IMMUNOCYTOCHEMICAL STUDY OF EZRIN IN CULTURED MADIN-DARBY CANINE KIDNEY CELLS

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ABSTRACT

Monoclonal antibody for ezrin was used to study the intracellular localization and distribution of ezrin in Madin-Darby canine kidney cells. Immunocytochemical study showed that ezrin was highly concentrated in microvillous and cell-to-cell adhesion sites. Using sorbitol gradient for cell fractionation, ezrin was mostly recovered in the plasma membrane-enriched fraction. Western blot analysis showed that the monoclonal antibody reacted only with the 81 K protein, which is one of ERM family proteins in MDCK cells. These data were consistent with immunofluorescence microscopic observation. Immunostaining pattern of cells using anti-ezrin mAb was very similar to that for F-actin as shown by a double-labelling technique. Treatment of the cells with cytochalasin B revealed disruption of the intracellular distribution of F-actin and ezrin.

Ezrin, an 81 kDa cytoskeletal protein isolated from chicken intestinal microvillous core (4), is present in actin-containing surface structures in a wide variety of cultured cells (5, 9, 19, 20). Recent studies have revealed that ezrin forms a family of proteins closely related with moesin and radixin (8, 22, 24). Sequence analysis has shown that these proteins are highly homologous. The ERM family potentially has an important role at the cortical cytoskeleton and may participate in the regulation of several cellular functions (2, 12). Using transient expression of the protein or of its truncated forms in mammalian cultured cells, Algrin *et al.* (1) showed that the NH₂-terminal domain of ezrin located near the membrane and the COOH-terminal part containing the alpha-helical domain was colocalized with actin filaments. These results suggest that ezrin links the cytoskeleton to the plasma membrane. However, the different distribution pattern of ezrin between apical and cell-to-cell adhesion in cultured cells has been reported (3, 6, 23). We are currently investigating the organization of microfilaments during the establishment of polarity in MDCK cells. These cells form a well polarized epithelium and developed apical microvilli and cell-to-cell adhesion when they reach confluency in

tissue culture. In fully polarized cells, the actin microfilaments are arranged along the apical and basolateral domains. In this work, we have further investigated histochemically and biochemically the localization of ezrin and actin filaments in isolated cells and in confluent cells using a monoclonal anti-ezrin antibody (mAb).

MATERIALS AND METHODS

Cells and Antibodies

MDCK cells were grown in plastic tissue culture dishes (Falcon) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum under humid atmosphere of 5% CO₂ at 37°C.

The monoclonal antibody against ezrin raised against a synthetic peptide comprising the region 575-585 of human ezrin, which was previously characterized (5, 10), was obtained from Chemicon International CA (Catalog No: MAB 3056). Anti-radixin polyclonal antibody, pAb-I1 (22), was a gift from Dr S. Tsukita.

Immunofluorescence Microscopy

For indirect immunofluorescence microscopy, cells

were grown on glass-bottomed chamber slides (Nunc) or glass coverslips plated on cultured dishes. In the case of cytochalasin B treatment, the cells were incubated with 1.0 μ M cytochalasin B in foetal bovin serum-free medium at 37°C for 1 h. The cells were washed in PBS and fixed in 4% paraformaldehyde/PBS (pH 7.4) for 20 min at room temperature. Then they were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After treatment with 1% BSA/PBS, the cells were incubated with anti-ezrin mAb diluted 1:100 for 60 min at room temperature. After washing with PBS, the samples were reacted with a second antibody, FITC-conjugated goat anti-mouse IgG, in 1% BSA/PBS for 1 h. For fluorescent double-labelling of F-actin and ezrin, the cells were incubated with a mixture of TRITC-labelled phalloidin and the FITC-conjugated second antibody. Specimens were then washed with PBS and viewed under a confocal laser microscope (Bio Rad MRC-600) or by fluorescence microscopy (Zeiss). In confocal laser microscope studies, optical sections 0.4 μ m in thickness were examined with an argon ion laser.

Subcellular Fractionation of the MDCK Cells

MDCK cells, cultured to confluence, were scraped directly into 2.5 ml isolation buffer, which contained 5% sorbitol, 5 mM histidine-imidazole buffer (pH 7.5), and 0.2 mM PMSF, and then homogenized with a Silverson homogenizer. Procedures for differential and density gradient centrifugation were similar to those described by Mircheff (17). Briefly, the homogenate was centrifuged at $2,000 \times g$ for 10 min at 4°C. The supernatant was saved and the pellets were again homogenized and centrifuged. The combined supernatant was mixed with 87.4% sorbitol, and the sample was made 55% with respect to sorbitol. The sample was loaded onto a hyperbolic gradient of 26.5%–55% and of 55%–70% sorbitol. The sample was centrifuged at 26,000 rpm for 5 h in a Beckman SW27 rotor and was collected in 12 equal fractions.

