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Characterization of *Bufo arenarum* oocyte plasma membrane proteins that interact with sperm

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Abstract

Sperm–oocyte plasma membrane interaction is an essential step in fertilization. In amphibians, the molecules involved have not been identified. Our aim was to detect and characterize oocyte molecules with binding affinity for sperm. We isolated plasma membranes free from vitelline envelope and yolk proteins from surface-biotinylated *Bufo arenarum* oocytes. Using binding assays we detected a biotinylated 100 kDa plasma membrane protein that consistently bound to sperm. Chromatographic studies confirmed the 100 kDa protein and detected two additional oocyte molecules of 30 and 70 kDa with affinity for sperm. Competition studies with an integrin-interacting peptide and cross-reaction with an anti-HSP70 antibody suggested that the 100 and 70 kDa proteins are members of the integrin family and HSP70, respectively. MS/MS analysis suggested extra candidates for a role in this step of fertilization. In conclusion, we provide evidence for the involvement of several proteins, including integrins and HSP70, in *B. arenarum* sperm–oocyte plasma membrane interactions.

Keywords: Fertilization; Amphibians; Oocyte plasma membrane; Sperm receptor

A number of animal and plant species have been employed as model organisms in fertilization research. Our current understanding of fertilization in vertebrates is based largely on data from amphibians, sea urchins, and mammals. The process of amphibian fertilization involves a cascade of cell–cell and cell-matrix interactions. First, sperm interact with the jelly coat and then bind to the vitelline envelope (VE), an extracellular matrix surrounding the oocyte. This interaction seems to trigger the acrosome reaction that, in turn, allows the sperm to penetrate the envelope. Finally, sperm approach, bind to, and fuse with the oocyte plasma membrane. While the details of the molecular interactions between sperm and the oocyte jelly coat [1–3] and the VE [4–6] are beginning to emerge, little is known about the molecular basis for the essential plasma membrane binding and fusion reactions that initiate embryonic development.

The amphibian oocyte has been the object of many studies in the areas of molecular and developmental biology during the last decades. Recently, the oocyte has gained increasing importance in studies of cell–cell adhesion, signaling, and other membrane-associated events [7–10]. In addition, one of the best exploited cell systems to study function, topology, and routing of wild-type and mutant channels and transporters is the heterologous expression in the *Xenopus laevis* oocyte [11,12]. It is clear that the amphibian oocyte provides an excellent model for these studies of more general biological interest, as well as for studies related to developmental and reproductive biology per se.

The plasma membrane of the amphibian oocyte has received little attention and is poorly understood at the biochemical level. Amphibian oocytes are voluminous cells (1.4 mm diameter for *Bufo arenarum*) equipped with large

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stores of nutrient materials and an abundant translation machinery [13]. These facts have been advantages for injection/expression experiments but complicate the isolation of reliable samples of plasma membranes free from cytoplasmic contaminants. The available information indicates that the oocyte is surrounded by the plasma membrane, coated on the outer surface with a highly developed array of external peripheral proteins collectively termed the VE. The VE has been studied in some detail [14–16]. It is known to contain glycoproteins, one or more of which may be involved in sperm–oocyte adhesion [16–18], and is involved in formation of the fertilization envelope after the oocyte is activated [4,16,19]. The cytoplasmic face of the plasma membrane is associated with arrays of specialized secretory vesicles, the cortical granules, which undergo exocytosis upon fertilization [20–22].

Earlier work in this laboratory has been focused on the jelly and vitelline coats [1,4,5,14,23]. As part of our ongoing studies of the cell surface complex and sperm–oocyte adhesion, we have now adjusted a plasma membrane isolation protocol to *B. arenarum* oocytes that renders plasma membranes free from VEs. Also, they retain their biological activity, as indicated by their ability to bind to spermatozoa. We also utilized these isolated plasma membranes combined with chromatographic techniques to detect and characterize oocyte molecules with binding affinity for sperm. Using different approaches we provide evidence for the involvement of HSP70, integrins, and other proteins in sperm–oocyte interactions at the plasma membrane level.

Materials and methods

Animals and gametes. Sexually mature *B. arenarum* specimens were collected in the neighborhoods of Rosario city and kept in a moist chamber at 12 °C until used.

