SPERM BINDING AND FERTILIZATION ENVELOPE FORMATION IN A CELL SURFACE COMPLEX ISOLATED FROM SEA URCHIN EGGS

GLENN L. DECKER and WILLIAM J. LENNARZ

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

ABSTRACT

An isolated surface complex consisting of the vitelline layer, plasma membrane, and attached secretory vesicles has been examined for its ability to bind sperm and to form the fertilization envelope. Isolated surface complexes (or intact eggs) fixed in glutaraldehyde and then washed in artificial sea water are capable of binding sperm in a species-specific manner. Sperm which bind to the isolated surface complex exhibit the acrosomal process only when they are associated with the exterior surface (vitelline layer) of the complex. Upon resuspension of the unfixed surface complex in artificial sea water, a limiting envelope is formed which, based on examination of thin sections and negatively stained surface preparations, is structurally similar to the fertilization envelope formed by the fertilized egg. These results suggest that the isolated egg surface complex retains the sperm receptor, as well as integrated functions for the secretion of components involved in assembly of the fertilization envelope.

KEY WORDS sperm binding · fertilization envelope · cortical reaction · cell surface complex

The major components of the surface and peripheral cytoplasm of the sea urchin egg are the vitelline layer, the plasma membrane and the cortical vesicles. During fertilization the cortical vesicles fuse with the plasma membrane and release their contents extracellularly, resulting in what has been termed the cortical reaction (1, 2, 8, 18). During this reaction, a protease is released which is thought to aid in the block against polyspermy by destruction of cell surface receptors for sperm (4, 24). In addition, secretory products of the vesicles, along with the vitelline layer, appear to interact and give rise to the fertilization envelope (12, 17, 19).

To facilitate elucidation of the biochemical events associated with the cortical reaction, we have devised a procedure for the isolation of a cell surface complex that consists of cortical vesicles attached to the plasma membrane, which is coated on its exterior face with the vitelline layer (7). We now report that this cell surface complex retains a number of functions which are characteristic of the cell surface of the intact egg. The cell surface complex induces the acrosomal reaction in sperm, and binds them in a species-specific manner. Concomitant induction of the cortical reaction in the surface complex results in the detachment of bound sperm and the formation of a fertilization envelope. Thus, it is apparent that early events in fertilization, i.e., sperm attachment, breakdown of cortical granules, detachment of excess sperm, and formation of the fertilization envelope, are
primarily functions of components of the cell-surface complex, and are demonstrable in the absence of other organelles of the egg.

MATERIALS AND METHODS

Isolation of the Cell Surface Complex and Induction of the Cortical Reaction

From a 2.0-ml suspension of surface complex preparations obtained from 0.6 ml of packed dejellied eggs, 0.2-ml aliquots were sedimented in a microfuge operated at 60 V via a variable transformer (7). Pellets of the surface complex were carefully rinsed once with ice-cold 0.54 M NaCl to remove the EGTA, and then resuspended in 300 μl of artificial sea water (ASW) to activate the cortical reaction. Control preparations were resuspended in Ca2+-, Mg2+-free ASW containing EGTA (7). After resuspension, the reaction was stopped at 1, 5, 10, and 30 min by dilution with equal volumes of 6.0% glutaraldehyde in the medium used for each test, and then sedimented as before in the microfuge. All solutions used had a pH value in the range of 8.0-8.3. To obtain individual preparations, the entire final pellet of the surface complex obtained from 0.6 ml of dejellied eggs was resuspended to 2.0 ml in ASW for activation of the cortical reaction and subsequently centrifuged in the clinical centrifuge for 30 s at maximum voltage (3,000 rpm).

