The Membrane-associated Cytoskeleton in Cultured Lens Cells. Electron Microscopical Visualization in Cleaved Whole-Mount Preparations and Platinum Replicas

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(Received 20 December 1985 and in revised form 21 April 1986)

The membrane-bound cytoskeleton of bovine lens cells was investigated using different electron-microscopic methods. Cells were grown on glass coverslips and fixed in 0.3% glutaraldehyde, post-fixed and prepared for critical-point drying following standardized methods. Cells were broken open before post-fixation or after critical-point drying in order to expose the cortical cytoplasm. Cleaved, critical-point-dried cells were subsequently rotary replicated. Cells were also cleaved on grids before post-fixation. These cells were examined directly in the microscope. All methods showed the presence of a cytoskeletal network. Bundles and starlike foci of microfilaments and microtubules were usually found in close association with the membrane. Occasionally also intermediate filaments were observed, in a few cases running close to the microtubules.

The reliability of the methods to visualize the membrane-bound cytoskeleton of lens cells is discussed.

Key words: lens cell cultures; cytoskeleton; dry cleavage.

1. Introduction

It is commonly accepted that the cytoskeleton, in combination with the plasma membrane, may control interactions between the cell and its environment (Geiger, 1983). Especially the membrane-bound cytoskeleton is apparently involved in processes such as cell movement, the formation of cellular contacts, endocytosis, organization of the membrane etc. (see for example Small, Rinnerthaler and Hinssen, 1982).

The intracellular filamentous matrix of bovine lens cells has been studied extensively using biochemical, immunochemical and electron-microscopical techniques (Ramaekers et al., 1980; Ramaekers, Boomkens and Bloemendal, 1981). Some constituents of this cytoskeleton have partly been identified as actin, vimentin and tubulin, while their localization has been studied. Furthermore, it has become clear that some of these components are attached to the lens plasma membrane, which may have implications for their function (Ramaekers, Dunia, Dodemont Benedetti and Bloemendal, 1982a).

The present study was undertaken in order to obtain more information concerning the membrane-associated cytoskeleton in cultured lens cells at an ultrastructural level.

Different techniques have been introduced that allow the visualization of the membrane-adherent cytoskeleton in its three-dimensional form. Thus, quick-freeze deep-etch techniques, but also methods including chemical fixation and critical-point drying or negative staining, have been extremely useful in providing a detailed view of the organization of the subplasmalemmal cytoplasm (Bridgman and Reese, 1984; Hirokawa and Heuser, 1981; Porter and Anderson, 1982; Small, 1981). Methods

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involving critical-point drying appear to be particularly suited to the study of the membrane-bound cytoskeleton. These techniques usually require cleaving of the cells in order to obtain samples of exposed membrane with adherent structures (Boyles and Bainton, 1979; Mesland, Spieksma and Roos, 1981; Aeggler, Taxemura and Werb, 1983). However, the moment of cleaving varies from just before fixation till after drying. We have tested two different procedures, i.e. those essentially described by Mesland

**Figs 1-3.** Cultured lens cells cleaved before drying, and examined in the microscope without replication. **Fig. 1.** Cell showing great differences in organization of the microfilament network. Microfilament bundles are radiating out from a focus at the edge of the cell. At the right, bundles are absent and only a diffuse network is visible. × 20 000. **Fig. 2.** Detail showing a microtubule (arrows) and an accompanying filament (arrowheads), probably of the intermediate size type. × 80 000. **Fig. 3.** Often microtubules (thin arrows) are seen close to the membrane end running parallel to the large stress fibers (thick arrows). × 38 000.
Figs 4-7. Star-like foci of microfilaments associated with the plasma membrane. Figs 4, 5. Low magnifications showing patterns of foci. Fig. 4, × 4590; Fig. 5, × 9900. Fig. 6. Immunofluorescence preparation showing actin filament bundles. The same patterns as in Figs 4 and 5 can be recognized, suggesting that these star-like patterns are formed by actin filaments. × 720. Fig. 7. Stereomicrographs showing that foci of microfilaments are lying in the plane of the membrane. × 36,000.
et al. (1981) and Aggerl et al. (1983) in order to define the optimal conditions for the visualization of the membrane-adherent cytoskeleton in cultured bovine lens cells. The results show that these techniques—involving chemical fixation and critical-point drying—are particularly suited to study the membrane-bound cytoskeleton in these cells.