Testes were dissected from male toads, and spermatozoa were obtained by mincing the organs in Ringer–Tris solution (0.11 M NaCl, 2 mM KCl, 1.4 mM $CaCl_2$, and 10 mM Tris, pH 7.2) at 4 °C. The homogenate was filtered through gauze and the suspension was centrifuged for 10 min at 130*g* at 4 °C to remove blood cells and tissue debris. The sperm suspension was centrifuged for 10 min at 650*g* at 4 °C. Pelleted spermatozoa were resuspended in Ringer–Tris solution and the concentration of cells was estimated by measuring absorbance at 410 nm.

Acrosome reaction was induced by the addition of calcium ionophore A-23187 to the suspension of sperm in Ringer–Tris to reach a concentration of 10 μM [24]. After 60 min at 4 °C, the suspension was extensively washed by centrifugation 10 min at 650*g* at 4 °C and resuspension in Ringer–Tris for three times.

Female specimens were kept in a moist chamber at 20–22 °C for 1 day before stimulation with one homologous hypophysis homogenate injected intracoelomically. After 10–12 h, oocyte strings were collected from ovisacs [16]. Dejelling was performed by a short exposure to 0.3% β-mercaptoethanol in Ringer–Tris, pH 8.9, and the oocytes were thoroughly washed with ice-cold Ringer–Tris solution.

Biotin labeling of oocyte surface proteins. Dejellied oocytes were washed four times with PBS and then biotinylated with sulfo-NHS biotin (Sigma Chemical Co., St. Louis, USA), a cell-impermeable biotin analogue. Briefly, 200 oocytes were labeled with sulfo-NHS biotin (500 μg/ml) in PBS for 30 min at 20 °C with occasional gentle agitation. The oocytes were washed twice with PBS/NH_4Cl 50 mM to quench free biotin and then three times with PBS. The biotinylated oocytes were stored at −70 °C until used.

Oocyte plasma membrane isolation. Plasma membranes were separated by ultracentrifugation on a discontinuous sucrose gradient, following a procedure that was previously carried out to isolate light and heavy plasma membranes on *X. laevis* oocytes [25]. In order to eliminate contamination with VE proteins, an additional filtration step was introduced before centrifugation. Also, the original procedure included an extra sucrose layer of 22% that we eliminated to ensure that all (light and heavy) functional plasma membranes were isolated together. Briefly, 300 oocytes were lysed by repeatedly pipetting in ice-cold 2× TNE buffer (10 mM Tris–HCl, 150 mm NaCl, and 5 mM EDTA, pH 7.4) with 1 mM PMSF and 10 μg/ml TPCK. Oocyte lysates were filtered through a double sheet of 30-mesh screen to eliminate vitelline envelopes and then they were sonicated for 1 min in a cold bath at 13% watt scale (Vibra Cell™, Sonics & Materials Inc). The lysates were added to an equal volume of 80% sucrose to give a final equivalent density of 40% sucrose. They were then overlaid with 4 ml each of sucrose at concentrations of 35% and 10%. The sucrose gradients were centrifuged 3 h at 100,000*g* (SW40 rotor, Beckman Instruments). The plasma membranes in the 10–35% interface were obtained, diluted with water with PMSF 0.1 mM, and centrifuged 1 h at 45,000 rpm (Ti80 rotor, Beckman Instruments). The pellet was resuspended in PBS/PMSF 0.1 mM and sonicated 1 min at 20% watt scale.

Co-precipitation of biotin-labeled oocyte plasma membrane proteins with acrosome-reacted sperm. Bovine serum albumin was added to two hundred microliter of isolated oocyte plasma membranes prepared as described above (70–100 μg of protein) to reach a concentration of 0, 3, 6, and 10 mg/ml. These samples were sonicated (20% of watt scale) and centrifuged 650*g* at 4 °C. The supernatants were transferred to new tubes and aliquots of a suspension of acrosome-reacted sperm were then added at a final concentration of 2000 sperm/μl. After 30 min of incubation at 20 °C, sperm were washed four times with PBS by centrifugation, and then SDS–PAGE sample buffer was added to the resulting pellet and they were boiled for 5 min.

