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Biochemical and Biophysical Research Communications 318 (2004) 148–155
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Spatio-temporal organization of Vam6P and SNAP on mouse spermatozoa and their involvement in sperm–zona pellucida interactions☆

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Received 12 March 2004

Abstract

Acrosomal assembly during spermatogenesis and acrosome reaction during sperm–oocyte interaction are unique events of vesicle synthesis, transport, and fusion leading to fertilization. SNARE complex formation is essential for membrane fusion, and vesicle-associated (v-) SNARE intertwines with target membrane (t-) SNARE to form a coiled coil that bridges two membranes and facilitates fusion. We detected messages of Vam6P and SNAP in mammalian testis and epididymis. Vam6P and SNAP were detected in a temporally organized fashion on the spermatozoa from testis and epididymis, which showed accumulation on the principal acrosomal domains during capacitation. Vam6P and SNAP were shed off from the principal acrosomal domain after acrosome reaction, but the equatorial and the post-acrosomal domains retained these proteins. Antibodies to VAMP and SNAP inhibited sperm–zona pellucida interaction, suggesting their possible involvement in sperm membrane vesiculation.

The acrosome is a large vesicle localized as a cap like structure on the head of spermatozoa, sharing similarities with lysosomes [1,2], though typical lysosomal markers are not present on the acrosome [3]. Nevertheless, the acrosome is a complex organelle that includes several structurally and biochemically distinct regions [4–7]. During mammalian spermiogenesis several distinct stages of spermatid differentiation reflect differences in nascent acrosome [8,9]. Most of the cytoplasm and organelles are discarded in the cytoplasmic droplet/residual body when acrosome reaches its final shape [8,9].

In acrosome formation, active trafficking from the Golgi apparatus is involved, both by delivering acrosomal contents to the nascent secretory vesicles and by controlling organelle growth and shaping [10]. The growth and shaping of acrosome relies on intense membrane trafficking, possibly involving clathrin coated vesicles [11,12]. The synthesis of acrosomal proteins may take place before spermiogenesis [4]. The acrosome is initially formed in close association with the Golgi apparatus in Golgi-phase spermatids, with a continuous and intense flow of membranes in the endoplasmic reticulum–Golgi–acrosome direction [5,13–17]. The acrosome maturation proceeds throughout spermiogenesis and is completed during the epididymal transit [4].

Calcium-triggered exocytosis of neurotransmitter or hormone-filled vesicles has developed as the main mechanism for cell-to-cell communication in animals [18]. Release of acrosomal contents during acrosome reaction, an exocytic event resulting in the fusion of the acrosomal membrane and the sperm plasma membrane that primes the sperm for zona penetration and sperm–egg binding/fusion [19], shares heavy analogy with classical calcium-triggered exocytosis. This event occurs

☆ *Abbreviations:* DMSO, dimethyl sulfoxide; Chaps, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonyl fluoride; EGTA, ethyleneglycol-bis(aminoethyl)tetraacetic acid; RT-PCR, reverse transcription polymease chain reaction; HRP, horseradish peroxidase; SNARE, soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor; SNAP, soluble NSF attachment proteins; NSF, *N*-ethylmaleimide-sensitive fusion protein, VAMP, vesicle-associated membrane protein.

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0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.04.006

via specific receptors localized over the anterior head region of the spermatozoon. Zona pellucida binding stimulates the spermatozoa to undergo the acrosome reaction resulting in the release of hydrolytic enzymes and in the exposure of new membrane domains, both of which are essential for fertilization [20].

Thus, two stages in the life span of spermatozoa with predominant vesicle formation and membrane trafficking include early stages of sperm development in the testis (undergoing acrosomal assembly), and the acrosome reaction (when it meets an oocyte). Soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins of the vesicle-associated membrane protein (VAMP) and syntaxin families play a central role in vesicular trafficking through the formation of complexes between proteins present on vesicle and target membranes. Formation of these complexes is proposed to mediate aspects of the specificity of vesicle trafficking and to promote fusion of the lipid bilayers [21]. Synaptic exocytosis requires the soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins syntaxin 1, SNAP-25, and synaptobrevin (VAMP) [22]. In this study, we examined the testicular and epididymal expression of VAMP and SNAP-25 and spatio-temporal organization of these gene products on spermatozoa in relation to their progressive development.

Materials and methods

Reagents. Antibodies to VAMP and SNAP were purchased from Santa Cruz Biotechnology, CA. Anti-rabbit IgG-HRP and DC Protein Assay kit were purchased from BioRad Laboratories, CA. *N*-2-Hydroxethyl piperazine-*N*-ethanesulfonic acid (Hepes), progesterone, DMSO, Trizma hydrochloride, Trizma base, Chaps, PMSF, EGTA, poly-L-lysine, sodium orthovanadate, Tween 20, and Coomassie Brilliant Blue R-250 were from Sigma Chemical, WI. LIVE/DEAD kit was from Molecular Probes, OR.

Sperm preparation. The mature male mice of the age group 3–5 months were obtained from an inbred line of Swiss strain maintained in the institutional facility. Animals were killed by cervical dislocation. Spermatozoa from testis and different segments of epididymis were collected as described earlier [23]. Briefly, the testis was cleared of adhering fat and was decapsulated. The seminiferous tubules were freed carefully with two pairs of tweezers and washed in 5 ml fresh Hanks' balanced salt solution (HBSS) three times. The seminiferous tubules were mechanically teased and were allowed to stand for 10 min at 37 °C. The supernatant was collected and filtered through a 100 μM Nitex screen remove any fragments of seminiferous tubules left unsedimented, and a second filtration was performed through an 80 μM Nitex screen and the filtration was centrifuged at 3500 rpm for 10 min. The pellets were resuspended in 2 ml of fresh HBSS. Spermatozoa from cauda epididymidis and vas deferens were obtained after mincing these tissue and dispersion in HBSS. The suspension was allowed to stand for 10 min at 37 °C. The cloudy upper layer of the suspension was recovered and was passed through 80 μm Nitex screen to remove the cell debris. The spermatozoa were pelleted by centrifugation at 500*g*. The sperm pellets thus obtained were resuspended in HBSS such that the final sperm counts were 1 million/cm^3.