2. Materials and Methods

Cell culture

Bovine lens cells from three-month-old calves were brought into tissue culture as described before (Ramaekers, Jap, Mungyver and Bloemendal, 1982b) and grown in modified Eagles Medium and 10% newborn calf serum or fetal calf serum. For immunofluorescence and electron microscopy cells were grown on glass coverslips or in plastic Petri dishes. Cells of passage numbers 10–20 were used in this study.

Indirect immunofluorescence

Actin antibodies used in the indirect immunofluorescence technique have been described before (Ramaekers et al., 1980). Cells grown on coverslips were treated with methanol (5 min, -20 °C) and dipped in acetone. Then they were air-dried and incubated with the actin antibody. The indirect immunofluorescence procedure has been described earlier (Ramaekers et al., 1980).

Cell preparation for electron microscopy

Spread cells were rinsed in 100 mM phosphate buffer (pH 6.9) and subsequently fixed in 0.3% glutaraldehyde in buffer for 1 hr, rinsed in phosphate buffer and treated with tannic acid (1% in phosphate buffer) for 30 min. Then cells were rinsed thoroughly in phosphate buffer before post-fixation in OsO₄ (0.5% in distilled water). They were stained in 1% uranyl acetate in distilled water for 30 min and dehydrated in ethanol before critical-point drying. Carbon dioxide (dried by passage through a molecular sieve) was used as a drying agent.

In order to expose the membrane-adherent cytoskeleton two different procedures were used:

(a) Cleaning before post-fixation. Cells were cleared after treatment with tannic acid and placed poly-l-lysine-coated grids on top of them and pulling these away, essentially as described by Aggerl et al. (1983). After critical-point drying the cell material left on the grids was examined directly in a Philips EM 300 or 201 at 80 or 100 kV. Cell material on coverslips was first rotary shadowed with platinum at an angle of 25° and thereafter with carbon at an angle of 80°.

(b) Dry cleaning. Cells on glass coverslips were dry cleaved as described by Mesland et al. (1981), and Mesland and Spiels (1983) and subsequently rotary shadowed. Since the cells would not spread onto Formvar-carbon-coated grids, we were not able to examine dry-cleaved cells without rotary replication.

3. Results

Methods

All preparations showed large surfaces of intact membrane with adherent cytoplasmic structures.

Figs 8, 9. Replicas of cells cleaned before drying. Cytoskeletal filaments are visible as distinct, interconnected filaments. In Fig. 8 a microfilament bundle can be recognized (fb). Coated pits and vesicles are forming at the membrane (cp). A network of small filaments of unknown identity can be seen. Fig. 8, x36 000; Fig. 9, x72 000.

Fig. 10. Stereo micrograph of a replica of a dry cleaved cell. The appearance of the filaments is obscured by granular and amorphous material. Clusters of coated vesicles can easily be recognized. x 31 500.
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Cleaving before drying. The cells were easily cleaved in water. At least 20% of the cells was cleaved, leaving large surfaces of membrane and adherent structures on the grids or coverslips.

Cleaved cells on grids examined directly in the microscope without rotary replication showed membrane-associated cytoskeletal networks with adhering microtubules, microfilaments and intermediate filaments as judged from their morphology and organization (Figs 1–7). Many of the smaller filaments exhibited an irregular ('microtrabeculae-like') appearance (e.g. Fig. 7).

Replicas of cells cleaved before drying likewise showed membrane adherent filamentous networks (Figs 8–10). However, individual filaments had a more regular morphology, and could be visualized as discrete rods with a constant diameter over their whole length. In many cases small particles were associated with these filaments.

Dry cleaving. Dry cleaving resulted in a much higher recovery of membrane and associated structures, since the cell bodies could be removed almost quantitatively. The membrane-adherent cytoskeletal elements were, however, less readily discernible in such replicas, as a result of granular material which was present between the filaments (Fig. 10). Moreover, individual filaments were less distinct as if their morphology was obscured by amorphous material.

The morphology of the membrane and adherent structures

Membrane. The inner surface of the membrane showed a smooth appearance. Small granules, smooth vesicles, coated pits and coated vesicles were scattered over the entire surface.