Extraction of reacted sperm head proteins. Acrosome-reacted *B. arenarum* sperm heads were isolated by a modification of the method of San Agustin & Witman [26]. Briefly, reacted sperm in Ringer–Tris with PMSF 1 mM were sonicated on ice for 1 min using the sonicator equipped with a microtip, set at 6% watt scale. The sonicated sperm suspension was mixed with 2.2 M sucrose (final sucrose concentration 1.5 M) and laid on top of a step gradient prepared with 4 ml of sucrose 2.2 M and 4 ml of sucrose 2.05 M. The tubes were centrifuged at 91,000*g* (SW40 rotor, Beckman Instruments) at 4 °C for 1.5 h. The bottom of the tube contained mostly heads. We carefully removed the sucrose layers to prevent contamination with flagella (1.5–2.05 M interface) and intact sperm (2.05–2.2 M interface). The quality and correct distribution of the heads and tails were checked by microscopy (data not shown). The heads were dispersed with Ringer–Tris/PMSF and centrifuged at 4500 rpm (SS-34 rotor, RC-5 C Sorvall centrifuge, DuPont Instruments). The pellet was resuspended in Ringer–Tris/PMSF and centrifuged at 1500 rpm. This pellet was resuspended with Ringer–Tris 50%/NaCl 0.5 M/2% Dodecyl Maltoside/1 mM PMSF and incubated for 60 min at 30 °C with gentle agitation. Finally, the extract was centrifuged at 10,000*g* at 4 °C for 5 min and the supernatant was stored.

Sperm head proteins-Sepharose affinity chromatography. Solubilized sperm head proteins, prepared as described above, were dialyzed against 0.1 M $NaHCO_3$, pH 8.3, overnight and concentrated by ultrafiltration (Centricon, Millipore, Bedford, MA, USA). This extract (1 mg protein in 1 ml solution) was coupled to cyanogen bromide-activated Sepharose CL 4B following the manufacturer's instructions. The affinity column (1.25 ml bed volume) was stored at 4 °C.

Aliquots of isolated oocyte plasma membranes (containing 1–1.2 mg protein each) were applied to sperm head proteins-Sepharose affinity column equilibrated with PBS with 2 mM $MgCl_2$. The column was washed with 20 volumes of the same buffer. Bound proteins were eluted with 2 M NaCl. Fractions of 1 ml were collected dialyzed overnight against 0.5 mM Tris, pH 7.0, and concentrated by freeze-drying.

Alternatively, the isolated plasma membranes (2 mg of protein) were split. One-half was applied to the column in the presence of MgCl and RGD peptide 1 mM, while the other was the positive control without RGD. The RGD peptide (a known integrin subfamily binding motif) was purchased from Sigma (St. Louis, MO, USA).

Electrophoresis, electrotransfer, and Western-blot analysis. Polyacrylamide gel electrophoresis under denaturing conditions (SDS–PAGE) was performed essentially according to the method of Laemmli [27]. Samples were diluted with an appropriate volume of 5× sample buffer with β-mercaptoethanol, boiled for 5 min, and loaded onto 10% or 12% acrylamide mini-gels with 5% stacking gel, and electrophoresed for about 1 h at 20 mA/gel (MiniProtean II Gel System, Bio-Rad, Hercules, CA). The apparent molecular masses were estimated with molecular mass standards (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Gels were processed for Coomassie brilliant blue staining or electrotransferred to nitrocellulose membranes by the method of Towbin et al. [28].

Membranes were washed twice with PBS and then blocked with PBS buffer supplemented with 5% nonfat dried milk.

Biotinylated proteins were detected using ECL Streptavidin-HRP (Amersham) followed by chemiluminescence and X-ray film (Biomax XS, Kodak Co, Rochester, NY).

Na^+/K^+ ATPase was detected with a goat polyclonal antibody against rabbit renal Na^+/K^+ ATPase (Calbiochem–Novabiochem) diluted 1:2000 in PBS/milk. Membranes were incubated overnight at 4 °C with agitation. After being washed four times with PBS, the membranes were incubated with an anti-goat secondary antibody coupled to horseradish peroxidase (1:5000 dilution in PBS, Amersham). VE proteins were detected with a rabbit polyclonal antiserum developed in our laboratory diluted 1:2000 in PBS/milk. HSP70 was detected with a rabbit polyclonal antiserum diluted 1:1000 in PBS/milk The membranes were incubated for 1 h at room temperature with agitation. After being washed four times with PBS, the membranes were incubated with an anti-rabbit-HRP (1:5000 dilution in PBS, Amersham). In all cases, antibody binding was detected using chemiluminescence and the above-mentioned X-ray film.