Capacitation and acrosome reaction of mouse spermatozoa. For the induction of capacitiation, the spermatozoa (1 million cells/ml) were incubated for 2 h in Krebs–Ringer bicarbonate buffer (KRB) containing sodium pyruvate and sodium lactate and in an atmosphere of 95% air and 5% CO_2. The incubation was under paraffin oil in a plastic culture dish (Falcon Labware/USA). Sperm were confirmed to have undergone capacitation by observing their hyperactivation. Acrosome reaction was initiated with the introduction of 3.18 μM progesterone prepared in 0.05% DMSO (final concentration) into the suspension of capacitated spermatozoa in KRB. At every 30 min interval, a 10-μl aliquot of the sperm suspension was withdrawn from beneath the paraffin oil layer, which was assessed for motility, viability, and capacitation status. The percentage of motile sperm was determined microscopically by counting 200–250 sperm under differential interference contrast (DIC) optics on a stage maintained at 37 °C. Viability of sperm was determined using the LIVE/DEAD kit of Molecular Probe, Eugene, OR.

In experiments that tested the role of VAMP/SNAP in sperm–zona pellucida interaction, we added anti-VAMP or anti-SNAP antibodies (1:200 dilution) into the capacitation set-up 2 h after the start of the incubation. Control wells received normal rabbit serum and the incubations were continued at 37 °C for 1 h in an atmosphere of 95% air and 5% CO_2.

Protein extraction. The spermatozoa prepared as stated above were washed three times with 0.0625 M Tris–HCl, pH 6.8. The spermatozoa were solubilized by suspending in the sperm solubilizing buffer (187 mM Tris–HCl, pH 6.8, 2% SDS, 0.05% Chaps, 10% glycerol, 1 mM PMSF, 1 mM EGTA, and 1 mM vanadate) and sonicating at 15 μm amplitude in a MSE Soniprep 150 sonicator for 3 cycles of 10 s each. This extract was centrifuged at 14,000*g* for 5 min. The protein concentrations of the cellular homogenates prepared as above were determined by DC Protein Assay kit (BioRad, CA) using the standard protocol and protein concentrations were adjusted to 20 μg/10 μl.

The supernatants were diluted 1:1 with SDS–PAGE buffer (0.0625 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, and 2% of 2-mercaptoethanol) and heated at 100 °C for 4 min to denature. Electrophoresis was performed as described earlier [24]. The samples were loaded keeping the protein concentration to 30 μg/well, run on 7–15% SDS–PAGE gradient gel after denaturation, and subjected to electrophoresis at 30 mA constant current. The gels were stained with 0.1% Coomassie Blue R-250 in 10% acetic acid and 40% methanol, destained in 10% acetic acid and 40% methanol.

Western blotting. The separated proteins were electroblotted on PVDF membrane (0.2 μm; BioRad, Hercules, CA) in presence of 40% v/v methanol, 25 mM Tris, pH 8.2, and 190 mM glycine at 30 mA for 12–16 h using Mini Trans Blot cell (BioRad, Hercules, CA) as described [24].

For development of the blots, the membranes were pre-wet in methanol and incubated in 5% nonfat skim milk in Tris-buffered saline containing 0.1% Tween 20 (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) overnight followed by extensive washing in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature. The blots were incubated for 2 h at 20 °C in 1:2000 dilution of the respective antibodies. The blots were then incubated in anti-rabbit IgG-peroxidase at a dilution of 1:2000 for 1 h at room temperature. The blots were washed extensively as mentioned above. The immunoblots were developed by dipping the blots in 0.05% diaminobenzidine and 0.1% H_2O_2/0.04% nickel chloride in 50 mM Tris–HCl, pH 7.5, until the desired contrast is obtained. The blots were photographed on a Chemimager 4400 (Alpha Innotech, USA).

All the experiments were repeated for a minimum of five times. Western blots were subjected to quantitative analysis using Advanced Phoretix 1.0 (Nonlinear Dynamics, CA) and the results were averaged. All the observations were subjected to a one-way analysis of variance using Sigmaplot 4.0 (SPSS, CA).

Immunocytochemistry. Glass slides were washed thoroughly in detergent and rinsed several times in double distilled water followed by two rinses in ethanol. Then they were soaked in 0.01% solution of poly-L-lysine (MW 300,000; Sigma) for 10 min, rinsed several times in distilled water, dried at room temperature, and stored.

The spermatozoa collected from testes, caput, corpus, cauda, and vas deferens were smeared onto a clean glass slide coated with 0.05% poly-L-lysine and the smear was allowed to dry at room temperature. The slides were then dipped in 10% neutral formalin to fix the sperm cells. The slides were neutralized in 0.5 M ammonium chloride and the spermatozoa were permeabilized using 0.25% Triton X-100 and chilled ethanol. The slides were washed with PBS at room temperature. The slides were incubated overnight at 4 °C in a 1:200 dilution of the respective primary antibody. The slides were washed extensively with PBS and incubated with antirat IgG conjugated with FITC. After the incubation was over the slides were again washed extensively in PBS to remove the background. Then they were observed under the NIKON Microscope equipped with a Nikon B-2A filter for excitation of the FITC conjugates. The Cool Snap camera and the Chemimager system of the Alpha Innotech, USA, were used for capturing the images.

