Tight junctions (TJs) form paracellular barriers defining the permeability characteristics of epithelial and endothelial cell layers in our body. Tetraspanin integral membrane proteins, including occludin, tricellulin, MarvelD3, and a set of claudins, form a network of anastomosing strands bringing the membranes of neighboring cells into close contact. Occludin is assumed to play an important role in the regulation of TJ formation, structure, and function, and is tightly regulated by phosphorylation. We here summarize the role of occludin phosphorylation on assembly/disassembly and function of TJs and specifically focus on a cluster of 11 amino acids in the C-terminal cytoplasmic domain of occludin (Tyr398–Ser408), including highly conserved phosphorylation sites for c-Src, PKCs, and CK2. Phosphorylation by these kinases affects occludin localization, dynamics, and interaction with other TJ proteins. Interestingly, this phosphorylation hotspot is localized in an unstructured region close to the ZO-1 binding site, and a cysteine residue which is involved in intermolecular disulfide-bond formation thus contributing to occludin dimerization. We discuss potential consequences and open questions in respect to the functional role of this phosphorylation hotspot.

Keywords: occludin; phosphorylation; tight junction; barrier function

Introduction

The paracellular permeability characteristics of epithelial and endothelial tissues are defined by the protein composition of their tight junctions (TJs). Occludin was the first identified integral transmembrane protein within TJs, and now, together with tricellulin and MarvelD3, forms the tight junction-associated MARVEL (MAL and related proteins for vesicle trafficking and membrane link) protein family (TAMPs). All three proteins are inserted into the membrane by four transmembrane domains connected by two extracellular loops and one intracellular loop, which together form the MARVEL domain. Occludin and MarvelD3 are equally distributed in bicellular TJs, whereas tricellulin is preferentially localized at tricellular junctions forming a barrier for macromolecules. Recent studies revealed that the three TAMPs have overlapping but nonredundant functions that cannot be mutually compensated. After knockdown of the different TAMP members, tight junctional structures were still detectable indicating that none of the TAMPs is essential for TJ formation. The N- and C-terminal domains of full-length TAMPs are oriented into the cytoplasm and play an essential role in the regulation of transport, stability, and dynamics of these TJ proteins.

In addition, up to 27 claudins have been identified that make up the major TJ-forming components. Similar to the TAMPs, claudins are integrated into the membrane by four transmembrane domains but differ in their short intracellular N- and C-terminal cytoplasmic tails. There is evidence that different claudins can interact specifically and thereby form homo- and heteromeric complexes. These interactions define the barrier characteristics of individual tissues. Both cis-interactions within the same membrane and trans-interactions between opposing cells have been reported. Detailed analyses
Occludin phosphorylation hotspot

Figure 1. The phosphorylation hotspot in the C-terminal cytosolic domain of occludin. Alignment of amino acid sequences around the phosphorylation hotspot in occludin from different species. Amino acids targeted by the indicated kinases are marked in orange. A conserved cysteine residue marked in blue and located next to the C-terminus of the hotspot region was shown to be involved in occludin homodimerization by disulfide bond formation.54

identified tightening and pore-forming activities of individual claudins, as shown for claudin-1 or -4 and claudin-2 or -10, respectively.9,10

Further, more than 40 TJ-associated proteins have been identified, including additional transmembrane proteins, such as members of the immunoglobulin superfamily of adhesion receptors including the junctional adhesion molecules (JAMs),11 coxsackie adenovirus receptor (CAR),12 and endothelial cell-selective adhesion molecule (ESAM).13 These proteins cannot assemble TJ strands by themselves but have been shown to be involved in the assembly and modulation of TJ function. The cytosolic domains of all these transmembrane proteins form a platform for the assembly of a multitude of intracellular adaptor, cytoskeletal, and regulatory proteins important for TJ structure and function.14

