

A 70 kDa protein is transferred from the outer acrosomal to the plasma membrane during capacitation

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Received 3 October 1994; revised version received 20 November 1994

Abstract Bull sperm plasma and outer acrosomal membranes were analyzed by SDS-PAGE. Analysis of the plasma membrane proteins revealed the presence of a 70 kDa band the prominence of which is enhanced after capacitation. This protein was found to bind to zona pellucida intact oocytes. PAGE analysis of outer acrosomal membrane proteins also reveals the presence of a 70 kDa band, but its prominence decreases after capacitation. This protein also binds to zona pellucida intact oocytes. Furthermore, the 70 kDa outer acrosomal membrane protein is recognized in Western blot analysis by antibodies to plasma membrane proteins and vice versa. The results indicate that the 70 kDa acrosomal and plasma membrane proteins are the same. This 70 kDa protein would thus be a zona pellucida binding protein which is initially stored in the outer acrosomal membrane and transferred to the plasma membrane during capacitation, enabling it to function in egg-sperm binding.

Key words: Sperm; Ova; Fertilization; Protein trafficking; Membrane remodelling; Cell recognition

1. Introduction

It has long been known that when released from the testes, sperm are immotile and infertile. While they attain motility during passage through the epididymis (reviewed in [1,2]), sperm do not become fully fertile until they have undergone an additional maturation phase called capacitation (reviewed in [1,3]). In vivo, capacitation commences upon exposure of the sperm to the female reproductive tract, and it is a prerequisite for gamete interaction. Various species-dependent modifications have been reported to occur during capacitation, including changes in metabolism, intracellular ionic composition, acrosomal structure, adenylate cyclase activity, and membrane remodelling. After a capacitated spermatozoan has penetrated the cumulus oophorus surrounding the ovum, it binds to the zona pellucida (ZP). This gamete recognition and binding requires species-specific ZP-binding proteins on the sperm cell surface. To date such proteins have been identified in several systems. Among these are the trypsin inhibitor-sensitive site on the surface of hamster and mouse sperm [4,5], galactosyl transferase in mouse [6,7], proacrosine [8,9] and APZ [10,11] in the pig, rabbit sperm autoantigen [12,13], and PH-20 in the guinea pig [14,15]. In ejaculated sperm, ZP-binding proteins may be masked by epididymal or seminal secretions until after capacitation. This would prevent sperm-ZP binding before the spermatozoan has completed capacitation, at which time these 'decapacitating factors' are eluted from the cell surface.

The acrosome arises from the Golgi apparatus during spermatogenesis [16]: it possesses some functions of the Golgi, including sorting and selective export of proteins [17,18]. ZP binding induces the sperm to undergo the acrosome reaction, which involves multiple fusions between the outer acrosomal membrane and the overlying plasma membrane. This vesiculation releases the acrosomal contents, including a variety of hydrolytic enzymes, and is required if fertilization is to proceed further. We have recently shown [19] that membrane remodelling, which occurs during capacitation, is required for the membrane fusion event during the acrosome reaction.

In this report we describe a 70 kDa ZP-binding protein on the surface of bull sperm. The results indicate that this protein is transferred to the plasma membrane from the acrosome during capacitation. We propose that storing this protein prior to capacitation in the acrosomal membrane prevents premature sperm-ZP binding and acrosome reaction.

2. Materials and methods

Frozen bovine sperm pellets were obtained from The Artificial Insemination Service, 'Hasherut', Israel, and maintained in the laboratory in liquid nitrogen. Bovine ovaries were obtained from a local abattoir and maintained in the laboratory at -70°C . Heparin, benzimidazole, HEPES, phenylmethylsulfonyl fluoride (PMSF), and 3-[N-morpholino]propanesulfonic acid (MOPS) were purchased from Sigma. All other reagents used were of analytical grade.

2.1. Preparation of cells

In vitro capacitation was accomplished by the method of Parrish et al. [20]. Sperm pellets were thawed and washed three times in 155 mM NaCl, 10 mM histidine, pH 7.4, and finally suspended in glucose-free TALP (100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO_3 , 0.29 mM KH_2PO_4 , 21.6 mM lactic acid, 1.5 mM MgCl_2 , 1 mM pyruvate, 3 mg/ml bovine serum albumin, 10 mM HEPES, pH 7.4), containing 10 $\mu\text{g}/\text{ml}$ heparin. The cells were incubated in this medium for 4 h at 37°C with shaking. In control samples (uncapacitated sperm), heparin was omitted from the incubation medium.

