Stabilization of the cytoplasmic ground substance in detergent-opened cells and a structural and biochemical analysis of its composition

(cytoskeleton/microtrabecular network/microfilaments/heavy meromyosin decoration)

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ABSTRACT Treatment of epithelial BSC-1 cells with low concentrations of the detergent Brij 58 results in partial or complete removal of the plasmalemma and partial extraction of internal membrane-bound organelles without causing massive release of "cytosolic" proteins from the cytoplasmic ground substance. Stereoscopic high-voltage electron microscopy of such extracted and fixed cells demonstrates a system of slender (4–20 nm) strands in a three-dimensional "microtrabecular" arrangement similar to that observed in unextracted whole-mount preparations. Extraction of Brij-extracted cells with Triton X-100 dissolves many of the microtrabecular strands, leaving, as a more stable structure, a characteristic cytoskeletal network composed of various filaments and microtubules. Two-dimensional polyacrylamide gel electrophoresis of ³⁵S-labeled polypeptides performed concurrently with the morphological studies demonstrates that Triton extraction of Brij-extracted cells releases a large number of polypeptides. This release parallels the loss of structural components observed by electron microscopy. Labeling of Brij-extracted cells with heavy meromyosin subfragment 1 decorates actin filaments with characteristic arrowhead complexes which are readily visualized only after subsequent Triton extraction. These observations support the concept that many cytoplasmic proteins are structure-bound and, in addition to the components comprising the cytoskeleton, are structure-forming. We conclude that a metastable association of various proteins of the cytoplasmic ground substance exists whose morphological integrity is maintained, at least temporarily, after removal of the plasmalemma in solutions containing Brij 58.

In recent years, new techniques have been devised to study the architecture and biochemical composition of the cytoplasm. Extraction, with nonionic detergents, of the less-stable (often termed "soluble") components has been an attractive and experimentally rewarding approach in the analysis of the cytoplasm (e.g., refs. 1–5). The detergent-resistant residue, commonly referred to as the "cytoskeleton" (2), is composed primarily of microtubules and different filament types (refs. 3–6; unpublished data). The degree of preservation, complexity of organization, and structural interaction demonstrated in cytoskeleton preparations vary with both extraction buffer composition and techniques used for visualization. However, it is generally agreed that, in the cytoplasm of all cell types thus far studied, there is a system of relatively stable filamentous structures of rather complex organization that interact with each other in various ways. These resist detergent extraction.

Porter and his collaborators (7–9) have examined cytoplasmic organization in the most complex system available, the whole cell. On the basis of a combination of whole-mount preparations (both critical point and freeze dried) and stereoscopic high-volt-

age electron microscopy, an anastomosing system of filamentous strands comprising the cytoplasmic ground substance has been described. This network has been termed the "microtrabecular lattice." Despite observations that support the presence of such a cytoplasmic complex (8), its existence is controversial (3). The most provocative aspect of the microtrabecular concept is that it questions the existence of a truly soluble cellular protein fraction; virtually all cytoplasmic constituents except small metabolites are envisaged as structure-bound, thereby dividing the cytoplasm into a polymeric "protein-rich" and a separate "water-rich" phase.

We have been especially interested in this aspect of the microtrabecular concept. If a three-dimensional, cytoplasmic, proteinaceous network does exist, then it should be stabilizable, even if only temporarily, in plasmalemma-free *in vitro* preparations, in much the same way as the cytoskeleton is retained after Triton extraction. In this report, we present the identification of such conditions and the results of a series of experiments performed in the course of their application to the study of epithelial tissue culture cells. The results lend support to the concept that the cytoplasmic ground substance is organized as a three-dimensional network of slender (4–20 nm) strands.

MATERIALS AND METHODS

Cell Culture. Epithelial cells of the strain BSC-1 (African green monkey kidney) were used throughout these studies. They were grown in Dulbecco's medium supplemented with 10% fetal calf serum.

Cell Extraction, Fixation, and Electron Microscopy. Procedures of cell extraction and fixation were similar to those described (10). Briefly, cells were first washed with stabilization buffer (60 mM piperazine diethanesulfonic acid/25 mM Hepes/10 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid/2 mM MgCl₂, pH 6.9). Cells were then extracted with 0.1% Triton-X-100, 0.2% Brij 58, or other detergents for the desired time period, washed with buffer, and fixed with 1% glutaraldehyde in stabilization buffer. The subsequent procedures of osmium fixation, dehydration, critical point drying, and high-voltage electron microscopy are described elsewhere (11). Whole (unextracted) cells were processed by the same procedures except that the extraction step was omitted.