When membrane reprobing was necessary, stripping was performed by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2%w/v sodium dodecyl sulphate, and 62.5 mM Tris–HCl, pH 6.7) at 50 °C for 30 min.

MS/MS analysis of eluted proteins. To avoid any loss of biotinylated proteins and to eliminate contaminants present in the eluate that could affect the procedure, we decided to load the eluate onto a 10% typical SDS–PAGE. Run was carried out just enough to ensure that the sample enters the running gel but not to spread it out. The progress of the running was followed loading prestained molecular markers in an adjacent well. After stopping the electrophoresis, we also checked by silver staining the extent of migration of the sample. The band (that comprised the mix of eluted proteins) was excised from the gel. The fragment of the gel containing all the bands was then submitted to the John Leszik Proteomic Mass Spectrometry Laboratory facility from the University of Massachusetts for peptide sequencing.

Protein assays. Protein concentrations were determined according to Sedmak and Grossberg [29], using serum albumin as standard.

Results

Characterization of the isolated oocyte plasma membranes

It was our goal to develop a protocol for purifying the plasma membrane of the oocyte so that proteins involved in the interaction with sperm could be detected and studied. Fig. 1A shows the analysis of the filtered oocyte lysates and the isolated plasma membrane components by SDS–PAGE. Coomassie blue staining revealed the presence of a different pattern of major proteins in the isolated membranes compared with the lysates. Remarkably, isolated membranes seem to lack yolk proteins, which are the major bands seen in the homogenate at 100–120 kDa (lipovitellin I) and as a doublet at 30–35 kDa (lipovitellin II). In the isolated membranes, we also observed several biotinylated protein bands (Fig. 1B), Fig. 1C showing the presence of the α subunit of Na^+/K^+ ATPase (a plasma membrane marker of approximately 97 kDa) in the isolated membranes that could not be detected on the lysates under the same conditions indicating the presence of surface proteins. These results suggest that the isolated membranes derived mainly from the plasma membrane. Panel D shows the presence of bands when the no primary antibody is included. The band present in the lysates at approximately 100 kDa (probably lipovitellin I) and the doublet at approximately 60 kDa (that also can be seen in panel E) are not specific.

Finally, Fig. 1E shows no VE glycoproteins in our isolated plasma membranes, as judged for the lack of

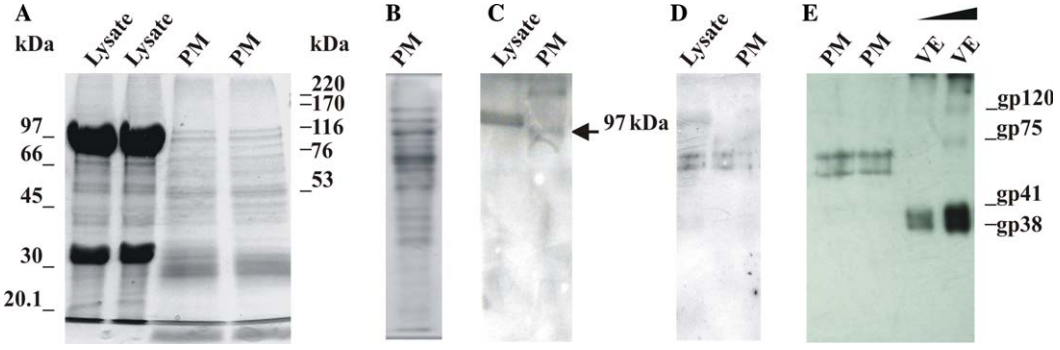

Fig. 1. Characterization of isolated plasma membranes. (A) Isolated plasma membranes (PM) and lysates (two different samples of each) were separated on a 10% SDS–PAGE and stained with Coomassie brilliant blue. (B) Biotinylated proteins in PM visualized with HRP-Streptavidin. (C) Na^+/K^+ ATPase, plasma membrane marker, in the lysates and PM. SDS–PAGE (12%) were run and transferred to nitrocellulose, and analyzed for the presence of Na^+/K^+ ATPase (97 kDa, arrow) by Western blot. (D) Samples of lysate and PM revealed without primary antibody. The doublet that appears in (D) and in the PM lanes of (E) are unspecific bands recognized by the secondary antibody. (E) Detection of VE glycoproteins in isolated membranes. Samples were loaded onto SDS–PAGE (12%) gels and transferred to nitrocellulose. VE glycoproteins were detected by Western blot. The molecular masses of the four major components of VE are displayed on the right. In all the cases, 10 μg of protein was loaded into each lane, except for VE samples that were 1 and 2 μg/lane, respectively.

