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REVIEW

Phosphorylation of tight junction transmembrane proteins: Many sites, much to do

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ABSTRACT

Phosphorylation is a dynamic post-translational modification that can alter protein structure, localization, protein-protein interactions and stability. All of the identified tight junction transmembrane proteins can be multiply phosphorylated, but only in a few cases are the consequences of phosphorylation at specific sites well characterized. The goal of this review is to highlight some of the best understood examples of phosphorylation changes in the integral membrane tight junction proteins in the context of more general overview of the effects of phosphorylation throughout the proteome. We expect as that structural information for the tight junction proteins becomes more widely available and the molecular modeling algorithms improve, so will our understanding of the relevance of phosphorylation changes at single and multiple sites in tight junction proteins.

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Introduction

Tight junctions form the paracellular epithelial barrier to ions and solutes and thus separate tissue spaces and enable directional transcellular absorption and secretion.[1] Many proteins have been localized to tight junctions,[2] but the critical barrier components include transmembrane proteins that physically form the sealing contacts, including members of the claudin (cldn), tight junction-associated MARVEL proteins (TAMPS) and junctional adhesion molecule (JAM or CTX, for cortical thymocyte marker in *Xenopus*)[3] family of proteins. The transmembrane proteins bind to scaffolding proteins, including ZO-1, -2 and -3 among others, that interact with cytoskeletal elements to regulate junctional integrity. Much information is available on in vitro binding interactions between these protein components,[4-10] but there is considerably less information available on how these binding interactions might be regulated in vivo. One mechanism likely to modulate protein-protein interactions is phosphorylation status.

Most proteins in mammalian cells are phosphorylated;[11] it is a dynamic post translational modification that can regulate protein folding, protein interactions, localization and stability.[12,13] Phosphorylation results in addition of two negative charges at physiological pH, which will alter the electrostatic environment and can alter the strength of protein-protein interactions, both within binding sites and through longer range changes in protein conformation.[12,13] Although the estimated stability of most protein complexes is not altered by phosphorylation, about a third are expected to be significantly stabilized or destabilized by phosphorylation.[12] The predominant influence is destabilization, but a significant minority of protein-protein interactions result in stabilization of protein complexes.[12] In spite of the fact that thousands of phosphorylation sites have been identified by mass spectrometry (MS),[14] relatively few of these have been analyzed in terms of how they affect structure and function.

All of the transmembrane tight junction proteins are reported to be phosphoproteins. In some studies, phosphorylation has been associated with functional changes, but only in a couple of cases has there been any detailed analysis of the relevant mechanisms. Rather than include an exhaustive and exhausting list of phosphorylation changes, the goal of this review is to focus on the best understood examples of how phosphorylation alters interactions or functions in the

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limited set of tight junction integral membrane proteins. In some cases, studies of closely related proteins will be included if they appear to provide relevant insights. By focusing on relatively few different approaches and examples, we hope to highlight some general themes and ongoing challenges as well as identify areas that are particularly promising for future study.

Cldn phosphorylation and PDZ-dependent interactions

PDZ-dependent interactions are a central feature of tight junction organization. Many tight junction scaffolding proteins, including ZO proteins,[15-17] MUPP1,[18] afadin/AF6,[19] MAGI-1, 2, 3,[20–22] PARD3,[23] PALS1[24] and PATJ[25] among others, contain multiple PDZ domains. PDZ domains form small globular structures of 80–90 residues;[26] which typically bind the 4–10 carboxy-terminal residues (PDZ binding motifs)[27,28] of transmembrane proteins and anchor them to the junction. The basic model for interaction is that the terminal hydrophobic residue of the PDZ binding motif-containing protein (numbered amino acid 0) inserts into a hydrophobic pocket in the PDZ domain, and then the upstream amino acids of the binding motif (-1 to -5) form an antiparallel beta strand to a beta strand in the PDZ domain.[29] Both the tight junction strand forming cldns[6] and the adhesion proteins, the JAMs[8,9] end in PDZ binding motifs and have been

Figure 1. Selected cldn cytoplasmic domains. Red text, phosphorylation sites identified by MS[14]; blue highlighted residues are referred to in text, purple highlight identified ubiquitination site blocked by adjacent phosphorylation. Double underline identified regions predicted to be disordered (see text).

shown to interact with the PDZ domain-containing scaffolding proteins.

