Phosphorylation of occludin correlates with occludin localization and function at the tight junction

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Wong, Vivian. Phosphorylation of occludin correlates with occludin localization and function at the tight junction. Am. J. Physiol. 273 (Cell Physiol. 42): C1859–C1867, 1997.—Multiple forms of occludin were found in Madin-Darby canine kidney (MDCK) cells. In the absence of cell-to-cell contacts, achieved by incubating cells in low-calcium growth medium, a cluster of lower-molecular-weight (LMW) occludin bands (−65,000−68,000) was present in both MDCK I and II cells. On formation of tight junctions, achieved by changing the low-calcium growth medium to normal-calcium growth medium, a cluster of higher-molecular-weight (HMW) bands (−72,000−75,000 for MDCK I cells and −70,000−73,000 for MDCK II cells) was also expressed. The HMW occludin bands could be eliminated by phosphatase treatment. Therefore, the HMW forms of occludin appeared to be the hyperphosphorylated product of the LMW forms. These HMW forms were Triton X-100 insoluble, which correlated with their localization at the tight junctions. Furthermore, depletion of tight junction-localized occludin by an occludin extracellular domain peptide (20) correlated with a decrease in the HMW forms of occludin. In conclusion, phosphorylation of occludin may be a mechanism by which occludin localization and function are regulated.

Madin-Darby canine kidney cells, strains I and II; “tight” and “leaky” tight junctions

THE TIGHT JUNCTIONS of epithelial and endothelial cells act as a physical permeability barrier that regulates the passage of ions and macromolecules through the paracellular pathway. This allows the separation of apical and basolateral compartments, resulting in the formation of biological barriers such as the blood-brain barrier and the blood-retinal barrier that are essential for the maintenance of the internal milieu in different tissues of the organism (16, 19). The tight junctions of epithelia of different tissues have different permeability properties to ions and macromolecules (15 A˚) (11). For example, the epithelia of the blood-brain barrier and blood-retinal barrier have high transepithelial electrical resistance (TER) (1, 10, 13, 18, 19), whereas the epithelia of the proximal tubule and gall bladder have low TER. The differences in the permeability properties of the tight junctions appear to be important for the respective physiological function of each epithelium.

The permeability of the tight junction has been proposed to correlate with its ultrastructure. In transmission electron microscopy (TEM) of freeze fracture replicas, the tight junction is seen as strands of intramembrane fibrils forming a network that circumscribes continuously and completely the apices of cells. These fibrils represent cell-to-cell contact points of adjacent plasma membrane that are seen in transmission electron micrographs of thin sections and that demarcate the regions where paracellular tracers such as horseradish peroxidase (HRP) were excluded. When the tight junction fibrils of epithelia with different TER were compared, the numbers and complexities of the organization of fibrils increased with increasing TER (5, 6). Therefore, it has been proposed that each fibril corresponds to a barrier and that an increasing amount and complexity of fibrils would create epithelia of increasing TER and decreasing paracellular permeability (5, 6).

However, the TER of an epithelium could also be changed without altering the number and organization of tight junction fibrils. The permeability of the tight junctions of intestinal epithelium has been shown to be increased by luminal glucose and amino acids after food intake without any observable changes in tight junction ultrastructure (2, 4). In addition, similar tight junction fibril structures were found in two strains of Madin-Darby canine kidney (MDCK) epithelial cells (MDCK I and II) that have very different TER (17). Therefore, it has been proposed that the permeability of the tight junctions could also be regulated within the fibrillar structure (17).

Tight junction fibrils have been shown to be regulated in various physiological processes such as leukocyte transmigration across an endothelium (14, 15). During these dynamic processes, the tight junction permeability barrier is temporarily disrupted but subsequently resealed. The resealing process is completed relatively quickly, usually within 1 h, suggesting that resealing is accomplished by assembly of preexisting elements rather than by resynthesis of new proteins. In addition, tight junction fibrils were found to be present after the permeability barrier was disrupted, supporting the notion that the tight junction sealing element is only temporarily uncoupled. To begin to understand the dynamics of the tight junction, it is important to understand the regulation of the sealing element of the tight junction permeability barrier.

