

CHAPTER 2

Occludin, a Constituent of Tight Junctions

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Abstract

Occludin was the first tight junction (TJ) integral membrane protein identified. This ~65 kDa protein specifically localizes at TJs of epithelial and endothelial cells and is incorporated into the network of TJ strands. Occludin has a predicted tetraspan membrane topology with two extracellular loops and a large COOH-terminal cytoplasmic domain. Both extracellular domains are enriched with tyrosine residues, and in the first domain, more than half of the residues are tyrosines and glycines. Multiple domains of occludin are responsible for its localization and functions. Occludin interacts with many structural as well as signaling molecules and participates in the regulation of TJ functions. Although occludin-deficient mice show various phenotypes such as significant postnatal growth retardation, chronic inflammation, hyperplasia of the gastric epithelium, and calcification in the brain, the roles of occludin *in vivo* remain unclear since many epithelia in occludin null mice seem unaffected.

Identification of Occludin from Different Species

The first TJ integral membrane protein was identified in 1993 by Tsukita's laboratory. Furuse et al¹ used the membrane fraction of adherens junctions isolated from the chick liver as antigen and obtained monoclonal antibodies (mAb) against a ~65 kDa protein named occludin. Immunofluorescence and immunoelectron microscopy revealed that occludin was exclusively localized at TJs between both epithelial and endothelial cells. Subsequently, Tsukita's group cloned occludin cDNAs from human, mouse, dog, and rat-kangaroo.² By sequence analysis, the three mammalian (human, murine, and canine) occludins are highly homologous to each other, but diverge considerably from those of chicken and rat-kangaroo. Three years later, Cordenonsi et al³ obtained *Xenopus laevis* occludin cDNA, which showed ~58% sequence identity to mammalian occludins and ~41% identity to chicken and rat-kangaroo. Table 1 compares the occludin sequence homology from different species to human occludin at the amino acid level.

Several occludin splice variants have been reported. Muresan et al⁴ isolated a transcript encoding an alternatively spliced form of occludin (occludin 1B) from MDCK cells. Occludin 1B transcript contains a 193 base pair (bp) insertion encoding a unique amino terminal sequence of 56 amino acids. Occludin 1B is co-expressed with occludin in cultured MDCK and T84 cells as well as in various mouse tissues by indirect immunofluorescence method.⁴ Another alternative spliced isoform of occludin has been reported.⁵ This occludin variant (occludin TM4⁻) lacks the fourth transmembrane domain containing 162 bp, which coincides precisely with occludin exon 4. Therefore, occludin TM4⁻ could be generated by the exon 4 deletion with the reading in frame with the downstream exon 5. Occludin TM4⁻ is present at low levels in human Caco-2 cells, monkey BSC1 cells, canine MDCK cells, murine CMT64/61 cells and lung tissue by immunoblot analysis.⁵ Mankertz et al⁶ found by PCR using human

Table 1. Comparison of occludin sequence identity from different species to human occludin

Species	Amino Acids in ORF (Open Reading Frame)	Sequence Identity (%)	Accession Number
Human	522	100	U49184
Dog	521	91.9	U49221
Mouse	521	91.5	U49185
Rat	523	88	NM_031329
Xenopus	493	58	AF170275
Chicken	504	45.6	D21837
Rat-kangaroo	489	45	U49183

colon cDNA that there are three additional forms of occludin mRNAs with different in frame deletions in addition to the original full-length occludin mRNA. These products show altered subcellular distribution when expressed in human intestinal HT-29/B6 cells.⁶ The functions of these occludin variants are unknown.

The identification of occludin presents the first experimental evidence supporting the model that TJ strands contain integral membrane proteins. This has opened the door for studying TJ structure and function at the molecular level and led to the discovery of the claudin family (see Chapter 3).

Localization of Occludin at TJs

Immunoelectron microscopy, freeze-fracture immunoreplica electron microscopy and indirect immunofluorescence microscopy have been applied to visualize the location of occludin at TJs. Using a monoclonal antibody specific for occludin, Furuse et al¹ showed that occludin was precisely localized at the TJ region between chick intestinal epithelial cells as revealed by immunogold labeling (Fig. 1A). On the freeze-fracture image, the immunogold particles were clustered along the TJ fibrils in chick hepatocytes (Fig. 1B)⁷ and in MDCK cells (Fig. 1C), indicating that occludin is a structural component of the TJ network. Figure 2 shows the immunofluorescence staining of occludin in cultured cells as well as in tissues. Occludin is localized at the cell-cell junction of LLC-RK1 cells (Fig. 2A), at the apical surface of blastomeres of early *Xenopus* embryo (Fig. 2B), in the epithelial cells lining the kidney tubules (Fig. 2C), and in the blood vessels of adult mouse brain (Fig. 2D). Besides its expression in brain endothelial cells, occludin is also present at cell-cell contacts of cultured endothelial cells⁸⁻¹⁰ and localized at TJs of retinal pigment epithelial cells (RPE) in chick and mouse.¹¹⁻¹³

