted sperm (B). Controls using normal rabbit serum, the secondary antibody alone, or nonpermeabilized sperm are also devoid of the immunofluorescence signal. Bar, 2 μm.

8). The lack of this complex in acrosome-reacted sperm (Fig. 6, lane 4) and the absence of VAMP in VAMP immunoprecipitates of reacted sperm (Fig. 6, lane 3) support the loss of both proteins during acrosomal exocytosis. A normal rabbit serum control did not react with solubilized sperm proteins (Fig. 6, lane 1). In lane 6, a similar normal rabbit serum control precipitated a minor reacting band on anti-syntaxin immunoblots. When compared to lanes 7 and 8, this cross-reactivity is minor. Lane 2 shows that antibodies to VAMP immunoprecipitate VAMP from unreacted sperm.

To investigate the association of VAMP and syntaxin, detergent-solubilized sperm proteins were separated by sucrose gradient velocity sedimentation. Syntaxin and VAMP had overlapping but nonidentical sedimentation patterns (Fig. 7, a and b) with estimated sedimentation coefficients of 2.1S and 0.7S, respectively. Boiling the sample with SDS in SB resulted in shifts of both syntaxin and VAMP to fractions of lower density (Fig. 7, c and d). Enough ARV material was obtained from acrosome-reacted sperm to fractionate the detergent-solubilized membrane proteins on sucrose gradients. In contrast to the sedimentation pattern from unreacted sperm (Fig. 7, a and b), syntaxin and VAMP from ARVs had overlapping patterns of sedimentation and sedimented farther than the unreacted samples with an estimated sedimentation coefficient of 6.2 S (Fig. 7, e and f). Syntaxin and VAMP cosedimentation was labile to boiling with SDS in SB resulting in patterns of sedimentation identical to those in Fig. 7, c and d (data not shown). The different patterns of syntaxin and VAMP sedimentation before and after the acrosome reaction indicate that larger complexes of these proteins have formed.

DISCUSSION

In this report, we have identified syntaxin and VAMP homologues in sea urchin sperm. These are the first protein

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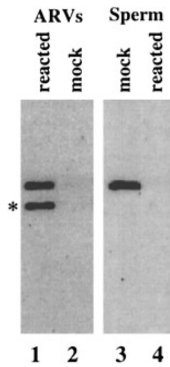

FIG. 4. Syntaxin is shed with membrane vesicles (ARVs) during acrosomal exocytosis. The 10,000*g* supernatants from acrosome-reacted sperm and mock-reacted sperm were pelleted at 180,000*g* to collect the ARVs. Equal volumes of the resuspended ARVs from the acrosome-reacted (lane 1) and mock-reacted control (lane 2) were immunoblotted using the syntaxin antiserum along with 12 μg of solubilized protein from the mock-reacted (lane 3) and acrosome-reacted (lane 4) sperm. Asterisk indicates apparent syntaxin degradation product (34 kDa) in the ARVs (lane 1).

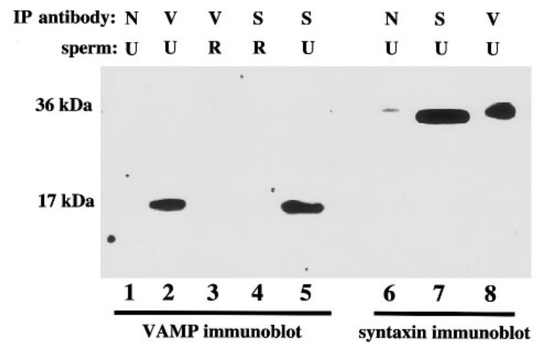

FIG. 6. VAMP is present in syntaxin immunoprecipitates of unreacted sperm. Detergent-solubilized sperm membrane proteins (200 μg) from unreacted (U:1,2,5,6,7,8) and reacted (R:3,4) sperm were immunoprecipitated with normal rabbit serum (N:1,6), antiserum against VAMP (V:2,3,8), and antiserum against syntaxin (S:4,5,7). VAMP (17 kDa) is present in the VAMP and syntaxin immunoprecipitates of unreacted sperm (2,5). VAMP is completely absent from immunoprecipitates of ionophore-reacted sperm (3,4). For the reciprocal combination, syntaxin (36 kDa) is enriched in the syntaxin and VAMP immunoprecipitates of unreacted sperm (7,8).