Isolation of Fertilization Envelopes from Eggs and from Surface Complexes

After fertilization of eggs or resuspension of the isolated surface complex in artificial sea water, the preparations were incubated for 5, 10, or 30 min at 0-4°C (Strongylocentrotus purpuratus) or at 22°C (Arbacia punctulata). The fertilized eggs or the surface complexes were then collected by centrifugation, diluted 1:10 with distilled water, and immediately homogenized by five strokes in a glass homogenizing vessel (7). The homogenates were then further diluted 1:10 with distilled water and sedimented in the clinical centrifuge at maximum voltage for 1 min. Envelopes from fertilized eggs were washed until the supernates were clear (3-4 washes). Envelopes from both sources were then processed for electron microscopy by fixation in glutaraldehyde followed by negative staining, or were embedded for thin sectioning. To test for solubility of the fertilization envelope, preparations were resuspended in 6 M guanidine HCl at 60°C for 20 min (10) or in 30% mercaptoethanol (16) at room temperature, respectively, and then examined in the phase or electron microscope.

Prefixation of the Cell Surface Complex and Eggs

Stock suspensions (2.0 ml) of prefixed surface complexes were prepared by addition of an equal volume of cold 6.0% glutaraldehyde in isolation medium. After 15 min at 0°C, the mixture was sedimented for 10 s in the clinical centrifuge at maximum voltage. The supernate was quickly removed and the pellets were immediately washed by resuspension in 5.0 ml of cold ASW and then sedimented for 10 s in the clinical centrifuge as before. The supernate was removed and the pellet was resuspended in 5 ml of ice-cold ASW and allowed to sit at room temperature or on ice for 30 min. The fixed surface complexes were again sedimented for 10 s and then resuspended with a Pasteur pipet in 1 ml of ASW at 0°C or at room temperature. Since it seemed likely that more glutaraldehyde was entrapped by fixed eggs than by fixed surface complexes, eggs were treated similarly except that the resuspension-wash step consisted of two 1:100 dilutions and gravity sedimentations after the initial fixation.

Attachment of Sperm

"Dry" sperm (60-130 mg protein/ml) were collected with minimal dilution by electrical shock (A. punctulata) or by injection of 0.5 M KCl (S. purpuratus) and immediately stored on ice. Just before use, aliquots of stock sperm were diluted 100-fold with 1.0 ml of ice-cold ASW or with Ca2+-, Mg2+-free ASW containing EGTA. Prefixed surface complexes (0.1-1.0 mg protein/ml) or 5 × 10^5 prefixed eggs were then added at room temperature or on ice, and allowed to interact for five min. Surface complexes or eggs which had not been pre-fixed were similarly mixed with sperm. The interaction of sperm with pre-fixed or untreated surface complexes was terminated by addition of an equal volume of 6.0% glutaraldehyde to kill the sperm. The specimens were then photographed through a Wild (Wild Heerbrugg Instruments Inc., Farmingdale, N. Y.) or a Zeiss (Carl Zeiss, Inc., N. Y.) phase microscope, or processed for electron microscopy.

Electron Microscopy Preparation for Thin Sectioning

Specimens of eggs, sperm, and surface complexes were fixed for electron microscopy by the addition of an equal volume of 6.0% glutaraldehyde freshly prepared in the particular specimen medium. The preparation was then sedimented in either the microfuge or the clinical centrifuge and allowed to sit on ice for 2 h. Subsequently, all samples were resuspended in the medium used before fixation and stored at 4°C for at least 1 h. Postfixation was accomplished in 2.0% OsO4 in ASW. Dehydration was done in increasing concentrations of cold ethanol (50, 70, 95%, and absolute) for 5 min each except for the absolute ethanol, which was added cold and then allowed to sit at room temperature for 15 min and then exchanged for absolute ethanol at room temperature for an additional 15 min. Infiltration was carried out in open vials in a 1:1 mixture of acetone and
Negative Staining

Pellets (0.05-0.1 ml) of the envelopes prepared from the surface complex or from eggs were resuspended in 1 ml of distilled water and then fixed by addition of 1 ml of 6.0% glutaraldehyde for 5 min and sedimented in the clinical centrifuge for 5 s at maximum voltage. The supernate was immediately removed and the pellet was resuspended in 5.0 ml of distilled water and sedimented as before. The resulting pellet was resuspended in distilled water, mixed with an equal volume of 2.0% ammonium molybdate (at pH 7.0 with ammonium hydroxide), and then an aliquot of it was placed on a formvar-coated grid. After 2 min, the edge of the grid was touched with filter paper to remove excess material and the remaining thin layer containing the specimen was air-dried for a few minutes and then viewed in the electron microscope.