Western-blot signal in two different preparations of membranes.

Association of oocyte plasma membrane molecules with sperm

A protein of the oolemma with high affinity for sperm is a strong candidate as a sperm receptor. In this study, we explored if any of the proteins from the isolated plasma membranes was able to interact with sperm. Oocytes were surface-biotinylated and their plasma membranes were isolated. These membranes were incubated with sperm as described in Materials and methods. Since all the detergents checked (Triton X-100, Brij-97, Tween 20, octyl glucoside, etc.) affected sperm viability (data not shown), we did not include any of them in the assay. Previous to the incubation, we carried out the following procedures: (i) addition of increasing amounts of BSA to avoid non-specific interactions, (ii) dilution and sonication of the sample to keep the plasma membranes soluble, and (iii) centrifugation of them at the speed used to pellet sperm to eliminate any unspecific co-sedimentation. Fig. 2 shows the result of a typical experiment. A biotinylated protein of approximately 100 kDa consistently co-sedimented with sperm independently of the amount of BSA added. This band was not present when plasma membranes were not included in the incubation (lane 5) or when the plasma membranes were prepared from oocytes that had not been biotinylated (lane 8). As the figure shows, the band has an electrophoretic mobility similar to that of a major biotinylated band in the isolated plasma membranes (lane 6). This band is still present in the post-assay supernatant (lane 7). As this fact may raise some issues regarding its role as a sperm binding protein despite its capacity to remain bound in the presence of 10 mg/ml of BSA, we made use of a different approach (i.e., affinity chromatography) to detect putative sperm receptors in oocyte plasma membranes.

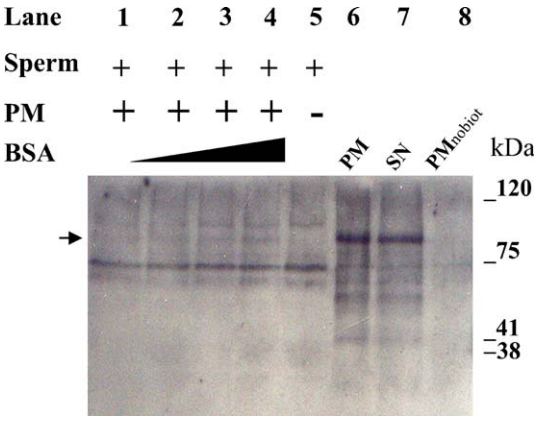

Fig. 2. Detection of oocyte plasma membrane proteins with affinity for sperm. Isolated plasma membranes (PM) from biotinylated oocytes were separated into aliquots (200 μl each, 70–200 μg prot) and incubated with sperm as described in Materials and methods. Sperm were lysed, and proteins were separated on 10% SDS–PAGE. After transference to nitrocellulose, biotinylated proteins that bound to sperm were visualized with HRP-Streptavidin and chemiluminescence. Lanes represent sperm associated with oocyte PM proteins at 0, 3, 6 y 10 mg/ml BSA (lanes 1–4), sperm incubated without PM (lane 5), PM (10 μl) not incubated with sperm (lane 6), PM supernatant (10 μl) after incubation with sperm (SN, lane7), PM from oocytes without biotinylation (PMnobiot, lane 8). The arrow indicates a 100 kDa oocyte surface protein with specific affinity for sperm.

Affinity chromatography

An affinity chromatography column containing proteins from acrosome-reacted sperm heads was used to search for oocyte plasma membrane molecules with sperm binding capacity. Only components of the acrosome-reacted sperm heads were used. Extracts were prepared from isolated sperm heads using the detergent dodecyl-maltoside. This detergent permits a fair solubilization preserving enzyme function and protein interactions in the membrane [30]. The extract was dialyzed, ultrafiltered, and coupled to the matrix. The isolated plasma membranes obtained from biotinylated oocytes were diluted and applied to the affinity column. Since divalent cations have been involved in this step in amphibian fertilization [31], 2 mM $MgCl_2$ was added to the sample and washing buffer. After extensive washing, bound proteins were eluted with 2 M NaCl. The result of this chromatography is shown in Fig. 3A. In the eluted samples, we found three main biotinylated bands with apparent molecular weights of approximately 100, 70, and 30 kDa.