Occludin as TJ regulator

Although occludin was the first reported integral membrane protein of TJ strands and is now known for nearly 20 years, its function is still not completely understood. Human occludin initially was characterized as a 522 amino acid protein expressed in epithelial and endothelial cells but is not detectable in fibroblasts. Western blot analyses detected a major band with an apparent molecular mass of 60 kDa and a set of additional bands defined as phosphorylation or alternative splicing products.15 Expression of occludin in Madin-Darby canine kidney (MDCK) cells induced an increase in transepithelial resistance (TER) and unexpectedly enhanced paracellular flux.16,17 These initial findings suggested that occludin is essential for TJ formation and function. Therefore, it was surprising that mice after inactivation of occludin by gene knockout were viable, formed normal TJ strands, and showed only minor signs of pathological disorders.18,19 Similarly, knockdown of occludin in MDCK cells did not prevent TJ formation, however, changes in the expression of specific claudins and reduced extrusion of apoptotic cells was observed.7

It is now well accepted that claudins compose the major TJ constituents, whereas occludin is postulated to play a regulatory role for TJ assembly/disassembly and function. Thereby occludin forms a kind of signaling platform for different stimuli and signaling pathways including growth factors, cytokines, or oxidative stress (for review, see Refs. 20–22). In line with the regulatory role of occludin, different proteins associated around the cytosolic domains of occludin, which are involved in the formation of the tight junctional plaque, have a dual function at the TJs as well as within the nucleus where they modulate gene transcription. These tight junctional proteins including...
Table 1. Amino acid residues within the human occludin C-terminal phosphorylation hotspot, which were identified as specific kinase targets

<table>
<thead>
<tr>
<th>Amino acid(s) modified by kinase</th>
<th>TER</th>
<th>Permeability</th>
<th>Junctional interaction with occludin</th>
<th>TJ-assembly</th>
<th>Interaction with TJ proteins</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Src</td>
<td>↓</td>
<td>↑ (inulin)</td>
<td>↓</td>
<td>↓</td>
<td>↓ (ZO-1, -2, -3)</td>
<td>32, 34, 35</td>
</tr>
<tr>
<td>Y398</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34</td>
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<tr>
<td>Y402</td>
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<td>34</td>
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<tr>
<td>Y398</td>
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<td>Y402</td>
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<td>34</td>
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<tr>
<td>nPKCγ</td>
<td>↑</td>
<td>↓ (inulin)</td>
<td>↑</td>
<td>↑</td>
<td></td>
<td>41, 48</td>
</tr>
<tr>
<td>T403</td>
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<td></td>
<td></td>
<td>48</td>
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<td>T404</td>
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</tr>
<tr>
<td>CK2</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>43, 49–51</td>
</tr>
<tr>
<td>T400</td>
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<td></td>
<td></td>
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<tr>
<td>T404</td>
<td></td>
<td></td>
<td>mobility&lt;sup&gt;c&lt;/sup&gt; →</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>S408</td>
<td></td>
<td></td>
<td>mobility&lt;sup&gt;c&lt;/sup&gt; ↑</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>S408</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>50</td>
</tr>
</tbody>
</table>

Note: The effects on tight junction structure and function induced by overexpression/stimulation of kinase are summarized for each kinase in the lines with gray background. In addition, effects induced by phosphorylation of specific amino acid residues are shown. For more detail, see references.

<sup>a</sup> Results obtained by mutation of corresponding amino acids in the mouse are comparable.

<sup>b</sup> Same amino acids also appear to be phosphorylated by aPKC<sub>S</sub>.<sup>42</sup>

<sup>c</sup> Mobility of occludin measured by FRAP.

ZO-1, ZO-2, symplekin, ZONAB, and huASH1 were defined as the nucleus and adhesion complexes (NACos) proteins (for review, see Ref. 23). Cingulin, another tight junctional plaque protein, can associate with molecules regulating RhoA and Rac1 signaling. The regulatory role of occludin was emphasized when it was recently shown that occludin is required for cytokine-induced modulations of barrier properties. There is clear evidence that phosphorylation/dephosphorylation of occludin is essential in the regulation of TJ structure and function.