RESULTS AND DISCUSSION

The objectives of these studies with the cell surface complex were to investigate the binding of sperm to receptors associated with the vitelline layer and/or the plasma membrane, and to determine whether this binding was species specific. An additional aim was to determine whether this complex contained the components necessary for the formation of the fertilization envelope.

In our earlier investigations on the cell surface complex, we found that calcium, which is required for the acrosomal reaction in sperm, causes the cortical vesicles in the cell surface to burst (7). Similar observations, using cortices attached by their outer surface to a culture dish, have been reported by Vacquier (22). In the current study, we initially found that, as an apparent consequence of this rupture of the cortical vesicles in artificial sea water, binding of sperm by the cell surface complex was only transient. Presumably, this is because of the release from the cortical vesicles of a protease reported by Vacquier et al. (24) to be involved in termination of polyspermy by destruction of receptor sites on the cell surface. This possibility is consistent with our earlier report in which we demonstrated that indeed a protease is highly enriched in the cell surface complex (7). It should be noted that sperm binding can readily be observed in isolated vitelline layers (which are devoid of cortical vesicles) prepared from *Xenopus laevis* (13) and *S. purpuratus* (11).

Sperm Binding to Pre-fixed Eggs

To develop and test methods for preventing breakdown of the cortical vesicles in the cell surface complex, we turned to intact eggs and examined procedures for preventing the cortical reaction. We found that brief pre-fixation in glutaraldehyde in Ca²⁺, Mg²⁺-free ASW, followed by washing with ASW to remove excess glutaraldehyde, yielded preparations of *S. purpuratus* and *A. punctulata* eggs that would bind and retain many homologous sperm (Fig. 1A and B). This binding was species specific, since the pre-fixed eggs bound and retained few if any heterologous sperm. Stable binding of sperm to eggs was very low (≤10 sperm/egg) when the eggs had not been pre-fixed, undoubtedly because of the onset of the cortical reaction. Binding was also low when the eggs had been pre-treated with trypsin before aldehyde fixation, which is consistent with the earlier conclusion that a receptor for sperm is cleaved from the egg by trypsin (20). This ability of pre-fixed eggs to bind sperm is not limited to *S. purpuratus* and *A. punctulata*, since after completion of this work Kimura-Furakawa et al. (15) reported sperm binding to pre-fixed eggs of *Pseudocentronus depressus*.

Examination of pre-fixed eggs in the electron microscope indicated that sperm which had attached to the vitelline layer had always undergone the acrosomal reaction. Moreover, many of these sperm were intimately associated with the microvilli (Fig. 2A and B). Furthermore, at higher concentrations of added sperm, sections cut transversely through the attached sperm indicated that the egg surface could be completely saturated with sperm (Fig. 2C). The head of each *S. purpuratus* sperm occupies a minimum area of 4.0 μm². Since the transverse slice (Fig. 2C) was made ~4.0 μm from the point of attachment of the sperm to the egg surface, the number of sperm attached was calculated relative to a sphere 88 μm in diameter. Based on these considerations, it appears that an *S. purpuratus* egg can bind 6,000 sperm. This value, using pre-fixed eggs, can be compared to a value of 1,500 estimated by scanning electron microscopy (9), or to a value of 3,000 reported by Vacquier and Payne (23). This latter method involved measurement of sperm bound to eggs that were fixed only after addition of sperm.
Figure 1. After brief aldehyde fixation and washing, *S. purpuratus* (A) or *A. punctulata* eggs (B) bind and retain many homologous sperm. Cessation of sperm motility and further preservation of specimens was accomplished by addition of glutaraldehyde 5 min after addition of $10^4$ sperm/egg. Bar, 25 μm. × 800.
FIGURE 2  Prefixed eggs induce the acrosomal reaction in homologous sperm that attach to the surface. The cortical reaction is inhibited (i.e., no exocytosis of the cortical vesicles) in both *S. purpuratus* (*A*) and *A. punctulata* (*B*), and sperm remain tightly adherent to the egg surface, often associated with the microvilli. At higher magnification (*insets a and b*), the reacted sperm appear to be attached to vitelline layer sites on the apices of the microvilli (*vl*) by material derived from the acrosome matrix (*acm*). After interaction of $4 \times 10^6$ sperm/egg for 5 min in ASW (*Fig. 2C*), a transverse section through attached sperm suggests that ~$6,000$ sperm can bind to an *S. purpuratus* egg (see text). (*A, B, and C*) Bar, 2.0 μm, × 10,000. (*insets*) Bar, 0.5 μm, × 20,000.
Sperm Binding to the Pre-Fixed Cell Surface Complex