In Figs 11–14 different stages of coated vesicle formation are represented. The diameter of the coated vesicles was highly variable, and ranged roughly between 60 and 200 nm.


The membrane-adherent cytoskeleton. In all preparations a part of the cytoskeleton remained associated with the membrane adhering to the grids or coverslips. The plane of cleavage was somewhat variable especially when cells were cleaved on grids. Thus, in some cases only coated pits, vesicles and some smaller filaments were left on the membrane, while in other preparations stress fibers and dense networks were still present.

In general microfilaments, intermediate filaments and microtubules could be seen in the preparations. Moreover a number of smaller (less than 5 nm-thick) filaments were present.

Microfilaments

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Microfilaments

In all preparations extensive networks of filaments were present having a diameter of 5–8 nm in non-replicated cells. Locally these filaments were organized in large bundles. Depending on the stage of spreading of the cells also many starlike foci were observed, in close proximity to, or in association with, the plasma membrane (Figs 4–7). Within one cell striking differences in the organization and density of the network were commonly observed in different regions of the cytoplasm. As judged from their morphology and organization, these filaments represent actin microfilaments. This conclusion was strongly supported by a comparison of the distribution of actin in immunofluorescence preparations and the distribution of 5–8 nm filaments in cleaved cells (Figs 4, 5 and 6).

Intermediate filaments. In most preparations a limited number of 10-nm filaments was observed in close proximity to the plasma membrane. Occasionally these filaments were seen in a close topographic relation to microtubules (Fig. 2).

Microtubules. In the membrane-adherent cytoskeleton microtubules were always present. They were usually not attached directly to the membrane, but somehow suspended in the cytoplasmic network. However, microtubules were also seen in close association with the membrane. These microtubules were mostly oriented strictly parallel to the stress fibers lying close by (Fig. 3).

4. Discussion

The cytoskeleton in lens cells

In fully differentiated lens cells in situ, the main cytoskeletal components, i.e., microfilaments and intermediate filaments of the vimentin type, have been found in close proximity to the cellular membrane (Kibbelaar et al., 1980; Ramaekers et al., 1982a, b). In these cells we have proposed a contractile function for microfilaments during visual accommodation, and a more supportive role for intermediate filaments (Kibbelaar et al., 1980; Ramaekers et al., 1982c).

The cytoskeletal proteins of the non-terminally differentiated epithelioid cells that occur on the lateral side of the lens have mainly been studied in vivo. In the lens these cells elongate at the equatorial zone to form the long lens fibers. During aging of tissue cultures from these cells, their morphology changes from a polygonal cell shape into a more elongated, spindle-shaped cell type. This change in cell morphology, which has been suggested as having its counterpart in lens-cell elongation in vivo, is paralleled by a reorganization of the microfilament cytoskeleton (Ramaekers and Bloemendal, 1981; Ramaekers et al., 1982b). From these and other data (Ramaekers et al., 1981) it was suggested that the assembly of microfilaments is one of the driving forces in lens-cell elongation, both in vivo and in vitro.

Such a functional aspect of microfilaments will have to involve their close interaction with the cellular membrane. The techniques described here provide us with a tool to study this interaction in a three-dimensional fashion and allow the direct examination of the membrane-cytoskeleton complex because of the stripping technique that is applied.

An important finding in our recent experiments was the fact that the focally centered, star-like microfilament bundles could be shown to be directly associated with the cellular membrane. These vertices have been suggested to act as nucleation sites for actin aggregation and microfilament elongation (Lazarides, 1976; see also Small.
et al., 1981), which in lens cells might result in the morphological changes observed during cell elongation in vivo and in vitro. Moreover, the arrangements of cytoskeletal filaments in net-like organizations and associated with the plasma membrane may be more accurately studied using the techniques described here instead of using plastic-embedded material.

**Methodology**

Both cleaving methods could be shown to be very effective, since large areas of the membrane and associated cytoskeletal elements of a great number of cells were exposed. Both replicas and cleaved cells invariably showed networks of discrete interconnected filaments. From their morphology and spatial organization, microfilaments, intermediate filaments and microtubules can be recognized.

The direct examination of cleaved cells has several advantages over the use of replicas. The preparation is rapid and the loss of material is very low so that most cleaved cells on the grid can be studied. High-resolution views of the cytoskeleton can be obtained especially when stereo micrographs are made. The most important disadvantage of these preparations is that they are vulnerable to moisture and electron-beam damage in the microscope (see also Bridgman and Reese, 1984).

