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CALCIUM-DEPENDENT BINDING OF EDTA-EXTRACTABLE PROTEINS TO CALF LENS FIBER MEMBRANE STRUCTURES

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Calf lens fiber membranes and fractions enriched in junction-like structures have been isolated in the absence and presence of EDTA. Their biochemical features have been studied. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting experiments have provided evidence that a distinct group of EDTA-extractable proteins, being one of the main protein components of calf lens fiber membranes and very likely also of junction-like structures, is bound to these membranes via calcium ions. In addition to these proteins, four polypeptides with apparent molecular weights between 14000 and 17000 are characteristic for detergent-insoluble lens fiber structures prepared in calcium-rich medium. The absence of EDTA-extractable proteins in the urea-soluble calcium-containing fraction implies that they are not components of the cytoskeleton and that the calcium-dependent binding of these proteins to the membrane is urea-resistant. The use of EDTA throughout the whole membrane isolation procedure results in their complete removal from the membranes which already starts during buffer washing. This indicates that EDTA-extractable proteins exclusively consist of extrinsic membrane proteins which probably are not involved in cytoskeleton binding.

Introduction

Earlier studies have shown that a distinct group of extrinsic membrane proteins can be partially extracted from urea-treated calf lens fiber membranes by means of EDTA or EGTA [1–3]. For this reason the EDTA-extractable proteins (EEP) were supposed to be bound to the inner surface of the fiber cell membrane via calcium ions, whereas the non-extractable part was thought to be more strongly bound.

In the present communication evidence is provided that EEP exclusively consists of extrinsic membrane proteins and that calcium very probably is the only prerequisite for their in vivo binding to calf lens fiber membrane structures.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EEP, EDTA-extractable proteins.

Materials and Methods

Isolation of lens membranes

Fiber membranes were prepared from whole decapsulated 4-month-old calf lenses according to Van Raaij et al. [3]. In short: the lens mass was repeatedly extracted with buffer and afterwards the pellet consisting of membrane-cytoskeleton complex was treated four times with 6 M urea in the same buffer. The final pellet consisted of cytoskeleton-free plasma membranes. For the isolation of lens fiber membranes in calcium-free medium, 50 mM Tris-HCl (pH 8.0) or 50 mM Tris-HCl/5 mM EDTA (pH 8.0) were used instead of Tris-$CaCl_2$ buffer throughout the whole procedure.

Fractions enriched in junction-like structures were isolated in the presence of detergent according to the method described by Goodenough [4]. Exactly the same procedure was followed for the

isolation of these structures in the absence of EDTA. However, in the initial wash buffer 5 mM EDTA was replaced by 1 mM $CaCl_2$ and EDTA-free buffer solutions were used during the next steps.

Analytical and immunological methods

Protein determinations and SDS-gel electrophoresis in 10 and 13% gels were carried out as reported by Van Raaij et al. [3]. Preparation of an antiserum, which is specific for EEP (anti-EEP antiserum) and the immunological detection of EEP on nitrocellulose sheets have been described [5].

Electron microscopy

Fiber membrane pellets were fixed for 1 h in Karnovsky's fixative [6] supplemented with 4% tannic acid. Afterwards, the pellets were cut into small pieces and then fixed for an additional 15–18 h. Following a rinse in 0.1 M cacodylate buffer (pH 7.2) overnight, the specimens were postfixed for 2 h in 2% osmium tetroxide in veronal-acetate buffer (pH 7.2), rinsed and stained for 1 h with 1% uranyl acetate prepared in the latter buffer. These specimens were then dehydrated through a series of graded ethanol and embedded in Epon.

Results

During the preparation of calf lens fiber membranes, no EEP is lost if calcium ions are present throughout the whole isolation procedure. This appears from Fig. 1 which shows the SDS-gel pattern of the urea-soluble calcium-containing fraction consisting of cytoskeleton components and crystallins. EEP appears to be absent even in case of overloading the gel. In addition, no radioactivity can be detected on the corresponding electroblot after incubation with anti-EEP antiserum and ^{125}I-labelled protein A (not shown).