Occludin, an integral membrane protein of the tight junction, has been shown to localize to the tight junction fibrils by TEM of immunogold-labeled freeze fracture replicas (9). Furthermore, occludin has been shown to be essential in the formation of the actual tight junction seal (3, 12, 20). Therefore, the element that is proposed to reside in the fibrils regulating the paracellular permeability could be the occludin protein.

The MDCK cells form a typical epithelial monolayer that has characteristic intercellular junctions, including the tight junctions. Two strains of MDCK cells (I and II) were found to form monolayers that have very different TER, thus providing a potentially useful system for the study of tight junction characteristics. The
difference in TER in MDCK I and II cells most likely reflects the distinct ion permeabilities of their tight junctions, because the electrical resistance of the plasma membrane is usually much higher than the electrical resistance of the paracellular pathway (5, 7, 8). Interestingly, MDCK I and II cells possess similar tight junction ultrastructures, as determined by TEM of thin sections and freeze fracture replicas (17). Both MDCK I and II cells form characteristic tight junction membrane contacts and intramembrane fibril strands that are indistinguishable between the two strains of cells. Therefore, it is possible that other factors, such as regulation of tight junction proteins such as occludin, play a role in determining the permeability properties of the tight junction.

To examine the regulation of occludin and its potential role in determining tight junction permeability properties, the basic biochemistry of occludin in MDCK I and II cells was studied.

METHODS

Cell culture, calcium switch assay, and measurements of TER. MDCK epithelial cell line strains I and II were gifts from Kai Simons (European Molecular Biology Laboratory). Cells were grown on Costar Transwell filters (Fisher, Santa Clara, CA) in Eagle’s minimum essential medium (MEM) supplemented with 5% fetal bovine serum and were maintained at 37°C and 5% CO₂. For the calcium switch assay, cells were initially grown to confluence in normal-calcium growth medium and were subsequently changed to low-calcium medium (calcium-free MEM supplemented with 10% dialyzed fetal bovine serum) for 48 h. At the end of the low-calcium switch, cell monolayers were replenished with normal-calcium medium and the formation of tight junctions was monitored by the generation of TER. The TER was measured directly in normal growth media in Transwell wells. A short, 4-µA current pulse was passed across the cell monolayer with the use of a pair of calomel electrodes via KCl salt bridges, and the voltage was measured by a conventional voltmeter across the same cell monolayer with the use of a pair of Ag-AgCl electrodes via KCl salt bridges. The TER was calculated from the measured voltage and was normalized by the area of the monolayer. The background TER of blank Transwell filters was subtracted from the TER of monolayers. Triton X-100 extraction, immunofluorescence microscopy, and antigen blotting. MDCK cells were grown on Costar Transwell filters (Fisher), and the TERs were measured before preparation of cells for indirect immunofluorescence microscopy and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For Triton X-100 (TX-100) extractions, cells were incubated in TX-100 extraction buffer (0.5% TX-100, 5 mM EDTA, and 0.15 M NaCl) for 3 h at 4°C. The TX-100-insoluble fraction of cells that was left attached to the Transwell filters was rinsed two times in 0.15 M NaCl and was subsequently processed for immunofluorescence microscopy and SDS-PAGE in parallel with nonextracted cells. For immunofluorescence, cells were fixed on filters with 100% methanol at -20°C for 30 min and were dried with 100% acetone at -20°C for 5 min. Filters were blocked with immunofluorescence staining buffer [1% nonfat dry milk in 0.5% TX-100, 5 mM EDTA, 0.15 M NaCl, and 20 mM N-2-hydroxyethylpiperazineN’-2-ethanesulfonic acid (HEPES), at pH 7.0] before incubation with primary antibodies. Rabbit anti-occludin antibodies were raised against a glutathione S-transferase (GST) fusion protein of the cytoplasmic domain of chick occludin (255–510 amino acids). Fluorescein isothiocyanate-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR). For SDS-PAGE, cells were extracted directly in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2.5 mM EDTA, 15% sucrose, 2% SDS, and 50 mM dithiothreitol) containing protease inhibitors [5 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml pepstatin A, 1 µg/ml Na-p-tolyl-l-lysine chloromethyl ketone (TLCK), 10 µg/ml leupeptin, 20 µg/ml aprotinin, 50 µg/ml antipain, 2 mM benzamidine, 50 µg/ml soybean trypsin inhibitor, and 2.5 mM iodoacetamide]. SDS-PAGE samples were boiled for 10 min and were cooled to room temperature before addition of iodoacetamide to achieve a final concentration of 125 mM. All SDS-PAGE were performed with 4–15% gradient gels. Western blots for occludin were done using the same primary antibodies as in immunofluorescence microscopy. Rabbit anti-ZO-1 (10153) and anti-ZO-2 (9989) antibodies were gifts from D. Goodenough. Rabbit anti-cingulin antibodies were gifts from S. Citi. Secondary antibodies conjugated with HRP (Bio-Rad) were developed by enhanced chemiluminescence (Amersham Life Sciences, Arlington Heights, IL). For blot competition, 20 µg/ml of the antigen (GST-occludin cytoplasmic tail) or 20 µg/ml GST were added to the blots along with anti-occludin antibodies.