One striking characteristic of whole-mount preparations is the more irregular shape of filaments as compared with replicas. This irregular appearance might well be caused by small particles, such as ribosomes or protein aggregates which are often associated with the filaments. In whole mounts these particles seem to form part of the cytoskeletal elements, having more or less the same electron density and giving the filaments a 'microradicular' appearance. In replicas, on the contrary, small particles are more easily recognized.

**Chemical fixation, critical-point drying and the appearance of the cytoskeleton.** Considerable scepticism has been raised against methods such as chemical fixation and critical-point drying (Heuser and Kirschner, 1980; Small, 1981; Kondo, 1983).

First, fixation and critical-point drying have been described to cause distortion and collapse of cytoskeletal filaments, obscuring their form and discreteness. Second, it has been argued that these techniques cause soluble, granular proteins to condense on cytoskeletal elements, thus forming filamentous microtubule structures. (Heuser and Kirschner, 1980; Hirokawa and Heuser, 1981; Hirokawa and Tilney, 1983).

Several observations, including our own, contradict this point of view (see also Porter and Anderson, 1982).

First, filaments appear as distinct rods of uniform diameter, most clearly in replicas, and there is no obvious sign of collapse of these elements (see also Aggeler et al., 1983). Second the bulk of the matrix material between the filaments does not condense onto the cytoskeletal elements upon chemical fixation and critical-point drying.

This material is still visible in replicas of dry cleaved cells (cf. Fig. 10) and is apparently removed when cells are cleaved in buffer. Although a large part of the filaments that we have observed can be identified as cytoskeletal filaments with known diameters, it cannot be excluded that some filaments are artefactual as has been argued by Kondo (1986). Therefore extreme caution must be taken in identifying all filaments as authentic structures present in the living cell.

We conclude that the methods involving chemical fixation, critical-point drying and cleaving form an excellent alternative to the study of cytoskeletal elements (see also Aggeler et al., 1983; Mesland et al., 1981; Bridgman and Reese, 1984; Ris, 1985), and allow the dimensional freedom of the cytoskeleton.
and allow the visualization of the membrane-adherent cytoskeleton in its three-dimensional form.

The results and methods described in this paper can serve as a basis for further studies on the characterization of those elements that play a role in the interaction of the cytoskeleton with the plasma membrane. Especially immunocytochemical procedures will play an important role in this respect.

Although Fey, Wan and Penman (1984a) and Fey, Capco, Kroehlmaier and Penman (1984b) have already demonstrated that different types of filaments can be identified using immunolabeling techniques at the EM-level, their procedures involved a selective extraction by detergents. As a result lipids and soluble membrane proteins may be removed. Therefore their method has two major disadvantages as compared with the technique described above. First, a direct interaction of cytoskeletal- or cytoskeleton-associated proteins with the lipid bilayer cannot be studied using the extraction procedures of Fey et al. (1984a,b). Such interactions have been suggested for vimentin intermediate filaments in lens fibers (Ramanesker et al., 1982a). Secondly, intramembranous proteins will be difficult to identify as such after application of the antibody techniques to detergent-extracted cells, or may even be completely absent after such treatments. Our method described above allows the study of the intact membrane-cytoskeleton complex. Dry-cleaved whole-mount preparations have already been used to label membrane components (Roos et al., 1985) and cytoskeletal elements (Mesland and Spieles, 1984).

Traas and Kengen (in press) could recently show that the use of cells cleaved in buffer solutions is even more suitable for immunogold-labeling experiments, since a thin layer of cytoplasm is obtained, which is easily accessible for antibodies.

In conclusion it can be anticipated that the cleaved whole-mount preparation technique will prove to be an important method in the study of the membrane-cytoskeleton complex, especially since more and more specific antibodies to proteins of this cellular domain become available.

ACKNOWLEDGMENTS

The authors wish to thank Anita Huysman for culturing of the lens cells. Yvonne Stammes is gratefully acknowledged for excellent secretarial help with the preparation of the manuscript. The study was partly supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) and the Dutch Cancer Foundation, Queen Wilhelmina Fund (K.W.F.).

REFERENCES


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