The Role and Regulation of Sperm Gelsolin Prior to Fertilization

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In order to acquire fertilization competence, spermatozoa should undergo several biochemical changes in the female reproductive tract, known as capacitation. The capacitated spermatozoon can interact with the egg zona-pellucida resulting in the occurrence of the acrosome reaction, a process which allowed its penetration into the egg and fertilize it. Sperm capacitation requires actin polymerization, while F-actin must disperse prior to the acrosome reaction. Here we suggest that the actin severing protein, gelsolin, is inactive during capacitation and is activated prior to the acrosome reaction. The release of bound gelsolin from phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) by PBP10, a peptide containing the PIP<sub>2</sub>-binding domain of gelsolin, or by activation of PLC which hydrolyses PIP<sub>2</sub>, caused rapid Ca<sup>2+</sup>-dependent F-actin depolymerization as well as enhanced acrosome reaction. Using immunoprecipitation assays, we showed that the tyrosine kinase SRC and gelsolin coimmunoprecipitate, and activating SRC by adding 8Br-cAMP enhanced the amount of gelsolin in this precipitate. Moreover, 8Br-cAMP enhanced tyrosine phosphorylation of gelsolin and its binding to PIP<sub>2,4,5p</sub>, both of which inactivated gelsolin, allowing actin polymerization during capacitation. This actin polymerization was blocked by inhibiting the Src-family-kinases, suggesting that gelsolin is activated under these conditions. These results are further supported by our finding that PBP10 was unable to cause complete F-actin breakdown in the presence of 8Br-cAMP or vanadate. In conclusion, inactivation of gelsolin during capacitation occurs by its binding to PIP<sub>2</sub> and tyrosine phosphorylation by SRC. The release of gelsolin from PIP<sub>2</sub> together with its dephosphorylation enables gelsolin activation, resulting in the acrosome reaction.

Mammalian sperm must undergo a series of biochemical modifications in the female reproductive tract prior to productive sperm-egg interaction and the occurrence of the acrosome reaction (AR). The mechanisms of these modifications, collectively called capacitation, are not well characterized. There is no clear recognizable marker of successful capacitation, although several cellular changes are known to have taken place, including cholesterol efflux from the plasma membrane, an increase in membrane permeability to bicarbonate and calcium ions, and an increase in cAMP and protein kinase A (PKA)-dependent protein tyrosine phosphorylation (reviewed by (1,2). Another important process that occurs during sperm capacitation is actin polymerization; however, at the end of the capacitation, the polymers must disperse in order to achieve the AR (3). It was suggested that an increase in F-actin creates a network between the plasma and the outer acrosomal membranes, and the dispersion of F-actin between the two membranes is needed to enable the AR (3-6).

Better understanding of the mechanisms responsible for actin remodeling requires a closer look at actin binding proteins. The presence of actin-
Moreover, we showed elsewhere that PIP2(4,5), the kinase and protein kinase C (PKC) (1,28). PLD activity, which is regulated by the crosstalk polymerization during sperm capacitation depends on interactions with PIP2(4,5) (23-27). It appears that actin signaling enzymes including PLC and PLD through that gelsolin modulates the activity of several PIP2(4,5), and to cause the subsequent release of gelsolin’s binding to the actin filaments (19). Previous work identified SRC in human spermatozoa, and it appears to be involved in regulating sperm capacitation, calcium fluxes, tyrosine phosphorylation and the acrosome reaction (20). In a recent study, we reported that the epidermal growth factor receptor (EGFR) is partially activated during sperm capacitation by the cAMP/PKA/SRC mechanisms. It was suggested that EGFR is phosphorylated at the SRC specific site, suggesting that SRC is active during capacitation (21,22). Additionally, EGFR-mediated signaling is known to activate phospholipase Cγ (PLCγ), to hydrolyze PIP2(4,5), and to cause the subsequent release of PIP2(4,5) bound proteins. In vitro studies have shown that gelsolin modulates the activity of several signaling enzymes including PLC and PLD through interactions with PIP2(4,5) (23-27). It appears that actin polymerization during sperm capacitation depends on PLD activity, which is regulated by the crosstalk between PKA and protein kinase C (PKC) (1,28). Moreover, we showed elsewhere that PIP2(4,5), the cofactor for PLD activation and F-actin production during sperm capacitation, is mediated by phosphatidylinositol-4-kinase (PI4K) but not by phosphatidylinositol-3-kinase (PI3K) activity (29).

Even though the role of gelsolin in somatic cells is well established, the presence and the possible role of this protein in the male gamete is not fully understood. In guinea-pig sperm, gelsolin and actin were detected in a mixture of plasma and outer acrosomal membranes, and both proteins were absent from the membranes of capacitated spermatozoa (30).