Heat shock proteins were found to be abundant in mouse oolemma [32] and have also been involved in fertilization [33,34], thus we re-probed the membranes containing the eluted proteins with anti-HSP70 antibodies. Fig. 4 shows that this antibody recognized a band at approximately 70 kDa, suggesting it corresponds to HSP70.

The few reports that have dealt with the subject of sperm receptors in the plasma membrane of the amphibian oocyte [31,35] pointed to integrins as candidate molecules for such a role. As antibodies against *B. arenarum* integrins are not available, we decided to check if the presence of the known integrin-interacting motif RGD had any effect when included in the loading buffer of the affinity column. If an integrin that recognizes the peptide RGD was present in the eluate, the RGD molecule would compete for the binding to the column and a biotinylated band would disappear in the blot. As shown in Fig. 4, two of the biotinylated bands are absent when RGD is present: the 100 and 70 kDa ones. This suggests that an integrin (the 100 kDa protein) is involved in the interaction with sperm in *B. arenarum*, as has been suggested for *X. laevis* [31].

Analysis of the eluate by tandem mass spectrometry

To identify all the proteins present in the eluate of the affinity column, we carried out tandem mass analysis. All the biotinylated proteins that specifically interacted with the column were interesting, but we were not certain that the bands detected by chemiluminescence were the

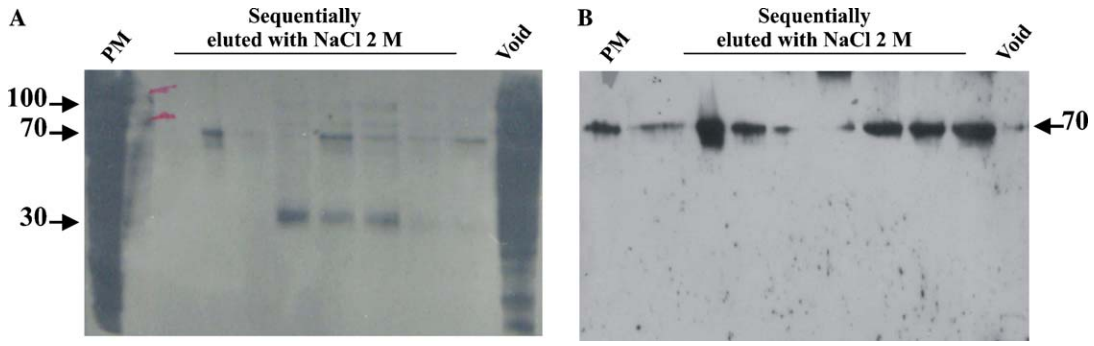

Fig. 3. Biotinylated proteins eluted from the sperm-affinity column. Samples were sequentially eluted with 2 M NaCl, (1 ml aliquots) dialyzed, concentrated, electrophoresed, and transferred to nitrocellulose. (A) Biotinylated proteins were visualized with HRP-Streptavidin and chemiluminescence. (B) Presence of HSP70 on the affinity column eluate. The blot presented in (A) was stripped (see Materials and methods) and re-probed with an anti-DnaK antibody. PM, plasma membrane sample loaded into the column, Void, plasma membrane sample after passing through the column.

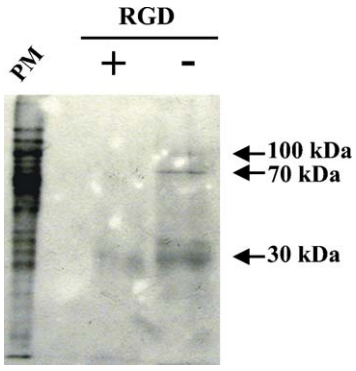

Fig. 4. Effect of the presence of RGD peptide on the affinity column. For details, see Materials and methods. PM, plasma membrane sample loaded into the column.