The staining pattern of occludin in tissues and the mechanism of occludin incorporation into TJs can be quite different in different tissues and cell types. Gonzalez-Mariscal et al¹⁴ observed very different immunostaining patterns of occludin in proximal and collecting tubules of rabbit kidney. In proximal tubules, occludin displayed a punctuated and discontinuous pattern along the cellular boundaries, while in the collecting tubules, occludin continuously labeled cellular borders. The epithelium of collecting tubules has much higher TER value than the proximal tubule, but the relationship of occludin distribution to this physiology is unknown. Expression of occludin was also found in primary and secondary cultures of astrocytes.¹⁵ Following treatment with 1% Triton X-100, occludin was completely extracted from astrocytic membranes, but not from MDCK cell membranes, suggesting a difference in the cytoplasmic and/or plasma membrane anchoring of occludin between these two cell types.

Developmental regulation of occludin expression and localization have been observed in several tissue types.¹⁶⁻¹⁸ For example, in the gastrointestinal tract of 3- to 21-day-old chick embryos, the immunoreactivity for occludin is not detected until day 4, and it gradually increases with

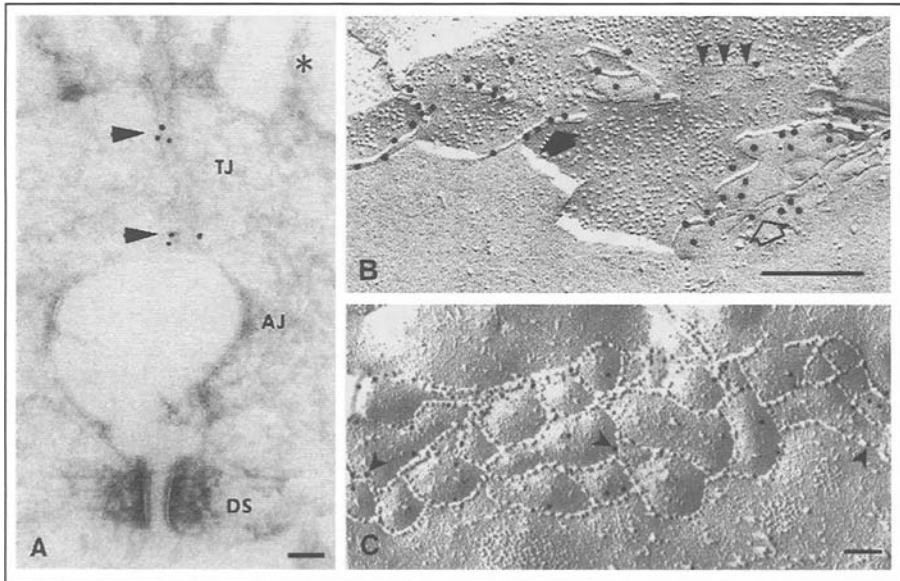


Figure 1. Localization of occludin at TJs by immunoelectron microscopy and freeze-fracture immunoreplica electron microscopy. A) Chick intestinal epithelial cells were fixed, ultrathin cryosectioned, and labeled with an anti-occludin mAb. The immunogold particles indicated by the arrowheads were only present in the TJ region, not in the adherens junction (AJ) and desmosome (DS) regions. *microvilli. (Reproduced from Furuse et al, *J Cell Biol* 1993; 123:1777-1788,¹ by copyright permission of the Rockefeller University Press.) B) On the freeze-fracture replica of chick hepatocytes, the immunogold labeling for occludin was observed along the network of TJ strands. Filled arrow: strand on the P-face; Open arrow: groove on the E-face; Arrowheads: groove on the P-face. (From Fujimoto *J Cell Sci* 1995; 108:3443-3449,⁷ with the permission of The Company of Biologists Ltd.) C) Freeze-fracture replica of MDCK cells labeled by immunogold for occludin. Arrowheads indicate the gold particles associated with TJ strands. (Courtesy of Dr. Eveline Schneeberger, Massachusetts General Hospital). Bars: 100 nm in A; 200 nm in B; 80 nm in C.

development.¹⁷ By day 11, occludin immunoreactivity is observed only at the apical surfaces of the epithelial cells. Interestingly, the immunoreactivity for occludin is clearly detected at the apical end of the lateral membrane of neuroepithelial cells throughout the chick neural plate, but this activity is lost during neural tube closure.¹⁶ Moreover, by injecting horseradish peroxidase into the amniotic cavity of mouse embryos, functional TJs are present in the neural plate, but not in the neural tube. The loss of occludin staining during the neural tube formation indicates the change of these neuroepithelial cells from the epithelial type into the neural precursor cell type.

