Role and Regulation of Sperm Gelsolin Prior to Fertilization

Maya Finkelstein, Nir Etkovitz, and Haim Breitbart
From The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

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 that allows its penetration into the egg and fertilization. Sperm capacitation requires actin polymerization, whereas 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 (PIP2) by PBP10, a peptide containing the PIP2-binding domain of gelsolin, or by activation of phospholipase C, which hydrolyzes PIP2, caused rapid Ca2+-dependent F-actin depolymerization as well as enhanced acrosome reaction. Using immunoprecipitation assays, we showed that the tyrosine kinase activity of SRC, the epidermal growth factor receptor (EGFR), was phosphorylated by pp60(c-SRC) and that this phosphorylation is enhanced during 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 C (PLCγ), to hydrolyze PIP2 and to cause the subsequent release of PIP2-bound proteins. In vitro studies have shown that gelsolin modulates the activity of several signaling enzymes, including PLC and phospholipase D through interactions with PIP2 (23–27). It appears that actin polymerization during sperm capacitation depends on phospholipase D activity, which is regulated by the cross-talk between PKA and protein kinase C (PKC) (1, 28). Moreover, we showed elsewhere that PIP2-bound gelsolin is the cofactor for phospholipase D activation and F-actin production during sperm capacitation, is mediated by phospho-

1 To whom correspondence should be addressed: The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan 52900, Israel. Fax: 972-3-6356041; E-mail: breith@mail.biu.ac.il.

2 The abbreviations used are: AR, acrosome reaction; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; 8-Br-cAMP, 8-bromo-cAMP; EGFR, EGF receptor; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’-tetraacetic acid tetrakis(acetoxymethyl ester); PSA, P. sativum agglutinin.
Gelsolin Regulation in Sperm

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 are 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 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 as follows: first, the binding of gelsolin to PIP_2(4,5)_(2) 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 here confirm this hypothesis and provide a better understanding of the regulation of gelsolin during sperm capacitation and in the acrosome reaction.

EXPERIMENTAL PROCEDURES

Materials—PBP10 (polyphosphoinositide-binding peptide, rhodamine B-conjugated), PP1, U73122, and A23187 were obtained from Calbiochem. Capacitation medium, F-10 (Ham’s) nutrient mixture with I-glutamine, was purchased from Biological Industries (Kibbutz Beit Haemek, IL). Goat polyclonal anti-gelsolin (C-20), mouse monoclonal anti-SRC, (H-12), anti-β-actin HRP-conjugated antibodies, secondary donkey anti-goat IgG, protein G PLUS-agarose (sc-53412) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor 568 donkey anti-goat IgG was purchased from Invitrogen. All other chemicals were purchased from Sigma, 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 6750 rpm at room temperature. The lower layer containing the sperm was collected and washed twice in Ham’s F-10 and 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 × 10^7 cells/ml) were capacitated by incubation in capacitation media (Ham’s F-10) supplemented with 3 mg/ml BSA. The cells were incubated in this capacitation medium for 3 h at 37 °C in 5% CO_2. 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 × 10^7 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 H_2O at 5-min intervals, and mounted with FluoroGuard Antifade (Bio-Rad). 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 mixture 1:100 (Calbiochem), 50 μM NaF, 50 μM pyrophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM Na_3VO_4 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 (31). 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 Biosciences).

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 min, 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’s F-10 (3 × 10^7/ml) for 3 h, and the cells were lysed as described previously (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 was 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 1 h 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 version 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 24 h 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 ImageJ 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 described previously. 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) 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, although 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 1400 rpm for 5 min, and the supernatant was removed, and the remaining pellet was SDS-extracted as described previously.

**Immunoprecipitation of Gelsolin-SRC/Gelsolin-PIP_2(4,5)/Gelsolin-p-Thy**—Sperm cells (5 × 10^7 cells/ml) were centrifuged at 4000 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 the following: 20 mM Tris-HCl, pH 7.5; 0.25 mM 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 7500 rpm for 5 min, and the supernatants were then incubated with 2 μg of 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 2 h 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.