We propose that during sperm capacitation, gelsolin is inactive in order to maintain actin polymerization; at some point prior to the AR, gelsolin is activated, leading to the occurrence of the AR. We further suggest that gelsolin inactivation occurs via two mechanisms: First, the binding of gelsolin to PIP2(4,5) and the second, the phosphorylation of tyrosine residues on gelsolin by SRC, which was shown to release gelsolin from the actin filaments (19). The data described in here confirm this hypothesis and provide a better understanding of the regulation of gelsolin during sperm capacitation and in the acrosome reaction.

Materials and methods

Materials

PBP10 (Polyphosphoinositide-Binding Peptide, Rhodamine B Conjugated), PP1, U73122, and A23187 were obtained from Cal-Biochem (San Diego, CA). Capacitation medium, F-10 (HAM) nutrient mixture with L-Glutamine was purchased from Biological Industries (Kibbutz Beit Haemek, IL). Goat polyclonal anti-gelsolin (C-20), mouse monoclonal anti-SRC, (H-12), anti beta-actin HRP-conjugated antibodies, secondary donkey anti–goat IgG, protein G PLUS-Agarose (sc-2002) and anti PIP2(4,5) (PIP2 2C11, sc-53412) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor 568 donkey anti–goat IgG was purchased from Invitrogen Molecular Probes. All other chemicals were purchased from Sigma (Sigma-Aldrich Israel Ltd., Rehovot, Israel), unless otherwise stated.

Sperm Preparation

Human semen was initially liquefied. The semen was then loaded on a Percoll gradient (80%, 40% and 20%) and centrifuged for 30 min at 6,750 rpm at room temperature. The lower layer containing the sperm was collected and washed twice in HAM F-10 then recentrifuged and allowed to "swim up" after the last wash at 37°C. The motile cells were collected and resuspended in capacitation medium, and the pellet was discarded. Only sperm preparations that contained at least 70% motile sperm were used in the experiments.

Sperm Capacitation

Human sperm (1×10⁷ cells/ml) were capacitated by incubation in capacitation media (HAM F-10) supplemented with 3 mg/ml BSA. The cells were incubated in this capacitation medium for 3 h at 37°C in 5% CO₂. The capacitation state of the sperm was confirmed after the 3 h incubation by examining the ability of the sperm to undergo the acrosome reaction.

Assessment of Sperm Acrosome Reaction

Washed cells (1×10⁷ cells/ml) were capacitated...
for 3 h at 37°C in capacitation medium. The inhibitors indicated for the various experiments were added after 3 h of incubation for 10-20 min, and the inducers were then added for another 60 min of incubation. The percentage of acrosome-reacted sperm was determined microscopically on air-dried sperm smears using FITC-conjugated Pisum sativum agglutinin (PSA). An aliquot of spermatozoa was smeared on a glass slide and allowed to air-dry. The sperm were then permeabilized by adding methanol for 15 min at room temperature. The cells were washed three times at 5-min intervals with TBS, air dried, and then incubated with FITC-PSA (60 mg/ml) at room temperature in the dark for 60 min, washed twice with H2O at 5 min intervals, and mounted with FluoroGuard Antifade (Bio-Rad Lab). For each experiment, at least 150 cells per slide on duplicate slides were evaluated (a total of 300 cells per experiment). Cells with green staining over the acrosomal cap were considered acrosome intact; those with equatorial green staining or no staining were considered acrosome reacted.

**Immunoblot Analysis**

Sperm lysates were prepared by the addition of lysis buffer that contained 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 6% SDS, protease inhibitor cocktail 1:100 (Calbiochem), 50 µM NaF, 50 µM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM Na3VO4, to the pellet. The mixture was vortexed for 10 min at room temperature. Lysates were then centrifuged at 14,000×g for 5 min at 4°C, the supernatant was removed, and the protein concentration was determined by the Bradford method (Bradford, 1976 #179). Sample buffer (2×) was added to the supernatant and boiled for 5 min. The extracts were separated on 10% SDS-polyacrylamide gels and electrophoretically transferred (200 mA for 1 h) to nitrocellulose membranes. Western blotting was performed using a buffer composed of 25 mM Tris (pH 8.2), 192 mM glycine, and 20% methanol. The nitrocellulose membranes were blocked with 5% milk (for anti gelsolin and anti actin) or 5% BSA (for anti p-SRC and anti SRC) in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBST), for 30 min at room temperature. Gelsolin and actin were immunodetected using a polyclonal anti-gelsolin antibody (diluted 1:3000), horseradish peroxidase (HRP)-conjugated anti-actin (1:3000) and a polyclonal anti-SRC antibody (diluted 1:3000). The membranes were incubated overnight at 4°C with the primary antibodies. Next, the membranes were washed three times with TBST and incubated for 1 h at room temperature with the appropriate secondary antibody diluted at 1:10,000. The membranes were washed three times with TBST and visualized by enhanced chemiluminescence (Amersham).

