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Occludin induces microvillus formation via phosphorylation of ezrin in a mouse hepatic cell line

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ABSTRACT

Apical and basolateral cell membranes are separated by tight junctions (TJs). Microvilli are limited to the apical cell membrane. TJs and microvilli are the landmarks for epithelial cell polarity. However, the direct relationship between TJ proteins (TJPs) and the components of microvilli remains unclear. In this study, we investigated whether occludin, which is considered to be a functional TJP, is involved in microvillus formation. In occludin knockout mouse hepatic cells (OcKO cells), the microvillus density was less than that in wild-type (WT) cells and the length of microvilli was short. Immunoreactivity of ezrin was decreased in OcKO cells compared with that in WT cells. Although there was no change in the expression level of ezrin, phosphorylation of ezrin was decreased in OcKO cells. The microvillus density and the length of microvilli were increased in OcKO cells by transfection of full-length mouse occludin and COOH-terminal domains of occludin. These results suggested that occludin induced microvillus formation via phosphorylation of ezrin and that the COOH-terminal domain of occludin, which is localized in non-TJ areas, might be able to induce microvilli formation. Our results provide new insights into the function of occludin.

1. Introduction

Tight junctions (TJs) are the apicalmost components of the intercellular junctional complex in mammalian epithelial cells [1,2]. They regulate the paracellular transport of ions and solutes through the paracellular space as a semipermeable barrier [3,4]. They also separate the apical from the basolateral cell surface domains to establish cell polarity [5]. TJs are formed by not only integral membrane proteins such as claudins, occludin, tricellulin, and junctional adhesion molecules (JAMs) but also a variety of subcellular scaffolding proteins [6–9]. TJs also play a role in the control of homeostasis involving cell growth, differentiation and apoptosis via various signal transductions [10]. The tissue- and cell-specific expression pattern of TJ proteins (TJPs) determines these functions. Aberrant and abnormal expression of TJPs causes dysfunction of TJs and is closely associated with various diseases including carcinomas [11,12]. Carcinogenesis is accompanied by the disruption or loss of functional TJs.

Occludin is the first identified integral membrane protein of TJs [13]. It is the most ubiquitously expressed in epithelial TJs, being the most reliable immunohistochemical marker for TJs [14]. Overexpression of occludin in mammalian epithelial cells suggests that occludin contributes to the barrier function of TJs [13–15]. However, well-developed TJs that exhibited no barrier dysfunction can be formed in occludin-deficient embryonic stem cells and occludin-deficient mouse hepatocyte cell lines [16,17]. Occludin-null mice exhibit various phenotypes such as growth retardation, inflammation and hyperplasia of the gastric mucosa, loss of cytoplasmic granules in striated duct cells of the salivary gland, thinning of compact bone, brain calcification, testicular atrophy and deafness [18,19]. These phenotypes suggest that occludin may be involved in an unexpected role other than TJ functions. Occludin is a tetraspan membrane protein with two extracellular loops, a short intracellular turn and N- and COOH-terminal cytoplasmic domains [20,21]. Among these domains, the long COOH-terminal domain is rich in serine, threonine and tyrosine residues, which are frequently phosphorylated by various protein kinases [22,23]. The COOH-terminal domain directly binds to ZO-1, which in turn associates with the actin cytoskeleton. In addition, occludin interacts with transforming growth factor (TGF) -β type I receptor [18], nonreceptor tyrosine kinase

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c-Yes, atypical protein kinase C (PKC), PI-3 kinase, protein phosphatases 2 A, small GTPase RhoA, mitogen-activated protein kinase (MAPK), and Akt signaling pathways [17,24–31]. Occludin interacts with a variety of cellular signaling molecules and may be involved in the signal transduction. However, the physiological functions of occludin remain unclear. Although four isoforms of occludin are produced by alternative splicing [32], their tissue distribution and the biological function of each variant is also unclear.

Microvilli are actin-based structures found on the apical cell membranes of various epithelial cells. The core structure of microvilli is comprised of parallel actin filaments and numerous actin-binding proteins such as villin, espin, fimbrin, and myosins as well as ezrin/radixin/moesin (ERM) proteins [32]. Some transmembrane proteins such as ERM-binding phosphoprotein 50 (EBP50) are also known to be components of microvilli [33,34]. The ERM proteins are expressed in a tissue-specific manner, and ezrin is a critical component of epithelial microvilli [32,35,36]. In ezrin knockout mice as well as EBP50-deficient mice, shortened and irregular microvilli are observed in enterocytes [37–39]. Phosphorylation on specific threonine residues in the C terminus (T567/T564/T568 in ezrin/radixin/moesin, respectively) is thought to regulate a head-to-tail interaction of ERM proteins [40,41]. This reversible conformational change causes activation of ERM proteins and their interaction with other molecules [42]. Phosphorylation of ezrin is caused by various signal pathways and regulates the formation of microvilli, epithelial-mesenchymal transition, migration and invasion [42–46].

Atypical PKC interacts with the COOH-terminal domain of occludin. Atypical PKC consists of isoform ι (and the mouse homolog, PKCλ) and isoform ζ and they have high homology. Atypical PKC controls cell survival via the PI-3 kinase signaling pathway, MEK and MAPK signaling pathways and NF-κB signaling pathway [47–50]. In addition, atypical PKC has critical roles in cell differentiation by polarity-dependent and independent mechanisms [51]. It was reported that PKCι/λ phosphorylates ezrin in epithelial cells [52–55].