Decoration with heavy meromyosin subfragment 1 will be described elsewhere; in the procedure used here, treatment with subfragment 1 lasted for 3–5 min.

Isotope Labeling and Gel Electrophoresis. Cell cultures were labeled with [³⁵S]methionine (1000 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels; New England Nuclear) at 300 μCi/ml for

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14–18 hr. Cells were washed twice with stabilization buffer and harvested with a rubber policeman. In extraction experiments, cultures were treated with detergent solutions for the desired time period. Supernatants of the extraction step were removed and either lyophilized immediately or first dialyzed against stabilization buffer diluted 1:9 with distilled water in the presence of a proteolysis inhibitor (phenylmethylsulfonyl fluoride). Cell residues (cytoskeletons) were washed with buffer, collected with a rubber policeman, centrifuged, and lyophilized. Immediately following lyophilization, samples were resuspended in lysis buffer (12) and subjected to isoelectric focusing electrophoresis.

Two-dimensional polyacrylamide gel electrophoresis was carried out according to the procedures of O'Farrell (12). Linear gradient slab gels were prepared as described (13).

RESULTS

Structure of Detergent-Treated Cells. Several detergents have been tested for their effect on the structure of the cytoplasmic ground substance. Triton-X-100, Nonidet P-40, polyoxyethylene ether (W1), and *n*-octyl glucoside (at 0.1%) all solubilize, within an exposure time <1 min, not only the plasmalemma and most of the internal membrane systems but also many of the cytoplasmic proteins. Many of the ribosomes are "dislodged" and end up in clusters. The detergent-resistant residue consists of various filament types, microtubules, and the nuclear remnant. Brij 58, however, has a different effect. At 0.2–0.3% Brij 58, the three-dimensional network comprising the cytoplasmic ground substance is not markedly affected within the first 5 min. Whereas Triton extracted many of the fibrous structures of the cytoplasmic ground substance, leaving only the open filamentous network of the cytoskeleton, the overall density of the cytoplasm in Brij-treated cells was virtually identical to that of untreated cells (Fig. 1). Not every filament present in an unextracted or Brij-treated cell was represented by a stable filament (F-actin, intermediate, or 2–3 nm) in the cytoskeleton. The only apparent difference between intact and Brij-treated cells was partial or complete extraction of some of the membrane-bound cytoplasmic organelles in the latter.

At higher magnification, Brij-extracted cells displayed a three-dimensional network of filamentous strands similar to that

of the microtrabecular network described for intact cells (Fig. 2 *a* and *b*). Because of this striking similarity, it was necessary to demonstrate that these cells had in fact been "extracted." Brij-treated cells therefore were shadowed with a thin film of platinum/palladium in order to render their upper surface visible (Fig. 2*c*). Instead of revealing a continuous surface lamina, as would be expected if the cell membrane were still intact (see figure 15 in ref. 8), it showed metal label on the surfaces of filamentous structures. In some areas, patches of an intact surface lamina may still have been present. The degree of extraction of the plasmalemma could be controlled by adding varying amounts of polyethylene glycol (M_r 20,000) to the extraction medium. Treatment with 0.2% Brij 58 for 3 min in the presence of 3% polyethylene glycol 20,000, for instance, created only small holes in the membrane (Fig. 2*d*). At lower concentrations of polyethylene glycol, or with a lower molecular weight polyethylene glycol, disruption of the plasmalemma was more complete.

We have tested a number of buffer modifications to determine whether or not *in vitro* stabilization of the cytoplasmic ground substance is unique to the extraction medium employed here. Virtually identical results were obtained with ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid concentrations in the range 1–10 mM, Mg^{2+} concentrations between 0.4 and 2 mM, or variations in pH between 6.7 and 7.3. Two completely different buffer systems, a sodium chloride-based buffer (14) and a sucrose buffer (15), also gave good preservation. In a low ionic strength buffer (25 mM piperazine-diethanesulfonic acid), extraction proceeded more rapidly.

Two-Dimensional Gel Electrophoresis. When Brij-extracted cells were extracted further with 0.1% Triton X-100, many of its filamentous components were readily solubilized within a few seconds, leaving a characteristic Triton cytoskeleton. This experiment demonstrates an important physicochemical property of the Brij cytoskeleton—namely, the existence of two subsets of fibers Brij-resistant (but Triton-labile), and Triton-resistant. We used two-dimensional gel electrophoresis in order to examine the polypeptide composition of the two fiber systems. Cells labeled with [^{35}S]methionine were extracted with 0.2% Brij 58 for 5 min. This treatment removed most of the plasmalemma and released approximately 10% of

FIG. 1. Low magnification ($\times 1900$) overview of unextracted and extracted BSC-1 cells. Whole-mount preparations were critical point dried and visualized in a high-voltage electron microscope. (a) Whole unextracted cell. (b) Cell extracted with Brij for 5 min. Note partial extraction of some of the membrane-bound organelles (arrows). (c) Cell extracted with 0.1% Triton-X-100 for 1 min. (Circular Insets) Higher magnifications ($\times 69,400$) of the respective preparations.

the total cellular protein. When such a Brij-skeleton was then extracted for 1 min with Triton, approximately 50% of the total cellular protein was released.