Enzyme Assays

Each fraction was assayed for Na⁺,K⁺-ATPase, NADPH-cytochrome *c* reductase, and protein concentration. Na⁺,K⁺-ATPase activity was determined as K⁺-dependent *p*-nitrophenyl-phosphatase activity as described by Murer *et al.* (18). Protein was determined by the Lowry method (15) with

bovine serum albumin as standard. NADPH-cytochrome *c* reductase and succinate dehydrogenase were determined with the procedures used in previous studies (16). All biochemical determinations were performed in duplicate.

SDS-Gel Electrophoresis and Immunoblotting

Total cell homogenates and subcellular fractions were collected and were electrophoresed in 9% SDS-polyacrylamide gels as described by Laemmli (13). Proteins were transferred to the nitrocellulose filters electrophoretically. The filters were incubated with 1% BSA/PBS (pH 7.4) and then with a primary antibody in 10 mM phosphate buffer (pH 7.4), 150 mM NaCl and 0.05% Tween 20. After several washes, the filters were incubated with 2 μ g/ml peroxidase-conjugates goat anti-mouse IgG to develop a color.

RESULTS

Ezrin Was Located at Cell-to-Cell Adhesion Sites, as well as in the Microvilli of MDCK Cells

Anti-ezrin monoclonal antibody was used in immuno-microscopical study of the antigen in cultured MDCK cells. For analysis of the cellular distribution of ezrin in MDCK cells, immunocytochemical examinations were done using laser confocal fluorescence microscopy (Fig. 1). The microscope was focused at three optical sections through the cultured MDCK cells that were stained for ezrin. Ezrin was enriched in apical microvilli (Fig. 1A) and also highly concentrated at the upper-lateral membrane domains and cell-cell adhesion sites (Fig. 1, A and B). However, fluorescence of the lower lateral, i.e., more basal, area was reduced (Fig. 1C). Staining the cells with an FITC-labelled second antibody only did not show any positivity at the apical microvillous membrane (data not shown).

Fig. 1 Confocal laser microscopic analysis of MDCK cells at three focal planes, upper surface (A, apical area, $\times 600$), cell-cell contacts level (B, lateral area, $\times 600$) and substratum level (C, basal area, $\times 600$) stained with anti-ezrin mAb. The ezrin is concentrated in the microvilli (arrowheads in A) and at the cell-cell adhesions sites (arrowheads in B). Note the immunofluorescence decreased at cell-cell contacts, and faintest in basal areas (C).

Fig. 1

Fig. 2 Staining of F-actin and ezrin with TRITC-phalloidin and with FITC-conjugated goat anti-mouse IgG, respectively. Double labelling, A: FITC-labelling anti-ezrin antibody ($\times 400$). B: TRITC-labelling phalloidin ($\times 400$). Ezrin colocalized with F-actin present in the plasma membrane at cell-cell adhesions sites (arrowheads in A) and apical (arrowheads in B).

Colocalization of Ezrin with F-Actin

To determine whether ezrin was colocalized with F-actin in cultured MDCK cells, we used an FITC-conjugated second antibody to stain for ezrin and TRITC-phalloidin to stain for F-actin. Roughly overlapping staining patterns were obtained for ezrin and F-actin (Fig. 2). Ezrin was present in close vicinity to the plasma membrane at apical and lateral sites where the actin filaments were abundant. Treatment of the cells with $1.0\ \mu\text{M}$ cytochalasin B for 1 h disturbed the distribution of phalloidin and antibody staining (Fig. 3). The parallel distribution of ezrin with F-actin was disrupted with $1.0\ \mu\text{M}$ cytochalasin B (Fig. 3, B and D).

Subcellular Fractionation of MDCK Cells and Western Blot Analysis for Ezrin

SDS-gel electrophoresis and Western blot analysis of the homogenate from cultured MDCK cells showed that the monoclonal antibody for ezrin specifically reacted with a single band at 81 kD (Fig. 4). On the other hand, a polyclonal antibody for radixin revealed two positive bands, one was ezrin and the other radixin, which was slightly smaller in molecular mass than ezrin as shown in Fig. 4.

