Ultrastructure of Detergent-Resistant Cytoskeletons in the Noncortical Domain of Sea Urchin Eggs as Revealed by the Quick-Freeze Deep-Etch Technique

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ABSTRACT. The ultrastructure of detergent-resistant cytoskeletons in the noncortical cytoplasm of sea urchin eggs was studied by quick-freeze, deep-etch electron microscopy. Two different cytoskeletal organizations were identified in the detergent-treated sea urchin eggs. They were distinguished by the presence or the absence of long actin filaments and probably correspond to the cortex and the noncortical cytoplasm, respectively. The non-cortical cytoplasm was composed of a complex network (designated here as the ground network) of filaments 6 to 13 nm in diameter, that interconnected aggregates of small globular materials, yolk granules and a meshwork of uniform filaments (8-9 nm in diameter). The 6 to 13 nm filaments comprising the ground network were branched and associated with filaments of the same or other sizes, resulting in the formation of an extremely complex network. The meshwork of 8-9 nm filaments was homogeneous in composition and constitutes a novel structure which has not been previously described. The 8-9 nm filaments were connected to one another at their ends, forming a meshwork of polygons. Meshworks, ranging up to 3 µm in diameter, were distributed throughout the non-cortical cytoplasm of the egg. Similar cytoplasmic structures were also observed in fertilized eggs.

The eggs of many species of sea urchins are large, round cells with a diameter of around 100 µm. The presence of a cytoplasmic structure supporting the round form has been suggested, but such an entity has not been documented. Many experiments have been conducted to elucidate properties of the egg cytoplasm, particularly in relation to early developmental phenomena (for review, see 10, 16). Iron and gold particles injected into the egg cytoplasm follow a zigzag course when a magnetic field or centrifugation force is applied, suggesting that the cytoplasm has an elastic and heterogeneous nature (14, 15, 26). When the force or magnetic field is terminated, the particles are seen to recoil for some distance, indicating that the cytoplasm has mechanoelastic properties.

The morphology of the cell is largely defined by the composition of its cytoskeleton which, in turn, is usually composed of three major filamentous systems: microtubules, intermediate and actin filaments (for review, see 33). However, these cytoskeletal structures are unlikely to be involved in the elastic and heterogeneous properties of the unfertilized egg cytoplasm. Microtubules are scarce in unfertilized eggs. Tubulin dimers polymerize into microtubules from the microtubule organizing center around the centriole only after fertilization (1). Although we cannot rule out the possibility that an unidentified class of proteins related to intermediate filaments may be present in echinodermal eggs, such filaments have not been detected morphologically, biochemically or immunologically in unfertilized mature sea urchin eggs. With respect to actin polymers, short filaments are reportedly present in the cortex (11, 35), though most of the actin is in an unpolymerized form (28). Although cortical actin filaments could participate in establishing the mechanical properties of the egg surface, it is unlikely that they contribute to the mechano-elastic properties in the noncortical area of the egg.

When sea urchin eggs are treated mildly with detergent in the presence of hexyleneglycol to remove the cell membrane, the eggs keep their spherical form, suggesting the presence of some structure which holds the cytoplasm together. Work on the detergent-resistant cytoskeleton, the cytomatrix (4, 5, 6, 31), has suggested that
there are, at least, two types of filaments present, 2-4 nm and 7-8 nm in diameter (9). The 2-4 nm filaments, whose properties are unknown, were assumed to be organized into a meshwork. The 7-8 nm filaments were identified as actin filaments, based on heavy meromyosin decoration. However, the resolution of traditional thin-section electron microscopy prevented a more detailed structural analysis of the cytomatrix.

Quick-freeze, deep-etch (QF-DE) electron microscopy is an excellent technique for observing the ultrastructure of cytoskeletons (12, 13). The technique has provided a wide range of information and has led to new ideas about the cytoskeletal architecture in many kinds of cells. For instance, microtubules in neurons have been shown to be extensively crosslinked by microtubule-associated proteins (17, 21). This technique has also been applied to sea urchin eggs. Microtubules of the mitotic apparatus were found to be associated with a hexagonally packed, button-shaped protein named buttonin (22, 23). Microvillar-associated actin in the unfertilized egg was shown to be composed of very short filaments arranged in a tight network and forming mounds that extend beyond the plane of the plasma membrane (3, 11, 32). However, these reports focused mainly on well-known cytoskeletal systems, leaving noncortical cytoplasmic structures to be studied in similar detail.