Expression of Occludin in Vitro

Occludin has the ability to form TJ-like structures when expressed in vitro. For example, when chicken occludin is overexpressed in Sf9 insect cells by recombinant baculovirus infection, multilamellar structures are induced in the cytoplasm.¹⁹ Thin section electron microscopy reveals that these multi-lamellar structures formed TJ-like structures. Moreover, on the freeze-fracture replica of these multi-lamellar structures, short TJ-like strands are specifically labeled by anti-occludin mAb.

When mouse occludin is co-transfected with claudin -1 or -2 into mouse L fibroblast cells lacking TJs, occludin is recruited to freeze-fracture strands at cell-cell contact sites.²⁰ McCarthy et al²¹ used an inducible system to transfect chick occludin into MDCK cells. By freeze-fracture analysis, they found that the number of parallel TJ fibrils shifts from three strands in control

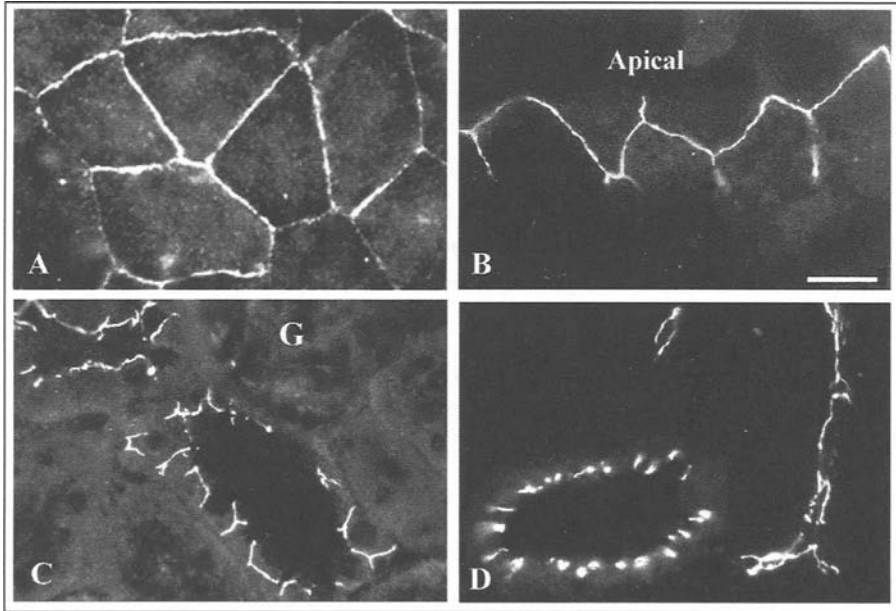


Figure 2. Immunofluorescent light microscopy of occludin localization in cultured cells as well as in tissues. A) LLC-RK1 cells (rabbit kidney proximal epithelial cells) were fixed in 100% methanol and immunostained with an anti-occludin antibody. B) Frozen sections of unfixed gastrulae of *Xenopus* embryos were labeled with an anti-occludin antibody. The fluorescent signal surrounded the apices of surface blastomeres in the partial side-view of the embryonic surface. (Reprinted from Merzdorf et al, Dev Biol 1998; 195:187-203,¹⁸ with permission from Elsevier.) C) Frozen sections of mouse kidney were fixed in 100% methanol and stained with an anti-occludin antibody. Anti-occludin signals were shown in the epithelial cells lining the kidney tubules. G, glomerulus. D) Frozen sections of mouse brain were fixed in 4 % paraformaldehyde and stained with an anti-occludin antibody. Occludin was observed in the endothelial cells of blood vessels and capillaries. Bar, 15 μ m in A, 20 μ m in B, C, and D.

cells to four strands in cells expressing chick occludin. The mean width of the TJ network increases from 175 ± 11 nm to 248 ± 16 nm. Overexpression of full length occludin in MDCK cells significantly increases the transepithelial electrical resistance (TER), suggesting that occludin is a functional component of TJs.^{21,22}

Occludin also confers adhesiveness when expressed in fibroblasts.²³ Stable expression of human occludin in NRK and Rat-1 fibroblast cells lacking endogenous occludin and TJ structure is able to induce cell adhesion in the absence of calcium. This indicates that the cell adhesion induced by the occludin expression is independent of cadherin-cadherin contacts. Synthetic peptides containing the amino acid sequences of the first extracellular loop inhibit the cell adhesion suggesting the direct involvement of the first extracellular loop of occludin in cell-cell adhesion.