When the pre-fixation procedure was applied to the isolated cell surface complex, similar stabilization of sperm binding was observed (Fig. 3A and B). Moreover, binding of these sites was species specific: when the pre-fixed surface complex prepared from *A. punctulata* eggs was mixed with *S. purpuratus* sperm, only a few sperm remained after washing (Fig. 3C). Similar results were obtained with the opposite cross, i.e., that between the surface complex of *S. purpuratus* and *A. punctulata* sperm (Fig. 3D). Although it is apparent that there is a low level of entrapment of both homologous and heterologous sperm (sometimes via the tail of the sperm), heterologous sperm were never found to have undergone the acrosomal reaction (data not shown). In contrast, electron microscope observations of homologous sperm bound to the complex revealed that those associated with the outer side of the complex always had undergone the acrosomal reaction (Fig. 4A). On the other hand, homologous sperm that were apposed to the inner side of the complex, as shown in Fig. 4B, were never observed to have undergone the acrosomal reaction. These findings are consistent with previous studies suggesting that sperm bind preferentially to the outer surface of isolated vitelline layers (11).

Formation of the Fertilization Envelope by the Cell Surface Complex

Perhaps the most morphologically readily distinguishable concomitant of fertilization is the elevation of the fertilization envelope. Others have established that this process does not require sperm since it can be accomplished by ionophore activation of eggs (5, 21). Earlier we reported that resuspension of unwashed preparations of the cell surface complex (still containing traces of EGTA) in ASW results in lysis of cortical granules and thickening of the vitelline layer (7). In the current study, using preparations of the complex that were washed to remove EGTA, we observed that resuspension in ASW in the presence or absence of sperm resulted in formation of a new refractive peripheral envelope (Fig. 5A). Formation of this envelope (as well as sperm binding) did not occur in Ca\(^{2+}\)- and Mg\(^{2+}\)-free ASW. As seen in thin sections, this envelope is trilamellar and is ~500 Å in thickness (Fig. 5B). Comparable results were obtained with surface complexes from *S. purpuratus* (Fig. 6). In both species, the envelope consists of two distinct layers of tightly packed particles which are roughly 150 Å across and are separated by a less dense space of 200 Å (Fig. 5B, inset, and Fig. 6). When envelopes from these preparations were examined further by negative staining using ammonium molybdate, it was apparent that envelopes isolated from surface complexes (Fig. 7A) or from fertilized eggs (Fig. 7B) contain a similar primary repeating subunit which is ~160 Å across. Like the fertilization envelope generated by intact, fertilized eggs, the fertilization envelope formed from the cell surface complex was not solubilized by mercaptoethanol (30%) or guanidine-hydrochloride (6 M).