If calf lens fiber membranes are isolated in calcium-free medium, the amount of EEP in the membranes strongly decreases (Figs. 2 a and b, lanes 3 and 4). This process of EEP extraction already starts during the initial buffer washings of the membranes (Figs. 2 a and b, lane 3). The addition of a chelator (EDTA) to the calcium-free wash fluids (urea-free and urea-containing) finally causes the complete removal of EEP from the membranes (Figs. 2 a and b, lane 6). The faint protein band at the site of EEP in the SDS-gel pattern of these EEP-free membranes has no immunological relationship with EEP.

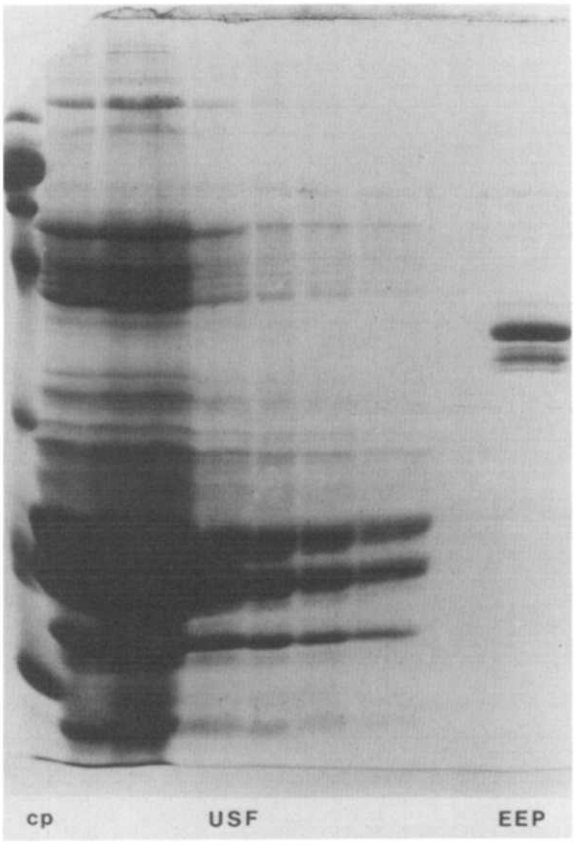

Fig. 1. SDS-gel electrophoresis (13% gel, 0.75 mm) of different amounts of the urea-soluble calcium-containing cytoskeleton fraction (USF; 250 μg, 500 μg, 50 μg, 25 μg, 18 μg and 9 μg total protein, respectively). The amount of EEP in the EEP lane is 10 μg. Calibration proteins (cp, from bottom to top): lysozyme (14.3 kDa), trypsin inhibitor (21 kDa, upper band of doublet), chymotrypsinogen A (25.7 kDa), ovalbumin (43 kDa), catalase (58 kDa), serum albumin (68 kDa) and phosphorylase A (92.5 kDa).

The use of excess EDTA in the buffer solutions also greatly affects the protein composition of membrane fractions enriched in junction-like structures. However, the morphological appearance of these fractions and those obtained in the absence of EDTA is quite similar as shown in Fig. 3. This figure represents cross sections of