Functional Analysis of Occludin Domains

Hydrophilicity plots predict that occludin contains four transmembrane segments, two extracellular domains, and a short intracellular loop. Both the NH₂- and COOH-termini are located in the cytoplasm.¹⁻³ Interestingly, the first extracellular domain of occludin contains high content of tyrosine and glycine residues (~ 60% in human, mouse, dog, rat-kangaroo, and rat occludins, 46% in *Xenopus* occludin). The two extracellular loops have no net charge at neutral pH consistent with their participation in an extracellular hydrophobic barrier.

Many studies indicate that different structural domains of occludin are important for different functions. It has been shown that the extracellular domains are critical for occludin localization and tight junction sealing.²⁴⁻²⁶ The TER of *Xenopus* kidney epithelial cells (A6) is greatly reduced following incubation with a synthetic peptide corresponding to the second extracellular loop of occludin.²⁴ This decrease in TER is associated with an increase in paracellular flux of membrane-impermeable tracers, indicating the disruption of TJ barrier function. However, there are no changes in cell morphology as examined by scanning electron microscopy. Lacaz-Vieira, et al²⁵ applied small synthetic peptides homologous to segments of the first extracellular domain of occludin to A6 cell monolayers, and found that these peptides impaired the TJ resealing. Occludin proteins lacking the second, or both, extracellular domains are absent from TJs and found only on the basolateral surface of MDCK cells.²⁶ This experiment suggests that the presence of the second extracellular domain is required for integration of occludin into TJs. Tavelin et al²⁷ reported that peptides corresponding to the NH₂-terminus of the first extracellular domain of occludin increase the permeability of TJs as judged by the increased paracellular flux of [¹⁴C] mannitol. Interestingly, these peptides have to be added to the basolateral side of the monolayer in order to have an effect. This could be partially due to the degradation of peptides by apical peptidases and aggregate formation since a lipopeptide, which protects the peptide from degradation and aggregation, is effective when added to the apical side of the monolayer.²⁷ Blaschuk et al²⁸ demonstrated that a LYHY sequence located in the second extracellular domain of occludin is an occludin cell adhesion recognition sequence. This short peptide inhibits the establishment of endothelial cell barriers in vitro and in vivo, and also prevents the aggregation of fibroblasts stably transfected with occludin cDNA.

The NH₂-terminal half of occludin also plays an important role in TJ assembly and barrier function. For example, Bamforth et al²⁹ showed that an occludin construct lacking NH₂-terminus and extracellular domains exerts a dramatic effect on TJ integrity. Cell monolayers transfected with this deletion construct have a lower TER and increased paracellular flux to small molecular tracers although the mutant protein is correctly targeted to the TJ. Furthermore, gaps are found to have been induced in the P-face associated TJ strands, as visualized by freeze-fracture electron microscopy. The study reported by Huber et al³⁰ indicated that the NH₂-terminal domain of occludin is important for the transmigration of neutrophils across epithelial sheets, but does not affect the paracellular permeability. Introduction of N-linked glycosylation sites into the two extracellular domains causes the glycosylated occludin unable to integrate into TJs.³¹ It was found that glycosylated occludin accumulate in the basolateral membrane, which suggests that occludin is first inserted into basolateral membrane.

Studies from different laboratories indicate that the COOH-terminus of occludin is required for TJ function.^{22,30-32} When COOH-terminally truncated chicken occludin is stably expressed in MDCK II cells, mutant occludin is incorporated into TJs, but exhibits a discontinuous junctional staining pattern as revealed by confocal immunofluorescence light microscopy.²² However, no abnormal TJ morphology is observed on thin section electron micrographs or freeze-fracture electron microscopic images. More interestingly, overexpression of this mutant occludin in MDCK II cells causes an increase of both TER and paracellular permeability. This paradox has not been resolved. One explanation could be that the overexpression of mutant occludin may lead to the increased numbers of paracellular pores formed by occludin that are permeable only to neutral molecules and the decreased numbers of paracellular channels formed by claudins that are permeable only to ions. In this scenario, both increased TER and paracellular permeability could be observed. Another explanation could be that recent transepithelial flux measurements have suggested that mannitol may not be a reliable reporter of tight junction small molecule flux since its flux across the epithelial cell lines does not correlate well with the TER.³³ Introduction of COOH-terminally truncated occludin into *Xenopus* embryos results in an increased paracellular leakage of low molecular tracers as shown in Figure 3.³² The leakage induced by the mutant occludin can be rescued by coinjection with full-length occludin mRNA. Immunoprecipitation analysis of detergent-solubilized embryo membranes reveals that the exogenous occludin is bound

