Disruption of Circumferential Actin Filament Causes Disappearance of Occludin from the Cell Borders of Rat Hepatocytes in Primary Culture without Distinct Changes of Tight Junction Strands

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ABSTRACT. We investigated the relationship of actin filament organization to occludin and tight junction strands in primary cultured rat hepatocytes using an actin depolymerizing agent, mycalolide B. In control cultures, well-developed circumferential actin filaments and occludin immunoreactivity were observed on the most subapical plasma membrane of the cells, and tight junction strands formed well-developed networks in freeze-fracture replicas. In hepatocytes treated with 3 μ M mycalolide B for 6 h, circumferential actin filaments and occludin immunoreactivity disappeared from the cell borders. However, there were no marked abnormalities of tight junction strands in freeze fracture replicas. Similar results were obtained from cells cultured in medium with 0.05 mM Ca^{2+} for 6 h. The close association of occludin with actin and the existence of intact tight junction strands that are virtually free of both occludin and actin suggest a physiological role of occludin, but not the other proteins forming the tight junction strands, in the linkage between actin cytoskeleton and tight junction.

Key words: tight junctions/occludin/actin filament organization/mycalolide B/low Ca^{2+} /primary rat hepatocytes

Tight junctions, the most apical component of intercellular junctions, divide the apical cell surface from the basolateral cell surface domain to create cell polarity and form the major barriers to solute and water flow through the paracellular space (11, 12, 29). Tight junctions have a particular net-like meshwork fibrils (31) and several peripheral and integral membrane proteins have been localized to tight junctions (4, 24). Tight junctions are now considered to be regulated by perijunctional actin (24).

Occludin is a 65 kD integral membrane protein lo-

cated at tight junctions (3, 9). It has been shown to localize to junctional strands by immunogold labeling of freeze-fracture replicas of the junctions (7). Occludin was first identified in the chicken using monoclonal antibodies, and its cDNA was cloned and sequenced (9). Recently, mammalian occludin was also reported (1). On the other hand, several peripheral membrane proteins such as ZO-1, ZO-2, ZO-3, cingulin and 7H6 antigen are also localized at tight junctions (2, 6, 11, 32, 35). It is known that the cytoplasmic tail of occludin binds to ZO-1 and ZO-2 (10), and actin filaments from the perijunctional ring attach directly onto the cytoplasmic surface of tight junctional contacts where occludin may be located (13). Furthermore, it is reported that a low Ca^{2+} concentration in the medium changes the distribution and phosphorylation of occludin in MDCK cells (28). Very recently, however, new membrane proteins of tight junction, claudins, were disclosed (8) and these proteins are suggested to form tight junction strands without occludin (27). Thus, the physiological roles of occludin in the formation of tight junctions be-

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Abbreviations: DMSO, dimethylsulfoxide; HBSS, Hanks balanced salt solution; BSA, bovine serum albumin; EGF, epidermal growth factor; PBS, phosphate-buffered saline; RT, room temperature; FITC, fluorescein isothiocyanate; DTT, dithiothreitol; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine.

come unclear again.

Mycalolide B was isolated from the marine sponge *Mycale* sp. It has antifungal and cytotoxic effects, and also inhibits Mg^{2+} -ATPase activity of native actomyosin (14). Furthermore, it was recently reported that mycalolide B quickly depolymerized F-actin but did not accelerate actin polymerization, while cytochalasin D accelerated actin nucleation and depolymerized F-actin at a slower rate (26). Accordingly, it is thought that mycalolide B may serve as a novel tool for analyzing actin-mediated cell function.

We previously showed that gap junction proteins connexin 32 (Cx32) and connexin 26 (Cx26) in primary cultures of rat hepatocytes are reexpressed and maintained in a medium containing epidermal growth factor (EGF) supplemented with 2% dimethylsulfoxide (DMSO) and 10^{-7} M glucagon (16, 17). Simultaneously, well-developed tight junction strands were observed in freeze-fracture replicas as well as gap junctions (18). In the present study, we examined changes of occludin expression and tight junction strands by disrupting actin filament organization in cultured hepatocytes using mycalolide B and a low- Ca^{2+} medium, clearly showing dependence of occludin localization at the cell borders on actin and independence of the strand integrity from actin and Ca^{2+} under certain conditions.