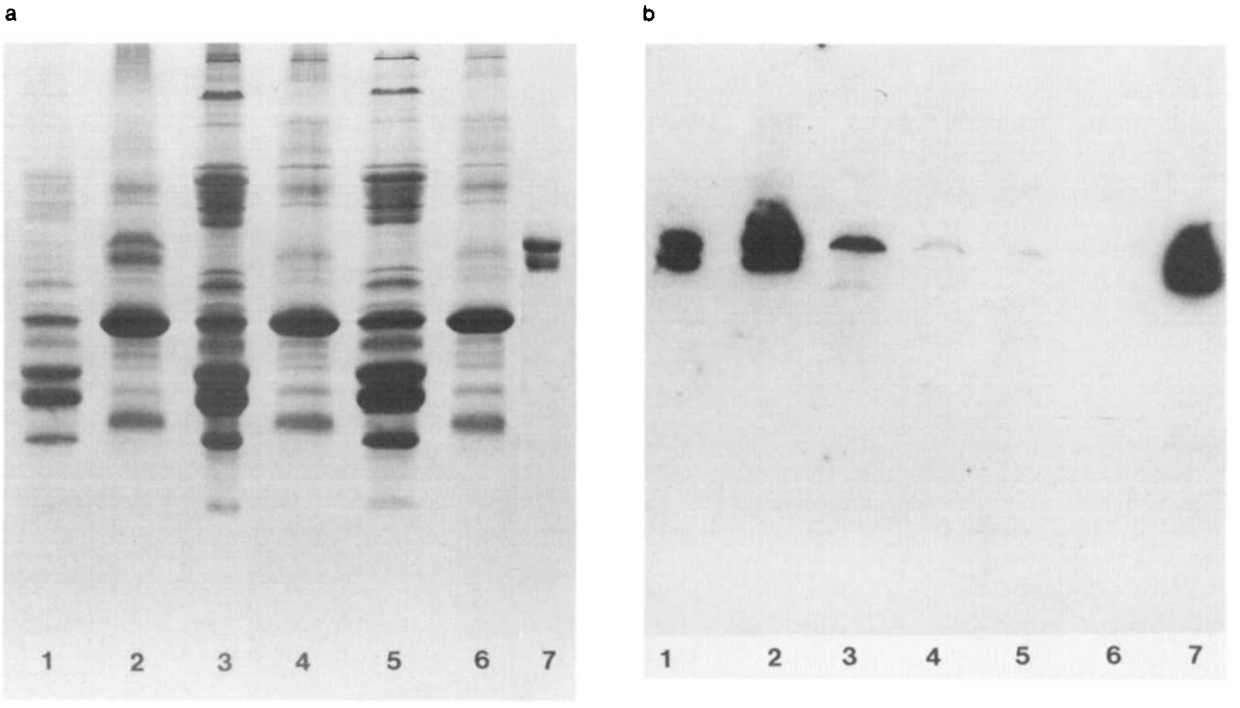

Fig. 2. (a) SDS-gel electrophoresis (13% gel, 0.75 mm) of buffer-washed calf lens fiber membranes (odd-numbered lanes) and urea-treated membranes (even-numbered lanes) isolated in Tris-$CaCl_2$ (lanes 1 and 2), in Tris (lanes 3 and 4), and Tris-EDTA buffer (lanes 5 and 6). Lanes 1–6 all contain 30 μg protein. Lane 7 contains 3 μg calf lens fiber EEP. (b) Autoradiograph of corresponding electroblot, after incubation with anti-EEP and ^{125}I-labelled protein A.

membrane pellets of the 30–41% sucrose layers prepared in the two different buffer systems. Both preparations contain many straight and undulating penta-layer and other multi-layer structures of various lengths. These structures are also present in the 0–30% sucrose layers from both protocols (not shown). Some amorphous and granular material (large arrows) as well as triple-layer membranes (arrowheads) are visible.

In Fig. 4a the SDS-polyacrylamide gel electrophoresis patterns of the 0–30% and 30–41% sucrose layers, obtained in the absence and presence of EDTA, are compared. If EDTA is used throughout the whole isolation procedure the fiber membranes show a major polypeptide at 25.5 kDa, flanked by a number of minor polypeptides with higher and lower molecular weights (lanes 6 and 7).

Detergent-resistant membrane structures isolated in calcium-rich EDTA-free buffer solutions contain EEP as a second main protein component (Fig. 4a, lanes 4 and 5). In addition, four polypeptides with molecular weights between 14000 and 17000 form part of the protein composition of the 30–41% sucrose layer. Possibly they are specific protein components of junction-like structures. In view of its molecular weight and binding via calcium, one of these bands may represent calmodulin (16.5 kDa). With exception of the intensity of these four polypeptide bands the protein pattern of the 30–41% layer is identical to that of the corresponding 0–30% sucrose layer (Fig. 4, lane 4) and that of urea-treated lens fiber membranes (Fig. 4, lane 3) prepared in the absence of chelator and detergent [3]. An intense 17.5 kDa protein band is present in all of the membrane preparations shown in Fig. 4a.

Following water extraction, treatment with EDTA of the 0–30% layer prepared by the EDTA-free procedure predominantly extracts EEP (Fig. 5, lane 2). If EDTA is used during the membrane preparation no EEP is present in the EDTA extract of the 0–30% sucrose layer (Fig. 5, lane 3).