**Fluorescence Staining of Actin Filaments**

Sperm cells were spread on microscope slides. After air-drying, the sperm cells were fixed in 2% formaldehyde in TBS for 10 min, placed in 0.2% Triton X-100 in TBS for 30 min, washed three times at 5-min intervals in distilled water, air dried, and then incubated with Phalloidin-FITC (4 µM in TBS) for 60 minutes, washed four times with distilled water at 10-min intervals, and mounted with FluoroGuard Antifade (Bio-Rad).

**Determination of Actin Incorporation into the Triton-Insoluble Cytoskeleton of Sperm, and Immunoblotting**

Sperm suspensions were incubated in HAM F-10 (3×10^7/ml) for 3 h, and the cells were lysed as previously described (29). Briefly, the sperm cells were washed once with TBS. An equal volume of lysis solution that contained 1.5% Triton X-100 was added, and the suspension vortexed vigorously (10 min at 4°C). At the end of this incubation, the mixture was centrifuged at 12,000×g for 5 min. The supernatant (Triton-soluble G-actin) was collected. The Triton-insoluble content was determined by the addition of 50 µl of lysis buffer containing 6% SDS. The mixture was vortexed vigorously for 10 min. The sample was centrifuged at 12,000×g for 5 min, and the supernatant (Triton-insoluble F-actin) was collected. Sample buffer was added, and the extracted proteins were separated by SDS-PAGE and immunoblotted, as indicated.

**Immunocytochemistry**

For immunocytochemistry, sperm cells were spread on glass slides, air-dried, fixed in formaldehyde (4%) for 10 min, dipped in 0.5% Triton X-100 in TBS for 30 min, and washed three times at 5-min intervals with TBS. Nonspecific reactive sites were blocked for 30 min at room temperature with TBS containing 10% donkey serum. The cells were incubated for 24 h at 4°C with goat polyclonal anti-gelsolin (C-20) antibody diluted 1:50 in TBS containing 1% donkey serum. Next, the slides were washed once in TBS-T and twice (for 5 min) in TBS. The bound antibody was detected using Alexa Fluor 568 donkey anti–goat IgG (a 1:200 dilution), incubated for 1h at 37°C and followed by one wash with TBS-T and two washes with H2O at 5 min intervals. The slides were then mounted with FluoroGuard Antifade. Nonspecific staining was
determined by incubating the sperm without the primary antibody. No staining was detected.

Microscopy

All images were captured on an Olympus AX70 microscope at 400× magnification. This microscope was equipped with an Olympus DP50 digital camera and with the Viewfinder Lite ver. 1 software (Pixera Corp., Los Gatos, CA). All fluorescence determinations were performed under nonsaturated conditions. Both the experiments and staining were performed on the same day, and the sperm were photographed within 24h to reduce the loss of fluorescence. All cell preparations from a single experiment were photographed in the same session and for the same exposure period. The fluorescence intensity was quantified using the MetaMorph Image J software (National Institutes of Health) and the background intensity was subtracted. For F-actin, all experiments were carried out in duplicate and at least 100 cells (5–7 pictures) per slide were quantified for fluorescence intensity. For gelsolin, at least 50 cells were quantified for fluorescence intensity.

Preparation of head and tail fractions of human sperm

Human spermatozoa (5×10^7 cells/ml) were isolated and capacitated as previously described. After incubation, the sperm samples were transferred into glass tubes and sonicated on ice for 1 min using the “Vibra Cell” (Sonics & Materials INC, Danbury, CT, USA) with an intensity setting of 40. The cells were then sonicated for 15 s to facilitate the separation of sperm heads and tails (32). Following sonication, the samples were layered over a 75% Percoll cushion in 2 ml tubes, and centrifuged at 700 rpm for 15 min to isolate the heads and tails in separate fractions. The pellet that formed contained the sperm heads, while the top layer contained the sperm membranes. The tails resided at the interface between the two liquid layers. The heads and tails were removed and diluted with F-10 media. The purity of each fraction was assessed by microscopy prior to analysis. The head and tail samples were then centrifuged at 1,400 rpm for 5 min, the supernatant removed, and the remaining pellet was SDS-extracted as previously described.

Immunoprecipitation of Gelsolin-SRC/ Gelsolin - PIP_{2(4,5)}/Gelsolin-p-Thy

Sperm cells (5×10^7 cells/ml) were centrifuged at 4,000 rpm for 10 min and washed with cold TBS. Equal amounts of protein (100 mg for spermatozoa) were sonicated three times in homogenization buffer containing: 20 mM Tris-HCl, pH 7.5; 0.25M sucrose; 2 mM EGTA; 2 mM EDTA; 1 mM benzamidine; 1 mM Na_3VO_4; 10% (w/v) glycerin; 5 mM NaF; 30 mM NaH_2PO_4; 25 μg/ml leupeptin; 4 μg/ml aprotinin; 1.5 μg/ml pepstatin; 2 μg/ml antipain; and 1 mM PMSF, and incubated for 30 min at 4°C. Next, the mixture was centrifuged at 14,000 rpm for 5 min. For immunoprecipitation, 50 μl of protein A/G plus -agarose was added for preclearing the suspension and the samples were incubated for 2 h at 4°C. The mixture was centrifuged at 7,500 rpm for 5 min and the supernatants were then incubated with 2 μg anti SRC/p-Thy/ PIP_{2(4,5)} overnight at 4°C. The next day, 50 μl of protein A/G agarose beads was added and further incubated for 2h with gentle agitation at 4°C. The beads were then subjected to three washes with 0.1% Triton in TBS buffer, and boiled for 5 min with SDS sample buffer.

