Lenticular intermediate-sized filaments: Biosynthesis and interaction with plasma membrane

(lens cytoskeleton/vimentin/α-crystallin/cytoskeletal protein biosynthesis)

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ABSTRACT Electron microscopical features of the lens fiber plasma membrane-cytoskeleton complex are suggestive of an intimate association between the intermediate-sized filaments (IF) and the lipid bilayer. Biochemical analysis of this complex reveals the occurrence of an appreciable amount of vimentin as a protein subunit of lenticular IF. Additional evidence for association between IF and membranes is provided by the observation that newly synthesized vimentin is associated with plasma membranes added to a reticulocyte lysate programmed with lens polyribosomes. Concomitantly α-crystallin polypeptide chains (αAαB) are also found associated with the plasma membrane together with a hitherto unidentified 47-kilodalton protein. Once associated with the bilayer, the vimentin polypeptide resists urea treatment, suggesting that it has become an integral constituent associated with part of the membrane.

Better insight into the function of the cytoskeleton in nonmuscle cells requires elucidation of the nature of the link between cytoskeletal motility proteins and the cell membranes (1). Whether or not protein subunits of the cytoskeleton are contained within the lipid membrane core as integral membrane components is still an open question.

Recently great interest has been focused on the intermediate-sized filaments (IF) (2), which, together with microtubules and actin filaments, constitute the intracellular matrix of eukaryotic cells. IF have been subdivided into five classes according to the embryonic origin of the cell type and the nature of their protein subunit (3).

IF have also been described in intact lens, where they contribute to the organization of the cytoskeleton of both the epithelial cells and the lens fibers (4–6). Moreover, we have demonstrated the presence of IF in cultured lens cells (6–8). Immunofluorescence studies and biochemical data indicated that the protein moiety of these IF is vimentin (6). Electron microscopical evidence has been provided for an end-on attachment of IF to the cytoplasmic site of the lens fiber plasma membranes (9). The link of IF with various types of membranes has been postulated as a nucleation site for filament formation. These reports, however, are mainly confined to the interaction of prekeratin IF with desmosomes (10), of desmin IF with the intercalated disks in muscle (11), and vimentin IF with the nuclear membrane (12).

In this paper we show that the lenticular IF are intimately associated with the plasma membranes. Moreover, we demonstrate that vimentin newly synthesized in vitro becomes associated with the lipid bilayer when purified lens fiber plasma membranes are added to the translation system.

MATERIALS AND METHODS

Isolation of Lens Fiber Plasma Membranes. Fresh calf lenses were decapsulated and homogenized by continuous stirring for 2 hr in either bicarbonate buffer (1 mM NaHCO₃/1 mM CaCl₂) or TKM buffer (50 mM Tris-HCl, pH 7.4/25 mM KCl/5 mM MgCl₂).

Plasma membranes were isolated by discontinuous sucrose gradient centrifugation as described earlier (13) in either bicarbonate or TKM buffer. The plasma membrane fractions obtained at sucrose densities 1.20–1.22 g/cm³, 1.18–1.20 g/cm³, and 1.16–1.18 g/cm³ were washed three times in the respective buffers and sedimented in a Beckman Ti 50 rotor.

In the isolation procedure using TKM buffer, a membrane-containing layer is formed only at 1.20–1.22 g/cm³. Protein concentrations were determined by the Lowry method (14).

De Novo Synthesis of Lens Proteins. Calf lens polyribosomes were isolated as described by Bloemendal et al. (15). Rabbit reticulocytes were prepared as described either by Evans and Lingrel (16) or by Pelham and Jackson (17). A 30,000 × g supernatant fraction of the lysed cells was used as the cell-free system, and incubations were performed at 30°C for 90 min (cf. ref. 15).

The lysate was made mRNA dependent by preincubation with micrococcal nuclease at 10 μg/ml in the presence of 1 mM CaCl₂ for 15 min at 20°C. After the incubation 2 mM EGTA was added to chelate the calcium ions. Polyribosomes were added in a concentration of 0.4–1 mg/ml (determined spectrophotometrically and assuming an extinction coefficient of 13 ml/mg per cm at 260 nm).