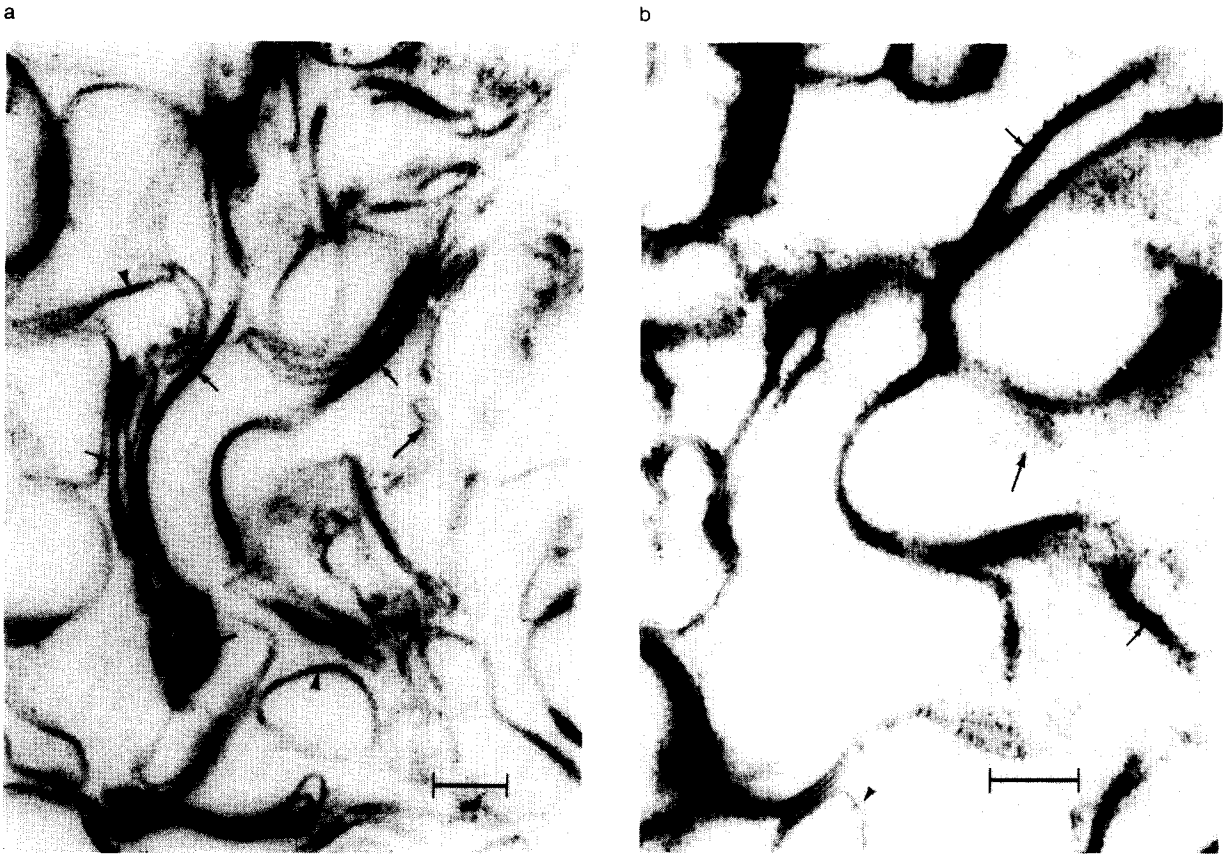

Fig. 3. Isolated junction-enriched calf lens fiber membrane fractions (30–41% sucrose layers) in thin section. The membranes are prepared in the absence (a) and presence (b) of EDTA. The preparations are enriched in pentalaminar structures (small arrows). Some amorphous and granular material (large arrows) as well as unit membranes (arrowheads) are visible. Bars indicate 100 nm.

Further evidence for the total absence of EEP in the 0–30% and 30–41% sucrose layers, prepared in EDTA-rich medium, was obtained from anti-EEP-treated electroblots of SDS-gels containing the same samples as in Fig. 4a. The results are shown in Fig. 4b. No radioactivity can be detected in lanes 8 and 9 excluding even the presence of traces of EEP.

These results again indicate that EEP is quantitatively removed from the membranes by the action of a chelator during the isolation. Furthermore, nor the faint 32 kDa protein band, visible in the protein pattern of the EEP-free sucrose layers (Fig. 4a, lanes 6 and 7), nor the four low molecular weight polypeptides characteristic for the EEP-rich 30–41% layer (Fig. 4a, lane 5) are immunologically related to EEP.