Materials and Methods

Isolation and culture of rat hepatocytes

Male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) weighing about 300–400 g were used to isolate hepatocytes by the two-step liver perfusion method of Seglen (30) with some modifications. Briefly, the liver was perfused in situ through the portal vein with 150 ml of Ca^{2+} , Mg^{2+} -free Hanks balanced salt solution (HBSS) supplemented with 0.5 mM EGTA (Sigma Chemical Co., St. Louis, MO, USA), 0.5 mg/L insulin (Sigma) and antibiotics. After the initial brief perfusion, the liver was perfused with 200 ml of HBSS containing 40 mg of collagenase (Yakult Co., Tokyo, Japan) for 10 min. The isolated cells were purified by Percoll iso-density centrifugation (20). Viability of the cells by the trypan blue exclusion test was more than 90% in these experiments. The cells were suspended in L-15 medium (GIBCO BRL, Gaithersburg, MD, USA) with 0.2% bovine serum albumin (BSA; Seikagaku Kogyo Co., Tokyo, Japan), 20 mM HEPES (Dojindo, Kumamoto, Japan), 0.5 mg/L insulin (Sigma), 10^{-7} M dexamethasone (Sigma), 1 g/L galactose (Sigma), 30 mg/L proline (Sigma), and antibiotics. The isolated hepatocytes were plated on 35 mm and 60 mm culture dishes (Corning Glass Works, Corning, NY, USA), which were coated with rat tail collagen (500 μ g of dried tendon/ml of 0.1% acetic acid) (23), and placed in a 100% air incubator at 37°C. Two to three hrs after plating, the medi-

um was changed to L-15 medium supplemented with 0.2% BSA, 20 mM HEPES, 0.5 mg/L insulin, 10^{-7} M dexamethasone, 1 g/L galactose, 30 mg/L proline, 20 mM $NaHCO_3$, 5 mg/L transferrin (Wako Pure Chemical Inc., Osaka, Japan), 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 0.5 mg/L $FeSO_4 \cdot 4H_2O$, 0.75 mg/L $ZnSO_4 \cdot 7H_2O$, 0.05 mg/L $MnSO_4$, 5 μ g/L Na_2SeO_3 , 10 ng/ml EGF (Becton-Dickinson Labware, MA), and antibiotics. The cells were then placed in a humidified, 5% CO_2 : 95% air incubator at 37°C. The medium was replaced with fresh medium every other day, and 2% DMSO (Aldrich Chemical Co., Inc., Milwaukee, WI, USA) and 10^{-7} M glucagon (glucagon novo, Yamanouchi, Tokyo, Japan) were added to the medium after 96 h of culture (17). The cells were maintained until day 10 and then treated with 3 μ M mycalolide B (Wako Pure Chemical Inc.) for 3 and 6 h. Some cells cultured in normal Ca^{2+} medium (1.8 mM $CaCl_2$) were washed three times with PBS and then transferred to the low- Ca^{2+} medium (0.05 mM $CaCl_2$) for 3 and 6 h.

Immunofluorescence Microscopy

Hepatocytes grown on type I collagen-coated glass coverslips (BIOCOAT, Becton-Dickinson Labware) were fixed with acetone for 30 min at $-20^\circ C$. After rinsing with PBS, the coverslips were incubated with a polyclonal anti-human occludin antibody (ZYMED, South San Francisco, CA, USA) at room temperature (RT) for 1 h. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (DAKO, Copenhagen, Denmark) at RT for 1 h. Some coverslips were incubated with rhodamine-phalloidin (Sigma). All samples were examined with a Nikon Fx epifluorescence photomicroscope (Nikon, Tokyo, Japan).