components of the vesicle fusion machinery to be identified in animal sperm. Immunoblotting demonstrates that both proteins are present in the sperm head, but are absent from the flagellum. Immunofluorescence localizes syntaxin to the acrosomal region, presumably to the plasma membrane covering the acrosomal vesicle. Immunoprecipitation with syntaxin and VAMP specific antibodies shows that the two are associated in a complex. Membrane vesiculation during the acrosome reaction releases both proteins in shed ARVs. ARVs were pelleted by ultracentrifugation and analyzed by immunoblotting and sucrose density gradient sedimentation. The qualitative absence of both proteins on immunoblots of adequately loaded gel lanes (Figs. 4 and 5) and from immunoprecipitates (Fig. 6) supports the claim that most, if not all, of both proteins are released from acrosome-reacted sperm. These data support the involvement of both proteins in the acrosome reaction. The release of both of these exocytosis regulatory proteins from acrosome-reacted sea urchin sperm is similar to the shedding of the IP3 receptor with the outer acrosomal membrane of mammalian sperm during the acrosome reaction (Walensky and Snyder, 1995). This receptor potentially regulates Ca^{2+} which triggers acrosomal exocytosis.

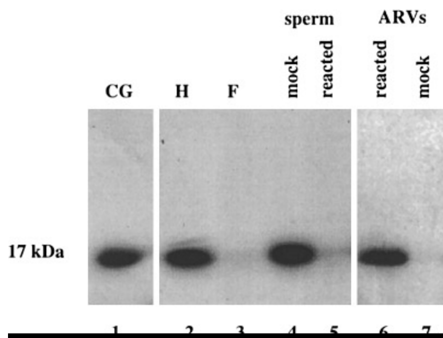

FIG. 5. VAMP (17 kDa; synaptobrevin) also localizes to the sea urchin sperm head and is shed from sperm during the acrosome reaction. Sperm fractionated into heads (lane 2) and flagella (lane 3) as well as mock-reacted (lane 4) and acrosome-reacted (lane 5) sperm were immunoblotted using antiserum to sea urchin VAMP along with cortical granule lawn protein as a control (lane 1). Each lane contains 10 μg of detergent-solubilized protein. As in Fig. 4, the 10,000*g* supernatants from acrosome-reacted sperm and mock-reacted sperm were pelleted at 180,000*g* to collect the ARVs. Equal volumes of the resuspended ARVs from reacted (lane 6) and mock-reacted control (lane 7) sperm were immunoblotted with the VAMP antiserum.

Sucrose gradient analysis of the ARV proteins solubilized by nonionic detergents showed that syntaxin and VAMP increased in complex size following the acrosome reaction. It is not known if the increases in the size of the complexes are significant to the exocytotic process or only a consequence. In either case, identification of the mechanism of complex formation of syntaxin and VAMP in ARVs, as well as identification and characterization of the other proteins in ARVs, is important for future studies of the mechanism of the acrosome reaction and exocytosis in general. The demonstration that sea urchin sperm acrosomes and egg cortical granules (Conner *et al.,* 1997) contain the same forms of syntaxin and VAMP (Figs. 1, 2, and 4) argues for the same exocytotic mechanism being operative in both types of gametes. The loss of these two proteins from sperm does not support a role for them in mediating membrane

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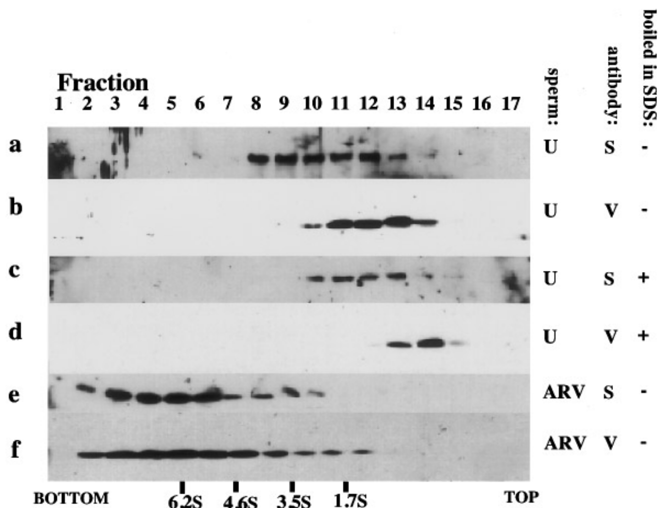