Cldns are small, tetraspan proteins, with short cytoplasmic tails that vary in length from 26-70 amino acid residues; these tail regions contain abundant serines, threonines and tyrosines (Fig. 1). All but three of the 23 (human) cldn family members end in a characteristic PDZ binding motif (for most cldns -XYV) and interact with the first PDZ domains of ZO-1, -2 and -3[6] and MUPP1,[30] among others. Although specificity of interaction between binding motif and binding domain is likely to be primarily regulated by primary amino acid sequence and protein localization,[29] regulation of this interaction is not well understood. One attractive possible regulatory mechanism is phosphorylation.

Mass Spectrometry data from tissues and cultured cells shows cldn C-terminal regions are abundantly phosphorylated,[14] including within the canonical C-terminal three amino acid PDZ binding motif. For example, much MS data[14] identifies phosphorylation of Y(-1) in cldn1-7, -9, -10 and -18.[14,31] Surprisingly, phosphorylation of this site might not affect interaction with ZO-1, since by analogy, tyrosine phosphorylation of the -1 residue in the PDZ binding motif of syndecan can be accommodated in the PDZ binding pocket in Tiam1 and does not alter binding affinity.[32] Syndecan, like cldns, can bind to a variety of PDZ containing proteins. The authors suggest that syndecan phosphorylation at this site might provide regulatory specificity, since both phosphorylated and unphosphorylated syndecan can bind Tiam1, but only unphosphorylated syndecan can bind to the syntenin PDZ domain. Additionally, binding of phosphorylated syndecan to Tiam1 dampened PDZ domain dynamics, and the authors suggested it might result changes in binding affinities at other sites in Tiam1.[32] Indirect evidence suggests that tyrosine phosphorylation of cldn4 at the -1 position decreases binding to ZO-1,[33] but the possibility that differentially phosphorylated cldns might have differing interactions with ZO-1 PDZ1 is intriguing but unexplored.

Cldn phosphorylation sites upstream from the C-terminal motif could also affect PDZ domain binding. For example, although only the last four residues of cldn1 are involved in interaction with PDZ1 of ZO-1, seven terminal residues of cldn2 contact PDZ1 of ZO-1, and Y-6 phenol group of cldn2 contributes to higher affinity binding to ZO-1 PDZ1 compared with that for cldn1.[34] We recently found that tyrosine phosphorylation of cldn2 at this -6 position decreased affinity for binding to PDZ1 of ZO-1,[34] suggesting a potential regulatory mechanism for their interactions. Similarly, phosphorylation of the analogous site in cldn3 increases its mobility in the membrane as determined by Fluorescence Recovery After Photobleaching (FRAP),[35] suggesting the possibility that phosphorylation of cldn3 Y -6 decreased scaffolding to ZO-1 at the tight junction. Along with possibly altering the affinity for PDZ1 of ZO-1, phosphorylation at other sites in cldn tails may promote binding specificity, which could be important given the presence of multiple PDZ domain-containing junctional proteins.[2]

Other cldn phosphorylation sites and tight junction association

There are many examples of cldn phosphorylation apart from the PDZ domain interaction that are thought to be important for tight junction localization.[31,36–41] For example, phosphorylation of cldn1 (T191)[39] and cldn2(S208)[31] is associated with enhanced tight junction strand formation or localization; Fujii et al.[39] found that hypotonic stress resulted in dephosphorylation at these sites and junctional removal by clathrin-dependent endocytosis. In support of a role for cldn1(T191) phosphorylation in enhancing junction localization, Shiomi et al.[40] found that AMPK activation by AICAR resulted in phosphorylation of T191 in cldn1 and stimulated the formation of ectopic tight junction formation on lateral membrane of Eph4 cells; ectopic fibril formation is also seen with a phosphomimetic mutant of the analogous site in cldn3(T192)[37] and after cldn4 phosphorylation by protein kinase C epsilon.[36] Shiomi et al.[40] further found that stimulated phosphorylation by AMPK activation was associated with decreased cldn1 ubiquitination at a nearby lysine (K189). These authors interpreted these findings to mean that cldn1 ubiquitination is normally required for turnover; blocking turnover by forced phosphorylation thus resulted in ectopic fibril formation. Many cldns have similar arrangements of serines/threonines close to ubiquitinylated lysines identified by MS,[14] so it is possible that this could be a common theme regulating cldn turnover.