Metabolic labeling, immunoprecipitations, phosphatase treatment, and fluorography. MDCK cells were grown on 75-mm Transwell filters (Fisher). For study of the phosphorylation of occludin, each monolayer was labeled with 100 µCi [³²P]orthophosphate in phosphate-free medium (supplemented with 5% fetal bovine serum) for 22 h. Each cell monolayer was extracted for immunoprecipitation immediately at the end of the labeling period with 2 ml 1% SDS containing 5 mM EDTA and protease inhibitors (5 mM PMSF, 5 µg/ml pepstatin A, 1 µg/ml TLCK, 10 µg/ml leupeptin, 20 µg/ml aprotinin, 50 µg/ml antipain, 2 mM benzamidine, 50 µg/ml soybean trypsin inhibitor, and 2.5 mM iodoacetamide). Each sample was boiled for 10 min before addition of TX-100, deoxycholate, NaCl, and HEPES, to a final concentration of 0.2% SDS, 1% TX-100, 0.5% deoxycholate, 0.15 M NaCl, and 20 mM HEPES (pH 7.4). Immunoprecipitations were performed with protein A Sepharose (Sigma) in the presence of anti-occludin, as described, or in the presence of preimmune serum. Immunoprecipitates were washed four times in immunoprecipitation buffer before extraction for SDS-PAGE. Phosphatase treatments of occludin were done by incubating occludin immunoprecipitates (washed 4 times in immunoprecipitation buffer) with 50 µg of potato acid phosphatase (Calbiochem, San Diego, CA) in 1 ml of 10 M HEPES, pH 6.0, for 2 h at 25°C. Phosphatase-treated immunoprecipitates were directly extracted in sample buffer for SDS-PAGE. For metabolic labeling of occludin, each monolayer was incubated with 1 mCi [³⁵S]methionine in methionine-free medium (supplemented with 5% fetal bovine serum) for 22 h. Immunoprecipitation and sample preparation for SDS-PAGE were performed as described. Polyclonal antibodies were fixed with 50% methyl alcohol and 10% acetic acid for 1 h and were incubated in Amplify (Amersham Life Sciences) for 45 min before being dried under a vacuum. Dried gels were exposed to Hyperfilm-MP (Amersham Life Sciences) at -80°C.

Peptide synthesis and peptide treatment of cells. Peptides OCC1 (44 amino acids = DYYGYGLGAYTGLGFFGY-SNYYSGLSLYSYGGYGVVNQRT) and OCC2 (44 amino acids = GVNPQAMSSGYYPLACQAGYSTLYN-QYIYHYCTVDPOEQ) correspond to the entire first and second
putative extracellular domains of chick occludin, respectively. OCC2 was modified by covalent linkage to acetamidomethyl groups at the two cysteine residues (underlined) to prevent formation of disulfide bond(s). Peptides were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO) and were added to both sides of the Transwell bathing wells at a final concentration of 10 µM for 24 h. All peptides were synthesized by the Microchemistry Core Facility at the Memorial Sloan-Kettering Cancer Center.