Statistical Analysis

Data are expressed as mean ± SD of at least three experiments for all determinations. Statistical significance was calculated by Student’s t-test or by ANOVA with Bonferroni’s post-hoc comparison test using SPSS software (Chicago, IL, USA).

Results

Localization of gelsolin in sperm capacitation

In our previous studies, we showed that actin polymerization occurs during sperm capacitation (3,28). Gelsolin, an actin-severing protein, is expected to be localized with the F-actin fraction in the cell when activated. The data in Figure 1 reveal that gelsolin levels during capacitation remained constant (Fig. 1A). The Triton X-100 sperm fractions revealed that before capacitation, most of the actin was in the form of monomers i.e. the G-actin form (soluble fraction), while at the end of capacitation most of the actin was in the form of polymers i.e. the F-actin form (insoluble fraction) (Fig. 1B). Thus, gelsolin is localized in the G-actin fraction before capacitation, while at the end of capacitation, gelsolin is localized in the F-actin fraction (Fig. 1 B and C).

We have shown elsewhere in bovine sperm, that before capacitation there is almost no F-actin in the sperm head, as most of it is localized to the tail-midpiece, whereas after capacitation, there is a significant increase in F-actin in the head (28). Here, we demonstrated that the amount of F-actin in human sperm heads was low before capacitation, increased in capacitated cells, and was reduced again in acrosome reacted cells (Fig. 2A). Moreover, immunocytochemical staining revealed an increase of gelsolin staining in the sperm head during capacitation (Fig. 2B). Interestingly, the increase of
Gelsolin in the head could not be seen when intracellular calcium was chelated by BAPTA/AM (Fig. 2B), indicating the importance of calcium ions for this translocation. Further support for the increase of gelsolin in the head is seen by western blot analysis of separated tail and heads, showing that most of the gelsolin was localized in the tail before capacitation whereas after capacitation there was a significant decrease in its amount in the tail and a significant increase in the head (Fig. 2C). These data suggest that gelsolin translocates from the sperm tail to the head during capacitation.

**Activation of gelsolin in capacitated sperm**

Gelsolin is an actin-severing protein which causes F-actin depolymerization, and its activation is regulated by calcium ions and phosphoinositides (14-16). Relatively low calcium concentrations cause conformational changes in the C-terminus of gelsolin which expose its binding site to F-actin, whereas higher calcium concentrations cause a second conformational change exposing the catalytic site (33). In order to test gelsolin activity, the cells were incubated under capacitation conditions to increase the intracellular levels of F-actin. At this point gelsolin was activated by increasing the intracellular calcium using the calcium ionophore A23187 or by activating the EGFR by EGF. The data in Fig. 3 reveal a rapid decrease in F-actin levels when intracellular calcium concentrations increased. This effect was blocked when intracellular calcium was chelated using BAPTA/AM. Moreover, a rapid decrease in F-actin was also induced by adding the peptide PBP10 (Fig. 3), which is a peptide derived from the PIP2(4,5)-binding domain of gelsolin. This peptide competes with gelsolin binding to PIP2(4,5) causing the release of gelsolin from PIP2(4,5) and enabling its activity on F-actin (34). F-actin depolymerization by PBP10 was also prevented by chelating intracellular calcium using BAPTA/AM (Fig. 3) suggesting the dependence of gelsolin activation on calcium ions.

In our previous study, we showed that F-actin depolymerization must occur in capacitated sperm prior to the acrosome reaction (3). Thus, the acrosome reaction cannot occur if F-actin is polymerized. Similarly, the F-actin depolymerization shown in Figure 3 occurs before the acrosome reaction (Fig. 4). Increasing the intracellular calcium concentration or adding PBP10 to capacitated sperm induced a significant increase in the acrosome reaction rate in sperm that was pretreated with the intracellular calcium chelator BAPTA/AM (Fig. 4).

The data in Figures 3 and 4 suggest that gelsolin is activated by an increase in the intracellular calcium levels of capacitated cells, enabling the acrosome reaction.

