THE VITELLINE LAYER OF THE SEA URCHIN EGG AND ITS MODIFICATION DURING FERTILIZATION

A Freeze-Fracture Study Using Quick-Freezing and Deep-Etching

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ABSTRACT

Eggs of the sea urchin Strongylocentrotus purpuratus were quick-frozen, freeze-fractured, and deep-etched to reveal the detailed structure of the vitelline layer (VL), an extracellular coat. The VL consisted of a network of fibers lying in a sheet raised 20 nm off the plasma membrane and connected to it by a series of short processes. Sperm attached to the fibers of this sheet and upon fertilization the VL rose off the egg surface to form the fertilization envelope (FE). By 1 min postinsemination (p.i.), the FE had become augmented by a new set of smaller fibrils, and the original fibers of the VL appeared to be undergoing degradation. The FE exhibited casts of microvilli the VL had once covered. These were rounded at 1 min p.i., but by 2 min they had become angular and coated with an orderly array of repeating macromolecular units. In areas between casts, the coating process was slower; incomplete rows of units were seen at 5 min p.i. and complete rows at 10 min. Deep-etching of FE isolated from eggs by homogenization and differential centrifugation showed that both top and bottom surfaces were coated. The coat pattern was made up of 17.5-nm wide rows of parallelogram-like units that repeated every 12.2 nm along the row axis. Units in adjacent rows were in register to produce a secondary axis 76° from the row axis. The results of this and previous studies suggest that the coating process plays a major role in “hardening” the FE to produce a tough barrier that protects the early embryo from chemical and mechanical injury.

KEY WORDS vitelline layer . fertilization envelope . cortical reaction . sea urchin eggs . fertilization . extracellular coats

The vitelline layer (VL) of the sea urchin egg is a thin, extracellular coat that plays several important roles in fertilization. Firstly, it is the structure to which sperm bind (15, 27, 29, 31, 32). Sperm binding is species specific (2, 28, 32), and is thought to be mediated by attachment of the sperm protein “bindin” (2, 32) to a glycoprotein component of the VL (16). Secondly, after fusion of one sperm with the egg, the VL becomes detached from the egg surface and the intervening perivitelline space (PS) is filled with secretory material that has been discharged from cortical granules (1, 11, 14). This secretory material includes a peroxidase that...
FIGURE 1 An electron micrograph of the cortex of a sea urchin egg. The long arrow points to the vitelline layer (VL), an extracellular coat lying just outside the plasma membrane. It is attached to the egg surface by a series of short processes, the vitelline posts (small arrows). The specimen was quick-frozen and freeze-substituted. × 95,000; bar, 0.1 μm.

FIGURE 2 An electron micrograph of an oblique section through the egg cortex. The VL appears as a network of fibers on the egg surface. In one case (arrow) the section followed this network as it climbed up the side of a microvillus. The specimen was quick-frozen and freeze-substituted. × 95,000; bar, 0.1 μm.

cross-links peptides in the VL (14, 17) and structural material that coats its surfaces (3, 19, 20). Within 5 min these modifications result in the formation of a tough fertilization envelope (FE) that is resistant to mechanical and chemical disruption (5, 26, 35), and serves to protect the embryo until the blastula stage. Recently, Kidd (21) used ruthenium red staining to show that the vitelline layer of unfertilized eggs consists of a sheet of fibers covering the plasma membrane but raised about 20 nm off its surface. This sheet is connected to the plasma membrane by an array of short processes that he called "vitelline posts." Much less is known about the struc-
FIGURE 3 Freeze-fracture replica of the surface of an unfertilized egg. The surface displays a regular array of protruding microvilli and is covered with a dense network of fibers, the VL. In the center of the figure the fracture plane dropped low enough to expose the P face of the plasma membrane and to cross-fracture five microvilli. The specimen was fixed, passed through distilled water, and quick-frozen, then fractured, etched deeply, and rotary shadowed with platinum-carbon. This figure and all subsequent freeze-fracture plates have been photographically reversed, and platinum deposits appear white. × 45,000; bar, 0.2 μm.

In this study we use the rapid-freezing technique developed by Heuser et al. (18) to freeze sea urchin eggs without the use of cryoprotectants. This leaves them amenable to extremely deep etching after freeze-fracture. Rotary deposition of platinum produces replicas that reveal in remarkable detail the three-dimensional structure of the vitelline layer before and during its modification.