RESULTS

Localization and expression of occludin in MDCK I and II cells. Localization of occludin in MDCK I and II cells was assessed by indirect immunofluorescence microscopy. MDCK cells were grown on Transwell filters to confluency, and TER was measured before the cells were fixed for immunofluorescence stainings (TER, 10,000 V cm⁻² for MDCK I cells and 50 V cm⁻² for MDCK II cells). Figure 1 shows that occludin was present at the tight junction in both MDCK I and II cells. However, the levels of stainings in the two strains of MDCK cells were not discernibly different. Therefore, it appears that the amount of occludin localizing at the tight junction is unlikely to account for the great difference in TER between MDCK I and II cells. Control immunofluorescence stainings for occludin of MDCK I and II cells that were devoid of cell-to-cell contacts (when the cells were grown in the low-calcium condition) are also shown in Fig. 1. Under the low-calcium growth condition, MDCK cells attached to the Transwell filters and grew to confluency as in normal-calcium growth conditions, although intercellular junctions were not formed. In the absence of tight junctions, occludin was not localized, which agrees with occludin localization studies in another epithelial cell line, A6 (20).

For further examination of the possible roles of occludin in determining TER, the basic biochemistry of occludin, including the expression levels and phosphorylation states, was analyzed. Polyclonal rabbit antibodies that were raised against the entire COOH-terminal cytoplasmic tail of chick occludin were used for antigen blots for occludin in MDCK cells. Figure 2A shows antigen blots for occludin of MDCK total cell lysates that were treated or not treated with the reducing agent dithiothreitol (see METHODS). Cell lysates were separated on a 4–15% gradient polyacrylamide gel before transfer to nitrocellulose paper. Multiple immunoreactive bands were observed at molecular weights of ~65,000–75,000 in both MDCK I and II cells, which were present in both the reduced and nonreduced samples, indicating that the multiple bands were not products of disulfide bond formation. As a control for specificity of occludin antibodies, antigen blots were performed in parallel using only secondary antibodies which show two lower nonspecific bands but not the clusters of occludin bands having a molecular weight of 65,000–75,000 (Fig. 2A). The specific band (small arrowhead) between the higher-molecular-weight (HMW)
and lower-molecular-weight (LMW) bands is not reproducible and is probably a degradation product of the HMW band.

To confirm that these multiple bands were antigenically related to chick occludin, antigen blot analysis was performed in the presence or absence of excess amounts of the immunogen (a GST fusion protein containing the COOH-terminal residues 255–510 of chick occludin) or GST only as control (Fig. 2B). Antibodies binding to the multiple occludin bands were successfully competed by the immunogen, indicating that these multiple bands were immunologically related to chick occludin cytoplasmic domain (Fig. 2B).

Comparison of antigen blots for occludin does not indicate significant differences in occludin expression levels in the two strains of MDCK cells (Fig. 2, A and B). In experiments in which total cellular protein was normalized between MDCK I and II cells, occludin expression levels between the two strains of cells were not significantly different. Therefore, the expression levels of occludin are also unlikely to account for the great differences in TER of MDCK I and II cells. However, if the multiple occludin bands are examined closely, a difference can be seen between MDCK I and II cells. Although the LMW clusters of occludin bands which migrated at molecular weights of ~65,000–68,000 were expressed in both strains of MDCK cells, the HMW clusters of occludin bands migrated differently (at molecular weights of ~72,000–75,000 in MDCK I cells and ~70,000–73,000 in MDCK II cells). These different HMW forms of occludin that are expressed in the two strains of MDCK cells might contribute to the formation of tight junctions that generate different TER.

Because the results suggest that neither the localization nor the expression levels of occludin could account for the great differences in TER in MDCK I and II cells, the functional significance of the HMW forms of occludin that are found in the two strains of MDCK cells was investigated.

HMW forms of occludin correlate with tight junction formation. For differentiation of the possible roles of these multiple forms of occludin, occludin expressions in MDCK I and II cells were studied before and after tight junction formation using the commonly used calcium switch assay (see METHODS and Fig. 2C). It is important to remark that the calcium switch experiments for the two strains of MDCK I and II cells were performed separately, and therefore comparison of occludin expression levels should be made only within each cell strain. Total protein loading for polyacrylamide gel electrophoresis was much lower for MDCK II cell lysates and had not been normalized to that of MDCK I cell lysates; therefore, occludin levels were much lower in the MDCK II cell samples. Antigen blot analyses of total cell lysates from MDCK I and II cells that were devoid of cell-to-cell contacts (achieved by growing cells in low-calcium medium) show that only the LMW (~65,000–68,000) forms of occludin were expressed (Fig. 2C). Both MDCK I and II cells barely expressed their respective HMW forms of occludin.