**Gelsolin activation depends on phospholipase C activity**

PIP2(4,5) can bind to gelsolin and the hydrolysis of PIP2(4,5) by phospholipase C (PLC) releases the bound gelsolin, resulting in gelsolin activation in Sertoli cells (35). Here, we demonstrate that F-actin depolymerization and acrosome reaction induced by the calcium ionophore, A23187, or EGF in capacitated sperm, was significantly reduced when PLC is blocked by U73122 (Figs. 5 and 6). However, when actin depolymerization or the acrosome reaction was induced by the peptide PBP10, there was no effect on F-actin depolymerization by the PLC inhibitor U73122 (Figs. 5 and 6). These data suggest that PLC mediates actin depolymerization prior to the acrosome reaction. In addition, sperm PLC requires micromolar levels of calcium for half maximal activation similar to the calcium concentration found after the sperm-zona interaction (6), suggesting the possible activation of PLC at this time. Stimulation of capacitated spermatozoa with progesterone or with isolated zona-pellucida leads to activation of calcium-dependent phospholipase C (36). Our results show that PLCγ was phosphorylated on Tyr-782 at the end of the capacitation period, an indication of its activation. This phosphorylation could also be seen when AR was induced by the Ca2+ ionophore, A23187, and was reduced after 1h (Fig. 5B). The induction of F-actin depolymerization by EGF, the physiological ligand of EGFR, a known activator of PLCγ, and the phosphorylation/activation of PLCγ indicates that the PLCγ isoform mediates F-actin depolymerization. The induction of F-actin breakdown by PBP10 suggests that this reaction is mediated by gelsolin.

**How is gelsolin activity inhibited during sperm capacitation?**

As shown above, gelsolin can be activated in capacitated sperm by PBP10, indicating that the intracellular Ca2+ concentration existing in capacitated sperm is sufficient to activate gelsolin. Thus, an elevation in intracellular calcium prior to the AR is needed to activate PLC, which then hydrolyzes PIP2(4,5) causing gelsolin release and activation. Since the level of F-actin increases during capacitation, it is likely that gelsolin is inactive during this period. The mechanism that keeps gelsolin inactive during sperm capacitation has yet to be elucidated. It has been shown in other cell types that gelsolin can be phosphorylated by c-SRC on tyrosine-438 resulting...
in its release from actin filaments (19). In human sperm, SRC is involved in regulating sperm capacitation, calcium fluxes, tyrosine phosphorylation and the AR (20,37,38). Previous studies indicated that SRC is not directly involved in tyrosine phosphorylation but rather inhibits protein phosphatase resulting in an increase of protein tyrosine phosphorylation (39). Moreover, it was shown that capacitation is regulated by two parallel pathways. One of them requires activation of protein kinase A leading to SRC activation, and the second one involves the inactivation of Ser/Thr phosphatases (37,40). SRC is also found in the sperm flagellum and head and is localized to membrane fraction (40). Thus, it is likely that SRC is the factor that inactivates gelsolin during sperm capacitation. In order to test this assumption, we first looked at the kinetics of SRC activation, and found that 8Br-cAMP causes fast F-actin formation which is inhibited by the SRC-family inhibitor, PP1 (Fig 7A). These data suggest that activation of SRC causes gelsolin inhibition resulting in the formation of F-actin. However, it is also possible that SRC directly mediates F-actin formation. To distinguish between these possibilities, we performed a series of experiments to determine whether SRC directly activates gelsolin. We found that PBP10 induced F-actin breakdown was inhibited in the presence of 8Br-cAMP (Fig. 7A). In addition, immunoprecipitation data revealed that SRC and gelsolin coimmunoprecipitate in human sperm (Fig. 7B). Furthermore, when SRC was activated by adding 8Br-cAMP which activates PKA, the amount of gelsolin in the immunoprecipitate was significantly enhanced (Fig. 7B) inding that activated SRC binds to gelsolin. We also showed that 8Br-cAMP enhanced tyrosine phosphorylation of gelsolin and elevated the amount of gelsolin bound to PIP$_{2(4,5)}$ (Fig. 7B). After 5 min of incubation under capacitation conditions, SRC was already phosphorylated at Tyr416, signifying the active form of the kinase (Fig. 7C). This suggests that the activation of PKA/SRC inhibits gelsolin, probably due to tyrosine phosphorylation of gelsolin during capacitation. These conclusions are further supported by showing that the activation of SRC-family by 8Br-cAMP or the inhibition of tyrosine dephosphorylation by vanadate, both inhibit PBP10 induced F-actin breakdown (Fig. 8).