**Phosphorylation of the occludin C-terminal domain**

Early studies by Sakakibara *et al.* already indicated that phosphorylation of occludin is of central importance for TJ formation. Meanwhile, different kinases and phosphatase have been identified that directly target occludin and thereby modulate barrier structure and function (for review, see Ref. 29). The Src-family kinases c-Yes<sup>30, 31</sup> and c-Src<sup>32</sup> have been shown to bind to the occludin C-terminal domain. Tyrosine residues corresponding to Tyr398, Tyr402, and Tyr474 in human occludin have been identified as c-Src phosphorylation sites,<sup>33, 34</sup> whereas no specific sites targeted by c-Yes have been reported. Currently, the precise role of occludin tyrosine phosphorylation is not fully understood. Nevertheless, much data suggest that occludin tyrosine phosphorylation contributes to TJ destabilization and disruption probably by abrogation of the interaction between the occludin C-terminus and ZO-1.<sup>32</sup> Moreover, Tyr473 in mouse
occludin recruits the p85α subunit of PI3K to the leading edge, activates it, and augments lamellipodia formation during cell migration. 33 In addition, an interaction of the cytosolic tyrosine kinase, the focal adhesion kinase (FAK), was observed. However, since a FAK phosphorylation site in occludin has not been identified, it is currently not clear whether the barrier disruption observed in response to knockdown of FAK 35 is a direct effect or a consequence of cytoskeletal rearrangements.

Two further phosphorylation sites in the occludin C-terminal domain have been identified for Rho kinase (RhoK) at amino acids T382 and S507 in mouse occludin. 36 In human occludin, a corresponding amino acid to T382 is missing. Inhibition of RhoK activity was shown to reduce occludin phosphorylation and monocyte transmigration across the blood–brain barrier 37 and to attenuate LPA-induced increases in endothelial cell permeability. 38

Extensive studies have been performed to analyze the role of protein kinases C (PKCs) on TJ function. Apparently, depending on the inhibitor/activator and the cellular system used, results obtained differed and sometimes were difficult to interpret. This can be explained by the fact that different PKC isoforms are expressed and become active on tight junctional proteins including occludin. 39 There is evidence now that classical (cPKCs) and novel PKCs (nPKCs) have antagonistic effects on TJ assembly and target different amino acids in the occludin C-terminal domain. 40–41 In this respect, Ser338 in mouse occludin was identified as a cPKC phosphorylation site. 40 42 Threonines 403, 404, 424, and 438 in human occludin were reported as potential PKCζ sites. 42 Mutation of threonine residues 424 and 438 to alanine resulted in delayed assembly of occludin into TJs in Ca2+ switch experiments.

Association of occludin with CK1 (formerly casein kinase 1) as another Ser/Thr kinase was shown in coimmunoprecipitation and pull-down assays and in vitro phosphorylation experiments suggested that occludin is a direct target of CK1. 43–44 In addition to the occludin C-terminal domain, we also have evidence that the N-terminal domain includes putative CK1 phosphorylation sites. 43 Whether all CK1 isoforms bind to occludin is not clear at the moment. Up to now, binding has only been shown for CK1α and ε. 44 Preliminary evidence suggests that multiple sites can be phosphorylated by CK1. It is not clear, however, whether different CK1 isoforms phosphorylate different sites in occludin.

Phosphorylation of Ser490 in response to vascular endothelial growth factor (VEGF) or platelet derived growth factor (PDGF) treatment attracted some research attention. Although the kinase involved is not identified yet, interesting functional consequences with respect to Ser490 phosphorylation have been observed. Expression of an occludin-S490D mutated protein resulted in diminished ZO-1 binding. 45 Furthermore, phosphorylation of Ser490 enhances binding of the ubiquitin-ligase Itch and subsequent occludin ubiquitination. 46 Interestingly, it was recently reported that Ser490-phosphorylation of occludin increases during mitosis and regulates mitotic entry. 47 Ser490-phosphorylated occludin colocalizes with γ-tubulin in centrosomes in mitotic cells and affects centrosome separation. Moreover, expression of a phosphomimetic occludin-S490D construct enhanced cell proliferation, whereas occludin-S490A had the opposite effect. 47