Two-dimensional polyacrylamide gel electrophoresis of this fraction demonstrated a complex polypeptide composition, with actin being the most prominent species (Fig. 3). In total, more than 400 polypeptides could be resolved. All the polypeptides of this Triton extract also were present in the preceding Brij extract, although in varying relative amounts. This result suggests that none of the polypeptides resists Brij extraction; therefore, with respect to protein extraction, the action of Brij 58 is basically similar to that of Triton X-100, albeit much slower. In agreement with this conclusion is the electron microscopic observation that it requires more than 10 min of treatment with Brij 58 to achieve a state of extraction similar to that of a 10- to 15-sec exposure to Triton X-100 (data not shown).

Fourteen polypeptides were present in the Brij extract but not in the subsequent Triton extract. These polypeptides may have been associated with the plasmalemma because this is the only cellular component that is at least partially solubilized by this detergent in the time and concentration used.

Of the more than 200 polypeptides present in Triton cytoskeletons, only 6 appeared to be unique to this structure. One of them corresponds to the intermediate filament protein vimentin; three other major polypeptides are believed to be degradation products of this protein (16). As expected, both vimentin and actin are major polypeptide species of cytoskeletons.

Heavy Meromyosin Subfragment 1 Decoration. To identify the presence of actin filaments, Brij-extracted cells were reacted with subfragment 1 for 3–5 min. The structure of cells treated in this way was similar to that of Brij-extracted cells not so treated in that they displayed an irregular meshwork of filamentous strands (Fig. 4). Periodic decoration of filaments with arrowhead structures was not readily detectable, although some filaments, notably those forming stress fibers, appeared to be studded with small lumps. However, when subfragment 1-decorated cells were further extracted with Triton X-100 (not containing subfragment 1), periodic arrowhead decoration of actin filaments was readily apparent, although decoration occasionally appeared to be irregular or incomplete.

DISCUSSION

This paper describes a procedure that allows stabilization of the cytoplasmic ground substance after partial or complete removal of the plasmalemma. The procedure involves the use of a detergent from the Brij group, polyoxyethylene glycol cetyl alcohol (Brij 58), to solubilize large portions of the plasmalemma without severely affecting the underlying cytoplasm. The cell residue remaining after Brij extraction has an organization similar to that of an unextracted cell—i.e., it consists of an intricate system of filamentous strands similar to, if not identical with, the microtrabecular network (7, 8). This organization is different from the cytoskeleton remaining after subsequent Triton extraction of a Brij-treated cell—an extraction that leaves an open

FIG. 2. Stereoscopic electron micrographs of BSC-1 cells extracted under different conditions. (a) Treatment with 0.1% Triton X-100 reveals a filamentous network of which microtubules are part. (b) Cell extracted with 0.2% Brij 58 for 5 min. The network of fibers displays a characteristic "microtrabecular" appearance. (c) Cell extracted with 0.2% Brij 58 for 5 min and then shadowed lightly with platinum/palladium after critical point drying. Small granular metal particles can be found on the surfaces of filamentous microtrabecular strands. (d) Cell extracted with 0.25% Brij 58 in the presence of 3% polyethylene glycol (M_r 20,000). The surface membrane is not completely removed but appears to be perforated with numerous small fenestrations (arrows). (a and b, $\times 32,000$; c and d, $\times 115,000$.)

network of filaments (the cytoskeleton). Coupled with our two-dimensional gel electrophoretic analysis performed in parallel to the structural studies, this suggests that a large number of cellular proteins are associated with cytoskeletal fibers. Thus, the microtrabecular network would appear to include two structural entities with different stabilities (i) a Triton-stable filamentous network consisting primarily of the well-known cytoskeletal filaments, and (ii) a Triton-labile, but relatively Brij-resistant, heterogeneous complex of proteins that is associated with, and essentially contains, the cytoskeletal filament network.

The observation that many of the proteins comprising microtrabeculae are readily released into the supernatant by Triton X-100 or similar detergents may be explained by detergent-protein interactions; such interactions may be different for Brij 58. Triton X-100 and Brij 58 differ in the length of their polyoxyethylene glycol chains and in the hydrophile/lipophile balance, with Brij 58 being more hydrophilic than Triton X-100 (17). However, it is not apparent whether such physical-chemical differences are responsible for the different effects of the two detergents in extraction experiments.