Discussion

We have previously shown that actin polymerization occurs during sperm capacitation and that F-actin breakdown must take place in order to achieve the acrosome reaction (AR) (3). In the present study, we describe the role of the actin severing protein, gelsolin, its regulation both during capacitation and during the AR, and how this regulation affects F-actin formation and breakdown. It should be mentioned that besides its severing activity, gelsolin is a powerful nucleator of actin filaments formation, however, we do not have so far any information regarding this activity in sperm. Our results show that the level of gelsolin remains constant during capacitation. Gelsolin is localized with the G-actin fraction at the beginning of capacitation, while at the end of capacitation, gelsolin localizes in the F-actin fraction (Fig. 1). Moreover, phalloidin-FITC staining revealed an increase of F-actin levels in the sperm heads during capacitation that decreased after the AR (Fig. 2A), as was previously shown in bovine sperm (28). Accordingly, cytochemical and western blot analysis of separated sperm heads and tails showed that gelsolin translocates from the tail to the head during capacitation (Fig. 2B-C). The localization of gelsolin with its substrate F-actin in the sperm head allows efficient F-actin dispersion which is a necessary step required to occur prior to the AR (3). In guinea pig spermatozoa, it was shown that in non-capacitated sperm, gelsolin is located in the postacrosomal regions and the flagellum, while in capacitated cells, gelsolin is found in the in the apical zone of the acrosome (30). This translocation of gelsolin to the sperm head was prevented by chelating intracellular Ca$^{2+}$ using BAPTA/AM, indicating the importance of calcium ions for this process (Fig. 2B). It was reported that Ca$^{2+}$ can induce the unfolding of gelsolin from a compact form to a more open form, in which the three actin-binding sites become exposed (41). Thus, it is possible that this conformational change is necessary for gelsolin translocation. The translocation of gelsolin towards the acrosome region reflects the importance of gelsolin localization in the sperm head in order for gelsolin to break down F-actin prior to the AR.

We have shown here that increasing the levels of Ca$^{2+}$ by adding the Ca$^{2+}$ ionophore A23187 causes a significant breakdown of F-actin in the sperm head (Fig. 2B) and initiation of the AR (Fig. 3,4). Moreover, when PBP10, a peptide that releases gelsolin from its binding to PIP$_{2(4,5)}$, is added, gelsolin becomes activated, as well. PBP10 causes F-actin breakdown and it does not affect Ca$^{2+}$ transport into the cells. This indicates that the intracellular Ca$^{2+}$ concentration at the end of the capacitation is sufficient for gelsolin activation. Furthermore, the
results seen with PBP10 indicate that the release of bound gelsolin from PIP_{2(4,5)} is the rate limiting step for the activation of gelsolin in F-actin breakdown. In addition, we have shown elsewhere that there is an elevation of PIP_{2(4,5)} during sperm capacitation prior to F-actin enhancement (29). Moreover, PIP_{2(4,5)} can bind gelsolin and thus inactivate it (16,42). Therefore, we suggest that gelsolin is inactive during sperm capacitation due to its sequestration by PIP_{2(4,5)}, and is activated at the end of capacitation upon its release. We suggest that PLC may be the factor responsible for the release of gelsolin from PIP_{2(4,5)}. Activation of PLC which hydrolyses PIP_{2(4,5)} causes the release of the bound gelsolin to the cytosol (16,35). Also, sperm PLC requires ~5 µM calcium for half maximal activation similar to the calcium concentration found after sperm-zona interaction (6), suggesting the possibility of gelsolin activation at this stage. Consistent with this hypothesis, we noticed that F-actin depolymerization and the acrosome reaction which are induced by calcium ionophore A23187 or EGF in capacitated sperm, were significantly reduced when PLC was blocked by U73122 (Figs. 5 and 6). However, when actin depolymerization or the acrosome reaction was induced by the peptide PBP10, there was no effect on these activities by the PLC inhibitor (Figs. 5 and 6). These results, which show that PLC mediates F-actin breakdown and the induction of AR by calcium ionophores or EGF but not by PBP10 (Fig. 5, 6), clearly indicate that the increase in Ca^{2+} ions prior to the AR is needed for PLC activity and not for direct activation of gelsolin. The activation of PLC which hydrolyzes PIP_{2(4,5)}, would release PIP_{2(4,5)}-bound gelsolin and allow its activity of breaking down F-actin resulting in the AR. Thus, PLC activity mediates F-actin depolymerization at the end of capacitation, leading to the AR. We suggest that the calcium ionophore activates PLC to hydrolyze PIP_{2(4,5)} whereas in the presence of PBP10, which competes with gelsolin in binding to PIP_{2(4,5)}, there is no need for PLC-dependent PIP_{2(4,5)} hydrolysis. Although PLC activity is not needed for the activation of gelsolin by PBP10, Ca^{2+} is required, as can be seen in Figures 3 and 4. These data further support the role of PBP10 as a factor which releases gelsolin from PIP_{2(4,5)} and allows its activation by Ca^{2+}. The fact that F-actin depolymerization can be induced by activating the EGFR by EGF, and the increase of PLC\(\gamma\) phosphorylation/activation under conditions in which F-actin is depolymerized, clearly indicates that the PLC\(\gamma\) isoform mediates PIP_{2} hydrolysis leading to gelsolin activation and F-actin dispersion.