In this paper, we report on the ultrastructure of the detergent-resistant cytoskeletons in the noncortical cytoplasm of sea urchin eggs as revealed by QF-DE electron microscopy. Cytoskeletons in noncortical cytoplasm were composed of complex networks of various sized filaments (6-13 nm diameter) interconnecting yolk granules, aggregates of small granules and masses of 8-9 nm filament meshworks. Meshworks, novel structures composed of homogeneous filaments (8-9 nm in diameter), were distributed throughout the cytoplasm. The nature and functions of these two cytoplasmic filamentous systems are discussed.

**MATERIALS AND METHODS**

**Preparation of the detergent-resistant cytoskeletons.** Detergent-resistant cytoskeletons were prepared as previously described (9). Eggs were obtained by injection of 0.5 M KCl into the body cavity of *Pseudocentrotus depressus*, *Hemicentrotus depressus* and *Tripneustes gratilla*. After washing with artificial sea water, the eggs were sedimented. Packed eggs were suspended in 50 vol of the lysis medium (1% Nonidet P-40, 10% dimethylsulfoxide (DMSO), 1 M glycerol, 10 mM Piperazine-N,N'-bis(2-ethanesulfonic acid) (Pipes), pH 6.6, 1 mM ethylene glycol-bis(2-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM MgCl₂ containing protease inhibitors (2.5 μg/ml leupeptin, 2.5 μg/ml antipain, 2.5 μg/ml pepstatin, 2.5 μg/ml chymostatin, 50 μg/ml kunitz trypsin inhibitor and 2 mM bannamidine) at 4°C). Extraction was performed by gently stirring the eggs.

Detergent-resistant cytoskeletons were also prepared from fertilized eggs. After insemination, the egg suspension was diluted with 9 vol of 1 M urea and passed through a nylon mesh (70 μm) twice. The eggs were then washed in Ca²⁺-free artificial sea water, and allowed to develop for 20 min or until the majority of the population had reached the metaphase of first mitosis. The eggs were then collected and lysed as described above.

**Morphological techniques.** Eggs extracted with detergent were fixed in 2% paraformaldehyde, 1% glutaraldehyde in PEM (0.1 M Pipes, pH 6.6, 1 mM EGTA, 1 mM MgCl₂) at 4°C overnight. After washing with distilled water, photomicrographs were taken of extracted specimens with differential interferential contrast optics.

Fixed cytoskeletal preparations were processed for thin-section electron microscopy as described previously (7). Specimens were postfixed with 1% OsO₄, dehydrated with ethanol and embedded in Rigolac (27). Sections were cut on a Sorvall MT6000 ultramicrotome, expanded with chloroform vapor, stained with uranylacetate and lead citrate, and observed with an H-7000 electron microscope (Hitachi, Tokyo, Japan).

Quick-freeze, deep-etch (QF-DE) was performed as described previously (17, 24) according to Heuser and Salpeter (12). Detergent-resistant cytoskeletons were washed once with PEM and centrifuged at 12000 rpm for 5 min. The pellets were collected and quick-frozen by slamming against a copper block cooled to liquid helium temperature. Fixed preparations were also quick-frozen after washing with distilled water. Frozen specimens were then freeze-fractured in a Balzers BAF 301, etched at -95°C for 7 min, and rotary-shadowed with platinum and carbon. Replicas were examined with a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan) operated at 100 kV.

**RESULTS**

**Preparation of the detergent-resistant cytoskeletons.** Sea urchin eggs (*P. depressus*, *H. pulcherrimus* and *T. gratilla*) were treated mildly with the lysis medium as described previously (9). The round shape of the egg was maintained after treatment with Nonidet P-40 containing DMSO and glycerol, suggesting that the cytoskeletal components responsible for the form of the egg retained their three-dimensional organization. Changes in the overall morphology of the egg were not detectable by differential interferential microscopy (Fig. 1). Elongated microvilli were also well preserved in fertilized eggs treated with detergent (Fig. 1b and c).