All epithelial cells can polarize to generate morphological distinct regions at the cell membranes. For example, the apical and basolateral cell membranes separated by TJs have distinct protein and lipid compositions, and microvilli are limited to the apical cell membrane. TJs and microvilli are the landmarks for epithelial cell polarity. However, the direct relationship between TJPs and the components of microvilli remains unclear. In this work, we undertook to confirm the hypothesis that occludin as a functional TJP is involved in the formation of microvilli in epithelial cells.

2. Materials and methods

2.1. Antibodies and reagents

Mouse monoclonal anti-occludin and rabbit polyclonal anti-occludin antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Rat monoclonal anti-ezrin, radixin, and moesin were purchased from IDIA Co., Ltd. (Tokyo, Japan). Rabbit polyclonal anti-EBP50 antibody and rabbit polyclonal anti-actin antibody were purchased from Sigma Chemical Co. (St Louis, MO). Mouse monoclonal anti-aPKCι/λ antibody was purchased from BD Transduction Laboratories (San Jose, CA). Rabbit polyclonal anti-aPKCζ antibody was purchased from Merck (Kenilworth, NJ, USA). Alexa 488 (green)-conjugated anti-rabbit and mouse IgG and Alexia 594 (red)-conjugated anti-mouse IgG antibodies were purchased from Molecular Probes Inc. (Eugene, OR). Horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgG was obtained from DAKO (A/S, Denmark). An enhanced chemiluminescence (ECL) Western blotting system was obtained from Amersham Corp. (Buckinghamshire, UK).

2.2. Cell lines and cell culture

Immortalized mouse hepatic cell lines were established from primary cultures of occludin knockout hepatocytes (termed OcKO-1, −2 and −3) and wild-type hepatocytes (termed WT-1, −2 and −3) as we previously described [17]. B16F10 cells (murine melanoma cells) and Detroit 548 cells (human fibroblasts) were purchased from ATCC (Manassas, VA). These cell lines were plated on 35 mm and 60 mm culture dishes (Corning Glass Works, Corning, NY, USA), which were coated with rat tail collagen (500 μg of dried tendon/ml 0.1% acetic acid) and were maintained with DMEM medium supplemented with 4% dialyzed fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) (Cansera International Inc.,Ontario, Canada) and antibiotics. The cells were incubated in a humidified 5% CO_2 incubator at 37 °C.

2.3. Transfection

Expression vectors of full-length murine occludin and occludin deletion mutants were used as we previously described [30]. Constructs of deletion mutants used to identify the gene regions responsible for cellular senescence were as follows: OcFL, full-length murine occludin cDNA (coding region from nucleotides 223 to 1788 [occludin 223–1788], encoding a 522-amino-acid polypeptide; GeneBank accession number, U49185); ΔC-S, occludin construct without 44 amino acids at the COOH-terminal end; ΔC-L, occludin construct without 158 amino acids at the COOH-terminal end; CD, occludin construct with only the cytoplasmic region cloned (coding region from nucleotides 1315 to 1788 [occludin 1315–1788], encoding a 158-amino-acid polypeptide). For transient transfection, OcKO-1 cells were transfected with 5 μg of each plasmid using LIPOfectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 5 h of incubation, the cells were transferred to DMEM medium containing 4% FBS. All cell cultures were maintained for 48 h after the transfection and were then examined by the following assay. Cells transfected with an empty vector were used as a control.

2.4. Electron microscopy

For transmission electron microscopy (TEM), cells were cultured on 60 mm dishes. The dishes were washed with phosphate-buffered saline (PBS), and the cells were scraped from the dishes and collected in microcentrifuge tubes with fixation by 2.5% glutaraldehyde in PBS overnight at 4 °C. After washing with PBS, they were postfixed in 1% osmium tetroxide (OsO_4) in PBS for 2 h. Samples were subsequently stained with uranyl acetate for 2 h at room temperature, washed, and dehydrated followed by embedding in Epon 812. Ultrathin sections were obtained with a Sorvall MT5000 ultramicrotome (DEDuPontUPOMT, New Castle, DE, USA). The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (JEM-1400; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV.

For scanning electron microscopy (SEM), cells grown on coverslips were fixed with 2.5% glutaraldehyde in PBS overnight at 4 °C. After several rinses with PBS, they were postfixed in 1% OsO_4 in PBS at 4 °C for 3 h and washed with distilled water followed by dehydration through a graded series of ethanol and freeze drying. Samples were sputter-coated with platinum and examined under a scanning electron microscope (JEM-1400; JEOL Ltd., Tokyo, Japan) operating at 10 kV.

The microvillus density in each cell was assessed as dense (3; c.f. Fig. 4A, OcFL), moderate (2; c.f. Fig. 4D, Detroit 548 CD), sparse (1; c.f. Fig. 4A, mock) or none (0; c.f. Fig. 4D, Detroit 548 mock) using SEM images. The ratios of the microvillus density were measured from at least fifteen separate SEM images (magnification: *800) using Adobe Photoshop CS4 extended version 11.0 (Adobe Systems Incorporated, USA). The lengths of microvilli were measured from at least 100 separate SEM images (magnification: *1200) using Image J version 1.47(National Institutes of Health, USA). The long microvilli lie