Western blot analysis and densitometry analysis

The dishes were washed with phosphate-buffered saline (PBS) twice and 1 ml of the buffer (1 mM $NaHCO_3$, 2 mM PMSF [Sigma] and 2 mg/L leupeptin [Sigma]) was added to 60-mm dishes. The cells were scraped and collected to eppendorf tubes and then sonicated for 30 sec. The sonicates were centrifuged at $4,500 \times g$ for 10 min. The final pellets were resuspended in Laemmli sample buffer (21) without dithiothreitol (DTT). Some of the resuspended samples were incubated for 1 h at 37°C in the presence of 5 units of calf alkaline phosphatase (Takara, Kyoto, Japan). For control reactions conducted in the presence of the phosphatase inhibitor, 10 mM sodium orthovanadate was added to the phosphatase reaction buffer. The protein concentration of the samples was determined using a protein assay kit (Bio-Rad, Richmond, CA). Twenty μ g of protein of each sample was treated with DTT (final concentration of 100 mM), boiled for 3 min and then loaded on 4–20% SDS-polyacrylamide gel (Daiichi Pure Chemicals Co., Tokyo, Japan). After electrophoretic transfer to a nitrocellulose membrane (Bio-Rad) using semi-dry blotting for 6 h (0.65 mA/cm²), the membrane was stained with Ponceau S (Sigma) and photographed. Thereafter, the

membrane was saturated overnight at 4°C with a blocking buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20, 4% skim milk) and incubated with a polyclonal anti-human occludin antibody (ZYMED) at RT for 2 h. The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA)

and 3,3'-diaminobenzidine (DAB) was used as a substrate.

Freeze-fracture analysis

For the freeze-fracture technique (34), cells cultured on cover slips were immersed in 40% glycerin solution after fixation in

Fig. 1. Localizations of actin, occludin, and ZO-1 in cultured hepatocytes; control (a, d, g), mycalolide B (b, e, h), low-Ca²⁺ condition (c, f, i); actin (a, b, c), occludin (d, e, f), ZO-1 (g, h, i). $\times 440$.

2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.3). The specimens were mounted on a copper stage, cooled in liquid nitrogen and fractured at -170°C to -180°C in a JFD-7000 freeze-fracture device (JEOL Ltd., Tokyo, Japan). Platinum-carbon replicas were made without etching. After thawing, the replicas were floated on filtered 10% sodium hypochlorite solution for 10 min in a Teflon dish. Replicas were washed in distilled water for 30 min, mounted on copper grids and examined at 80 KV with a JEM transmission electron microscope.

Results

Immunofluorescence microscopy

In control cultures at day 10, well developed circumferential actin filaments were observed (Fig. 1a), as we previously reported (Kojima *et al.*, 1997). In the hepatocytes treated with mycalolide B for 6 h, almost all circumferential actin filaments disappeared (Fig. 1b). In the hepatocytes cultured in low- Ca^{2+} medium for 6 h, circumferential actin filaments were unclear (Fig. 1c). Fluorescent immunocytochemistry was carried out to examine the changes in localization of occludin and ZO-1 in the hepatocytes. In the control at day 10, occludin and ZO-1 immunoreactivities were strongly observed as lines between adjacent cells (Figs. 1d and 1g). In the hepatocytes treated with mycalolide B at 6 h, almost all occludin immunoreactivity disappeared (Fig. 1e), while ZO-1 was observed as short broken lines on the cell borders (Fig. 1h). In the hepatocytes cultured in low- Ca^{2+} medium for 6 h, occludin immunoreactivity disappeared from the cell borders and was observed as small granular structures around nuclei in the cytoplasm (Fig. 1f). ZO-1 was observed on a small part of the cell border (Fig. 1i).

E-cadherin completely disappeared in the low- Ca^{2+} culture (data not shown). No increase of transaminase leaking from the cultures was observed during either experiment (data not shown).