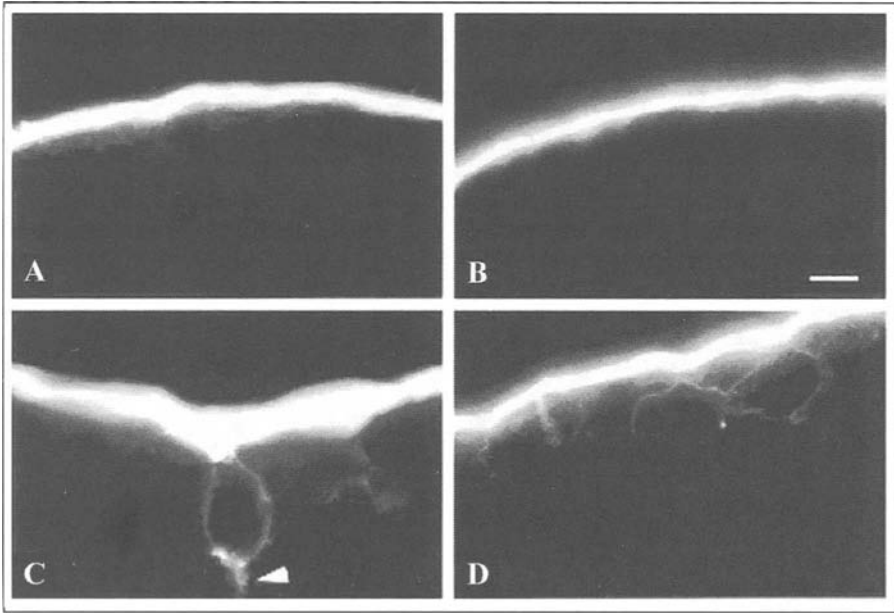


Figure 3. Expression of mutant occludin disrupted the barrier function of TJ in *Xenopus* embryos. mRNAs transcribed from full-length or mutant occludins were microinjected into the antero-dorsal blastomere of eight-cell embryos. 6 hours after injection (2,000 cell blastula), the embryos were labeled by incubation in 1 mg/ml NHS-LC-biotin for 12 minutes at 10°C, then washed and fixed. Frozen sections were stained with RITC-avidin. The staining of NHS-LC-biotin appeared as a thick continuous line on the surface of blastomeres. The TJs in the embryos injected with full-length (A, 504 amino acids), or the least COOH-terminally truncated (B, 486 amino acids) occludin mRNAs were impermeable to the biotin tracer. In contrast, in the embryos injected with two COOH-terminally truncated occludins (C, 386 amino acids, and D, 336 amino acids), the TJs were leaky and therefore, the biotin tracer penetrated into the intercellular spaces. Bar, 10 μ m. (Reproduced from Chen et al, J Cell Biol 1997; 138(4):891-899,³² by copyright permission of the Rockefeller University Press.)

to endogenous *Xenopus* occludin in vivo, indicating that occludin forms oligomers during the normal process of TJ assembly. These data demonstrate that the COOH-terminus of occludin is required for the correct assembly of TJ barrier function. COOH-terminal domain of occludin is also sufficient to mediate direct basolateral targeting of a reporter protein, indicating that it contains a basolateral-targeting determinant.³¹ Further support for the function of COOH-terminus of occludin in its TJ targeting comes from a connexin-occludin chimera study. Connexins are the integral components of gap junctions that are between the lateral membranes of epithelial cells. However, connexin-occludin chimeras are localized within TJ fibrils when expressed in MDCK cells as assessed by immunofluorescence and immunogold freeze-fracture imaging.³⁴ This chimera contains transmembrane and extracellular domains of connexin 32 and the COOH-terminal half of occludin. Therefore, it seems likely that the localization of chimeras at TJs depends on the COOH-terminal domain of occludin.

Occludin Interacting Proteins

Occludin is able to interact with many proteins. Table 2 is a list of known occludin interacting proteins.

Besides the above molecules, occludin also interacts directly with F-actin and does not require other scaffolding proteins for this interaction.⁴⁰ All proteins listed in Table 2 interact