Zona binding assay. Mature female mice (Swiss strain) of 3–4 months age were first checked for the proestrous stage and then superovulated [25] by injecting 5 IU pregnant mare serum gonadotropin at 4.00 PM and 5 IU of hCG 48 h later. The mice were killed and the fallopian tubes were excised, cleared of adherent fatty tissue, and minced in KRB containing 100 U/ml hyaluronidase for 20 min to disperse all the cumulus cells. The ova were collected and pooled.

A sterile 24-well Falcon tissue culture plate was prepared with each of its wells containing 1 ml of fresh Krebs–Ringer bicarbonate buffer maintained at 37 °C. The ova were distributed in the various wells in such a way that each well receives 10–15 ova. Two hundred microliters each of the sperm prepared as above was added to the ova suspension and the plate was left undisturbed for 15 min. The ova with attached spermatozoa were transferred into fresh wells having respective identical conditions. Three such transfers were made to release the unbound spermatozoa. The images were observed under the Nikon Microscope. Images were captured using the Cool Snap camera and Chemimager of Alpha Innotech, USA.

RT-PCR assays. mRNA from the round cells in the semen was extracted using QuickPrep mRNA purification kit (Amersham Biotech). The mRNA concentration was adjusted to 50 μg/ml and the preparations were immediately processed for first strand synthesis. The first strand synthesis was performed with 2 μg mRNA using Ready-To-Go T-primed first strand synthesis kit (Amersham Biotech) which utilized Moloney murine leukemia virus (M-MuLV) reverse trancriptase and *Not*I-d$(T)_{18}$ primer (5′-d[ACTGGAAGAATTCGCGGCCG CAGGAAT$_{18}$]-3′). PCR of the first strand was performed using VAMP-specific forward primer (GAG TGG GAA GAG TGG TTA CTG GCA as the sense primer and 5′-d[ACTGGAAGAATTCGCG GCCGCAGGAAT$_{18}$]- 3′ as the reverse primer). Beta actin was amplified using GGACTTCGAGCAAGAGATGG (forward) and CACCTTCACCGTTCCAGTTT (reverse) primer set. For SNAP amplification, the primer set used was 5′-CCATGGATAATCT GTCCCCAGAG (forward) and 5′-ACGATTCTTGTTGGTGTCA GC (reverse). The cycling conditions were: 1 min melting at 94 °C, 45 s annealing at 68 °C, and 1 min primer extension at 72 °C. The cycle was repeated 35 times, with 10 s increments in extension time per cycle. After the completion of the cycles, the reaction was held at 72 °C for 30 min, after which the samples were recovered. Five microliters of the reaction from each of the samples was mixed with 1 μl of 6× gel loading buffer (Sigma) and separated on 4% NuSieve 3:1 agarose (FMN) gel and 1× TAE buffer. Electrophoresis was performed at 5 V/cm^2 for 3 h. The gels were visualized and photographed on an Alpha Innotech imager station.

Results

Western blot assays

Protein extracts from spermatozoa collected from testis, caput epididymidis, corpus epididymis, and cauda epididymidis showed an immunopositive band with anti-VAMP antibody at an estimated molecular weight of 33 kDa (Fig. 1A). In order to confirm the identity of this molecule, we performed N-terminal microsequencing of this protein, which showed 100% homology with a docking protein involved in vesicle aggregation and collapse known as Vam6p/VPS39p (results not presented). While testicular spermatozoa possessed moderate levels of Vam6p, those from caput, corpus, and cauda epididymidal regions showed heavy association of this protein (Fig. 1A, lanes 2–4).

Since Vam6p is known to interact with SNAP, we performed a Western blot assay to evaluate the presence of this molecule in spermatozoa of various stages of development. A representative blot is presented in Fig. 1B. Testiscular spermatozoa showed the presence of a ~25 kDa band that stained positive with anti-SNAP antibody (Fig. 1B, lane 1). Protein extracts of spermatozoa from caput, corpus, and cauda epididymides showed a SNAP-positive band at ~50 kDa position, but no band was detected at 25 kDa position (Fig. 1B, lanes

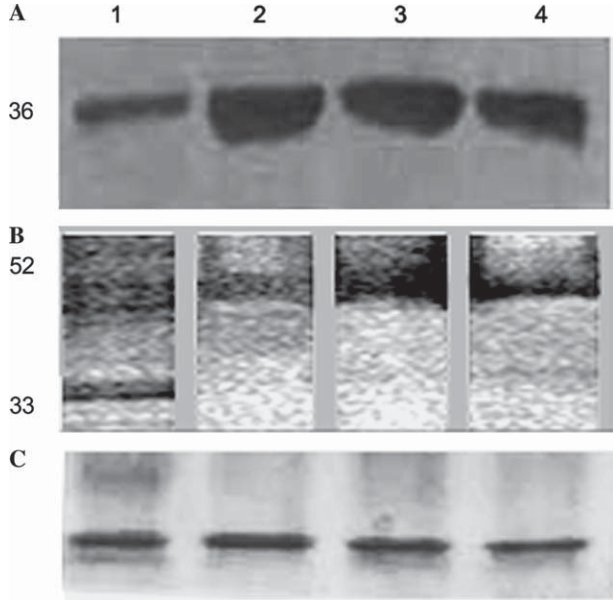

Fig. 1. Western blot analysis of VAMP (A) and SNAP (B) on spermatozoa collected from various segments of the male reproductive tract. Spermatozoa were collected from testis (lane 1), caput epididymidis (lane 2), corpus epididymidis (lane 3), and cauda epididymidis (lane 4) and protein extracts were prepared as discussed in Materials and methods. A 33 kDa band stained positive with VAMP antbody was present on the spermatozoa from all stages of development studied. Similarly, a 25 kDa band stained positive for SNAP in all the samples (B). A panel showing the levels of β-actin is presented (C).

