Increased Association of Synaptosome-associated Protein of 25 kDa with Syntaxin and Vesicle-associated Membrane Protein following Acrosomal Exocytosis of Sea Urchin Sperm*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF036902.

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‡ The abbreviations used are: ARV, acrosome reaction vesicle; SNAP-25, synaptosome-associated protein of 25 kDa; VAMP, vesicle-associated membrane protein; PCR, polymerase chain reaction; PEG, polyethylene glycol.

The sea urchin sperm acrosome reaction is triggered by interaction of a plasma membrane receptor (receptor for egg jelly) with a sulfated fucan in the egg jelly coat (8). The activated receptor regulates ion channels, resulting in the influx of Ca²⁺ and exocytosis (9). This results in the exposure of bindin and the elongation of the acrosomal process. Acrosomal exocytosis can be induced by ionophores (10), or by the addition of Ca²⁺ to digitonin-permeabilized sperm (11), making sperm an interesting model for studying Ca²⁺-triggered exocytosis.

To understand the mechanism of acrosomal exocytosis, we have identified homologues in sea urchin sperm of proteins believed to be key regulators of membrane fusion during exocytosis. Syntaxin, an intracellular protein integral to the plasma membrane (12, 13), and VAMP (synaptobrevin; Refs. 14 and 15), which is integral to the vesicle membrane, are expressed in sea urchin sperm and are shed with the ARVs during the acrosome reaction. Previous work demonstrated that sperm syntaxin and VAMP increase their association following acrosomal exocytosis (7). In neurons, these proteins form a ternary complex with SNAP-25 (16), which is postulated to regulate membrane fusion (17). Here, we describe a sea urchin SNAP-25 homologue expressed in a non-neuronal cell type. Sperm SNAP-25 is found in a complex with syntaxin and VAMP and is also shed with the ARVs. The amount of complex of these three proteins increases following the acrosome reaction. This increase may represent the post-exocytotic state of these proteins in the absence of an endocytic membrane retrieval cycle common to most cells.

**EXPERIMENTAL PROCEDURES**

Amplification of SNAP-25 from Testis cDNA—The complete sequence of SNAP-25 was obtained by PCR amplification using degenerate and exact primers from a Strongylocentrotus purpuratus testis cDNA library following a standard PCR protocol and variable annealing temperatures from 42 °C to 52 °C. Degenerate primers were first used to amplify PCR products encoding for SNAP-25 (S25F, 5'-GAGCTCGAGCATATGGAAGACCAGAATGAC-3'; S25R, 5'-GARGARG-AGATGGGGTCAGATGGAATGGG-3'; SNAF, 5'-GCATTTGTGTCTGTCCG-3'; SNCR, 5'-CCCATCTC-NAYNGCCCAT-3') (N = A, C, G, or T; H = A, C, or T; R = A or G; Y = C or T; K = G or T). These initial products were then used to design exact match primers to PCR amplify, along with library vector primers, the N- and C-terminal regions (SNDF, 5'-GCCATTTGTGTCTCGCG-TGG-3'; SNEDF, 5'-CAAGCCAAATGAGAATGGAGG-3'; SNCR, 5'-CCTCT-CGTCCTCTCCCAT-3'). The 5'-untranslated sequence contains one in-frame stop codon three codons prior to the start codon and the termination codon is followed by two in-frame stop codons (four and eight codons following the termination codon). The full-length sequence was amplified from a testis cDNA library using linker-containing primers (SP25F, 5'-GAGCTGAGATCAGATGGAAGACCAGAATGAC-3'; SP25R, 5'-TTGCATTCCGATCTTGGTGCTCAGTGCAGTCAG-3') to facilitate cloning into the His-tagged expression vector PET15b (Novagen). Sequences were analyzed using GeneDoc (18), and a neighbor-joining distance was constructed using the program MEGA (19).

Preparation of Proteins—Sea urchins (S. purpuratus) were spawned by intracoelemic injection of 0.5 mM KCl. Isolation of sperm heads and...
flagella was performed as described (20). Sperm were separated from coelomocytes and sedimented to remove seminal plasma proteins as described (20). Sperm were acrosome-reacted with the ionophore nigericin (final concentration 40 μM; Sigma) as described and pelleted at 10,000 × g for 30 min (10). Shed acrosome reaction vesicles, ARVs, were collected from the 10,000 × g supernatant by centrifugation for 30 min at 180,000 × g in an Airfuge. Soluble sperm and ARV proteins were prepared as described with 0.4% Nonidet P-40 (7).