The nature of association between the proteins forming the morphological image of the microtrabecular net is not understood. However, two sets of observations suggest that microtrabeculae do not represent a rigid skeletal framework. (i) Prolonged exposure of cells to Brij 58 will slowly dissociate the less-stable components from the stable cytoskeleton. Supernatants of Brij extractions contain all the proteins also released by Triton X-100; however, an approximately 50-fold longer exposure is required to attain a comparable degree of extraction. (ii) Brij-extracted cells, when treated with subfragment 1, show decoration of actin filaments with arrowhead patterns; this decoration, however, is only readily visualized upon subsequent Triton extraction. This observation may be interpreted to mean that the association between cytoplasmic proteins and actin filaments, as seen in a Brij-lysed cell, still allows access of subfragment 1 molecules to actin filaments. This finding is in agreement with results from physiological experiments in which treatment of Brij-lysed mitotic cells with a nondissociable analog of heavy meromyosin, *N*-ethylmaleimide-treated heavy meromyosin, blocked cytokinesis (17).

We interpret our results as supportive evidence for the concept that many cytoplasmic proteins, in addition to those forming the cytoskeleton, may be structure-associated and structure-forming. This association, which probably can best be described as "metastable," may be equated with the structure described as a microtrabecular network by Porter and coworkers (7, 8).

FIG. 3. Two-dimensional polyacrylamide gel electrophoretic patterns of ^{35}S -labeled proteins from unextracted (whole) cells and extracts and residues of detergent-treated cells. Isoelectric focusing was from left to right (IEF); electrophoresis in $\text{Na}_2\text{DodSO}_4$ /polyacrylamide slab gels was from top to bottom. Arrow, actin; large arrowhead, vimentin; small arrowheads, putative degradation products of vimentin. (a) Total ^{35}S -labeled polypeptides from BSC-1 cells (1.5×10^6 trichloroacetic acid-precipitable cpm loaded in the first dimension). (b) Fluorograph (270,000 trichloroacetic acid-precipitable cpm loaded in the first dimension) of ^{35}S -labeled proteins extracted from cells given a 5-min treatment with 0.2% Brij 58. Encircled polypeptides were not present in a subsequent Triton extract of this preparation (see c). (c) Autoradiograph (2×10^6 trichloroacetic acid-precipitable cpm loaded in the first dimension) of polypeptides removed by a 1-min treatment with 0.1% Triton-X-100 from a preparation previously extracted with Brij 58 (see b). (d) Autoradiograph (675,000 trichloroacetic acid-precipitable cpm loaded in the first dimension) of labeled polypeptides present in cytoskeletons. Gels a and c were exposed to x-ray film (Kodak NS2T) for 4 days; gel d was exposed for 10 days. Gel b was treated with EN 3 HANCE (New England Nuclear) for 1 hr and exposed at -80°C to Kodak X-O-Mat (XR-5) x-ray film for 12 days.

FIG. 4. Stereoscopic electron micrographs of BSC-1 cells labeled with heavy meromyosin subfragment 1. (a) Cell extracted with 0.2% Brij 58 for 3 min and treated with subfragment 1 for 3 min. (b) Cell extracted with 0.2% Brij 58 for 3 min, treated with subfragment 1 for 3 min, and then extracted with 0.1% Triton X-100 for 2 min in the absence of subfragment 1. Actin filaments are decorated with arrowhead complexes. Note limited occurrence of actin filaments relative to total microtrabecular network. Decoration may be incomplete or irregular in some places. Arrow, microtubule; arrowhead, 3-nm filaments not decorated. ($\times 42,000$.)

How and why many of the proteins present in the cytoplasmic ground substance should maintain such an association and how it changes in time and space under various physiologic conditions are unknown.

Our observations are of increased interest when considered against the physiological studies by Cande and coworkers (19–21) who used Brij-extracted cells as model systems to study chromosome transport in mammalian cells. Their results demonstrate that the Brij-lysed cell is a physiologically intact model system (at least for 8–10 min) able to support mitotic movements under *in vitro* conditions. Essential ingredients required for chromosome movements seem to be retained under the conditions of Brij lysis used in their studies but lost upon Triton extraction. Therefore it should be considered that detergents such as Triton X-100 release certain cellular components not because these components are truly soluble (as they should be readily released upon Brij-treatment as well) but because the detergents interact with certain cellular proteins in a way that leads to their differential liberation from a structured metastable state.

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