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OCCLUDIN PHOSPHORYLATION IN REGULATION OF EPITHELIAL TIGHT JUNCTIONS

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Abstract

Occludin is the first transmembrane protein of tight junction to be discovered. While numerous studies emphasized the important role of occludin in assembly and maintenance of tight junctions, occludin knockout studies indicated that it was not required for the assembly of tight junction in different epithelia. However, a detailed characterization of occludin knockout mouse concluded that the occludin gene is indispensable, rather played a complex role in regulation of epithelial tight junctions in different organs. This article describes the role of occludin phosphorylation in the regulation of epithelial tight junctions. Occludin is highly phosphorylated on Ser and Thr residues, while Tyr-phosphorylation is kept at minimum in the intact epithelium. During the disruption of tight junctions by various factors occludin undergoes dephosphorylation on Ser/Thr residues and elevated phosphorylation on Tyr residues. The phosphorylation of occludin on Tyr, Ser and Thr residues appears to be regulated by the balance between protein kinases such as c-Src, PKCζ and PKCλ/ι and protein phosphatases such as PP2A, PP1 and PTP1B. The precise mechanism of regulation of tight junction by occludin phosphorylation is unclear at this time. However, an *in vitro* study indicated that Tyr-phosphorylation of occludin C-terminal domain attenuates its interaction with ZO-1. Therefore, phosphorylation of specific Ser/Thr/Tyr residues in occludin may regulate the interactions between various proteins of tight junctions and adherens junctions. Therefore, it is likely that occludin plays a regulatory role in tight junctions rather than a role in the *de novo* assembly of tight junctions.

Keywords

tight junction; occludin; protein kinase; protein phosphatase; ZO-1; epithelium; barrier function; PKC; PP2A; Src

Occludin is the first transmembrane protein of epithelial tight junctions to be discovered [1], and therefore its structure is relatively well characterized. Numerous studies during the past fifteen years have indicated that occludin plays an important role in the regulation of tight junction integrity. Recent studies indicated that tight junctions are formed in the absence of occludin [2, 3] and questioned the role of occludin in the assembly of tight junctions. However, characterization of occludin knockout mouse established that the occludin gene is

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indispensable and suggested that occludin may play an important role in the regulation of tight junction integrity rather than in the assembly of tight junction. The potential role of occludin phosphorylation in the regulation of epithelial tight junction integrity is discussed in this article.

Structure and function of occludin in tight junctions

Occludin is a tetraspanin membrane protein with four transmembrane domains, two extracellular loops and one intracellular loop [1, 4, 5]; the C-terminal and N-terminal domains project into the cytoplasm. The first extracellular loop exhibits a unique amino acid sequence that is rich n Tyr and Gly residues (~60% of total amino acids). The extracellular loops of occludin interact with similar loops of occludin in the adjacent cells to form the tight junctions. Whether N-terminal domain of occludin plays a role in its function is not known. However, the C-terminal domain of occludin interacts with various intracellular proteins of tight junctions, including ZO-1, ZO-2 and ZO-3 [6]; this interaction is required for the assembly of occludin into the tight junctions [6, 7].

Expression of occludin in Xenopus eggs [8] or fibroblasts [9] confers adhesive property to these cells and results in the formation of primitive forms of tight junction strands. Over expression of full length occludin in MDCK cells results in enhanced transepithelial electrical resistance [10]. Expression of C-terminally truncated occludin increased paracellular permeability in MDCK and Xenopus embryo cells [8, 11]. Disruption of interactions between extracellular domains of occludin using synthetic peptides with the sequence corresponding to that of occludin extracellular loops disrupts tight junctions and increases paracellular permeability [12–17]. Therefore, occludin seems to play a crucial role in the assembly or maintenance of epithelial tight junctions. However, deletion of occludin gene in embryonic stem cells failed to prevent the assembly of tight junctions and differentiation into polarized epithelial cells [2]. Furthermore, the occludin knockout mouse showed the presence of normal tight junctions in the intestinal epithelium [3]. These studies raised the question whether occludin is required for the assembly of tight junctions. This doubt was further emphasized by the observation that claudin-1 and claudin-2, occludin-like transmembrane proteins, are localized in the tight junction strands [18]. The occludin knockout mouse, however showed that the tight junction integrity of epithelial tissues in gastric glands, seminiferous tubules and salivary glands were defective in the absence of occludin and exhibited a very complex phenotype, thus establishing that occludin gene is indispensable.