Table 2. Occludin interacting proteins

	M.W. kDa	Binding Site/ Method Used	Possible Roles	References
TJ associated proteins				
ZO-1	220	GUK domain/GST pull-down	Localization of occludin at TJ	Furuse et al ³⁵ Fanning et al ³
ZO-2	160	N-terminal dlg-like domain/Co-IP	Form a complex to Establish TJ	Itoh et al ³⁷ Peng et al ³⁸
ZO-3	130	Unknown/Affinity resin	Linkage between occludin and F-actin	Haskins et al ³⁹ Wittchen et al ⁴⁰
Cingulin	140-160	Unknown/GST pull-down	Unknown	Cordenonsi et al ⁴¹
Gap junction (GJ) proteins				
Connexin 32	32	Unknown/Co-IP	Regulation of occludin expression	Kojima et al ^{42,43}
Connexin 26	26	Unknown/Occludin bait peptide	Proximity of TJ and GJ	Nusrat et al, ⁴⁴
Signaling molecules				
PKC ξ (Protein kinase C)	80	Unknown/Occludin bait peptide	Regulation of actin function	Nusrat et al ⁴⁴
PKC	80	Unknown/Kinase assay; Peptide mass fingerprint	Regulation of occludin function at TJ	Andreeva et al ⁴⁵
c-Yes	62	Unknown/Occludin bait peptide; Co-IP	TJ formation and regulation	Nusrat et al ⁴⁴ Chen et al, 2002 ^{46,47}
c-Src	60	Unknown/GST pull-down	Disruption of TJ	Kale et al ⁴⁸
Subunit of PI3-kinase	85	Unknown/Occludin bait peptide; GST pull-down	Affecting actin polymeration; Oxidative stress-induced disruption of TJ	Nusrat et al ⁴⁴ Sheth et al ⁴⁹
CK2 (Casein kinase 2)	39	Unknown/Kinase assay; Peptide mass fingerprint	Regulation of occludin phosphorylation	Cordenonsi et al ³ Smales et al ⁵⁰
CPE (Clostridium perfringens enterotoxin)	90	Unknown/IP	CPE-induced cytotoxicity	Singh et al ⁵¹
E3 ubiquitin-protein ligase Itch	120	WW motifs/Yeast two-hybrid screen; Co-IP	Ubiquitination of occludin	Traweger et al ⁵²
VAP-33	33	Unknown/Yeast two-hybrid screen; Subcellular fractionation	Vesicle targeting; Regulate occludin localization	Lapierre et al ⁵³

with the COOH-terminal cytoplasmic domain of occludin except the E3 ubiquitin-protein ligase Itch, which binds to NH₂-terminal portion of occludin.⁵²

Roles of Occludin Phosphorylation

Occludin migrates as a cluster of multiple bands (62–82 kDa) on SDS gels resulting from multiple phosphorylation of serine and threonine residues.^{3,54,55} Using a monoclonal antibody specific for phosphorylated occludin, Sakakibara et al⁵⁴ found that highly phosphorylated occludin selectively concentrates at the TJ region of chick intestinal epithelial cells. Occludin can be directly phosphorylated by several protein kinases including PKC and CK2 *in vitro*.^{45,50} Smales et al⁵⁰ used a recombinant COOH-terminal fragment of occludin as a substrate, and incubated the fragment with a kinase obtained from crude extracts of brain. This kinase was later identified as CK2 by peptide mass fingerprinting, immunoblotting, and mutation of CK2 sites within the occludin sequence.

Phosphorylation of occludin may be important for tight junction formation since occludin phosphorylation and dephosphorylation are closely associated with TJ assembly and disassembly.^{46,56} In a calcium-switch model, when monolayers of MDCK cells are exposed to prolonged Ca^{2+} starvation, the TJ complex is disassembled.⁵⁶ In this case, occludin is no longer localized at cell surface, and the phosphorylated 71 kDa band of occludin disappears on western blots. For endothelial cells, Kevil et al⁵⁷ reported that occludin is heavily phosphorylated on serine residues upon H_2O_2 administration. The H_2O_2 -mediated elevation in endothelial solute permeability and alterations in occludin localization and phosphorylation, are all blocked by MEK-1 inhibitor, PD 98059. This suggests the involvement of ERK1/ERK2 signaling pathway in the regulation of endothelial TJ integrity.

Occludin is phosphorylated upon the addition of the PKC activators PMA or diC8 to MDCK cells incubated in low calcium medium.⁴⁵ The redistribution of occludin from the cytoplasm to the lateral plasma membrane is observed after the treatment of MDCK cells with PMA or diC8. The PKC inhibitor GF-109203X markedly inhibits both the phosphorylation of occludin and its incorporation into TJs induced by Ca^{2+} -switch. *In vitro* experiments show that the recombinant COOH-terminal domain of murine occludin can be phosphorylated by purified PKC. Further experiments identify Ser (338) of occludin as an *in vitro* PKC phosphorylation site by using peptide mass fingerprint analysis and electrospray ionization tandem mass spectroscopy. These findings indicate that PKC may regulate the phosphorylation and cellular localization of occludin at TJs. However, other studies show that PKC activation by TPA or PMA leads to dephosphorylation of occludin, and an increase in TJ permeability in LLC-PK1 and human corneal epithelial cells.^{58,59} Since there are so many PKC isoforms, different isoforms could have opposite effects on occludin phosphorylation and TJ function in different types of cells.