A phosphorylation cluster within the occludin C-terminal domain

In addition to the phosphorylation sites summarized above, a sequence of 11 amino acids from Tyr398 to Ser408 in human occludin was identified, which includes six residues that are targeted by different kinases (Fig. 1). The previously mentioned c-Src sites Tyr398 and Tyr402 lie within this cluster, as well as Thr403 and Thr404 functioning as sites for the novel PKCζ. 48 Overexpression of PKCζ or expression of a phosphomimetic variant of occludin (Occ-T403D/T404D) enhanced tight junctional localization of occludin, increased TER, and reduced paracellular permeability (Table 1). In contrast, expression of occludin-Y398D/Y402D resulted in its reduced junctional localization and TER, impaired ZO-1 binding, and delayed assembly of TJs in Ca2+ switch experiments.

Amino acid residues Thr400, Thr404, and Ser408 have been identified as casein kinase 2 (CK2) phosphorylation sites. 49–51 Inhibition or knockdown of CK2 resulted in increased TER, reduced paracellular Na+ flux and enhanced tight junctional localization of occludin. 50 An important finding in this context was, that inhibition of CK2 reduces the mobile fraction of occludin. Moreover,
CK2-dependent phosphorylation appears to affect occludin dimerization and heterodimerization with claudin-1 and -2. Taken together CK2 appears to induce mobile occludin homodimers, whereas inhibition of CK2-dependent phosphorylation promotes formation of heteromeric complexes with ZO-1, claudin-1, and -2.\(^2\)\(^\text{50}\) Our own studies using an occludin-T400D/T404D/S408D construct surprisingly resulted in an increase in the paracellular resistance (unpublished data). Currently, we have no explanation for these discrepancies, which may be caused by the use of different cell lines. All observations available in the context of the Tyr398-Ser408 sequence motif and its kinase-dependent posttranslational modification suggest that this region may be a phosphorylation hotspot for occludin.

**Future perspectives**

A number of open questions remain with respect to the role of this phosphorylation hotspot for occludin. The Tyr398-Ser408 motif is located close to the coiled-coil sequence regions in the occludin C-terminal domain that mediate occludin dimerization and the interaction with ZO-1.\(^2\)\(^\text{52}\) It is assumed that changes in surface charge distribution and concomitant structural alterations induced by the phosphorylation of amino acids within this hotspot region modulate occludin homomeric and heteromeric interactions. Structural analysis of the distal C-terminal domain of occludin (amino acids 383–522) revealed that the N-terminal 34 amino acids of this construct including the phosphorylation hotspot region of occludin are disordered.\(^2\)\(^\text{53}\) Since the recombinant protein used for structural analyses was expressed in *Escherichia coli*, it is assumed that none of the amino acids within the hotspot region was phosphorylated. It is currently not known if phosphorylation of amino acids within the hotspot region induces a switch to an ordered structure. Moreover, it would be interesting to know if and how phosphorylation of the hotspot region affects the ordered structure of occludin residues 416–522, which form three α-helices that build two antiparallel coiled-coils with an N-terminal loop.\(^2\)\(^\text{53}\)

It is completely unclear at the moment whether phosphorylation of Tyr residues within this sequence cluster excludes Ser/Thr phosphorylation and vice versa, or if both types of phosphorylation can occur simultaneously, may be by sequential actions of kinases. In consequence, the different phospho-signatures of occludin may define its dynamic and binding behavior.

Another highly interesting aspect in this context is the role of Cys408 in mouse occludin (Cys409 in human occludin), which was shown to be involved in occludin dimerization by forming disulfide bridges.\(^2\)\(^\text{54}\) Thus redox-dependent changes in occludin dimerizations are assumed to contribute to changes in TJ assembly and maintenance under physiological and pathological conditions. It will be interesting to see whether and how phosphorylation of this occludin kinase hotspot affects the redox-sensitivity of occludin dimerization.

Finally, it cannot be excluded that further kinases can target amino acid residues within the Tyr398-Ser408 cluster. At least at the sequence level, a potential consensus motif for CK1 can be found within this cluster.

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**Conflicts of interest**

The authors declare no conflicts of interest.

**References**

Occludin phosphorylation hotspot


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