**Immunoblots and Immunoprecipitations**—Hen egg yolk antibodies (IgY) were generated against the His-tagged sea urchin SNAP-25 fusion protein. Antibodies were purified from egg yolks as described (21). Briefly, egg yolks were resuspended in 30 ml of 0.01M potassium phosphate (pH 7.2) and 0.1 M NaCl per yolk. To the resuspended sample was added 30 ml per yolk 7% (w/v) PEG 8000 (Sigma) in the same buffer. The sample was centrifuged at 14,000 × g for 10 min, and the supernatant was brought to 12% PEG 8000 and centrifuged at 14,000 × g for 10 min. The pellet was resuspended in the above buffer (20 ml/yolk) and precipitated by addition of an equal volume of buffer containing 24% PEG and centrifugation at 14,000 × g for 10 min. Pellets were resuspended and dialyzed against the same buffer.

Antisera to recombinant sea urchin syntaxin and VAMP were kindly provided by G. M. Wessel and S. Conner (7, 22). A polyclonal antibody directed against mammalian SNAP-25 was obtained from Alomone Laboratories. Antibodies were used at a dilution of 1:1000 for immunoprecipitation and 1:1000 for immunoblotting. An antibody directed against mammalian SNAP-25 (Ray-25, Table I) was aligned to human SNAP-25 (GenBank accession no. AF036902) was aligned to human SNAP-25 isoform a (Human 25a; accession no. L19760), human SNAP-25 (Human-23; accession no. U55936), electric ray SNAP-25 (T. marmorata; accession no. L22020), and leech SNAP-25 (H. medicinalis; accession no. U58806). Black shading indicates positions of identity in all sequences, dark shading indicates positions of identity in four out of five sequences, and lightest shading indicates positions identical in three of the five sequences. Gaps (bars) were introduced to improve the alignment. A consensus sequence is provided below the aligned sequences, where identity in all five sequences is denoted by uppercase letters and four out of five by lowercase letters.

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**RESULTS**

**Identification of SNAP-25 cDNA in Sea Urchin Testis**—Multiple overlapping PCR products were amplified from a sea urchin testis cDNA library that encoded a single isoform of SNAP-25. Based on the sequences of these products, primers were designed to amplify the entire sequence encoding sea urchin testis SNAP-25. The sequence encodes a 212-amino acid protein and, when aligned to other SNAP-25 family members, is conserved throughout the sequence (Fig. 1). The length of the protein is conserved with other invertebrate SNAP-25 sequences (Fig. 1, e.g., Leech-25). Sea urchin SNAP-25 is 59% identical to electric ray SNAP-25 (Ray-25, Table I) and 58% identical to human SNAP-25. It is 51% identical to human SNAP-23 (23). Neighbor-joining distance analysis was performed with SNAP-25 protein sequences (Fig. 2). Sea urchin SNAP-25 falls between vertebrate and invertebrate sequences and is an outgroup to a SNAP-25/-23 clade consisting exclusively of vertebrate members.

**Identification of SNAP-25 in Sea Urchin Sperm**—Other invertebrate SNAP-25 homologues have been identified in neurons at recombinant SNAP-25 dilution standards, and the resulting autoradiograms were scanned and analyzed using the NIH Image program. To quantitate the amounts and molar ratios of SNAP-25 and syntaxin in ARVs and Nonidet P-40 sperm protein extracts, a dilution series for each of the recombinant proteins was created and used on immunoblots with the sperm protein samples. Linear regressions for the syntaxin and SNAP-25 standards were used to calculate the molar ratios of SNAP-25 and syntaxin present in sperm sources from the same exposure used to calculate the regression as described above.

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**Table I**

| Urchin 25 | Human 25a | Human | Human | Ray 25 | Leech 25 |
|-----------|-----------|-------|-------|--------|----------|
| 58        | 51        | 59    | 54    | 51     | 51       |
| 73        | 53        | 74    | 51    | 52     | 52       |
| 64        | 69        | 56    | 54    | 52     | 52       |
| 72        | 68        | 62    | 68    | 68     | 68       |

The Image program was developed at the National Institutes of Health and is available by FTP (zippy.nih.gov).
either the mRNA or protein level (24, 25). To determine if sea urchin SNAP-25 is present in sperm (a non-neuronal cell type), antibodies generated against sea urchin SNAP-25 were used to identify SNAP-25 in sperm. These antibodies react with the expressed protein with the His tag removed by thrombin digestion (32 kDa; Fig. 3, lane 1). SNAP-25 (32 kDa) is present in the ARVs released from sperm during the acrosome reaction (Fig. 3A, lane 3). Sea urchin and leech (Hirudo medicinalis) SNAP-25 (25) have apparent molecular masses larger than mammalian SNAP-25 homologues. The signals for recombinant and ARV SNAP-25 are blocked (Fig. 3A, lanes 2 and 4) by pretreatment of the antibody with the SNAP-25 fusion protein.