only ones present. To perform a complete analysis by standard in gel digestion protocols and not to lose any potentially interesting protein, we ran a SDS–PAGE gel with our eluted sample and ran it just enough to ensure that the proteins enter the separating gel. The gel was silver-stained and the bands were excised and sent to MS/MS analysis. The resulting spectra were used to perform Mascot search including the biotin modification in NCBInr database. We focused on the peptides having the biotin modification because this ensures us that the peptide comes from the surface-biotinylated oocyte plasma membrane sample and not from any possible contamination of the column matrix. Six hundred twenty-four peptides were analyzed and less than 100 of them were biotinylated. This search resulted in only two biotinylated peptides matching to proteins (see Table 1) including one that matched to an uncharacterized protein from *Ferroplasma acidarmanus* (an extremophile). The rest (80) could not be assigned to any protein. We decided to try the EST_others *X. laevis* database and look for homology by manual BLAST search. Using this database we found 14 peptides that matched EST sequences. Table 1 shows the matched EST sequences that showed homology to known proteins using BLAST. We could not find any direct match to integrins or heat shock proteins. Interestingly, however, we found EST sequences with homology to proteins involved in signal transduction: Rho-guanine nucleotide exchanging factor 7 and protein tyrosine phosphatase. Also with cytoskeletal elements: intermediate filaments, proteins with spectrin repeats, girdin (a regulatory actin-binding protein), and Hook-related protein (a protein that anchors organelles to filaments). Finally a protein with EF-hand: a low affinity Ca^{2+}-binding motif present in CREC family secretory proteins [36] and recently found in integrins [37].

Discussion

Isolation of plasma membranes from B. arenarum oocytes

Preparation of plasma membrane from ovarian, unfertilized, and fertilized oocytes of *Xenopus* has been reported previously. In the protocols described by Richter et al.

Table 1
List of proteins identified by MS/MS with their respective NCBI#

DATABASE	NCBI #	Peptides matched (biotinylated)	Homology to
NCBInr	CAI12049	3 (1)	Vertebrate spectrin repeat containing, nuclear envelope 1 (SYNE1)
	ZP_00307284	1	Uncharacterized conserved protein [*Ferroplasma acidarmanus*]
NCBI Est_others *Xenopus laevis*	BQ731533	3 (1)	Intermediate filaments
	BQ732437	3 (1)	Citokeratin
	BF232581	2 (1)	Hook-related protein 1 and girdin
	CD303583	2 (1)	rcn2 protein (EF-2 repeats)
	BM191435	2 (1)	Rho-guanine nucleotide exchanging factor 7
	BJ081419	2 (1)	Protein tyrosin phosphatase non-receptor type

[38,39], nylon mesh filtration was used to remove VEs from homogenates, followed by low-speed centrifugation to collect plasma membrane and cortex fragments as a fluffy layer on top of the yolk platelet pellet. With this method, however, the preparation is contaminated with yolk proteins. The method described by Young et al. [40] did not include filtration of the oocyte extracts to eliminate VE proteins. Later, a method was developed by Wall and Patel [41], but again plasma membrane was isolated without filtration and VE proteins were present in the isolated membranes. Kamsteeg et al. [11] reported an efficient and high-yield method for isolating plasma membranes from *X. laevis* oocytes. However, the protocol involves the stripping of the VE of each oocyte, which makes the procedure time-consuming and laborious. These authors did not inform the presence of VE or yolk proteins in their samples. Recently a method similar to the one described by Young et al. [42] has been published. Filtration was not included in this procedure and VE protein contamination was not informed.

We are confident that the membranes we isolated are from the plasma membrane based on the results of cell surface biotinylation and the presence of the plasma membrane marker Na^+/K^+ ATPase. In addition, when this type of discontinuous sucrose gradient was used on other cell types, plasma membrane fractions were also isolated [25,43]. We present evidences here that the membranes isolated with our method lack VE glycoproteins that may interfere in binding or affinity assays. Besides, the yolk platelet proteins do not appear in SDS–PAGE gels of the isolated membranes, suggesting that they were also eliminated. The method that we describe in this report is a modification of the one published by Luria et al. [25]. In that report, the authors strengthened the fact that one of the two plasma membrane fractions that they isolate was very similar to plasma membrane domains known as detergent-resistant-membranes or ''rafts''. With our modifications we get total plasma membranes free from VE proteins and from yolk proteins, and useful for the search for sperm receptors.