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**Fig. 2.** Multiple forms of occludin are present in MDCK I and II cells. A: antigen blots of total cell lysates. Clusters of lower-molecular weight (LMW) bands (~60,000–65,000) are present in both MDCK I and II cells. In addition, a cluster of higher-molecular weight (HMW) bands that are different in MDCK I and II cells are present (~72,000–75,000 in MDCK I cells and ~70,000–73,000 in MDCK II cells). These multiple occludin bands were found under reducing and nonreducing conditions, indicating that they were not products of disulfide bond formation. Control blot using only secondary antibodies (2oAb) was performed in parallel. B: multiple bands are competed by excess glutathione S-transferase (GST) occludin protein and therefore are immunologically related to chick occludin. Antigen blots of total cell lysates of MDCK I and II cells were performed in presence or absence of 50 µg/ml of antigen (GST-OCC, a GST fusion protein containing cytoplasmic tail of chick occludin) or GST alone. C: expression of HMW bands correlates with formation of tight junctions, whereas LMW bands are constitutively expressed in presence and absence of cell-to-cell contacts. Confluent MDCK monolayers that were incubated in low-calcium medium for 48 h (t = 0) were induced to form intercellular junctions by switching to normal-calcium medium for 2 days (t = 2d). Positive (+) controls were monolayers that were grown in parallel but had not been subjected to calcium switch manipulation. TER, transepithelial electrical resistance.
when tight junctions were not present. However, when tight junctions were induced to form by switching of the low-calcium medium to normal-calcium medium for 48 h, both the LMW and HMW forms of occludin were expressed (TER changed from ~30 to ~3,300 Ω cm² for MDCK I cells and did not change for MDCK II cells, at ~30 Ω cm²). The pattern of bands that was induced after the calcium switch manipulation was essentially identical to that of control MDCK cells that were continuously maintained in normal calcium in a parallel experiment, demonstrating that the HMW bands induced by tight junction formation were the normally expressed forms of occludin. The expression of the HMW forms of occludin only in the presence of tight junctions suggests that the HMW forms that participate in the formation of the actual contact seal might be functionally significant. It is worth mentioning that the HMW forms of occludin in both strains of MDCK cells represent a minor fraction of total occludin forms in these cells and therefore were usually difficult to notice. However, when MDCK cells are grown on filter supports, the HMW forms of occludin are much more prominently expressed, as shown for the calcium switch experiments in Fig. 2C.

HMW forms are the hyperphosphorylated forms of occludin. To examine whether the multiple forms of occludin in MDCK cells are the result of expression of occludin isoforms or of posttranslational modifications of the occludin protein such as phosphorylation, the phosphorylation patterns of the occludin protein were analyzed. Immunoprecipitations of occludin from [32P]orthophosphate metabolically labeled cells showed that both the LMW and HMW forms of occludin were phosphorylated in MDCK I and II cells (Fig. 3). These multiple phosphorylated bands were not immunoprecipitated proteins of occludin because the cells were extracted directly in boiling 1% SDS to dissociate any occludin-binding protein from occludin; the bands therefore represented only the occludin protein. The HMW forms of occludin in both MDCK I and II cells appeared to be hyperphosphorylated compared with the LMW forms because the ratio of 32P activities per amount of occludin protein was much greater for the HMW forms than for the LMW forms (as indicated in a parallel experiment of [35S]methionine metabolically labeled cells) (Fig. 3). In that case, the HMW forms of occludin could be the hyperphosphorylated products of the LMW forms. Indeed, in vitro treatment of occludin immunoprecipitates with phosphatase showed that when most of the 32P radioactivity in the HMW forms was removed, the 35S-labeled HMW forms were also eliminated. In addition, antigen blot analyses of phosphatase-treated 32P-occludin and 35S-occludin. In addition, antigen blot analyses of 32P-labeled immunoprecipitates showed that dephosphorylation was sufficient to eliminate HMW forms of occludin protein. LMW bands of occludin were masked by co-migrated immunoglobin (IgG) band.