2–4). Corresponding β-actin levels are shown in Fig. 1C, lanes 1–4.

RT-PCR analysis

cDNA prepared from testis, and the three segments of the epididymis showed the presence of Vam6p in all these regions (Fig. 2A, lanes 1–4). But, the expression of SNAP showed more regional variations. Thus, testis and the caput epididymidal segments showed relatively higher levels of SNAP expression (Fig. 2B, lanes 1–2), whereas the corpus and cauda regions showed weaker expression of this gene (Fig. 2B, lanes 3–4). Corresponding β-actin amplification is presented in Fig. 2C. Lane 5 represents negative control in this panel.

Immunolocalization of Vam6p and SNAP on spermatozoa

Spermatozoa from the testis showed moderate and well-defined localization of VAMP on the principal acrosomal region of the head, and the mid-piece and tail regions also stained positive, though the intensity was weak all over (Fig. 3A). The spermatozoa from caput epididymidis were comparable with those from the testis, though the tail stained negative in the latter (Fig. 3B). There was a remarkable enhancement in the amount and distribution of this protein on the spermatozoa from the corpus and cauda epididymides. The corpus spermatozoa showed a distinct enhancement of VAMP on the post-acrosomal domains (Fig. 3C), which got enhanced to another order as the spermatozoa moved to the cauda region (Fig. 3D). Moreover, the corpus and cauda spermatozoa showed marked increase in the levels of Vam6P on their mid pieces. However, tail regions stained negative for VAMP (Figs. 3C and D). Capacitation of spermatozoa in vitro brought about topographical redistribution of Vam6P from the post-acrosomal domain onto the principal acrosomal domain (Fig. 3E). In a progesterone-induced acrosome reaction assay, the spermatozoa lost a substantial part of VAMP from their surfaces, though many of the spermatozoa that underwent showed a streak of fluorescence at their equatorial domains (Fig. 3F). The corresponding phase contrast images are shown in Figs. 3G–L.

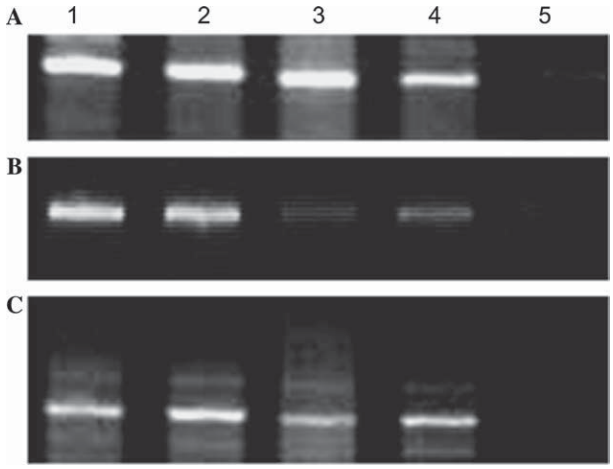

Fig. 2. RT-PCR analysis of VAMP and SNAP messages in testis (lane 1), caput epididymidis (lane 2), corpus epididymidis (lane 3), and cauda epididymidis (lane 4). Representative amplifications of VAMP from cDNA prepared from various regions of the male reproductive tract are shown (A). Amplification of SNAP messages is shown in (B). Corresponding β-actin levels are represented in (C). Lane 5 is of negative control.

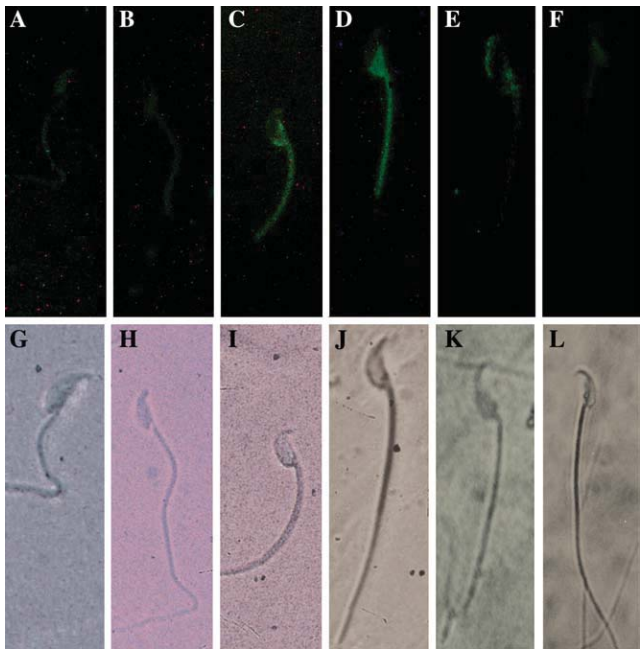

Fig. 3. Immunofluorescence analysis of the localization of VAMP on mouse spermatozoa. Representative immunofluorescene localization of VAMP (A–F) and corresponding phase contrast images (G–L) are shown. Spermatozoa were collected from testis (A,G), caput epididymidis (B,H), corpus epididymidis (C,I), and cauda epididymidis (D,J). The cauda spermatozoa were capacitated in vitro (E,K), and the capacitated spermatozoa were exposed to progesterone (F,L).