2.2. Preparation of sperm membranes

Plasma membranes were prepared as previously described [21]. Cells were suspended in 10 mM histidine, 0.5 mM EDTA, 1 mM PMSF, 1 mM benzimidazole, pH 7.4, brought to 4°C and their plasma membranes removed by ultraturax. The membranes were collected by differential centrifugation and suspended in HBS (150 mM NaCl, 10 mM HEPES, pH 7.0). Outer acrosomal membranes were then removed from the same cells by a modification of the procedure of Zahler and Doak [22,23]. The cells were centrifuged twice through 1.3 M sucrose in HBS and incubated for 30 min in 0.2 M sucrose, 10 mM HEPES, and 5 mM EDTA, at 37°C with shaking, to loosen the outer acrosomal membrane. The cells were brought to 4°C and the outer acrosomal membranes were then removed by ultraturax. The membranes were then collected by differential centrifugation and suspended in HBS. The plasma and outer acrosomal membranes were further purified on a 1.3 M/1.75 M sucrose step gradient made in HBS. The plasma membranes were collected from the HBS/1.3 M interface, and the outer acrosomal membranes from the 1.3/1.75 M interface. The purified

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membranes were collected, washed once and resuspended in HBS. Electron microscopic examination of the cells and membranes after the first ultraturax treatment shows that the plasma membranes are removed, but not the acrosomes or mitochondria [21]. After the second treatment, the outer acrosomal membranes are removed, but not the inner acrosomal membranes or mitochondria [23]. The purity of these membrane preparations have further been established by two enzyme markers. There is an ATP-dependent calcium pump associated with the plasma membranes [21] not present on the acrosomal membranes (our unpublished observations). It has previously been shown that Ca_2^+ -ATPase is associated with the plasma membrane, but not the acrosomal membrane [24]. In addition, acrosin activity is present on the acrosomal membranes but not on the plasma membranes [22], and our unpublished observations). The electrophoretic maps of the membrane proteins (Fig. 1) and Western blot analysis (Fig. 2) also show that there is no detectable cross-contamination.

2.3. Collection of bovine oocytes

Oocytes were collected from bovine ovaries by the method of Florman and First [25]. Ovaries were brought to 37°C in harvesting medium (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4 , 1.47 mM KH_2PO_4 , 0.59 mM EDTA, 1.1 mM glucose, and 4 mg/ml polyvinylpyrrolidone), and gently minced in a blender. The oocytes were then collected by successive sieving until they were retained on a screen having spacings of 39 μm . Microscopic examination of the oocytes showed that the zona pellucidae were intact.

2.4. PAGE analysis of membrane proteins

Membranes containing 70 μg protein (determined by the method of Lowry [26]) in 50 μl HBS were added to 12.5 μl denaturation buffer (150 mM Tris, pH 7.5, 10% SDS, 25% β -mercaptoethanol, 50% sucrose, and 0.5% Phenol red). The mixture was boiled for 10 min and then electrophoresed on a 5–15% SDS polyacrylamide gradient gel with a 5% stacking gel. The molecular weight standards used were carbonic anhydride (29 kDa), ovalbumin (45), bovine plasma albumin (66), phospholipase B (97.4), and β -galactosidase (116). The gels were stained with Coomassie blue.

2.5. Extraction of membrane proteins

Membranes were suspended in cytosolic medium (110 mM KCl, 5 mM NaCl, 10 mM MOPS, pH 6.8), and the proteins were extracted with 0.5% sodium cholate for 15 min. The proteins were then precipitated by the addition of 30% polyethylene glycol 4000 (PEG). After 1 h, the proteins were collected by centrifugation at 10,000 rpm for 10 min. About 85% of the total membrane protein is recovered after precipitation with PEG. The electrophoretic profiles of the membrane proteins after precipitation with PEG are somewhat different from those obtained by the PAGE analysis described above, indicating that not all proteins are equally recovered after precipitation with PEG.