Phosphorylation/ubiquitinoylation may also be important in cldn16 removal from the tight junction. Phosphorylation of S217 in cldn16 by protein kinase A pathway results in tight junction localization.[41,42]

This group found that phosphorylation at this site is required for interaction with syntaxin-8 and recycling to the membrane[42] and that dephosphorylated cldn16 was associated with the E3 ubiquitin ligase, PDZRN3, a protein containing both PDZ and ring finger domains.[43] Marunaka et al. suggest this interaction was likely involved in the endocytosis of dephosphorylated cldn16; interestingly, PDZRN3 has also been implicated in regulation of the stability of endothelial cell junctions through targeting the multiPDZ protein MUPP1 for proteasomal degradation.[44]

Cldn phosphorylation has also, although less frequently, been reported to play a role in removal from the tight junction. For example, Cong et al. found that carbachol stimulated ERK phosphorylation of cldn4 and its removal from tight junctions in a rat submandibular epithelial cell line, by promoting its interaction with β-arrestin2 and clathrin endocytosis.[45]

JAM family phosphorylation

The JAM family of proteins are single span integral membrane proteins that are members of the immunoglobulin superfamily; they are broadly distributed in immune cells as well as epithelial and endothelial cells.[46] JAM-A is the principal form expressed on mucosal epithelial cells and is known to be an important regulator of cell polarization, migration, cell-cell adhesion and paracellular permeability. It, like the other JAMs, has a fairly short cytoplasmic C-terminal tail (~40 amino acids) that contains conserved consensus phosphorylation sites (Fig. 2). Like the cldns, JAM cytoplasmic tails end in PDZ binding motifs that interact with PDZ3 of ZO1, as well as with other PDZ domain containing proteins, including afadin, MUPP1 and PAR3.[47]

The carboxyl terminal of human JAM-A, -B and -C all end in the conserved sequence S(295)-S(296)-F(297)-I/V/L(298)-V/I(299). Structural analysis demonstrated that S(296) is a critical component of interaction with ZO-1[48] and high throughput MS has identified this as a phosphorylation site. Although there is no evidence for an effect of phosphorylation at S(296)[49] on interaction with ZO-1 or other PDZ domain containing proteins, possible phosphorylation at this site might regulated the affinity or specificity of interaction with tight junction PDZ containing protein.

In epithelial cells, JAM-A recruits atypical Protein Kinase C (aPKC) to nascent tight junctions where it phosphorylates JAM-A at S285 (S284 in human JAM-A). Total JAM-A is normally present at tight junctions and also to a variable extent along the lateral membrane, but JAM-A S285P is exclusively localized to tight junctions.[50] This phosphorylation is required for the full development of the paracellular barrier and is negatively regulated by Protein Phosphatase 2A (PP2A), which is also involved in de-phosphorylation of other tight junction proteins.[50,51] JAM-A S285 phosphorylation increases as cell contacts mature,[50] and Burridge and colleagues[52] reasoned that tension associated with junction formation might stimulate JAM-A phosphorylation at this site. They used anti JAM-A antibody-coated (to the extracellular domain)

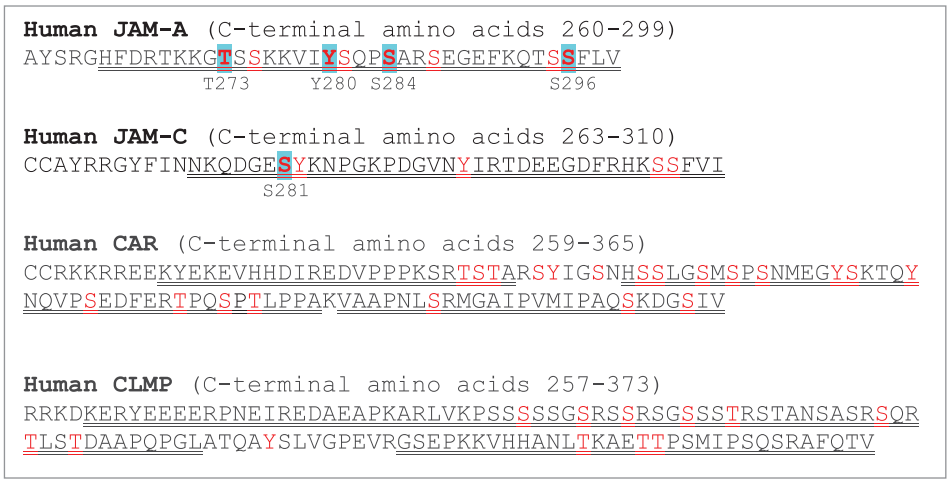

Figure 2. JAM-A, CAR and CLMP cytoplasmic domains. Red text, phosphorylation sites identified by MS ([14]; blue highlighted residues are referred to in text, double underline identified regions predicted to be disordered (see text).

paramagnetic beads to apply tension to JAM-A and found tension increased JAM-A phosphorylation at S284; further, tension applied to JAM-A activated RhoA in a phosphorylation-dependent fashion. Inhibition of RhoA activity is associated with barrier disruption,[53] which might explain in part how JAM-A phosphorylation regulates barrier function. However, the exact mechanism is unclear.