Since there is no evidence for the existence of multiple isoforms of occludin in mammalian epithelial cells, it is unlikely that redundant proteins compensate for the role of occludin. It was suggested that occludin may play a role in the regulation of tight junction integrity rather than in the assembly of tight junctions [3]. The cytoplasmic domain of occludin may be involved in regulation of tight junctions through intracellular signaling. Occludin is highly phosphorylated on Ser/Thr residues [19–21]. Additionally, numerous signaling molecules are associated with the tight junction [22]; occludin directly interacts with c-Src [23], ERK1/2 [24], PP2A and PP1 [19]. Therefore, occludin is likely to play a regulatory

role in tight junction integrity, an area that need to be explored to further understand the occludin-based signaling in tight junction regulation.

Ser/Thr-phosphorylation of occludin

Occludin is highly phosphorylated on Ser and Thr residues in the resting epithelium [19–21]. Assembly and disassembly of tight junctions are associated with reversible phosphorylation of occludin on Ser and Thr residues [19, 21, 25], indicating that Ser/Thr-phosphorylation of occludin plays a crucial role in the regulation of tight junction integrity. Several studies indicated that occludin undergoes dephosphorylation on Ser and Thr residues during the disruption of tight junctions by various factors. Disruption of tight junctions by incubation with low calcium medium resulted in a rapid reduction in p-Ser and p-Thr contents of occludin [19, 21]. Occludin was rephosphorylated on Ser/Thr residues during calcium-induced reassembly of tight junctions in MDCK cells. However, the precise role of Ser/Thr-phosphorylation of occludin is not known at this time. A recent study showed that rapid disassembly of tight junctions in Caco-2 cell monolayers by EGTA-induced calcium depletion caused occludin dephosphorylation on Thr residues without affecting the Ser-phosphorylation of occludin [20]; reassembly of tight junctions by calcium replacement rapidly restored the Thr-phosphorylation of occludin. This study suggested that Thr-phosphorylation rather than Ser-phosphorylation of occludin is involved in recruitment of occludin during tight junction assembly.

Studies also indicated that Ser/Thr-phosphorylation of occludin is dynamically regulated by protein kinases and protein phosphatases. The specific protein kinases involved in occludin phosphorylation is unclear. Localization at the tight junctions and association with tight junction proteins suggested that atypical protein kinase C (PKC) isoforms, PKCζ and PKCλ/ι, may play a role in phosphorylation of occludin. PKC activity is required for hydrogen peroxide-induced disruption of tight junctions and barrier dysfunction in human tracheal epithelial cells and intestinal epithelial monolayers [26–28]. Hydrogen peroxide activated PKCδ and PKCλ, and knock down of these PKC isoforms attenuated the hydrogen peroxide -induced barrier dysfunction [26–28], while PKC activity was not required for oxidative stress-induced barrier disruption in renal tubular epithelial cells [29]. PKC activity however was required for epidermal growth factor (EGF)-mediated protection of tight junctions from hydrogen peroxide [30] and acetaldehyde [31]. Phospholipase Cγ-dependent activation of PKCβI and PKCε are involved in EGF-mediated protection of tight junction from acetaldehyde in Caco-2 cell monolayers [31]. Therefore, the type of influence exerted on tight junction integrity may depend on the isoform of PKC involved.

Protein phosphatases such as PP2A [20, 32] and PP1 [20] are implicated in the regulation of Ser/Thr phosphorylation of occludin in MDCK and Caco-2 cell monolayers. A combination of *in vitro* and *in vivo* studies indicated that PP2A and PP1 directly interact with occludin and dephosphorylate it on Ser/Thr residues. Knock down of PP2A or PP1 by siRNA enhanced the integrity of tight junctions and accelerated the calcium-induced reassembly of tight junctions in Caco-2 cell monolayers [20]. Interestingly enough, PP2A dephosphorylated occludin preferably on p-Thr residues, while PP1 was more active in dephosphorylating occludin on Ser residues. These studies suggest that occludin

phosphorylation on Ser and Thr residues may play distinct roles in the regulation of occludin function. In general, phosphorylation of occludin on Ser and Thr residues is associated with the assembly of tight junction and dephosphorylation of occluding is associated with the assembly of tight junction. Therefore, the balance between protein kinases such as atypical PKC isoforms and protein phosphatases such as PP2A and PP1 may determine the state Ser/Thr-phosphorylation of occludin.