Incubation and Reisolation of Lens Plasma Membranes. Ninety minutes after addition of lens polyribosomes the reticulocyte cell-free incubation mixture was centrifuged for 10 min at 10,000 × g and supplemented with purified lens plasma membranes, isolated in either bicarbonate or TKM buffer. In some experiments membranes isolated in bicarbonate that were exhaustively washed with 6 M urea were used. The total mixture was incubated as indicated at 30°C with occasional stirring. When membranes isolated in TKM buffer were added to the reticulocyte cell-free system, the ionic conditions of this incubation were adjusted to the salt concentrations of the TKM buffer. Then the membranes were reisolated in bicarbonate or TKM buffer as described above or simply by centrifugation in a minicentrifuge. The membrane pellets were dissolved for gel electrophoretic analysis.

Abbreviations: IF, intermediate filaments; kDal, kilodalton(s); IEF, isoelectric focusing.

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Polyacrylamide Gel Electrophoresis. Analysis of the polypeptides was performed by NaDodSO₄/polyacrylamide gel electrophoresis according to Laemmli (19) with the modification that slab gels instead of gel rods were used. The gels were 12 cm long and contained either NaDodSO₄/13% acrylamide gel or a 7–18% acrylamide gradient with 0.4% methylenebisacrylamide and 0.1% NaDodSO₄. In this method a stacking gel was applied. Staining and destaining were performed as described by Weber and Osborn (20).

Gels were processed for autoradiography. For the detection of the labeled proteins the procedure of Bonner and Laskey (21) was used in combination with the drying procedure described by Berns and Bloemendal (22). Two-dimensional gel electrophoresis was performed according to O'Farrell (23).

Electron Microscopy. Electron microscopic observations on the isolated lens fiber plasma membrane cytoskeleton complex were carried out on thin sections fixed in glutaraldehyde/osmium tetroxide and embedded in Vestopal (24) or by the negative staining technique. Freeze-fracture experiments were performed on isolated fractions that were rapidly frozen in liquid Freon 22. The freeze-fracture replica was obtained in a Balzers BAF-301 apparatus. The unfixed specimens were fractured at −150°C. The replica was made immediately after the fracture by evaporation of platinum and carbon, using an electron gun (25). A Philips EM 400 microscope was used.

RESULTS

Electron microscopical observation demonstrates a striking difference when the lens plasma membranes instead of being isolated in sodium bicarbonate are fractionated under cytoskeleton stabilizing conditions (TKM buffer) (9). In the former method the membranes are practically devoid of filamentous structures. Conversely, when the cytoskeletal organization is preserved IF and actin filaments are clearly visualized in close association with the cytoplasmic site of the membrane (Fig. 1a).

In Fig. 1b a high-resolution electron micrograph of isolated IF is shown. From this picture and at higher magnification in

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![Image of electron micrographs](image-url)
Fig. 1. The subunit organization of these structures becomes clearly visible, showing that each IF is built up probably by four subfilaments.

The intimate association between IF and membrane leaflets is further demonstrated by freeze-fracture aspects of the lens fiber plasma membrane cytoskeletal complex. In freeze-fracture replicas rodlike structures, most probably corresponding to longitudinal fractures of IF, appear mainly associated to the protoplasmic membrane fracture face (PF), where they are closely packed with intramembraneous particles (Fig. 1d). The rods are continuous with filaments found in the cytoplasmic space where the fracture aspects of the cytoskeleton structure are visible.

NaDodSO₄ gel electrophoretic analysis of the cytoskeleton membrane complex shows a very strong band migrating in the molecular weight region of vimentin (Fig. 2). Less heavily stained bands represent the α-crystallin subunits, actin, and a 47-kdalton (kDa) polyepitope. The lens fiber membrane proteins MP26 and MP34 (26) are masked by the predominance of the cytoskeletal polyepitopes. Only after removal of the cytoskeleton by bicarbonate washings and subsequent reisolation by flotation of the membranes do the MP26 and a doublet in the 34-kDa region become clearly visible on the electropherogram (Fig. 2).