2.6. Binding of membrane proteins to oocytes

30 μg of membrane protein were solubilized by sonication for 1 min [27] to yield a dispersion that remained in suspension for several hours, and then combined with 20 oocytes in NKM (110 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1 mM PMSF, 1 mM benzimidazole, 10 mM MOPS, pH 7.4). The mixture was incubated for 3 h at room temperature with shaking. The oocytes were then removed by centrifugation, and the proteins remaining in the supernatant (the non-ZP-binding proteins) were precipitated with 10% cold trichloroacetic acid and collected by centrifugation. In control experiments (total extracted protein), oocytes were omitted from the incubation mixture. The specificity of the protein binding to the ZP was evaluated in other control experiments in which the oocytes were replaced with 4000 dog red blood cells which have a combined surface area about equal to that of 20 oocytes [28]. This control supernatant contains the non-RBC-binding proteins.

2.7. Quantitation of the 70 kDa proteins

The amount of 70 kDa protein as the percentage of the total extracted protein, the non-ZP-binding protein or the non-RBC-binding protein, in each membrane species, was determined as follows. The total membrane protein, or the non-ZP-binding protein fraction, obtained as just described, was suspended in 50 μl distilled water. 12.5 μl denaturation buffer was added, and the protein samples electrophoresed as described above. Each lane in the Coomassie blue-stained gel was scanned on a

Hoefel Scientific Instruments GS 300 densitometer which was used in the transmittance mode. For each lane, the area under the 70 kDa peak was measured and divided by the total area under the scan.

2.8. Preparation of antibodies to sperm membrane proteins

Sperm membranes were suspended in PBS and emulsified with an equal volume of Freund's complete adjuvant. 200 μg membrane protein was injected into adult female rabbits. The animals were boosted twice at intervals of 30 days with the same amount of membranes using incomplete Freund's adjuvant. One week after the last boost, they were bled from the ear artery. The titer of the antiserum was determined by comparison with preimmune serum by ELISA.

2.9. Western blotting

Sperm membrane proteins were separated by PAGE and transferred to nitrocellulose using a buffer composed of 25 mM Tris, 192 mM glycine and 20% methanol. The nitrocellulose blot was blocked with 1% BSA in PBS with 0.1% Tween-20 for 1 h at room temperature. The blot was incubated with antibodies raised against sperm membranes diluted 1:10,000 for 1 h at room temperature. The blot was washed three times with PBS containing 0.1% Tween-20, and incubated with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody diluted 1:20,000. The blot was then washed 3 times and visualized by ECL (Amersham). Controls in which the antibodies were blocked with membrane proteins were routinely performed (not shown) and established the specificity of the labeling observed in the Western blots.

3. Results

Fig. 1 shows the PAGE patterns of the total plasma and outer acrosomal membrane proteins. In all cases a large number of bands were observed. The results indicate that major membrane protein remodelling does not occur in either membrane during capacitation. There is, however, a 70 kDa plasma membrane protein band the prominence of which is enhanced after capacitation while that of a 70 kDa outer acrosomal membrane protein band decreases.

We removed the ZP-binding proteins from the total membrane proteins by extracting the proteins from the membranes, and incubated them with ZP-intact oocytes. The oocytes and

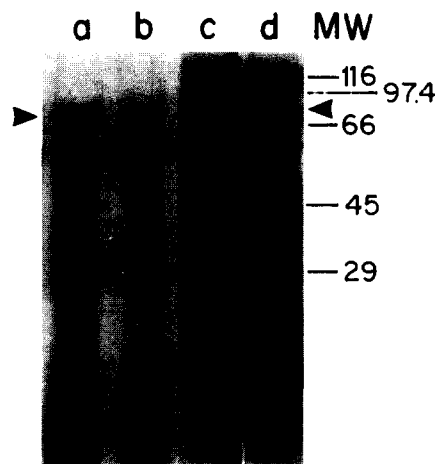


Fig. 1. PAGE analysis of sperm membrane proteins. Total outer acrosomal membrane proteins before (a) and after (b) capacitation. Plasma membrane proteins before (c) and after (d) capacitation. 70 μg membrane protein was loaded in each lane. The arrowheads indicate 70 kDa outer acrosomal and plasma membrane proteins. The prominence of the acrosomal protein decreases with capacitation, whilst that of the plasma membrane protein increases. Lines show the positions of the molecular weight standards. This experiment was repeated 3 times with membranes from three different preparations, and a representative result is shown.