Studies of tyrosine phosphorylation have also revealed contradictory data on TJ physiology. Inhibition of tyrosine phosphatase activity has been reported to result in decreases in TER and increases in paracellular permeability in both epithelial and endothelial cells.^{60–63} For example, the tyrosine phosphatase inhibitor, pervanadate, causes a concentration- and time-dependent decrease in TER in both MDCK and brain endothelial cells.⁶⁰ Tyrosine phosphorylation of occludin decreases its interaction with ZO-1, ZO-2, and ZO-3 in Caco-2 cells.⁴⁸ In other studies, PI3-kinase plays a role in oxidative stress-induced disruption of TJs.⁴⁹ PI3-kinase inhibitor, LY294002, prevents oxidative stress-induced tyrosine phosphorylation of occludin and dissociation of occludin from the actin cytoskeleton.

A number of studies have shown that tyrosine phosphorylation can be positively and temporally correlated with tight junction assembly and function.^{46,64,65} Tsukamoto and Nigam⁶⁵ provided the first evidence that occludin is tyrosine phosphorylated and that tyrosine kinase activity is necessary for TJ reassembly during ATP repletion. Chen et al⁴⁶ demonstrated that occludin is tyrosine phosphorylated when localized at TJs. When Ca^{2+} is depleted from the culture medium, occludin tyrosine phosphorylation is diminished in minutes from MDCK cells, which correlates with a significant reduction in TER. Reconstitution of Ca^{2+} restores occludin tyrosine phosphorylation that is temporally associated with an increase in TER. Moreover, occludin forms an immunoprecipitable complex with nonreceptor tyrosine kinase c-Yes,

and this complex dissociates when the cells are incubated in Ca^{2+} -free medium or treated with a c-Yes inhibitor, CGP77675. In the presence of c-Yes inhibitor after Ca^{2+} repletion, occludin tyrosine phosphorylation is abolished and the increase of TER inhibited. These results provide strong evidence that tyrosine phosphorylation of occludin is tightly linked to TJ formation in epithelial cells and that tyrosine kinase c-Yes is involved in the regulation of this process.

In epithelia of *Xenopus* embryos, occludin dephosphorylation is correlated with the de novo assembly of TJs.⁶⁶ Occludin migrates as a 61 kDa protein on the immunoblot in unfertilized eggs, but presents as multiple bands with 57-60 kDa in fertilized eggs and in early cleavages up to blastula stage 8. In gastrulae and tailbud stage embryos, occludin migrates as a 57 kDa protein. This mobility downshift was specifically reproduced by treatment of egg extracts with a phosphatase, indicating that it is due to dephosphorylation. These studies, together with the findings discussed above, suggest that occludin phosphorylation may play different roles in different biological systems. In addition, phosphorylation of specific residues by different kinases may have different functional consequences.

Regulation of Occludin by Signaling Molecules

Occludin localization and function can be regulated by many signaling molecules and pathways including the Rho family small GTPases,⁶⁷⁻⁷¹ Par proteins and aPKC,^{71,72} G proteins,⁷³ proinflammatory cytokines and cytotoxic necrotizing factor-1,⁷⁶⁻⁷⁹ hormones and growth factors,⁸⁰⁻⁸² ERK1/2 and p38 MAP kinase pathway,^{59,83-86} PKA- and PKC-dependent pathway⁸⁷⁻⁸⁹ and protein phosphatases and PLC γ .^{90,91} Not surprisingly, occludin in different cells responds differently to the activation and inactivation of different signaling pathways.

In MDCK cells, Rho signaling is required for TJ assembly.⁶⁸ Constitutive Rho activation causes an accumulation of occludin at the cell junction, while Rho inhibition results in decreased localization of occludin at the cell junction. Expression of an activating Rho mutant protein increases levels of occludin phosphorylation, indicating that occludin is a target for Rho signaling. In a different study, however, Jou et al⁹² reported that occludin distribution in MDCK cells is disrupted by constitutively active mutants RhoAV14 and Rac1V12, but not by dominant negative mutants RhoAN19 and Rac1N17. All RhoA and Rac1 mutants result in decreased TER and increased paracellular flux. Differences in occludin localization induced by Rho inhibition might result from different methods used in the studies: Rho inhibitor (C3 transferase) versus dominant negative construct (RhoAN19).