The general features of Japanese sea urchin detergent-resistant egg cytoskeletons appeared very similar to those of the French sea urchin egg (*Paracentrotus lividus*) when the former was examined by thin-section electron microscopy (Fig. 2). Cytoplasmic organelles were...
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Fig. 1. Differential interferential micrographs of *P. depressus* eggs treated with 1% Nonidet -40. (a) Unfertilized egg treated with detergent. (b) The detergent-treated egg at 20 min after fertilization. (c) The detergent-treated egg at metaphase. Note that microvilli are well preserved. Bar represents 25 μm.

sparsely distributed in the detergent-treated preparations. Yolk granules (white arrow in Fig. 2a), ribosome-like particles (arrowhead in Fig. 2b) and aggregates of small electron dense materials (arrows in Fig. 2a and b) were observed to be the major components of the preparations. Three types of filamentous structures were recognized. Many short filaments were observed to emanate from the aggregation of electron dense material (Fig. 2b). Meshworks of fine filaments (long arrow in Fig. 2b), which might correspond to the 2-4 nm filaments described by Foucault *et al.* (9), were observed to be distributed sporadically throughout the noncortical cytoplasm. In cortical areas, short thin filaments, possibly corresponding to actin filaments, were also observed (data not shown).

*Quick-freeze deep-etch observations of the noncortical cytoplasmic cytoskeletons in unfertilized eggs.* QF-DE examinations were performed with both unfixed and fixed, detergent-resistant cytoskeletons obtained from *P. depressus* and with fixed preparations from *T. gratilla*. In each case the results were essentially similar. It should be noted that the regions observed were cyto-

Fig. 2. Thin-section electron micrographs of detergent-resistant cytoskeletons prepared from unfertilized eggs. (a) Low-magnification view of the detergent-resistant cytoskeletons of *P. depressus* eggs. The vertical bar indicates a 5 μm depth of the egg cortex. A yolk granule and an aggregation of small electron dense particles are indicated by white and black arrows, respectively. (b) High-magnification view of the detergent-resistant cytoskeletons prepared from *H. pulcherrimus* eggs. Ribosome-like particles (arrowhead) and filamentous meshwork (long arrow), aggregates of small electron dense material (arrow) are indicated. Fine filaments emanated from the aggregates of the small electron dense material. Bar represents 200 nm.

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plasmic areas at a depth of less than 10 μm from the egg surface. This area corresponds to that which was frozen without forming large ice crystals. A depth of 5 μm from the egg surface is indicated by the vertical bar in Fig. 2a.

Detergent-extracted unfertilized eggs displayed two major domains, distinguished by the presence or the absence of long actin filaments. The region where abundant actin filaments were located may correspond to the cortex while the other may represent the subcortical cytoplasm. In the present investigation, we focused our observations on the noncortical cytoplasm. Fig. 3 shows a typical low-magnification view of the subcortical cytoplasm where long actin filaments were not observed. In contrast to the images obtained by the thin-section technique in which relatively little material was observed, substantial cytoplasm was observed in replicas. Yolk granules (white arrows) and aggregates of small granular material (arrows) were frequently present. Meshworks of fine filament were distributed throughout the noncortical cytoplasm (long arrows). Filaments of different sizes and coated vesicles (arrowheads) were recognized in these areas as well.