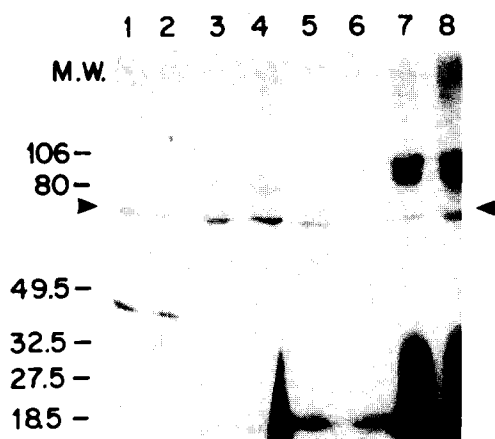


Fig. 2. Western blot analysis of sperm membrane proteins. Sperm membrane proteins were separated by PAGE as in Fig. 1, and blotted onto nitrocellulose. The proteins were detected using antibodies raised against outer acrosomal membranes (lanes 1–4) or plasma membranes (lanes 5–8) from uncapacitated cells. Lanes 1 and 5, uncapacitated acrosomal membranes; lanes 2 and 6, capacitated acrosomal membranes; lanes 3 and 7, uncapacitated plasma membranes; lanes 4 and 8, capacitated plasma membranes. The arrowheads point to 70 kDa proteins which are common to both membrane species. The positions of the molecular weight standards are shown at the left. This experiment was done 3 times from 3 different membrane preparations. Shown is a typical result.

the bound proteins were removed by centrifugation, and the non-ZP-binding proteins in the supernatant were electrophoresed. The gels were stained with Coomassie blue and scanned densitometrically. The results appear in Table 1. During capacitation, outer acrosomal membrane 70 kDa protein decreased from 19% to 8% of the total extracted outer acrosomal membrane protein. At the same time, plasma membrane 70 kDa protein increased from 12% to 22% of the total extracted protein of that membrane. Table 1 also shows that 70 kDa proteins constitute a much smaller fraction of the non-ZP-binding proteins than of the total protein in their respective membranes. The 70 kDa proteins thus preferentially bind to ZP-intact oocytes, in comparison to other proteins of the same membrane. The same proteins have relatively low affinity for the glycoprotein coat of dog red blood cells, demonstrating that the binding of these proteins to the ZP is specific.

Fig. 2 shows a Western blot of sperm membrane proteins using antibodies raised against sperm membranes. A 70 kDa plasma membrane protein is recognized by antibodies raised against acrosomal membranes and vice versa. This 70 kDa

protein is the most prominent protein which is common to both membranes. Also shown in the Western blot, the amount of this 70 kDa protein in the outer acrosomal membrane decreases during capacitation, while it increases in the plasma membrane.

4. Discussion

ZP-intact eggs were used to identify ZP-binding proteins extracted from sperm membranes. After incubation, eggs and bound proteins were removed by centrifugation. The egg cell thus serves as a handle on the ZP and its bound proteins. This method of affinity chromatography is much quicker and simpler than with columns of extracted ZP [11]. Another technique which has been used for identifying ZP-binding proteins involves monoclonal antibodies raised against sperm proteins [12,29–33]. An antibody which when bound to the sperm surface inhibits ZP-binding, implicates its ligand as a ZP-binding protein. This approach has been criticized because inhibition of ZP binding is not definitive proof of function [3]. Moreover, this method can not be used for identifying ZP-binding proteins in the acrosomal membrane of acrosome-intact cells. Another strategy uses radiolabeled ZP to probe sperm proteins that have been separated electrophoretically and then electroblotted to nitrocellulose [31,34,35]. With this approach, significant non-specific binding has been shown to occur, making interpretation of the results difficult [34–36]. We, however, extracted proteins from isolated sperm membranes, instead of whole cells. As has been suggested [3], such fractionated sperm preparations should reduce some of this non-specific binding.

We have recently shown [19] that remodelling of sperm membranes which takes place during capacitation is a prerequisite for membrane fusion during the acrosome reaction. PAGE analysis of sperm membrane proteins indicates that major remodelling of the protein phase does not occur (Fig. 1), implying that the major structural change in the membranes involves the lipid phase. This is consistent with the view that the major membrane alterations which occur during capacitation involve cholesterol efflux [37,38], redistribution of existing membrane proteins [39], and elution of coating proteins [40–42]. Thus, once the sperm leaves the epididymis, there is essentially no further change in the protein content of its plasma and outer acrosomal membranes. Exceptions to this are 70 kDa ZP-binding proteins in both membranes. Table 1 shows that during capacitation an increase in the presence of a plasma membrane 70 kDa ZP-binding protein occurs simultaneously with the decrease in a 70 kDa ZP-binding outer acrosomal membrane protein.