The distribution of SNAP followed a different pattern. The spermatozoa from testis, caput epididymidis, corpus epididymidis, and cauda epididymidis showed moderate levels of SNAP all over the sperm surfaces (Figs. 4A–D). But, during capacitation, the localization became more patterned, with the principal acrosomal domain and the mid-piece accumulating heavy levels of this protein (Fig. 4E). Induction of acrosome reaction brought about a remarkable loss of SNAP from sperm surfaces (Fig. 4F). Corresponding phase contrast images are shown in Figs. 4G–L.

Sperm–zona pellucida interaction assay

The binding of spermatozoa to the zona pellucida in control preparations where spermatozoa were incubated with normal rabbit serum before insemination is shown

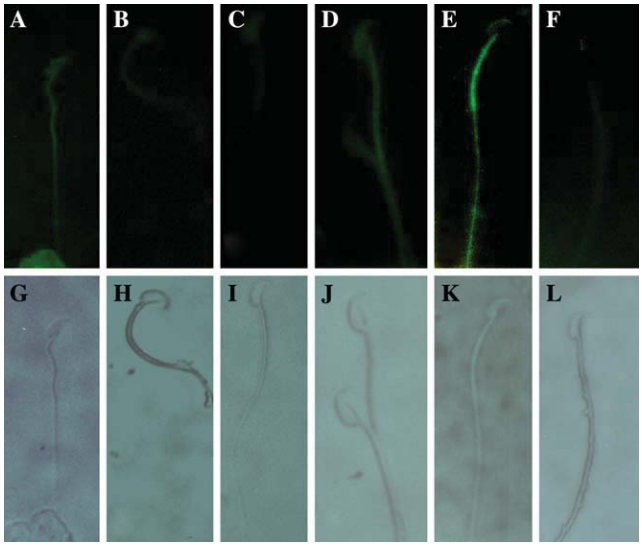

Fig. 4. Immunofluorescence analysis of the localization of SNAP on mouse spermatozoa. Representative immunofluorescene localization of VAMP (A–F) and corresponding phase contrast images (G–L) are shown. Spermatozoa were collected from testis (A,G), caput epididymidis (B,H), corpus epididymidis (C,I), and cauda epididymidis (D,J). The cauda spermatozoa were capacitated in vitro (E,K), and the capacitated spermatozoa were exposed to progesterone (F,L).

in Fig. 5A, where a heavy attachment of spermatozoa to the oocyte could be visualized. Incubation of mouse spermatozoa with anti-VAMP or anti-SNAP antibody followed by insemination into mouse oocyte cultures revealed significantly reduced zona pellucida binding under in vitro conditions (Figs. 5B and C).

Discussion

The fusion of cellular membranes during secretion, endocytosis, and organelle inheritance is essential for cellular compartmentalization. Highly conserved proteins are involved in fusion events throughout the cell and across species. The first such protein to be isolated was called NSF (*N*-ethylmaleimide-sensitive fusion) protein. It is a soluble cytosolic protein that binds to membranes as a complex with other proteins called SNAPs (soluble NSF attachment proteins). NSF and SNAPs bind to families of specific membrane receptors called SNAP receptors or SNAREs. The SNARE hypothesis propagates interactions between specific SNAREs on the vesicle (v-SNAREs) and target (t-SNAREs) membranes are responsible for the specificity of vesicle fusion. The *trans*-pairing of cognate v- and t-SNAREs is a central event in docking membranes before fusion. These interactions are additionally regulated by the Rab GTP-bound proteins, which are also essential for vesicle transport. After specific vesicle–target interaction, the SNARE complex recruits NSF and SNAPs, leading to fusion of the vesicle and target membranes. The ATP-driven chaperone NSF/Sec18p and its partner proteins α-SNAP/Sec17p, and a large family of Rab GTPases thus participate in the fusion events [26].

During sperm development in the testis, *N*-ethylmaleimide sensitive factor, soluble NSF attachment proteins, and homologs of the t-SNARE syntaxin and of the v-SNARE VAMP/synaptobrevin, as well as members of the rab family of small GTPases, are associated with the acrosome in round and elongated spermatids [27]. The participation of sperm SNAREs during membrane fusion events at fertilization in mammals has been suggested [28]. Sperm binding to the zona pellucida occurs via specific receptors on the sperm surfaces, localized over the anterior head region of the spermatozoa, and the zona pellucida glycoproteins. This binding stimulates the spermatozoa to undergo the acrosome

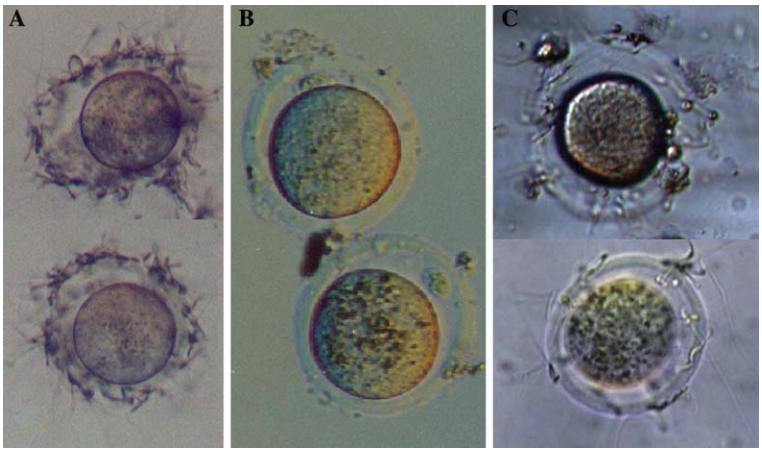

Fig. 5. Effect of VAMP and SNAP antibodies on sperm–zona pellucida interaction in vitro. Spermatozoa treated with normal rabbit serum (A) shows heavy binding of spermatozoa to the zona pellucida. The spermatozoa pre-treated with anti-VAMP antibody showed no spermatozoa attached to the zona pellucida (B). Spermatozoa pre-treated with anti-SNAP antibody showed significant reduction in the number of spermatozoa attached to the zona pellucida (C), 200×.

reaction. During this secretory event, the exocytic vesicle (the acrosome) fuses with the overlying plasma membrane [29]. The multiple fusions between the outer acrosomal membrane and the plasma membrane result in the exposure of new membrane domains both of which are essential for fertilization to proceed.