Discussion

This study shows the effect of the presence or absence of EDTA during the preparation of calf lens fiber membrane structures on their protein composition.

EEP is one of the principal protein components of calf lens fiber membranes and very likely also of junction-like structures. Our SDS-gel protein patterns and immunoblotting experiments provide clear evidence that EEP is absent in the urea-soluble calcium-containing cytoskeleton fraction. The use of calcium-free buffer solutions throughout the whole membrane isolation causes dissociation of EEP from the membranes. Furthermore, the presence of excess chelator (5 mM EDTA) during the isolation of urea-treated membranes or fractions,

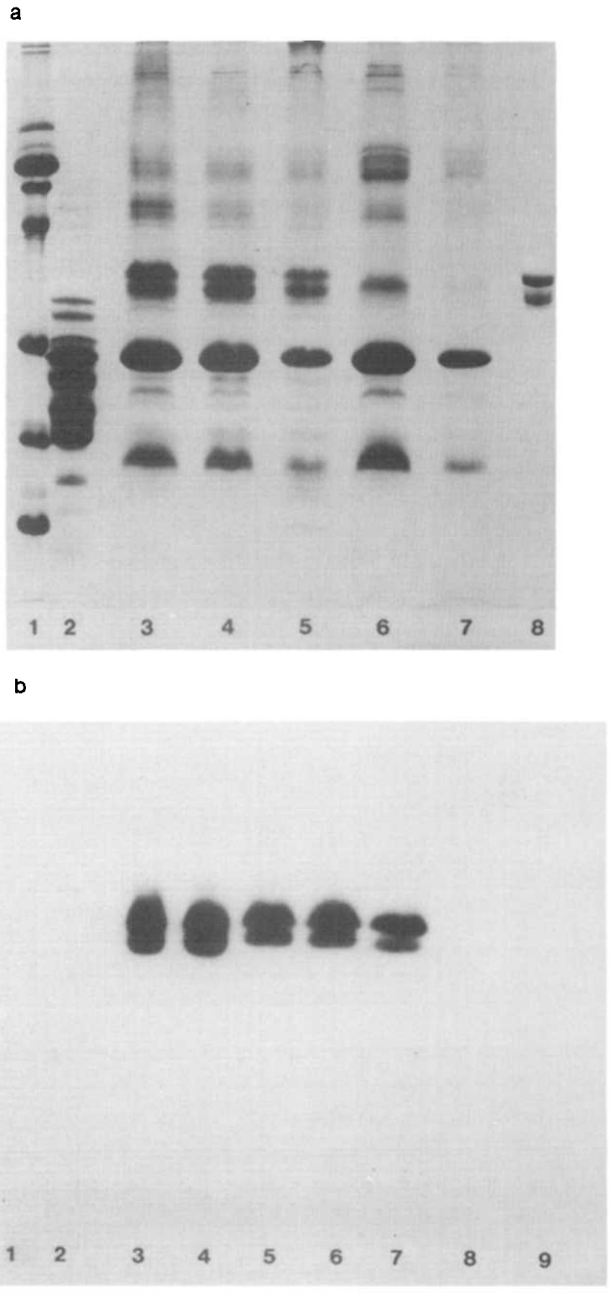

Fig. 4. (a) SDS-gel electrophoresis (13% gel, 0.75 mm) of (1) calibration proteins (as in Fig. 1); (2) total calf lens crystallins; (3) urea-treated lens membranes prepared according to Van Raaij et al. [3]; (4) and (5) 0–30% and 30–41% sucrose layer, respectively, obtained in the absence of EDTA; (6) and (7) 0–30% and 30–41% sucrose layer, respectively, obtained in the presence of EDTA, (8) calf lens fiber EEP. (b) Autoradiograph of an anti-EEP-treated electroblot of a SDS-gel, loaded with the same samples as in (a). (1) Calibration proteins (as in Fig. 1); (2) lens crystallins; (3) and (4) calf lens fiber EEP (8 μg and 12 μg, respectively). Lanes 5–9 contain the same samples in the same order as shown in (a), lanes 3–7.