**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 Fig. 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), although 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, although 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 that 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). 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 (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...

---

**Figure 1. Colocalization of gelsolin and actin before and after capacitation.** Human sperm were incubated in Ham’s F-10 for 3 h. 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 (A and B) represent one experiment, typical of at least three repetitions performed with sperm from three experiments from different donors. The filled bars represent actin, and the empty bars represent gelsolin content. The data in C represent the means ± S.D. of these three experiments using different donors. **, significant difference from time 0, 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’s F-10 for 3 h. After 3 h of incubation, calcium ionophore A23187 (10 μM) was added for an additional 1 h, and 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’s F-10 for 3 h with or without BAPTA-AM (3 μM added for last 2 h). 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’s F-10 for 3 h. 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 the blots were then stained with anti-gelsolin. These data represent one experiment, typical of at least three repetitions performed, with sperm from three experiments using different donors.

**Figure 3. Induction of Ca2+-dependent F-actin depolymerization.** Human sperm were incubated in Ham’s F-10 for 3 h with or without BAPTA-AM (3 μM added for last 2 h and then either A23187 (10 μM), EGF (1 ng/ml), or PBP10 (1 μM) were added for an additional 1 h). Aliquots were removed, and F-actin levels were determined using phalloidin-FITC as described. The fluorescence at zero time (5.7 units (a.u.)) was subtracted from each measurement. The data represent the means ± S.D. of three experiments using different donors. **, significant difference compared with the control, p < 0.01.
Gelsolin Regulation in Sperm

FIGURE 4. Induction of Ca^{2+}-dependent acrosome reaction. Human sperm were incubated in capacitation medium (F-10) for 3 h with or without BAPTA/AM (3 μM added for the last 2 h). At the end of the incubation, the AR inducers, Ca^{2+} ionophore A23187 (10 μM), EGF (1 ng/ml), or PBP10 (1 μM), were added for an additional 1 h of incubation. Acrosome-reacted cells were identified by PSA staining, as described. The percentage of spontaneous acrosome-reacted cells at the end of the 4-h incubation (22%) was subtracted to obtain the induced percentage. The data represent the means ± S.D. 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’s F-10 for 3 h. At the end of this time, 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 phalloidin-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 means ± S.D. of three experiments using different donors. Time 0 represents 3 h of incubation. B, human sperm were incubated in Ham’s F-10 for 3 h. 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’s F-10 for 3 h. Afterward, 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 4 h by PSA staining, as described. The percentage of spontaneously acrosome-reacted cells at the end of the 4-h incubation (21.5%) was subtracted to obtain the induced percentage. The data represent the means ± S.D. of duplicates from at least five experiments using different donors. **, significant difference from the corresponding control, p < 0.01.
How Is Gelsolin Activity Inhibited during Sperm Capacitation?—As shown above, gelsolin can be activated in capacitated sperm by PBP10, indicating that the intracellular Ca\(^{2+}\) 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 PIP\(_{2}\) causing gelsolin release and activation. Because 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 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. The first one 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. To test this assumption, we first looked at the kinetics of SRC activation and found that 8-Br-cAMP causes fast F-actin formation that 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 8-Br-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 8-Br-cAMP, which activates PKA, the amount of gelsolin in the immunoprecipitate was significantly enhanced (Fig. 7B) indicating that activated SRC binds to gelsolin. We also showed that 8-Br-cAMP enhanced tyrosine phosphorylation of gelsolin and elevated the amount of gelsolin bound to PIP\(_{2}\) (Fig. 7B). After 5 min of incubation under capacitation conditions, SRC was already phosphorylated at Tyr-416, 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 the SRC family by 8-Br-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 to achieve the AR (3). In this 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 filament formations; however, so far we do not have 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, although at
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 hydrolyzes 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 (Figs. 5 and 6), clearly indicate that the increase in Ca^{2+} ions prior to the AR is needed for PLC activation 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 Figs. 3 and 4. These data further support the role of PBP10 as a factor that 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 PLCγ phosphorylation/activation under conditions in which F-actin is depolymerized, clearly indicates that the PLCγ 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 h 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 8-Br-cAMP, which activates PKA, increased the amount of gelsolin in the immunoprecipitate (Fig. 7B). Moreover, 8-Br-cAMP enhanced the phosphorylation of gelsolin and the amount of gelsolin bound to
Gelsolin Regulation in Sperm