F-actin levels are known to increase during capacitation, and it is assumed that gelsolin is inactive during this period of time. We therefore asked whether there is an additional mechanism that keeps gelsolin inactive during sperm capacitation. In previous studies, SRC was shown to be active 1 hour after the beginning of capacitation (38). Activation of SRC can be seen 5 min into the capacitation process (Fig. 7C). In addition, immunoprecipitation assays revealed that SRC and gelsolin coimmunoprecipitate in human sperm and that SRC activation by the addition of 8Br-cAMP which activates PKA, increased the amount of gelsolin in the immunoprecipitate (Fig. 7B). Moreover, 8Br-cAMP enhanced the phosphorylation of gelsolin and the amount of gelsolin bound to PIP_{2(4,5)}, both of which processes inactivated gelsolin during capacitation (Fig. 7B). In order to confirm that SRC keeps gelsolin inactive during capacitation, we induced F-actin depolymerization by PBP10 in cells treated with vanadate (a tyrosine phosphatase inhibitor) or 8Br-cAMP (a PKA activator) and showed that PBP10 cannot cause complete F-actin breakdown in the presence of 8Br-cAMP. This suggests that the activation of PKA/SRC causes inhibition of gelsolin, probably due to its tyrosine phosphorylation. These results confirm our hypothesis that gelsolin is inhibited during capacitation as a result of SRC phosphorylation.

Our results thus suggest the following model. During capacitation, the Ca^{2+} concentration rises, leading to conformational changes in gelsolin and revealing the F-actin binding site. As a result, gelsolin is translocated to the head of the sperm. Nevertheless, the elevation of PIP_{2(4,5)} levels and SRC phosphorylation maintain gelsolin in an inactive state and actin polymerization can occur. Immediately prior to the acrosome reaction, the intracellular Ca^{2+} concentration is elevated, PLC is activated and hydrolyzes PIP_{2(4,5)}, resulting in the release of gelsolin to the cytosol. The free gelsolin is now activated as a result of elevated levels of Ca^{2+} and tyrosine dephosphorylation by tyrosine phosphatases, leading to F-actin dispersion and the occurrence of the AR.
References


Figure 1: Colocalization of gelsolin and actin before and after capacitation. Human sperm were incubated in HAM F-10 for 3h. At the beginning of this incubation, (A) sperm were lysed and separated by SDS-PAGE. At the end of the incubation (B) sperm were separated to Triton-x-100 soluble and insoluble fractions and then lysed and separated by SDS-PAGE. The blots were stained with anti-gelsolin and anti-actin antibodies and quantification was done by densitometry (C). The data (in A and B) represent one experiment, typical of at least three repetitions with sperm from three experiments from different donors. ** Significant difference from time zero, P<0.01.

Figure 2: Actin remodeling and gelsolin localization before and after sperm capacitation and the acrosome reaction. (A) Human sperm were incubated under capacitation conditions in HAM F-10 for 3h. After 3h of incubation, calcium ionophore A23187 (10 µM) was added for additional 1h, the cells were stained with FITC-phalloidin, photographed under a fluorescence microscope and analyzed for fluorescence intensity in the sperm. (B) Human sperm were incubated under capacitation conditions in HAM F-10 for 3h with or without BAPTA-AM (3µM-added for last 2h). At the beginning and at the end of the incubation, the cells were fixed and stained with anti-gelsolin, followed by rhodamine RED-X-conjugated antibody. (C) Human sperm were incubated in HAM F-10 for 3h. The sperm were sonicated and Percoll-purified to separate sperm heads and tails. The sperm heads and tails were lysed, and the extracted proteins were separated by SDS-PAGE, and then the blots were stained with anti-gelsolin. This data represent one experiment, typical of at least three repetitions performed, with sperm from three experiments using different donors. **Significant difference from the corresponding control, P<0.01.

Figure 3: Induction of Ca2+ - dependent F-actin depolymerization. Human sperm were incubated in HAM F-10 for 3h with or without BAPTA-AM (3µM-added for last 2h); then, either A23187 (10 µM), EGF (1 ng/ml) or PBP10 (1 µM) were added for an additional 1h. Aliquots were removed and F-actin levels were determined using Phalloidin-FITC as described. The fluorescence at zero time (5.7 a.u.) was subtracted from each measurement. The data represent the mean ± SD of three experiments using different donors. ** Significant difference compared to the control, P <0.01.

Figure 4: Induction of Ca2+ - dependent acrosome reaction. Human sperm were incubated in capacitation medium (F-10) for 3h with or without BAPTA-AM (3µM-added for last 2h); then, either A23187 (10 µM), EGF (1 ng/ml) or PBP10 (1 µM) were added for an additional 1h of incubation. Acrosome-reacted cells were identified by PSA staining, as described. The percentage of spontaneous acrosome-reacted cells at the end of the 4h incubation (22%) was subtracted to obtain the induced percentage. The data represent the mean ± SD of duplicates from at least five experiments using different donors. **Significant difference from the corresponding control, P<0.01.