MATERIALS AND METHODS
Pacific sea urchins, Strongylocentrotus purpuratus, were obtained commercially (Pacific Bio-Marine Laboratories, Inc., Venice, Calif.) and kept at 7°C in artificial seawater (“Instant Ocean,” Aquarium Systems, Eastlake, Ohio). Shedding of gametes was induced by removing the buccal cavity and introducing 1–3 ml of 0.5 M KCl into the coelom. Eggs were shed into seawater, cleaned of jelly by passing them through a 150-μm mesh nylon
FIGURE 4 Freeze-fracture replica of the unfertilized egg surface showing the VL and short processes that attach it to the plasma membrane. In the foreground, just above the two cross-fractured microvilli, is a faint boundary (arrow) where the fracture plane abruptly left the interior of the plasma membrane. All structures above this line are on the cell exterior and would have been obscured by ice if the sample had not been deeply etched. The specimen was prepared as described in Fig. 3. x 125,000; bar, 0.1 μm.

cloth, and stirred gently in fresh seawater at 10°C until ready for use. Sperm was collected "dry" and kept at 5°C. At 10 min before fertilization, sperm was diluted 1:100 with seawater, and then both eggs and sperm were brought to room temperature for all experiments.

Fertilization was carried out by mixing 1 ml of a 1% sperm suspension with 6 ml of an egg suspension that would pack down to about 0.2 ml of eggs on centrifugation. Then, at the appropriate times, the fertilized eggs were fixed by rapid addition of 7 ml of 3.7% glutaraldehyde in diluted seawater (80% of normal tonicity). Aldehyde fixation proceeded for 1 h at room temperature, after which the eggs were washed in seawater and processed either for embedding or for freeze-fracture.

Eggs to be embedded were postfixed in 1% osmium tetroxide in diluted seawater (75%) for 1.5 h at room temperature, washed in seawater, and block stained with 1% uranyl acetate in 50 mM sodium acetate buffer, pH 5.0, for 2 h in the dark at room temperature. They were dehydrated with graded ethanol and embedded in Araldite. Silver sections were cut on a Porter-Blum MT-2B ultramicrotome (Du Pont Co., Sorvall Biomedical Div., Wilmington, Del.), stained with uranyl acetate and lead citrate as we described in a previous study (9), and viewed at 60 kV with a JEM 100 B microscope.

Eggs to be freeze-fractured were washed in 50% seawater, then in four changes of distilled water. Pellets of the washed eggs were placed either on a cover glass coated with protamine sulfate as described in reference 9, or on a small piece of filter paper and the sample quick-frozen using the machine developed by Heuser et al. (18). Briefly described, the sample was placed at the tip of a plunger that dropped by gravity to press the tissue against a copper block cooled to 4°K with liquid helium. Freezing was so rapid that the tissue ultrastructure remained undamaged even in the absence of cryoprotectants. Samples were fractured in a Balzers 301 unit (Balzers Corp., Hudson, N. H.) at a pressure of 2 x 10⁻⁵ torr and allowed to etch for 3 min with the cold microtome covering the sample. Replication was made by platinum-carbon from an electron beam gun positioned at 25°, followed by carbon applied from overhead. The sample was rotated during replication, resulting in multidirectional application of both platinum and carbon. Replicas were cleaned in sodium hypochlorite and viewed at 80 kV.

Some samples, where noted, were quick-frozen without prior fixation and either freeze-etched as described above or freeze-substituted. The substitution procedure consisted of placing the frozen sample on a frozen mixture of 4% osmium tetroxide in acetone, and storing it at −90°C for 2-5 d, then at −20°C for 4 h. The samples were washed in cold acetone twice, in ethanol twice, block stained with 1% uranyl acetate in ethanol for 2 h in the dark at room temperature, and, after passage through propylene oxide, embedded in Araldite.
RESULTS

The VL of quick-frozen sea urchin eggs was seen in thin sections as a darkly stained sheet (large arrow, Fig. 1) lying 20–30 nm outside the plasma membrane. The sheet was connected to the external surface of the egg by an array of short supports (small arrows, Fig. 1) called vitelline posts (21). In sections cut obliquely to the egg surface (Fig. 2), the vitelline sheet appeared as a dense network of fibers that coated the cell surface including the microvilli.