Pip2(4,5) and both processes inactivated gelsolin during cap- 
acitation (Fig. 7B). To confirm that SRC keeps gelsolin inac-
tive during capacitation, we induced F-actin depolymerization 
by PBP10 in cells treated with vanadate (a tyrosine phospha-
tase inhibitor) or 8-Br-cAMP (a PKA activator) and showed 
that PBP10 cannot cause complete F-actin breakdown in 
the presence of 8-Br-cAMP. This suggests that the activation 
of PKA/SRC causes inhibition of gelsolin, probably due to its 
tyrosine phosphorylation. These results confirm our hypothe-
sis that gelsolin is inhibited during capacitation as a result of 
SRC phosphorylation.

Our results thus suggest the following model. During cap-
acitation, the Ca2+ concentration rises, leading to conforma-
tional changes in gelsolin and revealing the F-actin-binding 
site. As a result, gelsolin is translocated to the head of the 
tailpiece (positive regulatory binding site). As a result, gelsolin 
is translocated to the head of the 

actin dispersion and the occurrence of the AR.

REFERENCES
1. Breitbart, H. (2003) Cell. Mol. Biol. 49, 321–327
2. Visconti, P. E., Moore, G. D., Bailey, J. L., Leclerc, P., Connors, S. A., Pan, 
D., Olds-Clarke, P., and Kopf, G. S. (1995) Development 121, 1139–1150
3. Brener, E., Rubinstein, S., Cohen, G., Shternall, K., Rivlin, J., and Breit-
bart, H. (2003) Biol. Reprod. 68, 837–845
4. Breitbart, H., Cohen, G., and Rubinstein, S. (2005) Reproduction 129, 
263–268
5. Spungin, B., Margalit, I., and Breitbart, H. (1995) J. Cell. Sci. 108, 
2525–2535
6. Arnoult, C., Kazam, I. G., Visconti, P. E., Kopf, G. S., Villaz, M., and Flo-
orman, H. M. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 6757–6762
7. von Bulow, M., Heid, H., Hess, H., and Franke, W. W. (1995) Exp. Cell 
Res. 219, 407–413
8. von Bulow, M., Rackwitz, H. R., Zimbemlann, R., and Franke, W. W. 
(1997) Exp. Cell Res. 233, 216–224
9. Tanaka, H., Yoshihara, Y., Nishina, Y., Nozaki, M., Nojima, H., and 
Nishimune, Y. (1994) FEBS Lett. 355, 4–10
10. Howes, E. A., Hurst, S. M., and Jones, R. (2001) J. Androl. 22, 62–72
11. Pelletier, R., Trifaro, J. M., Carabajal, M. E., Okawara, Y., and Vitale, M. L. 
(1999) Biol. Reprod. 60, 1128–1136
12. Heid, H., Figge, U., Winter, S., Kuhn, C., Zimbemlann, R., and Franke, 
W. (2002) Exp. Cell Res. 279, 177–187
13. de las Heras, M. A., Valcarcel, A., Perez, L. J., and Moses, D. F. (1997) 
Tissue & Cell 29, 47–53
14. Gremm, D., and Wegner, A. (2000) Eur. J. Biochem. 267, 4339–4345
15. Yin, H. L., Zaner, K. S., and Stossel, T. P. (1980) J. Biol. Chem. 255, 
9494–9500
16. Yin, H. L. (1987) BioEssays 7, 176–179
17. Janmey, P. A., and Stossel, T. P. (1987) Nature 325, 362–364