Western blot analysis of occludin protein

Figure 2 shows the changes of occludin protein in Western blot analysis. In the control cultures at day 10 (control), a broad band above the occludin protein (65 kD) was observed as previously reported (28). In the hepatocytes treated with mycalolide B at 3 h and 6 h, no differences were observed compared to those of control (Fig. 2).

Freeze-Fracture

Ultrastructural analysis of the cultured hepatocytes was carried out using the freeze-fracture technique. In the control hepatocytes at day 10, typical tight junction

Fig. 2. Western blot analysis for occludin protein in the hepatocytes treated with mycalolide B.

strands, interconnected networks of parallel strands with cross-bridges, were observed on freeze-fracture replicas (Fig. 3a). In the hepatocytes treated with mycalolide B and cultured in low- Ca^{2+} medium for 6 h, virtually normal-looking tight junctions composed of typical strands were observed on the luminal surfaces (Fig. 3b and c) though circumferential actin had completely disappeared. Additionally, the fragmentation and internalization of tight junction in the cells in low- Ca^{2+} medium for 6 hrs were observed, but the incidence was very low. The strands without any marked breakdown ran parallel to the interconnections, clearly showing that the morphological maintenance of tight junction integrity always requires neither actin nor Ca^{2+} under our condition.

Discussion

To determine the relationships among actin, occludin and tight junction strands, DMSO/glucagon-treated hepatocytes with high expression of occludin protein, networks composed of many typical tight junction strands and well developed circumferential actin filaments (18, 19) were treated for 6 hrs with either a novel actin depolymerizing agent, mycalolide B, or cultured in low- Ca^{2+} medium. Drastic changes in actin filament organization and the disappearance of occludin from the cell borders were induced by these treatments, while there were no marked abnormalities of tight junction strands. Our results show that the localization of occludin is more dependent on actin organization than the other tight junction membrane proteins forming the strands, suggesting a physiological role of occludin in the linkage between tight junction and actin cytoskeleton.

Many studies on tight junctions of cultured epithelial cells using cytokines and actin-disrupting drugs such as cytochalasin, have shown that any perturbation of perijunctional actin disrupts the paracellular barrier created by tight junctions (5, 22, 33). Recently, it was reported that the rho protein, one of the small GTP-binding proteins, regulates actin filament organization

Fig. 3. Freeze-fracture replicas of the hepatocytes treated with mycalolide B and cultured in low Ca^{2+} medium. (a): control (DMSO/glucagon-treated hepatocytes at day 10), (b): treatment with mycalolide B for 6 h, (c): in low Ca^{2+} medium for 6 h. Figures are the same magnification. a: $\times 13,000$, b: $\times 20,000$, c: $\times 18,000$.

and influences the organization and permeability of associated apical tight junctions (25), suggesting the dependence of tight junction integrity on actin organization.

In contrast, our results clearly showed that tight junction strands were well maintained free of both occludin and circumferential actin, though only under the limited conditions we used so far. Recent evidence

from transgenic ablation of the occludin gene indicated that occludin is not required for the structure and function of tight junctions (27). Claudins, novel integral membrane proteins of tight junctions, form tight junction strands, probably even when no occludin exists (8). These observations, consistent with our results, clearly show that occludin is not a major structural protein for the tight junction strand. However, it is possible that the dependence of tight junction integrity on actin organization results from a close association of occludin with actin.

It is now generally considered that ZO-1 stands between occludin and actin filaments (10), though the details have not been clarified. In the present experiment, low- Ca^{2+} culture and an actin-depolymerization agent partly erased ZO-1 on the cell border. However, circumferential actin bundles of the cells completely disappeared as a result of the treatments. Since ZO-1 is found at both tight and adherens junction in hepatocytes (15), ZO-1 remaining on the cell border may bind to proteins forming tight junction strands, presumably resulting from the differences in binding affinities of ZO-1 to actin, occludin and the other proteins. Thus, further study is required to clarify the relationship between actin and tight junction proteins.

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