Knowing that SNAP-25 is present in ARVs, we investigated the fate of SNAP-25 in sperm that had been acrosome-reacted by ionophore. SNAP-25 is present in sperm prior to the acrosome reaction (Fig. 3B, lane 2), but is qualitatively lost from acrosome-reacted sperm (Fig. 3B, lane 3). This loss is comparable to the loss of the exocytosis regulatory proteins syntaxin and VAMP from acrosome-reacted sperm with shed ARVs (7).

Isolation of SNAP-25 in a Complex with VAMP and Syntaxin—As SNAP-25 forms a ternary complex with syntaxin and VAMP in neurons, and sea urchin SNAP-25 is present in ARVs isolated from acrosome-reacted sperm, we wished to determine if SNAP-25 is associated with syntaxin and VAMP in sperm. SNAP-25 was affinity-purified from solubilized sperm proteins using an anti-SNAP-25 IgY affinity column (Fig. 4A, lane 3) and compared with the eluate of a control column containing normal IgY (Fig. 4A, lane 2). Multiple bands co-eluted specifically with SNAP-25. To confirm the identity of the co-eluting proteins (Fig. 4A, lane 3), the eluate was immunoblotted with SNAP-25 antibodies (Fig. 4B, lane 1) and syntaxin and VAMP antibodies (Fig. 4B, lane 3). Syntaxin and VAMP co-elute from the affinity column with SNAP-25 and are greatly enriched (Fig. 4A, lane 3). SNAP-25 antibodies reacted with the full-length protein (32 kDa) as well as a breakdown product (28 kDa) present in the starting material (Fig. 4B, lane 2). The identity of the breakdown product was confirmed by N-terminal protein sequencing, corresponding to a truncation of the N terminus commencing at residue 19 (Gln) of the SNAP-25 sequence. Multiple proteins co-eluted from the SNAP-25 affinity column in addition to syntaxin and VAMP (Fig. 4A, compare lanes 2 and 3, asterisks), suggesting that SNAP-25 is involved in multiple protein interactions (either directly or indirectly) prior to the acrosome reaction.

Following acrosomal exocytosis, there is a shift in the sedimentation patterns of syntaxin and VAMP on sucrose density gradients to denser fractions having an estimated sedimentation coefficient of 6.2 S (Ref. 7; Fig. 5). To compare this with SNAP-25, detergent-solubilized proteins from uncleaved sperm and ARVs were analyzed by sucrose gradient velocity sedimentation. The sedimentation patterns of the proteins from both sources are consistent with each other, and the sedimentation patterns of the major proteins present in both uncleaved sperm and ARV protein gradients (the similar data are not shown). A proteolytic degradation (7) product co-sediments with full-length syntaxin following the acrosome reaction (Fig. 5, ARV/syn), suggesting that this breakdown product is able to associate with the same proteins as is full-length syntaxin. SNAP-25 solubilized from ARVs does not undergo a complete shift; only a portion of SNAP-25 sediments to denser fractions following the acrosome reaction (Fig. 7, ARV/S25), suggesting that...
SNAP-25 is not limiting. To test this, the amount of SNAP-25 and syntaxin present in ARVs and a detergent extract of sperm were calculated (see “Experimental Procedures”). SNAP-25 is present at 30.5 ng/10 μg of ARV protein and 11.1 ng/10 μg of protein in the sperm detergent extract. Syntaxin is present at 4.9 ng/10 μg of ARV protein and 1.8 ng/10 μg of protein in the sperm detergent extract. The molar ratios of SNAP-25 to syntaxin were calculated for both ARVs and the detergent extract of sperm. SNAP-25 is 6.9-fold more abundant on a molar basis than syntaxin in both sources, suggesting that SNAP-25 may have multiple binding partners in addition to syntaxin and VAMP. Using syntaxin antibodies, SNAP-25 was co-immunoprecipitated from sperm proteins prior to and following the acrosome reaction. The estimated sedimentation coefficient of syntaxin (ARV/syn) is 6.2 S (7). A proteolytic degradation product of syntaxin (34 kDa; Ref. 7) co-sediments with full-length syntaxin (36 kDa) following the acrosome reaction. The sedimentation pattern of SNAP-25 following the acrosome reaction (ARV/S25) is extended, but not completely shifted, to denser fractions. Arrows indicate fractions used as a source for immunoprecipitations in Fig. 6.