Higher magnification views of the subcortical cytoplasm are shown in Fig. 4. Although abundant granular components made it difficult to reveal the overall aspects of the filamentous networks, many filaments were observed linking them together. Many fine filaments emanated from the aggregations of small granules (Fig. 4a). These filaments might correspond to those associated with the aggregates of electron dense material observed in thin sections (Fig. 2b). Association of these filaments with yolk granules is shown in Fig. 4b. A region containing relatively little granular material is shown in Fig. 4c. The filamentous network (referred to here as the ground network) was composed of various sizes of fine filaments. Some of the finest filaments, indicated by arrows in Fig. 4c, were about 6 nm
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Fig. 4. High-magnification views of the noncortical cytoplasmic cytoskeletons. (a) Aggregates of small granules and fine filaments. The latter extend from the aggregations into the surrounding cytoplasm. (b) Contacts of cytoplasmic filaments with a yolk granule. (c) Region of noncortical cytoplasmic cytoskeleton where granules were relatively sparse, consisting of a network of fine filaments (ground network). The various filaments depicted within the ground network were associated with each other, resulting in the formation of a complex network. Arrows indicate the finest filaments (~6 nm in diameter) that are present within the ground network. (d) A typical meshwork that is present in the noncortical cytoplasm. Granular materials are absent from the meshwork. Filaments of the meshwork extended into the surrounding cytoplasm, possibly to interconnect with the ground network. Fig. (a), (b) and (d) are from fixed, unfertilized eggs of *T. gratilla*; Fig. (c) is from an unfixed, unfertilized egg of *P. depressus*. Bar represents 200 nm.

Filaments showed branching, anastomosis and associations with other filaments of the same or different sizes.

A meshwork is shown in Fig. 4d. The overall size of these structures was variable, ranging up to 3 μm. Although granular materials were excluded from the area
of the meshwork, they showed connections to surrounding materials via fine filaments. A stereo pair depicting a meshwork is shown in Fig. 5. In contrast, the meshwork were composed of filaments with smooth surfaces whose diameters were rather constant at 8–9 nm. Their length ranged from 14 to 76 nm. Because both ends of the filaments formed junctions with one another, meshworks appeared as an assemblage of polygons.

Comparison of filaments observed in the noncortical cytoplasm with cortical actin filaments. Because the biochemical properties of the filaments comprising the meshwork and the ground network are unknown at present, it was of interest to compare their ultrastructure with that of actin filaments. Higher magnification views of these filamentous systems are shown in Fig. 6. The diameter of actin filaments is 7–8 nm when observed in thin-section electron micrographs. In Fig. 6d, the cortical, actin filaments appear to be about 11 nm when coated with platinum. Meshworks were composed of a homogeneous assemblage of 8–9 nm filaments (Fig. 6b). While cortical actin filaments were long and unbranching, meshwork filaments had a mean length of 33 ± 12 nm (n = 77) with both ends connected to other filaments, resulting in the formation of an assembly of polygons. At first glance the filaments seemed to be connected at regular intervals. Thus far, we have not elucidated factors which would contribute to this uniformity of structure, or how the meshwork is formed.

The ground network consisted of several kinds of filaments, each of which appeared to be thinner and shorter than filaments in the meshwork. The finest filaments were about 6 nm in diameter. The ends of these filaments were associated with other filaments or granular substances. These interactions appeared to be quite random, and the associations with the granular materials made the network considerably complex.

Filaments having a braid like appearance were sometimes observed in the noncortical cytoplasmic domain (Fig. 6c). They seemed to be composed of several subfilaments woven about one another. Their overall appearance and thickness of about 23 nm did not correspond to either that of actin (11 nm) or of microtubules (30 nm). Although the diameter of these filaments is somewhat larger than that (15–18 nm) of the neurofilaments (24), they might correspond to cytokeratin-like filaments which reportedly are present in sea urchin oocytes and disappear during maturation (2).

Detergent-resistant cytoskeletons of fertilized eggs. Fertilized eggs were treated with Nonidet P-40 in DMSO-glycerol solution in a manner similar to that used for the unfertilized eggs. Eggs were treated either 20 min after fertilization or at metaphase to observe the cytoskeletal appearance in interphase and mitotic eggs, respectively. Generally, images of the noncortical cytoplasm of fertilized egg were similar to those of the unfertilized eggs. Meshworks of 8–9 nm filaments were distributed sporadically through the cytoplasm, and there was extensive crosslinking with aggregates of granular material surrounding the meshworks (data not shown).