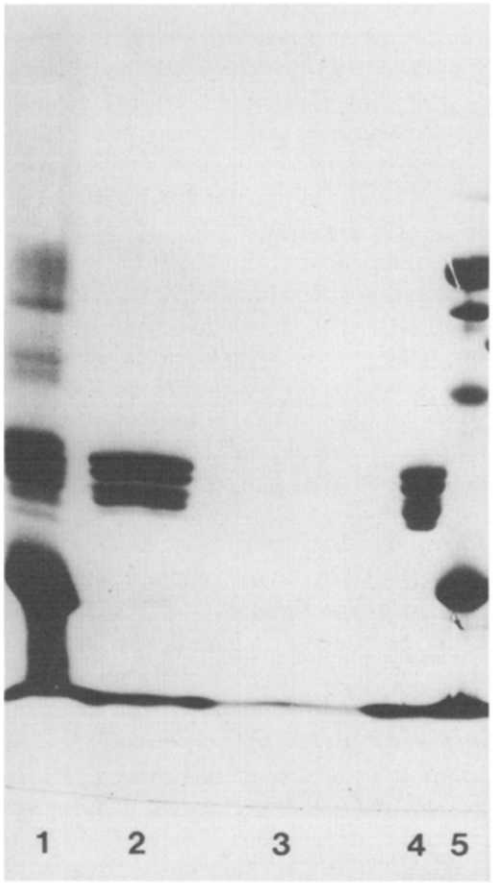

Fig. 5. SDS-gel electrophoresis (10% gel, 0.75 mm) of (1) urea-treated lens membranes (as in Fig. 4); (2) and (3) EDTA extracts of the membrane preparations shown in Fig. 4a, lanes 4 and 6, respectively; (4) calf lens fiber EEP; (5) calibration proteins as in Fig. 1 (lysozyme and trypsin inhibitor at the front).

detergent-enriched in junction-like structures, results in the total removal of EEP. This extraction is already almost complete after buffer-treatment of the lens fiber cells.

From these results we conclude that EEP nor interacts with the cytoskeleton nor is one of its protein components, but exclusively consists of a distinct group of extrinsic proteins which is bound to the membrane via calcium ions. The latter statement is confirmed by the possibility to remove EEP from the membrane by means of EGTA,

which has a very high affinity especially for calcium [3]. A similar calcium-dependent membrane binding has been described for delta-crystallin of chick lens [7]. The binding of EEP to the membranes is not sensitive to urea, but is effectively disrupted if calcium is removed. The incomplete extraction of EEP from urea-treated membrane preparations leaving a considerable fraction in the membrane [3] probably is the effect of insufficient removal of calcium ions rather than due to a second type of interaction of EEP with the membranes.

EEP is missing in SDS-gel patterns of membrane preparations in many studies as a consequence of the addition of EDTA to the homogenization buffers in order to avoid proteolytic degradation [4,8,9]. Comparison of the SDS-gel patterns of membranes obtained in the presence of EDTA (Fig. 2a, lane 6; Fig. 4a, lanes 6 and 7) with those prepared in EDTA-free medium (Fig. 2a, lane 2; Fig. 4a, lanes 4 and 5) does not reveal a detectable increase in the amount of the degradation products of the 25.5 kDa protein band. This indicates that no significant proteolytic degradation occurs, even if EDTA is not present.

Dunia et al. [10] sometimes observed a 34 kDa polypeptide as the only major protein component of their junction preparations isolated in EDTA-free buffer solutions. The absence of a 26 kDa polypeptide in these preparations probably is due to the addition of several enzymes during the membrane isolation rather than to activation of a Mg^{2+}-dependent protease associated with lens tissue [4].

The use of detergents in both isolation procedures results in an enrichment of the membrane preparations in penta-layer and other multi-layer structures (Fig. 3). If isolated in chelator-free, calcium-rich medium these membrane fractions contain EEP as one of the main protein components indicating that EEP might be bound to junction-like structures.

About the methods for preparing junction-like structures and about their architectural aspects no unanimity exists. Immunocytochemical experiments have shown a general distribution of the 26 kDa protein (MIP 26) in both unit and pentalaminar calf lens fiber membranes [11,12]. These findings, however, contrast with those recently published by Paul and Goodenough [13] who localized MIP 26 only on the cytoplasmic surface of trilaminar, and not on pentalaminar (junctional) membranes. Because of these confusing results and in view of the contamination of our junction-like membranes with trilaminar structures, further studies are needed to investigate whether unit plasma membranes and junctional structures exhibit a similar protein composition, as previously has been suggested [14].

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