It seems likely that other phosphorylation sites are important in JAM-A function, but they are less well studied. For example, T273 is required for the development of hepatocyte polarity[49] and Y280 development of tube formation by endothelial cells [54]. In addition, tyrosine phosphorylation of JAM-A in platelets modulates integrin signaling;[55] it is not clear if a similar modification is relevant in epithelial cells. However, given that JAMs play myriad roles and bind to a variety of proteins, it is likely that other phosphorylation sites are important, perhaps in altering the affinity for specific binding partners and signaling pathways.

JAM-C, which has been localized both to desmosomes[56] and tight junctions[57] is also found in a variety of tumor cell lines[58] where its function is unknown. Mandicourt et al.[59] demonstrated that exogenous expression of JAM-C in a tumor cell lacking the endogenous protein improved epithelial barrier function. Further, these authors demonstrated that phosphorylation of JAM-C S281 was required for improved tight junction function, since mutation of this residue to alanine blocked the epithelial phenotypic change.

A JAM-related protein, the Coxsackie and Adenovirus Receptor (CAR) has also been identified as a tight junction protein[60] that interacts with both ZO-1[60] and MUPP1.[61] Although addition of peptides corresponding to the extracellular domain of CAR interfere with recovery of TER after junction disruption following calcium removal,[60] it seems likely that CAR may also play a role at the adherens junction. Morton et al.[62] demonstrated that unphosphorylated CAR was involved in E-cadherin endocytosis and that when two residues in the cytoplasmic tail of CAR were phosphorylated by PKCδ, this blocked the endocytosis stimulated by over-expression of wild-type CAR. These results suggested that CAR normally plays a role in E-cadherin recycling and that CAR phosphorylation might stabilize E-cadherin at the membrane. One caveat to this study is that the fluorescent tag used to follow CAR localization is likely to block interaction with PDZ domain binding and thus may alter localization. These two sites are not found in the canonical JAMs, but another JAM family member, CLMP (CAR-like membrane protein) has a serine and threonine-rich tail with potential phosphorylation sites in the same region. CLMP is required for intestinal development and its absence results in short bowel syndrome.[63,64] It has been shown to localize at tight junctions[65] and it can mediate cell-cell adhesion[65] but more study is required to understand its specific functional role and the role of phosphorylation.[66]

TAMP family phosphorylation

Occludin Phosphorylation

Occludin (Ocln) was the first identified tight junction transmembrane protein;[67] like cldns, it is a tetraspan protein and most phosphorylation sites are found in the C-terminal cytoplasmic domain[14] (Fig. 3) It was early identified as being multiply phosphorylated.[68] Ocln, although normally concentrated at tight junctions, is found all along the lateral membrane; in an elegant study, Tsukita and colleagues demonstrated that ocln at the lateral membrane was phosphorylated at a much lower level than the highly phosphorylated junctional ocln.[68] Following this initial observation, there were a large number of studies that correlated the degree of ocln phosphorylation with its localization; in most cases, ocln dephosphorylation, as measured by changes in migration in SDS-PAGE, was correlated with barrier loss,[69–73] reviewed in.[74] A large number of kinases and phosphatases have been implicated in regulation of ocln phosphorylation, including CK2,[75] cYES,[76] CK1,[77] PKC,[78] Rho kinase,[79] Src,[80] G-coupled receptor kinases (GRK),[81] PP2A,[51] density enhanced phosphastase 1[82] and PTPN2 (non-receptor protein tyrosine phosphatase N2).[83]

Although correlations between ocln phosphorylation with localization and with different signaling pathways were noted soon after the discovery of ocln, mechanistic studies on the importance of specific phosphorylation sites are relatively recent. An elegant pair of studies from the Antonetti laboratory identified a number of ocln phosphorylation sites[84,85] and important functional consequences associated with phosphorylation of one of them. These authors found S490 was phosphorylated in response to VEGF treatment and that this phosphorylation attenuated interaction with ZO-1, suggesting a molecular mechanism

Figure 3. Ocln, tricellulin and marvelD3 cytoplasmic N- and C-terminal domains. Red text, phosphorylation sites identified by MS[14]; blue highlighted residues are referred to in text, double underline identified regions predicted to be disordered (see text). Yellow highlighted area in ocln C-terminal domain identifies area with structural information.[89,107]

for ocln dissociation from the tight junction. In addition, mutation of S490 to a non-phosphorylatable alanine suppressed VEGF-induced ubiquitination, tight junction protein trafficking and the increase in permeability associated with VEGF treatment. More recent work from this group identified PCKβ as the relevant kinase.[86] Unexpectedly, Antonetti and colleagues also has implicated this phosphorylation site in ocln-dependent regulation of mitotic entry[87] and in control of VEGF-induced neovascularization.[88] These data suggest that phosphorylation of ocln at S490 could be an important site in the coordination of endothelial proliferative response to injury associated with tight junction barrier changes.