FIG. 7. Syntaxin and VAMP sediment as larger complexes following acrosomal exocytosis. Detergent-solubilized proteins of unreacted sperm (U: a–d) or shed ARVs (ARV: e, f) were resolved on 7.5–25% sucrose gradients by ultracentrifugation. Gradients were run in the presence (c,d) or absence (a,b,e,f) of 0.1% SDS. Collected fractions were immunoblotted using anti-syntaxin (S: a,b,e) and anti-VAMP (V: b,d,f) sera. In unreacted sperm, syntaxin (a) and VAMP (b) sediment with estimated sedimentation coefficients of 2.1S and 0.7S, respectively. When the samples are boiled and run in the presence of 0.1% SDS, both syntaxin (c) and VAMP (d) remain in lighter fractions. Following acrosomal exocytosis and collection of the ARVs, syntaxin and VAMP cosedimented to heavier fractions (e, f) with peak amounts having an estimated sedimentation coefficient of 6.2S. Sedimentation coefficients were estimated from the sedimentation patterns of standard proteins and samples on both 7.5–25 and 5–55% sucrose gradients (not shown).

fusion between the gametes themselves (Allen and Green, 1997).

In mammals, multiple syntaxin isoforms exist (Bennett *et al.,* 1993). Not all isoforms localize to the plasma membrane; some may function in intracellular membrane and vesicle transport. Comparative sequence analysis of sea urchin syntaxin with mammalian isoforms shows that the sea urchin form is more closely related to mammalian types 1 and 2 than to types 3–5. In agreement with the sea urchin data, mammalian types 1 and 2 are plasma membrane proteins (Bennett *et al.,* 1993; Garcia *et al.,* 1995).

In nerve terminals, syntaxin, VAMP, and SNAP-25 (Oyler *et al.,* 1989) are associated as a complex (Hayashi *et al.,* 1995; Rothman, 1996). This complex serves as a receptor for NSF and SNAPs (Söllner *et al.,* 1993b). ATP hydrolysis by NSF results in the disruption of the complex, presumably during the vesicle priming step. Whether the sperm acrosomal vesicle has undergone an ATP-dependent priming step when the sperm are spawned and begin to interact with the egg remains unknown. A cDNA encoding a SNAP-25 homologue in the sea urchin testis has been identified (Sasaki, Schulz, and Vacquier, manuscript in preparation). ARVs may be an important material for obtaining post-acrosome-reacted SNAP-25.

In animal sperm, the acrosome reaction involves the influx of Ca^{2+} and Na^{+}, efflux of H^{+} and K^{+}, and depolarization of the plasma membrane, the culmination being the Ca^{2+} triggering of acrosomal vesicle exocytosis (Arnoult *et al.,* 1996; Espinosa and Darszon, 1995; Florman, 1994; Florman *et al.,* 1992; Gonzalez-Martinez *et al.,* 1992). Recent evidence has linked syntaxin, SNAP-25, and VAMP with the influx of Ca^{2+}-triggered synaptic vesicle exocytosis at the presynaptic membrane (Martin *et al.,* 1996). In neurons, syntaxin binds directly to a voltage-dependent Ca^{2+} channel in the presynaptic membrane thereby directly linking regulated ion fluxes to the fusion machinery (Martin *et al.,* 1996; Sheng *et al.,* 1994, 1996). Sperm syntaxin could associate with an acrosome-specific voltage-dependent Ca^{2+} channel during the final steps preceding vesicle exocytosis. This association would provide a link between the upstream transmembrane signaling events and acrosomal vesicle exocytosis. Such a Ca^{2+} channel protein might be found in the shed ARVs. Further study of the exocytosis regulatory proteins before and after the acrosome reaction will lead to a deeper understanding of this developmentally important event necessary for animal fertilization.

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