Activation of the MAP kinase pathway disrupts TJ structure and function in several cell lines, such as rat salivary gland epithelial cell line Pa-4, Ras-transformed MDCK cells, in addition to human corneal epithelial cells.^{59,83,84} Li and Mrsny⁸³ demonstrated that transfection of a constitutively active construct of Raf-1, a MEK-1 kinase, into Pa-4 cells results in a complete loss of TJ function. The cells transfected with Raf-1 display a stratified phenotype that lacked cell-cell contact growth control. The expression of occludin is downregulated in these cells. More importantly, introduction of occludin into Raf-1 transfected cells results in reacquisition of normal epithelial phenotype and functionally intact TJs. Similarly, in *ras*-transformed MDCK cells, TJ structure is absent and occludin is present only in the cytoplasm.⁸⁴ These cells show fibroblastic morphology and grow on top of each other. When Ras signaling is attenuated by the inhibition of MAP kinase pathway using MEK1 inhibitor, TJ structure and function are restored, occludin is concomitantly recruited to the cell-cell contact areas and cells resume their normal epithelial phenotype. Therefore, constitutive activation of MAP kinase pathway negatively regulates TJ assembly and function. However, Macek et al⁹³ recently reported that in claudin-1 expressing T47-D cells (low Ras activity) and claudin-1 negative MCF-7 cells (elevated Ras activity), no quantitative changes of mRNA or protein levels of occludin can be detected after inhibition of MAP kinase pathway by MEK1 inhibitor. Also, there is no difference in TER and paracellular flux between these two cell lines. It seems likely that the regulatory machinery of TJ is complex and different in different cell lines depending on the origin of the cell, the physiological and pathological state of the cell, and the genetic background of the cell.

Phenotypes of Occludin-Deficient Mice

Occludin-deficient mice display complex phenotypes.⁹⁴ These mice show significant postnatal growth retardation with ~75% of normal weight at 8 weeks of age. Interestingly, occludin^{-/-} females do not suckle their litters, resulting in neonatal death. Histological examination reveals a chronic inflammation and hyperplasia in the gastric epithelium of occludin null mice. Gastritis develops around 10 weeks of age and becomes very severe around 28 weeks of age. A progressive accumulation of calcium and phosphorus deposits is observed in the cerebellum and basal ganglia of occludin null mice using electron microscopy with energy dispersive X-ray microanalysis. Abnormalities are also found in the bone. In occludin null mice, the compact bone is significantly thinner than that of wild-type controls. However, despite these complex phenotypes, occludin-deficient mice form morphologically normal TJ strands. The TJ barrier function in intestinal epithelium is also normal as measured by TER. These results are consistent with the previous report that occludin-deficient ES cells can differentiate into polarized epithelial cells bearing a well-developed network of TJ strands.⁹⁵ It is clear from these studies that occludin is not required for the formation of TJ strands and its role in TJ barrier function can partially be compensated by other TJ membrane proteins *in vivo*.

Occludin in Cancer Cells

TJs are crucial for maintaining the cell polarity and junctional integrity. In many cancer tissues, cells lose their polarity and cell junctions are disrupted. These cells then become undifferentiated and begin to migrate. Occludin expression is often downregulated in human cancers.⁹⁶⁻¹⁰⁰ For example, in human prostate cancer, occludin expression is completely lost in unpolarized cells of high Gleason grade tumors (Gleason 4 and above), but remains expressed in cells facing a lumen in all grades of cancer.⁹⁹ Therefore, the loss of occludin expression is closely associated with the loss of cell polarity. Malignant brain tumors cause cerebral edema because they have leaky endothelial TJs, which allow plasma fluid to enter the brain from the microvessel lumen. Using immunohistochemistry and immunoblotting methods, Papadopoulos et al.⁹⁶ found that occludin expression is decreased in high grade (III or IV) brain tumors compared to that of non-neoplastic brain tissue samples. Similarly, in human gastric cancer, the expression of occludin mRNA in moderately and poorly differentiated groups is gradually reduced when compared with well-differentiated groups, suggesting a significant correlation between tumor differentiation and the expression of occludin mRNA.⁹⁷ Most recently, Tobioka et al.¹⁰⁰ also reported that occludin expression decreases progressively in parallel with the increase in carcinoma grade, and the decreased occludin expression correlates with myometrial invasion and lymph node metastasis. All these studies suggest that the loss of occludin expression is closely associated with the loss of cell polarity, the cell de-differentiated state and the progression of human tumors.

Summary

Occludin was the first integral membrane protein identified in TJ strands, but it is not required for the formation of TJ strands. Studies of occludin assembly, domain function, and regulation by phosphorylation and signaling molecules yield rich, although sometimes contradictory information that suggests its regulations in different biological systems are complex.

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<http://www.springer.com/978-0-387-33201-7>

Tight Junctions

González-Mariscal, L. (Ed.)

2006, XI, 224 p., Hardcover

ISBN: 978-0-387-33201-7