Fig. 3. HMW forms of occludin are hyperphosphorylated species of occludin. Autoradiograph (32P), fluorograph (35S), and antigen blot of phosphatase-treated (+) or untreated (-) occludin immunoprecipitates (IP). Phosphatase treatment eliminated HMW forms of both 32P-occludin and 35S-occludin. In addition, antigen blot analyses of 32P-labeled immunoprecipitates showed that dephosphorylation was sufficient to eliminate HMW forms of occludin protein. LMW bands of occludin were masked by co-migrated immunoglobin (IgG) band.

The solubility of the multiple forms of occludin in the nonionic detergent TX-100 was determined. Incubation of confluent monolayers of MDCK I and II cells with 0.5% TX-100 (see METHODS for details) fractionated the multiple forms of occludin into TX-100-soluble and TX-100-insoluble pools. The TX-100-soluble pool contains the LMW forms of occludin, whereas the TX-100-insoluble fraction contains the HMW forms and a small amount of the LMW forms (Fig. 4A). The results indicate that the LMW and HMW forms of occludin have different biophysical properties in the nonionic detergent TX-100. The TX-100-insoluble fraction of the HMW forms of occludin might reflect their incorporation into large protein complexes such as the cytoskeleton and/or junctional complexes.
in the TX-100-insoluble fraction in both MDCK I and II cells. Furthermore, the intensity of occludin stainings in both intact and TX-100-extracted cells was similar, suggesting that the TX-100-insoluble fraction of occludin indeed represented the tight junction-localized pool of occludin protein. Unfortunately, the TX-100-extractable pool of occludin was not detected by indirect immunofluorescence microscopy, which might be a result of lost proteins during methanol fixation procedures. If the LMW forms of occludin were not bound to other proteins to form a complex, the methanol fixation procedure, which depends on the inherent ability of proteins to precipitate in cold methanol, would not precipitate the LMW forms. Because methanol also solubilizes cell membranes, the methanol fixation procedure would probably wash away nonprecipitated LMW forms of occludin that reside in the plasma membrane or intracellular vesicles. Attempts to stain for occludin after formaldehyde fixation of cells to visualize the LMW forms were also unsuccessful. One possibility is that the polyclonal antibodies that were used were raised against chick occludin cytoplasmic tail. Therefore, only a fraction of the antibodies would recognize MDCK occludin. In that case, cross-linking of protein with formaldehyde might destroy the already low numbers of antibody recognition epitopes. A second explanation could be that the LMW forms of occludin were inaccessible to antibodies. Because the antibodies were raised to the cytoplasmic tail of occludin, the occludin binding protein that is specifically bound to the cytoplasmic tail of the LMW forms of occludin might mask antibody recognition sites. Nevertheless, the present results indicate that TX-100 insolubility correlates with occludin being incorporated into the tight junctional complex. Therefore, hyperphosphorylation of occludin might be a mechanism to regulate occludin localization and incorporation into tight junctions.

HMW forms of occludin participate in the formation of the tight junction permeability barrier. The expression of the HMW forms of occludin that correlates with the localization of occludin at tight junctions in the calcium switch experiments further suggests that the HMW forms of occludin are the junction-localized species (see Figs. 1 and 2C). To confirm that the junction-localized HMW forms of occludin indeed participate in the formation of the functional tight junction permeability barrier, the synthetic peptide OCC2 (corresponding to the second extracellular domain of chick occludin; see Ref. 20) that had been previously used to selectively deplete junctional occludin without disrupting other cytoplasmic tight junction proteins was used to deplete occludin in MDCK cells. Treatment of MDCK I cells (grown to confluency on Transwell filters) with OCC2 peptide decreased TER from $10,000$ to $200$ V cm$^{-2}$. Control peptide, OCC1, and solvent control, DMSO, had no effect on TER in parallel experiments. The decrease in TER in MDCK cells by OCC2 peptide is in agreement with the known mechanism of action of the OCC2 peptide, which is to specifically disrupt paracellular tight junction permeability by depleting junctional
occludin (20). Antigen blot analyses of total cell lysates from OCC2 peptide-treated and untreated MDCK I cells show that the HMW forms of occludin were much depleted, whereas the LMW forms were only slightly decreased (Fig. 5A). The reduction of the HMW forms of occludin was reproducible in three separate OCC2 peptide treatment experiments. For controls, two peripheral membrane proteins, ZO-1 and cingulin, were also blotted, and their expression levels were unaltered (Fig. 5A).