18. De Corte, V., Gettemans, J., and Vandekerckhove, J. (1997) FEBS Lett. 
401, 191–196
19. De Corte, V., Demol, H., Goethals, M., Van Damme, J., Gettemans, J., 
and Vandekerckhove, J. (1999) Protein Sci. 8, 234–241
20. Varano, G., Lombardi, A., Cantini, G., Forti, G., Baldi, E., and Luconi, M. 
(2008) Hum. Reprod. 23, 2652–2662
21. Etzkovitz, N., Tirosh, Y., Chazan, R., Jaldehy, Y., Daniel, L., Rubinstein, S., 
and Breitbart, H. (2009) Dev. Biol. 324, 447–457
22. Lax, Y., Rubinstein, S., and Breitbart, H. (1994) FEBS Lett. 339, 234–238
23. Banno, Y., Nakashima, T., Kumada, T., Ebisawa, K., Nonomura, Y., and 
Nozawa, Y. (1992) J. Biol. Chem. 267, 6488–6494
24. Cheng, J., Weber, J. D., Baldassare, J. J., and Raben, D. M. (1997) J. Biol. 
Chem. 272, 17312–17319
25. Sun, H., Lin, K., and Yin, H. L. (1997) J. Cell Biol. 138, 811–820
26. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C. S., and Cantley, L. C. 
(1994) J. Biol. Chem. 269, 21403–21406
27. Steed, P. M., Nagar, S., and Wenhole, L. P. (1996) Biochemistry 35, 
5229–5237
28. Cohen, G., Rubinstein, S., Gur, Y., and Breitbart, H. (2004) Dev. Biol. 
267, 230–241
29. Etzkovitz, N., Rubinstein, S., Daniel, L., and Breitbart, H. (2007) Biol. Re-
prod. 77, 263–273
30. Cabello-Aguieros, J. F., Hernández-González, E. O., and Mújica, A. 
(2003) Cell Motil. Cytoskeleton 56, 94–108
31. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
32. Nixon, B., MacIntyre, D. A., Mitchell, L. A., Gibbs, G. M., O’Bryan, M., 
and Aitken, R. J. (2006) Biol. Reprod. 74, 275–287
33. Kinosian, H. J., Newman, I., Lincoln, B., Selden, L. A., Gershman, L. C., 
and Peder, J. E. (1998) Biophys. J. 75, 3101–3109
34. Cunningham, C. C., Vegners, R., Bucki, R., Funaki, M., Kord, N., 
Hartwig, I. H., Stossel, T. P., and Janmey, P. A. (2001) J. Biol. Chem. 
276, 43390–43399
35. Guttman, J. A., Janmey, P., and Vogl, A. W. (2002) J. Cell Sci. 115, 
499–505
36. Baldi, E., Luconi, M., Bonaccorsi, L., and Forti, G. (2002) J. Reprod. 
Immunol. 53, 121–131
37. Baker, M. A., Hetherington, L., and Aitken, R. J. (2006) J. Cell Sci. 119, 
3182–3192
38. Mitchell, L. A., Nixon, B., Baker, M. A., and Aitken, R. J. (2008) Mol. 
Hum. Reprod. 14, 235–243
39. Krapf, D., Arcelay, E., Wertheimer, E. V., Sanjay, A., Pilder, S. H., Sal-
cioni, A. M., and Visconti, P. E. (2010) J. Biol. Chem. 285, 7977–7985
40. Lawson, C., Goupil, S., and Leclerc, P. (2008) Biol. Reprod. 79, 657–666
41. Ashish, Paine, M. S., Perryman, P. B., Yang, L., Yin, H. L., and Krueger, 
J. K. (2007) J. Biol. Chem. 282, 25884–25892
42. Janmey, P. A., Iida, K., Yin, H. L., and Stossel, T. P. (1987) J. Biol. Chem. 
262, 12228–12236