Oocyte surface molecules with affinity to sperm

Several proteins have been involved in sperm–oocyte interactions at the plasma membrane. In mammals, among others, integrins [44], calreticulin [45], and tetraspanins [46] have been mentioned as possible receptors for the sperm at the oocyte plasma membrane. In amphibians, indirect evidences point at ADAMs and integrins as the receptors mediating sperm binding to the oocyte plasma membrane: activation of *X. laevis* oocytes has been accomplished by RGD-containing peptides [31], and metalloproteases/disintegrins have been identified in *X. laevis* testes [35]. More recently, Uroplakin III has been involved in sperm–oocyte interaction as well as subsequent Src-dependent intracellular events of oocyte activation [47]. These findings provide a starting point, but many unresolved questions persist. To identify attachment proteins in amphibian's oocytes, we examined the molecules with affinity for sperm using our isolated plasma membranes. We showed the specific binding of a biotinylated oocyte plasma membrane protein to sperm. Only a 100 kDa oocyte plasma membrane protein showed a strong association with sperm although high BSA concentrations were included in the assay medium. To improve the sensitivity of the assay, we decided to try a different approach: affinity chromatography. It was shown again the presence of a 100 kDa protein but two more of approximately 30 and 70 kDa were observed. These evidences suggest the possible participation of these molecules in sperm–oocyte binding.

In a recent work [32], heat shock proteins were found to be very abundant in mouse oolemma. Also, sequence similarity of the sea urchin oocyte receptor for sperm and HSP70 has been reported [33] and antibodies against HSP70 have inhibitory effects on bovine fertilization [33,34]. These reports prompted us to check if any of the detected proteins (specially the one of approximately 70 kDa) is evidenced by an anti-HSP70 antibody. The results confirmed that we have a signal for a HSP70-related protein in our eluted proteins at an apparent molecular weight of 70 kDa. This finding suggests that as reported for mouse oolemma [32], *B. arenarum* oocytes have HSP70 in their plasma membrane and are in agreement with a role for this molecule in the interaction with the sperm.

RGD-containing peptides have been used previously to analyze the role of integrins during the interaction of sperm and the oocyte plasma membrane in *X. laevis* [31] and in mammals [44]. This strategy allowed us to avoid the use of non-specific antibodies that may or may not cross-react with *B. arenarum* integrins. One of the bands that disappear in the blot is the one of the largest molecular weight (approximately 100 kDa). It has been reported that integrins have different electrophoretic mobilities (90–200 kDa) depending on the conditions of the running and the isoform involved [48]. Also, the proteins were dependent on the presence of divalent cations for their interaction with the column. This agrees with the binding characteristics of some integrins that show complex behaviors regarding these ions to bind to their ligands [37]. All these evidences point to a role for integrins in the interaction at the plasma membrane between *B. arenarum* gametes. The 70 kDa protein also disappears in the presence of RGD peptide. As this protein seems to be HSP70, we suggest that perhaps there is an indirect interaction with sperm through the 100 kDa protein.

To further support our findings and to look for the identity of the other proteins present in the eluate, we decided to perform MS/MS analysis. Since *B. arenarum* is an organism with an unsequenced genome, we would have to rely on some of the peptides being identical or closely related to known proteins from other species. Although the analysis became complicated by the low number of biotinylated peptides matching to known

proteins, we obtained some interesting information from tandem mass analysis. The presence of multiple membrane-associated cytoskeletal elements and signal transduction machinery proteins further supported the fact that we isolated plasma membranes. More interestingly, the presence of a EF motif containing protein may relate to integrins, as EF-like domains have recently been found in these proteins [37]. Alternatively, a member of the CREC family, which is localized to the secretory pathway in mammalian cells [36], could be an interesting option. Recently, calreticulin, a Ca^{2+} binding chaperon previously located to the endoplasmic reticulum, has been shown to mediate transmembrane signaling linked to cell cycle resumption [45]. These hypothesis will be a matter of future studies in our laboratory, since this last option may account for the 30 kDa protein that we detect in the affinity column eluate.

In conclusion, this report provides a simple method to purify plasma membranes from amphibian oocytes free from VE and yolk proteins. This method could be very useful not only to study plasma membrane protein expression and affinity for ligands of endogenous proteins, but also for transgenic ones. Our results support the presence and involvement of heat shock proteins and integrins in the interaction of the oocyte plasma membrane with sperm. In addition, we have found some evidences that suggest that extra candidates for a role in this step of fertilization should be considered. The study of each protein and the search for its exact role will be the matter of further studies in our laboratory.

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