To examine whether the junction-localized occludin was indeed depleted by treatment with OCC2 peptide, MDCK I cells that were treated or not treated with the OCC2 peptide were analyzed by indirect immunofluorescence microscopy for occludin. Figure 5B shows that occludin was greatly reduced at the tight junctions after treatment with the OCC2 peptide in both MDCK I and II cells. These results confirm that the depletion of occludin at tight junctions correlates with a decrease in TER and a perturbation of the tight junction permeability barrier (20). Moreover, the specific depletion of the HMW forms of occludin by the OCC2 peptide suggests that the HMW forms and, to a much lesser extent, the LMW forms, are the functional and junction-localized species of occludin that participate in the formation of the actual permeability seal.

**DISCUSSION**

Occludin, an integral membrane protein of the tight junctions, plays an essential role in the formation of the tight junction paracellular permeability barrier (3, 12, 20). In this study, the potential function of occludin in determining the permeability properties of the tight junction was examined. Two strains of MDCK cells (MDCK I and II) that have very different TER were utilized for comparisons of expression levels, localization, and posttranslational modifications of the occludin protein. When total MDCK I and II cell lysates were analyzed by antigen blots, multiple forms of occludin with molecular weights of ~65,000-75,000 were observed. However, the overall expression levels of occludin in the two strains of MDCK cells were not very different. Both MDCK I and II cells expressed a cluster of LMW forms of occludin (~65,000-68,000) and a cluster of HMW forms of occludin (~70,000-75,000). The HMW forms of occludin in the two strains of MDCK cells reproducibly migrated at different molecular weights (~72,000-75,000 in MDCK I cells and ~70,000-73,000 in MDCK II cells). To examine the functional significance of these multiple occludin forms, the levels and phosphorylation states of occludin in cells that were devoid of cell-to-cell contacts (grown in low-calcium growth medium) were compared to those of cells that had already formed tight junctions (grown in normal-calcium growth medium). In both MDCK I and II cells, only the LMW forms of occludin were expressed in the absence of cell-to-cell contacts. However, on induction of tight junction formation, the respective HMW forms of occludin in both MDCK I and II cells were also expressed. These HMW forms of occludin in MDCK I and II cells could be eliminated by treatment of occludin immunoprecipitates with acid phosphatase in vitro and therefore appear to be the hyperphosphorylated products of the LMW forms. Detergent fractionation with the use of TX-100 showed that the LMW forms were mostly TX-100 soluble, whereas the HMW forms were TX-100 insoluble. The insolubility of the HMW forms of occludin suggests that they were biophysically different from the LMW forms, which might reflect the incorporation of the HMW forms of occludin into junctional complexes. When occludin was compared by indirect immunofluorescence microscopy in intact and TX-100-extracted cells, the localization and amount of occludin present at the tight junctions appeared to be similar, indicating that the portion of occludin that was localized to the tight junctions is likely to be accounted for solely by the TX-100-insoluble pool. Furthermore, depletion of junctional occludin by the addition of an occludin extracellular domain peptide (a method that has been previously used to deplete junctional occludin and disrupt tight junction barrier function (20)) greatly decreased TER and occludin at the tight junctions. The extracellular domain peptide
also depleted most of the HMW forms of occludin from the cells, although only a small fraction of the LMW forms was reduced. This demonstrates that the HMW forms of occludin are the functional forms of occludin that indeed participate in the formation of the tight junction barrier. In conclusion, the functional forms of occludin that localize to the tight junction appear to be represented mostly by the hyperphosphorylated HMW forms of occludin and, to a much lesser extent, the LMW forms of occludin. These results suggest that phosphorylation might be a mechanism by which occludin localization and function are regulated. In that case, the presence of different HMW forms of occludin in MDCK I and II cells might provide a possible explanation for the differences in TER and a potential role of occludin phosphorylation in dictating the permeability properties of the tight junction paracellular barrier.