Essentially all of the above mentioned properties have been described for the fertilization envelopes isolated from fertilized eggs (3, 10, 14, 16). Therefore, we conclude that the structural component produced upon exposure of the cell surface complex to artificial sea water is indeed the fertilization envelope. In this context, it should be noted that, although we previously found that the peroxidase responsible for cross-linking of the fertilization envelope (10) is highly enriched in the cell surface complex (7), examination of the reacted complex for dityrosine residues has failed to reveal the presence of this cross-bridging amino acid (C. Foerder and B. M. Shapiro, personal communication). Thus, although the fertilization envelope formed in vitro by the surface complex manifests the ultrastructural appearance of the fertilization envelope isolated from eggs, and is insoluble in mercaptoethanol or in guanidine hydrochloride, it apparently had not undergone the final "hardening" step involving formation of dityrosine cross-links. Since this step requires not only the peroxidase but also H\(_2\)O\(_2\) (10), it may be that this step is necessary for generating peroxide is not present in the complex or was inactivated by the isolation procedure.

These studies have established that an isolated cell surface complex can induce the acrosomal reaction in sperm and that it contains a receptor involved in the binding of sperm to the cell surface. In addition, it contains the components necessary to generate a fertilization envelope in vitro. In our previous studies, we demonstrated that the cell surface complex consisted of a multi-component "organelle" composed of the cortical vesicles attached to the plasma membrane, which
**FIGURE 3** Phase micrographs demonstrating species specificity for sperm binding to the isolated surface complex. (A) Pre-fixed *A. punctulata* surface complex with inset from same at a different focal plane showing refractive cortical vesicles. (B) Pre-fixed *A. punctulata* surface complex in ASW containing sperm exhibits the binding of large numbers of homologous sperm. Pre-fixed *A. punctulata* (C) or *S. purpuratus* (D) surface complexes exhibit little if any binding of heterologous sperm. (A, B, C, and D) Bars, 25 μm, × 800. (inset) Bar, 10 μm, × 1,350.
Figure 4 (A) S. purpuratus sperm are seen attached to the S. purpuratus complex in close apposition with the vitelline layer (vl) and microvilli. (B) Unreacted S. purpuratus sperm are occasionally seen apposed to the cytoplasmic face of the plasma membrane (plm); the acrosomal vesicle (av) is intact, indicating the absence of components for induction of the acrosomal reaction on the inner surface of the egg plasma membrane. (A and B) Bars, 0.5 μm, × 35,000.
Figure 5. (A) Phase micrograph of *A. punctulata* surface complexes that have undergone a cortical reaction in ASW, leading to formation of the fertilization envelope (fe), which is slightly detached. Bar, 25 μm, ×1,250. (B) As seen in thin sections of these surface complexes reacted in ASW for 10 min, limiting envelopes have formed. These envelopes (inset) are ~500 Å across, and are composed of two 150-Å thick particulate layers separated by a less dense space of 200 Å. Hyaline (hy), subjacent to the fertilization envelope (fe), is interspersed with vesiculated membranes (vm). Bar, 0.2 μm, ×10,000. (inset) Bar, 0.1 μm, ×72,000.
Figure 6  S. purpuratus surface complexes reacted for 5 min in ASW also exhibit spatially ordered fertilization envelopes (fe) and hyaline layers (hy). Bar, 2.0 μm, × 10,000.
(A) Negatively stained fertilization envelope isolated from *A. punctulata* surface complexes which were reacted in ASW for 30 min exhibits elongated structures resembling tubules which appear to be composed of particulate subunits which are 160 Å across. The tubular structures seem to interdigitate (arrows) across a 10-Å space which was penetrated by the electron-dense stain. (B) An envelope preparation isolated from *A. punctulata* eggs (30 min after fertilization) exhibits structural organization similar to that produced by the isolated surface complex shown in *A*. (*A* and *B*) Bars, 0.1 μm, × 135,000.
is coated on its exterior surface by the vitelline layer. It is clear from the results of the current investigation that this integrated cell surface complex, apparently independent of other intracellular components, can effect a number of the initial cell surface responses involved in fertilization.

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