The structure of this extracellular coat was brought out in greatest detail in replicas of eggs that had been freeze-fractured, deep-etched, and then shadowed with platinum-carbon while being rotated. The egg surface when viewed from above (Fig. 3) displayed a regular array of short microvilli, some of which had been broken off during fracturing. This surface was covered with a dense meshwork of fibers which at higher magnification (Fig. 4) appeared to be nearly a single sheet. Within the plane of this sheet, short fibers were connected in an angular but irregular pattern. The sheet contacted the external surface of the plasma membrane via a series of short “feet,” undoubtedly equivalent to the vitelline posts seen in thin sections (Fig. 1 and reference 21). These features of the vitelline sheet were identical, whether the eggs had been dejellied mechanically or by treatment in pH 4 seawater, suggesting that residual jelly contributed little to the structure we saw.

Identification of this fiber network as the VL was based not only on structural similarity but also on the fact that dithiothreitol (DTT), a reagent known to remove most of the VL of unfertilized eggs (13), also removed this fiber meshwork. As seen in Fig. 5, DTT treatment left the external surface of the egg free for observation; it displayed bare microvilli, somewhat irregular in shape, and was covered with numerous small bumps that could have been anchoring sites for the VL. Further identification was provided by the observation that VL, isolated from unfertilized eggs by the method of Glabe and Vacquier (15), consisted of a similar sheet of fibers including casts of the microvilli it had once covered (data not shown).

After insemination, numerous sperm were bound to the VL of each egg. As shown in Fig. 6, this involved direct contact between the acrosomal process of the sperm and the fibers of the vitelline sheet (see inset). One of these sperm fuses with the egg plasma membrane, thereby initiating fertilization and a wave of cortical granule exocytosis (1, 11). At the front of this secretory wave, the VL was seen to rise off the egg surface, bulging first at...
sites where individual cortical granules had fused with the plasma membrane (arrow, Fig. 7). In deep-etched eggs (Fig. 8), these exocytic depressions were filled with fibrous secretory material, probably hyaline protein, and were capped by the recently elevated VL (arrows, Fig. 8). Simultaneously, the VL detached from the surrounding plasma membrane (e.g., at the far right in Figs. 7 and 8). At high magnification, one could see that the vitelline posts which once held the VL to the plasma membrane were absent in areas where it had lifted (Fig. 9).

Within 1 min, cortical granule release was complete and the VL had become elevated about 10 μm from the egg surface (Fig. 10). The intervening PS was filled with fibrous material, probably hyaline protein, and numerous branched microvilli were present on the egg surface. The VL itself had undergone radical structural changes to become the FE. In thin sections, the newly elevated FE appeared much thicker than the original VL (Fig. 11) and within a few minutes it had become thinly coated with a darkly staining material on both the inside and the outside (Fig. 12). These coats appeared to be material added to the thickened FE, because in certain places they were incomplete (arrow, Fig. 12).

Deep-etching revealed the nature of these coats. In the first 30 s p.i., the elevated FE retained nearly the same appearance as the VL in unfertilized eggs. However, by 1 min, this network of large fibers had become incomplete (arrows, Fig. 13) and appeared to be undergoing degradation. Underneath this original structure there was a new network of much smaller fibrils, lace-like in appearance and of much finer mesh. This may be the material responsible for the thickened appearance of the FE in thin sections (Fig. 12).

By 2 min p.i., all vestiges of the former VL fibers had disappeared and the microvillar "casts" had become angular in appearance and coated with extremely well ordered rows of macromolecules (Fig. 14). These rows were usually parallel to one ridge of the cast and comprised units that appeared to aggregate linearly (Fig. 15). At high magnification, small groups of three to five units (circles, Fig. 15) were seen adhering to the FE surface between casts, although there were not enough of these to form rows. Many of these isolated groups appeared to be roughly parallel to
the adjacent rows on the casts, which suggests that
the underlying network of fibrils might somehow
determine their orientation.

At 5 min p.i., most of the FE surface between
casts had also become coated (Fig. 16). Even be-
fore completion, this coating was arranged into
parallel rows, this parallelism being maintained
over domains ranging from 0.2 to as large as 10
μm². In Fig. 16, the boundary between two do-
 mains is marked by a dashed line. After comple-
tion of the coating (Fig. 17), the rows between
casts consisted of long, linear aggregates of units
similar to those first seen at the casts. Interruptions

in these rows appeared to be a result of splits in
the coating, which may have occurred during fix-
atation or some later step in tissue processing.