The correlation between hyperphosphorylation of occludin and formation of tight junctions suggests that phosphorylation might be a mechanism to regulate occludin targeting, assembly, and/or function. Phosphorylation of occludin could result in stabilization of specific interaction(s) of occludin to occludin binding protein(s). A hypothetical model for the regulation of occludin function by phosphorylation is shown in Fig. 6. Two main pathways could be envisioned; one pathway involves recruitment of occludin from the plasma membrane, whereas the other pathway represents recruitment of occludin from intracellular vesicles. Three possible mechanisms could be used in the recruitment of occludin from the plasma membrane. Phosphorylation of occludin could result in polymerization of occludin in the plane of the plasma membrane and allow the formation of a continuous seal around the cells (A). Alternatively, occludin phosphorylation could stabilize the bindings of occludin extracellular domains and allow the formation of a functional paracellular permeability seal (B). In addition, phosphorylation of occludin could allow interaction of its cytoplasmic domain to tight junction proteins such as ZO-1 and ZO-2 and result in “trapping” of occludin at the tight junctions (C). The second pathway involves recruitment of occludin from intracellular vesicles, a mechanism that resembles docking of vesicles and that perhaps depends on interactions of occludin cytoplasmic tail to other tight junction proteins (D).

The hypothetical model in Fig. 6 that depicts localization of occludin at the apical membrane and intracellular vesicles is not supported by any substantial data. However, when OCC2 peptide was added apically to cells, a depletion of junctional and cellular occludin was observed. One explanation is that OCC2 peptide only recognizes junctional occludin that is accessible from the apical side. Alternatively, occludin present at apical plasma membrane and OCC2 peptide may bind to apically localized occludin to prevent incorporation of occludin into the tight junctions. Nevertheless, occludin may also localize to the basolateral plasma membrane. In addition, the experiment presented in Fig. 5 showed that a majority of the LMW form of occludin was unaltered after OCC2 peptide treatment. One possibility is that the LMW form was localized intracellularly in vesicles and was not accessible for OCC2 peptide binding. Alternatively, the LMW forms of occludin may be in conformations that are not recognized by OCC2 peptide. In any case, the present result does not address the localization of the LMW pool of occludin.

However, it is also possible that phosphorylation of occludin is secondary to its localization at the tight junctions. In that case, a tight junction-localized “occludin kinase” may specifically phosphorylate junctional occludin after occludin is targeted. Further studies on regulation of occludin phosphorylation will provide insight into the understanding of tight junction formation and the significance of occludin phosphorylation in occludin function.
The potential rapid regulation of the tight junctions provided by phosphorylation-dephosphorylation of occludin is consistent with the observation that tight junctions could be open and could reseal within a short time (~1 h) during dynamic physiological processes such as leukocyte transmigration across an endothelium (14, 15). Therefore, it is possible that the tight junction permeability barrier is regulated by assembly and disassembly of occludin at the tight junctions via phosphorylation and dephosphorylation of occludin. In addition, the presence of different HMW forms of occludin in the two strains of MDCK cells suggests that phosphorylation of occludin could also be involved in the regulation of the permeability properties of the physical seal that is formed by occludin. The current paradox is that, despite the enormous difference in TER, MDCK I and II cells have similar tight junction fibrilar structure (17). Therefore, the permeability properties of the tight junction might be influenced by elements within the tight junction fibrils (17). Because occludin is localized at the tight junction fibrils and has been implicated in the formation of the tight junction seal, it is a candidate for dictating the permeability properties of the tight junction. The results of this study are consistent with the paradox that occludin localization at tight junctions is similar in MDCK I and II cells. However, the different HMW forms of occludin that are found in MDCK I and II cells might provide an explanation for their difference in TER. Importantly, these HMW forms represented the junction-localized and functional forms of occludin and therefore were the structural components for the physical seal of the tight junction. The presence of distinct hyperphosphorylated HMW forms of occludin in MDCK I and II cells suggests that differential phosphorylation might be a mechanism to regulate the permeability properties of the tight junctions.

In conclusion, these results suggest that phosphorylation of occludin might be a mechanism to regulate occludin function and that differential phosphorylation of occludin might be important for generating tight junctions of different permeability properties.

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