The Vam6P homolog we have identified in the testis has 100% homology with VAM6P/VPS39, the human/yeast docking molecule involved in vesicle aggregation and collapse. The identification of VAM6P message and the localization of the Vam6P protein on the spermatozoa from the testis with predominant localization on the acrosome suggest its involvement in acrosome biogenesis. Further, the inhibition of sperm–oocyte interaction by Vam6P and SNAP antibodies might suggest a secondary role for this molecule in recognition and docking of the sperm to the zona pellucida. Further, the uniform distribution of Vam6P and SNAP over the entire head and mid piece on the spermatozoa from the testis and epididymis and the reorganization of these molecules onto the acrosomal crest during capacitation in vitro signify targeted protein transport onto membrane subdomains that would render these membranes fusible. We suspect that the Vam6P and SNAP might have been spatially separated on the outer acrosomal membrane and the plasma membrane overlying the acrosome to check premature acrosome reaction in epididymal spermatozoa. Interestingly, capacitation brought about heavy transport of both Vam6P and SNAP onto the outer curvature of the sperm head, and progesterone-induced acrosome reaction showed a complete disappearance of these proteins from the principal acrosomal domain. It is intriguing that the SNAP expressed in the testis had an estimated molecular size of 25 kDa, but that from the epididymis was twofold bigger in size. While the SNAP message amplified from all the regions should have predictably coded for a 25 kDa band, the shift in molecular size of SNAP extracted from the epididymal spermatozoa might be due to its SDS-resistant interaction with some of the yet unidentified partners.

It is quite likely that progesterone induced vacuolar collapse similar to the events occurring in homotypic membrane fusion in yeast vacuoles. In yeast, molecular basis of vacuolar biogenesis and morphogenesis is dependent on the function of over 50 members of the VPS (vacuolar protein sorting), PEP, VAM (vacuolar assembly molecule) [30–33], and others [34–36]. Some tethering/docking factors studied in *Saccharomyces cerevisiae* gene products involved in vacuole fusion are Vps11p, Vps18p, Vps16p, Vps33p, Vam2p/Vps41p, and Vam6p/Vps39p which assemble on the vacuolar membrane into a complex referred to as homotypic fusion and vacuole protein sorting (HOPS complex) [37–39]. This complex has been shown to cooperate with the yeast homolog of mammalian Rab7, Ypt7p, to enable vacuole fusion mediated by the SNAREs [37–41].

Vacuolar fusion has been shown to occur in a cascade of stages including priming, docking, and the final bilayer fusion. Priming starts with the preparation of individual vacuoles with the other vacuoles. It requires the action of Sec17p and sec18p [42] the vacuole having a *cis*-complex of SNAREs bound to it is disassembled and the t-SNARE is activated. The primed vacuoles come into contact during docking. Docking requires primed vacuoles, v- and t-SNAREs, and Ypt7p [43,44]. The interaction between vacuoles becomes irreversible through the association of SNARE proteins from the apposed vacuoles, forming a *trans*-SNARE complex. *trans*-SNARE pairing triggers the release of luminal calcium to interact with calmodulin and mediate downstream events that lead to fusion [45].

The VAM6 gene has been also known as VPS39 and CVT4 (cytoplasmic vacuole transport) [31,32,46]. The VAM6/VPS39 gene encodes hydrophilic protein of 122.9 kDa with 1049 amino acids [47]. Vam2p and Vam6p are required for normal vacuole morphology [30,47,48], for protein sorting to the vacuole [31,49,50], and for cytosol-to-vacuole protein targeting [46]. Though Vam2p and Vam6p are localized to the vacuole [30] and associated in a large complex [47], Vps41p/Vam2p also fulfills a specific role in transport vesicle budding from the Golgi [50] where it interacts with the AP3 coat. Vam2p and Vam6p are required for the docking stage of homotypic vacuole fusion and for the formation of stable *trans*-SNARE pairs [51].

A homolog of the vacuolar protein sorting gene product Vam6p/Vps39p has been identified in human and is designated as hVam6p. hVam6p promotes lysosome clustering and fusion in vivo by associating with the cytoplasmic face of the lysosomal membrane. The presence of a citron homology domain at the NH_2 terminus is the unique feature of human protein. An NH_2-terminal citron homology (CNH) domain and a central clathrin homology (CLH) repeat domain in hVam6p are required for lysosome clustering and fusion. This suggests its function as a tethering/docking factor specifically involved in lysosome fusion [52]. Deposition of hVam6p onto the membranes of lysosomes and late endosomes likely enhances their adhesiveness, leading to the formation of clusters. This in turn, increases the probability of fusion resulting in the generation of large vacuoles [52]. Overexpression of Vam6p at specific domains might augment the process by which lysosomes and late endosomes normally exchange materials, which may have a direct significance in mediating acrosome reaction.