To confirm the nature of this coat pattern in
unfixed FE and to visualize the underside of the
FE (i.e., the side facing the PS) we homogenized
eggs in distilled water 10 min after fertilization
and isolated their FE by differential centrifugation
using the method described by Decker et al. (10).
Deep-etching of these isolated FE quick-frozen
without fixation revealed an even more orderly
pattern (Fig. 18). This confirmed that the regular
pattern described above was not a fixation artifact.
FIGURE 9  Elevation of the VL from nearby microvilli. Vitelline posts, the short processes that once attached the VL to the cell, are missing. The specimen was prepared as in Fig. 3. \( \times 65,000 \); bar, 0.2 \( \mu \)m.

FIGURE 10  “Aerial” view of the egg 2 min p.i. The fertilization envelope (FE) is elevated about 10 \( \mu \)m from the surface of the egg (E), and the intervening perivitelline space (PS) is filled with secretory material. Numerous, highly branched microvilli have appeared on the egg surface. These are white as a result of electrons being deflected off platinum on both their front and back sides. The specimen was prepared as in Fig. 3. \( \times 8,000 \); bar, 2 \( \mu \)m.

In fact, in unfixed FE the rows were more closely packed and consecutive rows were in better register.

A similar pattern was seen on the underside of isolated FE (Fig. 19) except that the coating appeared to be more rigid and plaquelike. Fibrous tangles that still adhered to the undersides of these isolated FE (Fig. 19) were probably remnants of the hyaline protein that filled the PS.

At higher magnification, the pattern and shape of platinum deposits on the repeating coat units could be seen clearly (Fig. 20). This pattern was
analyzed by Dr. Michael Klymkowsky using image-reconstruction techniques described by Ross et al. (25). Briefly described, well-ordered areas on electron micrograph negatives ($\times$ 90,000 or 180,000) were analyzed with a densitometer and the data were Fourier transformed by an on-line computer. This transform was masked, leaving only observed “reflections,” then back-transformed into real space to give a reconstructed image of the coat pattern. An example of one of these images (Fig. 21) suggests that the parallelogram-shaped unit is bilobed, with one lobe apparently receiving more platinum than the other. Phase analysis of the reflections confirmed that the unit cell did not have a twofold axis of symmetry. The two axes that form the subunit “cells” subtend an angle of $76^\circ \pm 0.8^\circ$ (mean $\pm$ SE, $n = 5$) with the pattern repeating every $17.5$ nm $\pm 1.4$ nm from one axis (dimension A, Fig. 21) and every $12.2$ nm $\pm 1.0$ nm from the other axis (dimension B, Fig. 21).

DISCUSSION

Elevation of the VL and its subsequent modification to become the FE has received considerable attention from cell biologists and biochemists. This study describes a new way to view this structural transformation.

Elevation of the VL is initiated by a protease, vitelline delaminase, which is released from cortical granules and promptly cleaves the VL at the sites attaching it to the plasma membrane (7, 8). Our new viewing technique reveals these attachment sites quite clearly, and shows that they are missing in areas where the VL has elevated (Fig. 9). After the VL elevates, a second protease removes the glycoproteins in it that are responsible for sperm binding (7, 34). This results in detachment of supernumerary sperm (33) and of concanavalin A binding sites as well (36). Our new view shows that by 1 min p.i. the dense mass of relatively thick fibers that composed the original VL appears to be coarsened and disorganized (Fig. 13), and by 2 min its original fibers appear to have been completely replaced (Fig. 14). Unfortunately, it is not possible to decide from these surface views whether the fibers that formed the original VL are actually gone or whether they are transformed or simply hidden by the denser network of more delicate filaments that appears at this time. Radiolabeling studies indicate that in spite of the proteolysis that takes place, much of the original VL...
FIGURE 13 Freeze-fracture replica of the FE surface as seen from above, 1 min p.i. Three microvillar casts protrude straight toward the observer so only their tops can be seen; two of these have been cross-fractured. Coarse fibers of the original VL appear white (arrows), whereas a new network of lace-like fibrils appears grey. The specimen was prepared as in Fig. 3. x 55,000; bar, 0.2 μm.

FIGURE 14 The FE surface 2 min p.i. The casts are angular and coated with an ordered sheet of macromolecules. The specimen was prepared as in Fig. 3. x 55,000; bar, 0.2 μm.
is retained in the mature FE. Over 40% of the surface proteins that can be labeled with $^{125}$I by the lactoperoxidase technique remain in the FE (6, 30).