To test whether the changes in sedimentation patterns for all three proteins correlates with the formation of a ternary complex, gradient fractions were used as a source to immunoprecipitate the complex. Anti-syntaxin antibodies were used to precipitate the complex, gradient fractions were used as a source to immunoprecipitate the complex. Anti-syntaxin antibodies were used to co-immunoprecipitate VAMP and SNAP-25 from fractions 6 and 9 (Fig. 6B). SNAP-25 does not co-precipitate with syntaxin from fraction 9 (Fig. 6B, UnR/S25) prior to the acrosome reaction despite the abundance of SNAP-25 (Fig. 5) in these samples. VAMP is also absent from syntaxin immunoprecipitates of fraction 9 samples prior to the acrosome reaction (Fig. 6B, UnR/V). However, SNAP-25 and VAMP are co-precipitated with syntaxin from fraction 6 (Fig. 6B, ARV/V, S25) samples following the acrosome reaction (ARVs), correlating with the change in syntaxin’s sedimentation pattern. SNAP-25 and VAMP are also co-precipitated to a lesser extent with syntaxin from fraction 6 samples prior to the acrosome reaction (Fig. 6B). Similarly, anti-SNAP-25 antibodies co-precipitated syntaxin and VAMP from gradient fraction 6 samples both prior to and following the acrosome reaction (Fig. 6C). The syntaxin breakdown product present following the acrosome reaction was also co-precipitated from fraction 6 (Fig. 6C) in agreement with its co-sedimenting with full-length syntaxin on sucrose gradients. While the ternary complex (syntaxin, VAMP, and SNAP-25) is present prior to the acrosome reaction, it appears to increase over 4-fold in abundance following the acrosome reaction based on the amount of SNAP-25 present in syntaxin immunoprecipitates of the fraction 6 (6.2 S) gradient samples (see “Experimental Procedures”). Similarly, VAMP increases in abundance in syntaxin immunoprecipitates following the acrosome reaction (Fig. 6B), correlating with the observed shifts in the syntaxin and VAMP sedimentation patterns following acrosomal exocytosis (7).

**DISCUSSION**

SNAP-25 is an axonally transported protein in mammalian neurons where it localizes to nerve terminals (26, 27). At the presynaptic membrane, SNAP-25 regulates neurotransmission by forming a ternary complex with syntaxin and VAMP (28–31). In this report, we have identified a sea urchin homologue of SNAP-25. This is the first report of SNAP-25 being expressed in the sperm of any animal. Phylogenetic analysis of SNAP-25 protein sequences suggests that the mammalian SNAP-25 homologue, SNAP-23 (23, 32), arose by a gene duplication in the vertebrate lineage to function in non-neuronal cell types. In agreement with this idea, only a single isoform of SNAP-25 was amplified from a sea urchin testis cDNA library despite the use
of degenerate primers designed to amplify both SNAP-25 and
-23. This suggests that the sea urchin testis does not express a
SNAP-23 homologue. The identification of SNAP-23 in mouse
testis (32) raises the possibility that SNAP-23 has evolved to
function in non-neuronal cell types in vertebrates, in agreement
with the phylogenetic sequence analysis presented in Fig. 2.

Sea urchin sperm SNAP-25 shares many properties with
syntaxin and VAMP. All three proteins are shed from sperm
with the ARVs during the acrosome reaction. These proteins
can be isolated as a complex by immunoprecipitation and af
finity chromatography prior to the acrosome reaction and
the absence a subsequent endocytic cycle.

The presence of the ternary complex in ARVs may represent
acrosomal exocytosis, there is greater than a 4-fold increase
in the amount of ternary complex present, as demonstrated by
the changes in the sedimentation patterns of syntaxin, VAMP,
and SNAP-25 and by the immunoprecipitation of gradient frac
tions. Interestingly, only a portion of SNAP-25 sediments into
denser fractions following the acrosome reaction. The 6.9-fold
molar excess of SNAP-25 over syntaxin in sperm extracts and
ARVs suggests that SNAP-25 is not limiting for ternary com
plex formation. However, all the syntaxin present in ARVs
completely shifts to denser fractions after the acrosome reac
tion. The estimated sedimentation coefficient for the syntaxin
peak fractions following the acrosome reaction is 6.2 S (7),
which correlates with the size of the ternary complex based on
the sedimentation of molecular weight standards.

The presence of the ternary complex in ARVs may represent
the post-exocytic state of these proteins in the same lipid
bilayer, and the increase in complex formation following the
acrosome reaction may be an exocytotic intermediate formed in
the absence a subsequent endocytic cycle.

As the amount of ternary complex increases following acro
somal exocytosis, it will be of interest to determine what reg
ulates complex formation prior to the acrosome reaction and
how this correlates with the influx of Ca^{2+}, which triggers
acrosomal exocytosis.

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