Subcellular fractions from MDCK cells were collected as described in Materials and Methods. Each fraction was collected and enzyme activities were determined. The distribution of Na^+, K^+ -ATPase, a marker for basal-lateral plasma membrane, and NADPH-cytochrome *c* reductase, a marker for endoplasmic reticulum membrane, are shown in Fig. 5 (upper panel). The Na^+, K^+ -ATPase peaked in fractions 3 and 4. These findings suggested that the fractions 3 and 4 were enriched in basal-lateral membranes, although these fractions were not free from contamination with intracellular membranes, including endoplasmic reticulum and Golgi membranes. The result of immunoblotting with anti-ezrin mAb is shown in Fig. 5 (lower panel). Densitometric scanning analysis demonstrated that ezrin was enriched in fractions 2, 3 and 4, being highest in the fraction 3 (Fig. 5).

DISCUSSION

Studies of the ERM family (22, 24) have suggested that at least one of these members is concentrated at specific regions where actin filaments are closely associated with plasma membranes (3, 6, 22). Recent experiments with antisense oligonucleotides have shown that these ERM family members were involved in the formation of cell-to-cell and cell-

Fig. 3 Effect of cytochalasin B on the localization of ezrin and F-actin in MDCK cells. A, B: Staining of ezrin, the cells were treated with (A, $\times 200$) or without (B, $\times 200$) $1.0\mu\text{M}$ cytochalasin B. C, D: Phalloidin staining, the cells were treated with (C, $\times 600$) or without (D, $\times 600$) $1.0\mu\text{M}$ cytochalasin B. Phalloidin staining was significantly affected with cytochalasin B. Nonspecific fluorescence aggregations were seen in cells treated with cytochalasin B (B), decrease in phalloidin staining at lateral area was observed (D).

to-substrate adhesion and microvilli (23). The present study indicated that anti-ezrin mAb reacted strongly in these regions where actin filaments have a tight association with plasma membranes. Other researchers have reported that the ezrin mAb used in our current study recognized several bands around 81K in immunoblot analysis of some cultured cells (5, 9). Since highly homologous ERM family were coexpressed in various types of cells and tissues, the ezrin mAb could recognize several bands around 81K. As shown in this study, pAb-II antibody recognized two bands (Fig. 4; 81K and 75K) in MDCK cells, suggesting the presence of two members at least, ezrin and moesin estimated

from molecular mass. However, the mAb recognized only one band of 81K and did not cross-react with other proteins in MDCK cells. Western blot analysis showed that the antibody recognized only 81K band in some other cells, LLC-PK₁ cells (derived from pig kidney) and mouse stomach cells (data not shown).

Confocal analysis showed that the expression of ezrin in the lower lateral or basal area was weaker than that at the apical surface and upper lateral adhesions. By Western blot analysis of subcellular fractions obtained from MDCK cells, we found that ezrin was concentrated in plasma membrane-enriched fractions, in agreement with the immuno-

Fig. 4 Immunoblot analysis of the MDCK cell extract by using the antibodies. Cell homogenate was SDS-gel-electrophoresed on 9% gel and was transferred onto a nitrocellulose. Then the filters were immunostained with anti-ezrin monoclonal antibody and anti-radixin polyclonal antibody. Only one positive band was recognized at 81 K with the anti-ezrin antibody, whereas the other 75 K band was also found with pAbI1, anti-radixin polyclonal antibody.

cytochemical findings. However, widespread distribution of ezrin was also found in other fractions, including the soluble fraction (data not shown). Recent data support the hypothesis that the ezrin protein family functions as membrane-cytoskeleton linking proteins (1, 10, 23). This concept arose because of the observation that the NH₂-terminal half of ERM members was similar to the NH₂-terminal half of band 4.1 protein (7, 9, 11, 14, 21, 23), and the ERM members were characterized by intracellular locations similar to actin filaments (3, 4, 6, 10, 19, 22, 24, 25).

In the present study with cultured MDCK cells, it was found that ezrin was present in close vicinity to the plasma membrane and actin filaments. However, treatment of the cells with cytochalasin B caused degradation of cellular distribution of F-actin and ezrin. This suggests that ezrin might be important in maintaining lateral and surface polarity in MDCK cells by tethering specific membrane proteins to the actin cytoskeleton.

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Fig. 5 Cell fractionation of MDCK cells and Western blot analysis. Upper panel: Sorbitol density-gradient was carried out as described in Materials and Methods. ●, Na,K-ATPase; □, cytochrome *c* reductase; ▲, densitometric recording of data shown in the lower panel. Lower panel: Each fraction was applied on SDS-PAGE (9% gel) and proteins were transferred onto a nitrocellulose. Then the nitrocellulose membrane was immunostained with anti-ezrin antibody. Ezrin was mainly recovered in fractions 2, 3 and 4, being most abundant in the fraction 3. Molecular mass standards in kilodaltons are shown on the right.

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