Scanning electron microscopy has shown that soon after the VL is elevated, the casts that it formed over microvilli change in shape from cylindrical to angular (35). The current viewing technique has sufficient resolution to show that this shape change results from the formation of highly ordered plaques of surface structures on the slopes of the microvillar casts. Such plaques eventually cover the entire inside and outside surface of the FE. Because they appear first on the microvillar casts, we considered the possibility that the casts act as "initiation centers" for further crystallization of the plaques. If this were so, the plaques ought to extend out from the bases of the casts and gradually spread to cover the intervening areas. In actuality, however, the units composing the plaques appeared to be added to the intervening areas piecemeal, and to increase gradually in concentration and order. At 2 min, they formed small groups of three to five in a row, too low in concentration to touch each other but already appearing to be roughly parallel. By 5 min, they formed long rows much more closely packed and in register, but often incomplete. By 10 min, broad plaques as perfectly ordered as the ones seen initially on the slopes of the microvillar casts had finally filled in all the intervening areas. Thus, it would appear (a) that the surface structures have a strong tendency to associate with each other to form long rows, and (b) that the underlying fibrous mat plays a role in orienting these rows relative to each other.

The elaboration of these surface structures coincides in time with the hardening of the FE, in which the envelope becomes extremely resistant to
chemical and mechanical damage. The newly elevated FE is soluble in mercaptan solutions but within 3–5 min becomes insoluble (24, 26); over the same time span it becomes resistant to breakage during filtration (22) and to deformation as a result of osmotic pressure differences (12).

Evidence has existed for years to suggest that hardening results from the addition of new material to the VL. Endo (12) observed that hardening occurs first over the point of sperm fusion and gradually spreads around the egg. He noted that this pattern mimics the wave of cortical granule discharge that results from sperm fusion, and suggested that hardening of the VL could be mediated by material released from the granules. Bryan (3) has shown that material released from the granules does indeed coat the FE. In addition, there has been good evidence that the added material must polymerize to complete hardening. Bryan (4) has shown that the added material does tend to self-aggregate, so long as calcium is present. When calcium is removed from the seawater around eggs, hardening is prevented (4, 23) and the FE remains very fragile (26). This tendency to self-aggregate also depends on the formation of disulfide bonds. Reducing agents such as cysteine block it and prevent the hardening process as well (20, 35). Now it should be possible to use the viewing technique described here to determine exactly how these treatments alter the polymerization that normally occurs during hardening.

The hardening process is thought also to involve the formation of covalent linkages, because the final envelope is stable in mercaptan solutions and in high concentrations of urea and salts (29). A likely candidate for this role is the ovoperoxidase released from cortical granules. It is known to remain associated with the FE throughout hardening, and to catalyze the formation of dityrosine linkages within the envelope (14, 17). Chemicals that inhibit it also inhibit hardening (14). Our next step will be to examine eggs treated with these chemicals to see what effect they have on the orderly polymerization we have observed. Our working hypothesis is that noncovalent linkages are important in the initial association of the sur-
**Figure 17** The FE surface 10 min p.i. The coating between casts is now complete. The specimen was prepared as in Fig. 3. × 55,000; bar, 0.2 µm.

**Figure 18** The external surface of an isolated FE. FE were isolated from eggs 10 min p.i. (10), stored in distilled water at 4°C for 24 h, and then quick-frozen. The coating units are packed in a tight array. × 85,000; bar, 0.2 µm.
FIGURE 19 The interior surface of an isolated FE. Plates of coating units incompletely cover indentations at the microvillar casts (arrow). A fibrous network, probably residual hyaline protein from the PS, contacts this surface. The specimen was prepared as described in Fig. 18. × 85,000; bar, 0.2 μm.

FIGURE 20 The array of coating units on the interior surface of an isolated FE. The specimen was prepared as described in Fig. 18. × 430,000; bar, 20 nm.

FIGURE 21 A reconstructed image of the coat pattern from computer-assisted densitometry of electron micrograph negatives of isolated, unfixed FE. The parallelogram-shaped unit cell is formed by two axes subtending an angle of 76°. The pattern repeats every 17.5 nm from one axis (dimension A) and every 12.2 nm from the other axis (dimension B).
face molecules into extended rows, and that the final tight cross-linking of these rows and their attachment to the underlying fibrous mass require covalent modifications.

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