Table 1

Changes in the relative abundance of 70 kDa proteins due to capacitation or oocyte binding

	Outer acrosomal membranes		Plasma membranes	
	Uncapacitated	Capacitated	Uncapacitated	Capacitated
Total extracted protein	0.19	0.08	0.12	0.22
Non-ZP-binding protein	0.05	0.06	0.05	0.04
Non-RBC-binding protein	0.16	0.09	0.10	0.18

30 μ g extracted membrane protein were incubated with 20 oocytes (non-ZP-binding proteins), 4000 dog red blood cells (non-RBC-binding protein), or buffer alone (total extracted protein), as described in section 2. The cells were then removed by centrifugation and the proteins in the supernatant were separated by PAGE. The gel was stained with Coomassie blue, and each lane was scanned densitometrically. The table gives the relative abundance of 70 kDa proteins present as the fraction of total, non-ZP-binding protein and non-RBC-binding protein in each membrane species. Each value is the average of two determinations.

Several lines of reasoning suggest that the 70 kDa plasma and outer acrosomal membranes are the same. Both of these proteins are ZP-binding proteins, and the relative abundance of the plasma membrane 70 kDa protein before and after capacitation is complementary to that of the outer acrosomal membrane 70 kDa protein. Furthermore, a 70 kDa outer acrosomal membrane protein is recognized by antibodies to plasma membrane proteins and vice versa (Fig. 2). The relative amounts of this protein before and after capacitation in both membrane species, as revealed by Western Blot analysis, shows the same complementary pattern observed in the Coomassie-stained gel (Fig. 1). Moreover, this protein is the most prominent protein common to both membranes.

Several reports have described changes in the protein composition of the sperm plasma membrane during epididymal maturation and capacitation. Peterson et al. [11] have identified three proteins (30, 45 and 70 kDa) in caudal epididymal boar sperm plasma membranes which are not present in caput sperm. Esbenshade and Clegg [43], working with boar sperm, found a 70 kDa plasma membrane protein which increases from 10.1% to 13.5% of the total membrane protein during in utero incubation. Berger [44] found a 78 kDa protein which became exposed on the boar sperm surface during capacitation, the presence of which correlated with increased ability of the cells to fuse with ZP-free hamster ova. The locations of these proteins prior to their appearance in the plasma membrane were not determined. The increase in the abundance of a plasma membrane protein during capacitation can not be due to de novo synthesis because protein synthesis in sperm ceases in the early stages of spermatogenesis in the testis [16]. This implies that any protein which appears in the plasma membrane during capacitation had to have been transferred there from some other location. Our results with bovine sperm, however, constitute the first time that the appearance of a protein in the plasma membrane during capacitation has been correlated with its disappearance from the outer acrosomal membrane.

The observation that remodelling of the sperm plasma membrane continues through capacitation, although glycoprotein synthesis ceases much earlier, implies that these proteins which appear in the plasma membrane after the inactivation of the Golgi must previously have been stored in some other subcellular compartment. Our results support the conclusion that the acrosomal membrane fills this function. Transfer of proteins from the acrosome to the cell surface has been shown to occur during spermatogenesis and epididymal maturation. PH-20 is a 60 kDa guinea pig sperm protein present on the acrosomal membrane of round spermatids which is subsequently transferred to the plasma membrane [14,15]. APZ is a 55 kDa boar sperm surface protein present in the acrosome of testicular sperm, which is observed in both the acrosome and plasma membrane of ejaculated sperm [10,27]. The distribution of these proteins after capacitation has not been reported. The 70 kDa bovine sperm protein which we describe here is the first demonstration that the transfer of a protein from the acrosomal to the plasma membrane can occur as late as capacitation. APZ, PH-20, and our 70 kDa bovine sperm protein are all ZP-binding proteins. Taken together, these observations suggest a general mechanism in which ZP-binding proteins are stockpiled in the acrosome as long as the Golgi apparatus is active. They would initially be retained in the acrosome in order to prevent premature gamete interaction. Their appearance on the sperm

surface during capacitation would then allow sperm binding to the ZP and the initiation of the sequence of events leading to acrosome reaction and fertilization.

Acknowledgements: This work was supported in part by grants from the Pollack and Ihel Foundations to H.B.

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