Many newly synthesized vacuolar proteins require transport from the endoplasmic reticulum to the vacuole by the secretory pathway. At the *trans*-Golgi network, vacuolar proteins are diverted to the

endosomal compartments, which ultimately fuse with the vacuole [53]. This process is regulated by more than 40 proteins designated vacuolar protein sorting (VPS) proteins. Vps mutants have been divided into six major subgroups based on distinct hydrolase missorting, vacuole morphology, and growth phenotypes [31]. VPS39 is a class B VPS gene and its deletion results in the cytoplasmic accumulation of endosomal compartments and vesicles, which fail to fuse with the vacuole, fragmentation of the vacuole, and severe hydrolase missorting phenotypes [30,31]. It has been demonstrated that the class C-VPS complex of vacuolar protein sorting (vps) proteins (vps 11, vps18, vps16, and vps33 also contains two additional proteins, vps39 and vps41 [38]. The COOH-terminal 148 amino acids of vps39 bind to vps11 and thus get associated with class C-vps complex. A large protein complex containing vps39 and vps41 functions as a downstream effector of the active, GTP-bound form of Ypt7, a rab GTPase required for the fusion of vesicular intermediates with a vacuole [51]. It has been shown that the C-vps complex also functions to stimulate nucleotide exchange on Ypt7. Vps39 directly binds the CDP-bound and nucleotide-free forms of Ypt7 and purified Vps39 stimulated nucleotide exchange on Ypt7. Class C-VPS complex also acts as a Ypt7 effector that tethers transport vesicles to the vacuole [51]. Thus, this complex directs multiple reactions during the docking and fusion of vesicles with the vacuole, thus each contributes to the overall specificity and efficiency of this process [38].

With the identification of Vam6p and SNAP on mouse spermatozoa and their temporal concentration onto the principal acrosomal domains during capacitation, we feel that a model of v-SNARE interacting with a complementary t-SNARE would explain an acrosome reaction model. Although many additional protein/components might modulate this process as in the case of somatic cells [54,55], SNAREs are important in defining membrane trafficking events in germ cells as well. SNARE homologs can serve as markers for the acrosome of late round spermatids [2,56]. Thus these findings have been extended here to describe the continuing of these proteins throughout acrosome biogenesis and beyond, thus suggesting a possible role for SNARE proteins in membrane trafficking necessary for the maturation and vesiculation of this organelle. The expression of Vam6p message in the testis and epididymis might suggest that the testicular spermatozoa might inherit testis-expressed form of this protein, but could also accept the epididymal variant during maturation process. The mechanistic aspects of the mobilization of Vam6p/SNAP during capacitation in vitro cannot be explained at present. However, the identification of Vam6p and SNAP variants on spermatozoa, and their involvement in acrosome reaction and sperm–oocyte interaction necessitate further investigations to understand the interplay of SNARE complexes in gamete interaction.

Acknowledgments

This work was supported by DBT Grant No. BT/PR1316/Med/09/212/98 to Pradeep G. Kumar. M. BrahmaRaju and Mohammed Shoeb were awarded Junior Research Fellowship from Council of Scientific and Industrial Research, New Delhi, vide award Nos. F.NO.9/301(99)/2002-EMR-1 and F.NO.9/301(98)/2002-EMR-1, respectively.

References

[1] A.C. Allison, E.F. Hartree, J. Reprod. Fertil. 21 (1970) 501–515.
[2] R.D. Moreno, J. Ramalho-Santos, E.K. Chan, G.M. Wessel, G. Schatten, Dev. Biol. 219 (2000) 334–349.
[3] J.A. Martinez-Menarguez, H.J. Geuze, J. Ballesta, J. Histochem. Cytochem. 44 (1996) 313–320.
[4] R.N. Peterson, J. Bozzola, K. Polakoski, Tissue Cell 24 (1992) 1–15.
[5] M.H. Burgos, L.S. Gutierrez, Anat. Rec. 216 (1986) 139–145.
[6] G.E. Olson, V.P. Winfrey, V.A. Westbrook, M.H. Melner, Mol. Reprod. Dev. 50 (1998) 103–112.
[7] G.E. Olson, V.P. Winfrey, S.K. Nagdas, Biol. Reprod. 58 (1998) 361–370.
[8] C.P. Leblond, Y. Clermont, Ann. NY Acad. Sci. 20 (55) (1952) 548–573.
[9] Y. Clermont, C.P. Leblond, Am. J. Anat. 96 (1955) 229–253.
[10] M.S. Santos, J. Barbosa Jr., G.S. Veloso, F. Ribeiro, C. Kushmerick, M.V. Gomez, S.S. Ferguson, V.F. Prado, M.A. Prado, J. Neurochem. 78 (2001) 1104–1113.
[11] G. Griffiths, G. Warren, I. Stuhlfauth, B.M. Jockusch, Eur. J. Cell Biol. 26 (1981) 52–60.
[12] R.D. Moreno, J. Ramalho-Santos, P. Sutovsky, E.K. Chan, G. Schatten, Biol. Reprod. 63 (2000) 89–98.
[13] F.R. Susi, C.P. Leblond, Y. Clermont, Am. J. Anat. 130 (1971) 251–267.
[14] L. Hermo, A. Rambourg, Y. Clermont, Am. J. Anat. 157 (1980) 357–373.
[15] X.M. Tang, M.F. Lalli, Y. Clermont, Am. J. Anat. 163 (1982) 283–294.
[16] G. Thorne-Tjomsland, J.C. Jamieson, Anat. Rec. 244 (1996) 15–27.
[17] Y. Clermont, A. Rambourg, L. Hermo, Anat. Rec. 240 (1994) 469–480.
[18] J.B. Sorensen, Pflugers Arch. (2004) in press.
[19] J.P. Nolan, R.H. Hammerstedt, FASEB J. 11 (1997) 670–682.
[20] H. Breitbart, B. Spungin, Mol. Hum. Reprod. 3 (1997) 195–202.
[21] R.J. Advani, H.R. Bae, J.B. Bock, D.S. Chao, Y.C. Doung, R. Prekeris, J.S. Yoo, R.H. Scheller, J. Biol. Chem. 273 (1998) 10317–10324.
[22] C.G. Schuette, K. Hatsuzawa, M. Margittai, A. Stein, D. Riedel, P. Kuster, M. Konig, C. Seidel, R. Jahn, Proc. Natl. Acad. Sci. USA 101 (2004) 2858–2863.
[23] S. Chatterjee, M. Laloraya, G.P. Kumar, Biochem. Biophys. Res. Commun. 197 (1993) 450–456.
[24] P.G. Kumar, M. Laloraya, C.Y. Wang, Q.G. Ruan, A. Davoodi-Semiromi, K.J. Kao, J.X. She, J. Biol. Chem. 276 (2001) 41357–41364.
[25] H. Mizoguchi, W.R. Dukelow, Biol. Reprod. 23 (1980) 237–241.
[26] S.C. van Ijzendoorn, K.E. Mostov, D. Hoekstra, Int. Rev. Cytol. 232 (2003) 59–88.