Fig. 5. Stereo pair of a QF-DE preparation depicting the meshwork. Detergent-resistant cytoskeletons of unfertilized P. depressus eggs were quick-frozen without fixation and processed for deep-etch as described in Materials and Methods. Meshworks were composed of 8–9 nm filaments with smooth surfaces. The filaments are 14 nm to 76 nm in length and appear to be connected with other filaments at both ends, resulting in the formation of polygonal assemblies. Bar represents 100 nm.
DISCUSSION

When detergent-resistant cytoskeletons of sea urchin eggs were investigated by QF-DE electron microscopy, two different regions were distinguished based on either the presence or the absence of long actin filaments. This observation does not necessarily mean that the egg cytoskeletal architecture consists of a mixture of these two cytoskeletal domains. We think that these two regions represent the cytoskeletal features at different depths of the egg cytoplasm, i.e., the distance from the egg surface. The actin-filament-containing regions might correspond to cortical areas of the egg, while the regions containing fewer long actin filaments might represent non-cortical cytoplasm.

Since the organization of actin filaments in the cortex has been extensively studied by many techniques, including QF-DE, we directed our attention toward cytoplasmic structures underlying the cortex. In contrast to the cortical region, the subcortical cytoplasm was mainly composed of two novel filamentous networks: (a) the ground network containing 6 to 13 nm filaments and (b) the meshwork consisting of 8–9 nm filaments. There have been no reports, at least to our knowledge, describing the meshwork of 8–9 nm filaments observed here. We believe that these cytoskeletal structures are not artifacts produced during fixation and subsequent procedures, as the same structures were observed in the unfixed specimens. We used lysis medium containing 10% DMSO and 1 M glycerol to preserve these structures. This medium was originally developed for the large-scale isolation of the mitotic apparatus by Endo et al.
The mitotic apparatus isolated with this medium contained microtubular arrays similar to those observed in vivo by other morphological methods (22, 25). Furthermore, the organization of actin in the cortex was very similar to that observed in cortical preparations isolated without use of DMSO and glycerol (11, 32). These supporting data suggest that the lysis medium yields cytoskeletal preparations representative of the in vivo condition of the egg cytoplasm. Although the detergent employed extracted some components, specifically those associated with membrane organelles, we believe that cytoskeletal filaments observed here are novel and are an integral part of the in vivo structures present in the egg cytoplasm.

The ground network might correspond to the crosslinker system observed in other cells. Some of the reported crosslinkers have been shown to be composed of cytoskeleton-associated proteins such as microtubule-associated proteins, the carboxyterminal tail domains of higher molecular mass subunits of neurofilament proteins and non-erythroid spectrin (19, 20, 21, 24, 34). The filamentous system in the noncortical cytoplasm might differ from these cytoskeleton-associated proteins as the filaments crosslinked aggregates of granular materials, meshesworks, and yolk granules, but not the three major filamentous systems that compose the cytoskeleton of many cells. That the filaments in the ground network possessed different diameters suggests they may be composed of different proteins.

What might be the function of the meshwork? Cuticular plates in chick ear hair cells have been shown to consist of a meshwork of actin polymers and 3 nm actin binding filaments (18). On the other hand, the meshwork in the egg cytoplasm was composed of homogeneous filaments that are thinner than actin filaments. Furthermore the aggregations of 2–4 nm filaments which are observed in thin sections by electron microscopy, and may correspond to the meshwork seen here, are not decorated with S1 (9). Thus, it is unlikely that the meshworks observed in the sea urchin egg noncortical cytoplasm contain actin and actin binding proteins, although we cannot rule out this possibility entirely.

We have recently reported that a protein similar to connectin, an elastic protein in skeletal muscle cells (29), is present in the detergent-resistant cytoskeletal fraction of sea urchin eggs (30). The egg connectin-like protein has a high molecular weight and is immunoreactive with antibodies to muscle connectin. Immunofluorescent microscopy indicated a spotty localization of the connectin-like protein in detergent-treated eggs. The elastic properties of egg cytoplasm have also been shown by experiments in which iron particles recoil for some distance when a magnetic field is interrupted (14, 15). The meshwork reported here may be the structural component that contributes elastic properties to the egg cytoplasm. However, further studies will be required to elucidate the nature and relation of the meshwork and ground network.

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