Figure 5: PLCγ activity mediates F-actin depolymerization. (A) Human sperm were incubated in HAM F-10 for 3h. At the end of this incubation, the cells were incubated with or without U73122 (1 µM) for 10 min. Next, A23287 (10 µM), EGF (1 ng/ml) or PBP10 (1 µM) were added for an additional 60 min. Samples were removed at different times as indicated, and F-actin levels in the cells were determined using Phallloidin-FITC, as described. The different treatments are indicated by the following symbols: (●) control, (●) EGF, (○) U73122 with EGF, (■) A23187, (▲) U73122 with A23187, (▲) PBP10, (▲) U73122 with PBP10. The data represent the mean ± SD of three experiments using different donors. Time zero represents 3h capacitation. (B) Human sperm were incubated in HAM F-10 for 3h. After this incubation, the cells were additionally incubated with A23287 (10 µM). At the times indicated, samples were lysed and the proteins were separated by SDS-PAGE. The blots were stained with anti-P-PLCγ (Tyr-783) and anti-actin. The data represent one experiment, typical of at least three repetitions performed with sperm from three experiments using different donors.

Figure 6: PLCγ activity is required for the acrosome reaction. Human sperm were incubated in HAM F-10 for 3h. Afterwards, the cells were incubated with or without U73122 (1 µM) for 10 min. A23287 (10 µM), EGF (1 ng/ml) or PBP10 (1 µM) were then added for an additional 60 min. Acrosome-reacted cells were identified at 4h by PSA staining, as described. The percentage of spontaneously acrosome-reacted cells at the end of the 4h incubation (21.5%) was subtracted to obtain the induced percentage. The data represent the mean ±SD of duplicates from at least five experiments using different donors. **Significant difference from the corresponding control, P<0.01.
Figure 7: SRC-family proteins mediate F-actin depolymerization. (A) Human sperm were incubated for 30 min in HAM F-10 with or without the SRC-family inhibitor, PP1 (10 µM) for 10 min; afterwards, 8Br-cAMP (1 mM, for 20 min) or PBP10 (1 µM, for 10 min) was added. F-actin levels were determined using Phalloidin-FITC as described in Materials and Methods. The data represent the mean ± SD of three experiments with different donors. * Significant difference compared to the control (30 min), P < 0.1. (B) Human sperm were incubated in HAM F-10 for 10 min with or without 1 mM 8Br-cAMP. The immunoprecipitation (IP) was performed with anti-SRC, anti-P-Tyr or anti-PIP2(4,5) antibody as described in the Materials and Methods. The immunoprecipitates were subjected to western blot analysis, and the blots were stained with anti-gelsolin and anti-SRC. The data represent one experiment, typical of at least three repetitions performed with sperm from three different donors. (C) Human sperm were incubated in HAM F-10 and at the indicated times, sperm samples were subjected to western blot analysis, and the blots were stained with anti-phospho-SRC and anti-actin.

Fig. 8: F-actin depolymerization is mediated by SRC-Family-Kinase and tyrosine phosphatase. Human sperm were incubated in HAM F-10 for 3h. Next, the cells were incubated with or without vanadate for 10 min, and then A23287 (10 µM), 8-Br-cAMP (1 mM), PP1 (10 µM) or PBP10 (1µM) were added for an additional 10 min. Samples were removed at this time, and F-actin levels in the cells were determined using Phalloidin-FITC as described in Materials and Methods. The data represent the mean ± SD of three experiments using different donors. * Significant difference compared to the control 30 min, P < 0.1.
Figure 1

A

Gelsolin
Actin
Time (h): 0 3

B

Triton soluble Triton insoluble
Gelsolin
Actin
Time (h): 0 3 0 3

C

insoluble / soluble ratio

0 1 2 3 4 5 6 7 8

Time (h) 0 3 **
Figure 2

A  Time (h)  

| A23187 | 0 | - | 4 | + |

B  Time (h)  

| BAPTA-AM | 0 | - | 3 | + |

C  

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Figure 3

Fluorescence intensity (a.u.)

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**
**Figure 4**

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<td>BAPTA/AM</td>
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*Note: The table indicates the acrosome reacted cells (%) for different treatments.*
Figure 5

A

Fluorescence intensity % of control vs. Time (min)

B

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Legend:
- P-PLCγ (Tyr-783)
- Actin
Figure 6

Acrosome reacted cells (%)
Figure 7

A

Fluorescence intensity (a.u.)

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</table>

B

- IB: Gelsolin -
- IB: SRC -
- IB: Gelsolin -
- IB: Gelsolin -
- IP: p-Tyr
- IP: PIP2

C

Time (min): 0 5 10 30 60

- p-SRC -
- Actin -
Figure 8

Fluorescence intensity (% of control)

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The role and regulation of sperm gelsolin prior to fertilization
Maya Finkelstein, Nir Etkovitz and Haim Breitbart

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