[27] J. Ramalho-Santos, R.D. Moreno, G.M. Wessel, E.K. Chan, G. Schatten, Exp. Cell Res. 267 (2001) 45–60.
[28] J. Ramalho-Santos, R.D. Moreno, P. Sutovsky, A.W. Chan, L. Hewitson, G.M. Wessel, C.R. Simerly, G. Schatten, Dev. Biol. 223 (2000) 54–69.
[29] R. Yanagimachi, Zygote 2 (1994) 371–372.
[30] Y. Wada, Y. Ohsumi, Y. Anraku, J. Biol. Chem. 267 (1992) 18665–18670.
[31] A. Reymond, S. Schmidt, V. Simanis, Mol. Gen. Genet. 234 (1992) 449–456.
[32] J.H. Rothman, C.T. Yamashiro, P.M. Kane, T.H. Stevens, Trends Biochem. Sci. 14 (1989) 347–350.
[33] D.J. Klionsky, S.D. Emr, J. Biol. Chem. 265 (1990) 5349–5352.
[34] H. Wichmann, L. Hengst, D. Gallwitz, Cell 71 (1992) 1131–1142.
[35] V. Dulic, H. Riezman, EMBO J. 8 (1989) 1349–1359.
[36] K. Kitamoto, K. Yoshizawa, Y. Ohsumi, Y. Anraku, J. Bacteriol. 170 (1988) 2687–2691.
[37] G. Eitzen, E. Will, D. Gallwitz, A. Haas, W. Wickner, EMBO J. 19 (2000) 6713–6720.
[38] A.E. Wurmser, T.K. Sato, S.D. Emr, J. Cell Biol. 151 (2000) 551–562.
[39] C. Ungermann, A. Price, W. Wickner, Proc. Natl. Acad. Sci. USA 97 (2000) 8889–8891.
[40] C. Ungermann, W. Wickner, Z. Xu, Proc. Natl. Acad. Sci. USA 96 (1999) 11194–11199.
[41] C. Ungermann, G.F. von Mollard, O.N. Jensen, N. Margolis, T.H. Stevens, W. Wickner, J. Cell Biol. 145 (1999) 1435–1442.
[42] A. Mayer, W. Wickner, A. Haas, Cell 85 (1996) 83–94.
[43] A. Mayer, W. Wickner, J. Cell Biol. 136 (1997) 307–317.
[44] C. Ungermann, B.J. Nichols, H.R. Pelham, W. Wickner, J. Cell Biol. 140 (1998) 61–69.
[45] C. Peters, A. Mayer, Nature 396 (1998) 575–580.
[46] T.M. Harding, K.A. Morano, S.V. Scott, D.J. Klionsky, J. Cell Biol. 131 (1995) 591–602.
[47] N. Nakamura, A. Hirata, Y. Ohsumi, Y. Wada, J. Biol. Chem. 272 (1997) 11344–11349.
[48] B. Zheng, J.N. Wu, W. Schober, D.E. Lewis, T. Vida, Proc. Natl. Acad. Sci. USA 95 (1998) 11721–11726.
[49] C.R. Cowles, G. Odorizzi, G.S. Payne, S.D. Emr, Cell 91 (1997) 109–118.
[50] P. Rehling, T. Darsow, D.J. Katzmann, S.D. Emr, Nat. Cell Biol. 1 (1999) 346–353.
[51] A. Price, W. Wickner, C. Ungermann, J. Cell Biol. 20 (148) (2000) 1223–1229.
[52] S. Caplan, L.M. Hartnell, R.C. Aguilar, N. Naslavsky, J.S. Bonifacino, J. Cell Biol. 154 (2001) 109–122.
[53] T.A. Vida, G. Huyer, S.D. Emr, J. Cell Biol. 121 (1993) 1245–1256.
[54] B.J. Nichols, H.R. Pelham, Biochim. Biophys. Acta 1404 (1998) 9–31.
[55] S.R. Pfeffer, Nat. Cell Biol. 1 (1999) E17–E22.
[56] P. Sutovsky, J. Ramalho-Santos, R.D. Moreno, R. Oko, L. Hewitson, G. Schatten, Hum. Reprod. 14 (1999) 2301–2312.