Tyrosine-phosphorylation of occludin

In the intact epithelium, very low level of Tyr-phosphorylated occludin is present [33]. However, numerous studies indicated that occludin undergoes Tyr-phosphorylation during the disruption of tight junctions by various factors. Tyrosine kinase activity is required for the hydrogen peroxide -induced disassembly of tight junctions [23, 33–36]. Tyrosine kinase inhibitors prevent hydrogen peroxide [23, 33, 35] and acetaldehyde [34]-induced disruption of tight junction in Caco-2 and T84 cell monolayers. Hydrogen peroxide and acetaldehyde-induced disruption of tight junction was associated with a rapid Tyr-phosphorylation of occludin along with that of ZO-1, E-cadherin and β-catenin [33], suggesting that the Tyr phosphorylation of tight junction and adherens junction proteins may play a role in the disruption of these junctional complexes. Hydrogen peroxide caused oxidation of glutathione (GSH) leading to accumulation of oxidized-GSH (GSSG) and reduction of protein thiols [37]. GSH and N-acetyl cysteine effectively ameliorated hydrogen peroxide-induced barrier disruption [37]. Hydrogen peroxide treatment also resulted in inhibition of PTPase activity, which may very well represent one of the mechanisms involved in Tyr-phosphorylation of occludin and other proteins of tight junction and adherens junction; this was supported by the observation that GSSG inactivates PTPase activity *in vitro* [37] and PTPase inhibitor, pervanadate, effectively disrupts the tight junctions and barrier function. Acetaldehyde also directly inhibits PTPase (PTP1B) activity to increase protein Tyr-phosphorylation [34]. Therefore, inhibition of PTPase activity and resulting increase in Tyr-phosphorylation of occludin and other tight junction proteins may be one of the mechanisms involved in the regulation of tight junction integrity.

Tyrosine kinase involved in occludin phosphorylation is unknown. A recent study demonstrated that c-Src plays a role in the regulation of tight junction integrity in Caco-2 and MDCK cell monolayers [23]. Hydrogen peroxide rapidly activated c-Src and an Src kinase inhibitor attenuated hydrogen peroxide-induced disruption of tight junction [23]. The expression of a kinase-inactive c-Src ameliorated hydrogen peroxide-induced disruption of tight junctions, while over expression of wild type c-Src exacerbated hydrogen peroxide effect. Similarly, LPS induced an activation of c-Src in cholangiocyte monolayers and the Src kinase inhibitor attenuated LPS-induced tight junction disruption [38]. Therefore, c-Src activation may lead to disruption of tight junctions and this process may involve Tyr-phosphorylation of occludin. The specific role of protein tyrosine phosphorylation in the mechanism of oxidative stress-induced increase in paracellular permeability is not clear. However, evidence suggests that tyrosine phosphorylation of junctional proteins may play a role in regulation of cell-cell adhesion [39–42].

The precise role of occludin Tyr-phosphorylation in tight junction disruption is not clear at present. However, hydrogen peroxide-induced Tyr-phosphorylation was accompanied by a loss of interaction between occludin and ZO-1 [33]. The regulation of direct interaction between occludin C-terminal domain and ZO-1 was confirmed by *in vitro* studies using recombinant GST-fused C-terminal domain of occludin [43]. However, when the GST-fused occludin C-terminal domain was Tyr-phosphorylated *in vitro* by incubation with c-Src the ZO-1 binding was dramatically reduced, suggesting that the Tyr-phosphorylation of occludin may attenuate its interaction with ZO-1 at the tight junctions, leading to disruption of tight junctions.

Protein Tyr-phosphorylation also plays an important role in the regulation of adherens junctions. Hydrogen peroxide and acetaldehyde-induced disruption of tight junctions and adherens junctions was associated with a rapid increase in Tyr-phosphorylation of E-cadherin and β-catenin [44]. The recent study demonstrated that interaction between E-cadherin and β-catenin is attenuated by Tyr-phosphorylation of β-catenin [44]. Therefore, hydrogen peroxide and acetaldehyde-induced Tyr-phosphorylation of β-catenin is likely to play a crucial role in the dissociation of E-cadherin-β-catenin complexes. The E-cadherin-based cell-cell adhesion is essential for the assembly and maintenance of adherens junctions. Disruption of adherens junction is known to disrupt the tight junctions. Therefore, Tyr-phosphorylation of both tight junction and adherens junction proteins is important in the regulation of tight junction integrity and epithelial barrier function.

Although it appears that phosphorylation-mediated regulation of tight junction and adherens junction is quite complex, recent studies have clarified some of the issues in this aspect. It is clear that tight junction and adherens junction proteins are phosphorylated on Tyr, Ser and Thr residues, and that protein kinase and protein phosphatase activities certainly regulate the integrity of tight junction and adherens junctions. Recent studies also distinguished the differences between Tyr-phosphorylation and Ser/Thr-phosphorylation of occludin. Tyr-phosphorylation of occludin is clearly associated with the disruption of tight junctions, while Ser/Thr-phosphorylation may be required for the assembly of occludin into the tight junctions. Therefore, it is crucial to further investigate the role of specific Tyr, Ser and Thr residues in occludin to understand the tight junction regulation by phosphorylation. It is also important to recognize the likely possibility that phosphorylation of other proteins of tight junction and adherens junction such as ZO-1, ZO-2, ZO-3, claudins, junction adhesion molecule may be involved in tight junction regulation.

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