The cytoskeletal polyepitopes were further characterized by two-dimensional gel electrophoresis (Fig. 3). It can be seen that vimentin is the most abundant component and that the 47-kDa polyepitope differs from actin in its isoelectric point. The 100-kDa polyepitope coincides with α-actin.

Because the morphological evidence was suggestive for an intimate association between plasma membranes and IF, we examined the fate of newly synthesized vimentin in the presence of purified lenticular plasma membranes in vitro. For this purpose lens fiber polysomes were translated in a reticulocyte lysate. Two-dimensional gel electrophoresis showed that the polyribosomes direct the synthesis not only of crystallins but also of cytoskeletal polyepitopes. Among the latter polyepitopes vimentin is again predominant (Fig. 4a). Thereafter the following recombiant experiments were carried out. The lens polysome/reticulocyte lysate mixture was supplemented, upon 90 min of incubation, with purified plasma membranes from which the cytoskeleton was detached by bicarbonate washings. Incubation was continued for 1 hr and the membranes were re-isolated by flotation. Two-dimensional electrophoretic analysis yielded the pattern shown in Fig. 4b. Obviously, only two components have been selected from the protein population synthesized de novo, namely the major α-crystallin chain α₂ and the IF constituent vimentin. In addition the yet-unidentified 47-kDa protein also becomes associated with the plasma membranes.

When urea-extracted membranes were used in the reconstitution experiments it could be shown that these membranes also are capable of selectively sorting out newly synthesized translation products (Fig. 5). Of the products of translation shown in lane b, vimentin preferentially interacts with the membranes (Fig. 5, lane c). Also actin, MP47, α₁ and β₁ are sorted out, but although other β-crystallins were synthesized de novo in appreciable amounts, they were not found to be associated with the membrane. This latter result indicates that...
there is, at least for this class of proteins, no exchange between preexisting and newly synthesized polypeptides but a selective association. The gel pattern (Fig. 5) also shows that the newly synthesized vimentin becomes attached to the lipid bilayer and resists further urea extraction (Fig. 5, lane d). Crystallins, on the other hand, are partially solubilized by this treatment.

An interesting feature of this association of newly synthesized protein is shown in Fig. 6. This gel pattern shows the time dependence of the association process for the individual translation products. Whereas there is little or no increase of the vimentin and the MP34 doublets after 10-min incubation, there is a clear increase in the sorting out of αA, αB, and βB<sub>2</sub> polypeptides with increasing time of incubation. Unlike vimentin, actin association becomes detectable only after 60 min of incubation; thereafter it remains virtually constant. The same holds true for a high molecular weight component that coincides with α-actinin.

**DISCUSSION**

Our present reconstitution experiments provide evidence for an intimate association of newly synthesized motility protein, in particular vimentin, with the membrane. The same holds true for MP34, which is an integral urea-insoluble plasma membrane protein found in both lens epithelium and fiber cells. These proteins vanish after protease treatment (27). However, the fact that they are not affected by urea favors the assumption that vimentin and MP34 interact strongly with the lipid bilayer, although the inserted sequence of the molecule may be rather small. Thus, sensitivity to proteolytic enzymes may not necessarily mean that newly synthesized polypeptides do not become associated with the membrane as an integral constituent.

A still unresolved problem concerns the mechanism of interaction. There is as yet no evidence that the proteins sorted out from the bulk of translation products contain a "topogenic" amino acid sequence (28) that would favor interaction with the hydrophobic core of the membrane. At least for α-crystallin chains whose complete primary structure is known (29), association with the membrane does not seem to require a specific signal sequence. We are aware that in other membrane systems the assembly of protein components synthesized de novo may follow a different design principle. In one model, membrane protein assembly may occur even when the translation is completed at a distance from the membrane target (30). In the other model, close proximity of the lipid bilayer is necessary shortly after initiation of the protein (31). At least the lenticular proteins vimentin and MP34 follow the first model, because they rapidly...
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