Antonetti and colleagues went on to analyze the structural contacts between ZO-1 and ocln and identified a potential stabilizing phosphorylation site within the ocln tail, S471.[89] In a more recent study, they demonstrated that over-expression of ocln S471A blocked monolayer maturation and normal tight junction protein localization, consistent with a requirement for phosphorylation at this site in meditating normal interactions with ZO-1 and cell packing.[81]

In addition to these phosphorylation sites, Dorfel and Huber[90] identified a phosphorylation hotspot within the ocln c-terminal domain, an 11 amino acid stretch from 398-408. Within this region, Raleigh et al.[91] had found that inhibition of CK2-mediated

phosphorylation of ocln at S408 resulted in increased TER. Dephosphorylated ocln interacted with ZO-1 and through ZO-1 with cldn 2; these interactions were attenuated by S408 phosphorylation. The authors speculated that phosphorylation decreased interaction between ZO-1, cldn2 and ocln, affecting the dynamic behavior among these proteins and resulting in decreased paracellular permeability by disrupting the cation-permeable paracellular pores formed by cldn2. In contrast to the findings of Raleigh et al., ocln T400 and T404 were also identified as CK2 phosphorylation sites and when T400, T404 and S408 were mutated to phosphomimetic amino acids, Huber and colleagues found diminished interaction with ZO-2 and increased TER;[92] some differences were seen between the effects of the mutations on interactions with ZO-1 and ZO-2. Also within this region is T403, which with T404 has also been reported to be a phosphorylation site for PKCη;[93] expression of wild-type or dominant active PKCη enhances tight junction assembly, similar to what was reported by Huber and colleagues.[92] Phosphorylation of T403/4 was associated with tight junction assembly, since T403/4A ocln mutants fail to localize to tight junctions, while T403/4D mutants block the ability of a PKCη inhibitor to disrupt ocln localization. The reason for the different effects of phosphorylation in this small region is unclear, but may reflect differences in cell lines and/or relative cldn expression levels and requires further investigation.

Along with serine and threonine ocln phosphorylation, tyrosine phosphorylation of ocln has also been implicated in both tight junction disassembly;[71,94] and assembly[76] however, most studies have reported that tyrosine phosphorylation is predominantly associated with diminished junctional ocln. Both Y398 and 402 phosphomimetics have been shown to block binding to ZO-1 and to destabilize ocln at tight junctions.[95] In one unusual example, ocln Y473P was localized to the leading edge of wounded epithelial monolayers; where is was reported to recruit the phosphoinositol-3-kinase regulatory subunit, p85α and thus activate Rac1 to promote lamellipodial formation and migration.[80]

Tricellulin and MarvelD3 phosphorylation

Both tricellulin and marvelD3 are ocln-related proteins; marvelD3, like ocln, is distributed along bicellular tight junction contacts[96,97] while tricellulin is concentrated at tricellular contacts. Tricellulin is it critical for regulation of paracellular flux of larger molecules[98] while the role of marvelD3 is more complex.[99] Both tricellulin and marvelD3 are reported to be phosphoproteins,[14] but in contrast to ocln, tricellulin and marvelD3 have relatively extended intracellular N-terminal domains which contain a significant number of phosphosites; in the case of marvelD3, most phosphorylation sites lie in this domain (Fig. 3). All phospho sites to date within both tricellulin and marvelD3 have been identified solely by high throughput MS analysis, without direct experimental verification, so that the importance of these sites remains to be explored. As an aside, tricellulin is concentrated at tricellular contacts through interaction with angulin1/LSR, an immunoglobulin superfamily transmembrane protein. Although little is known about tricellulin phosphorylation, a recent study demonstrated the requirement for JNK1/2-mediated phosphorylation of angulin 1/LSR at S288 for its localization, and thus localization of tricellulin, to the tricellular junctions.[100]

Future approaches

Although it is possible to deduce some general rules from the above studies, for example, that serine/threonine phosphorylation of the cldn and JAM C-terminal domains is (mostly) associated with localization at the tight junction, while tyrosine phosphorylation of either cldns or ocln results in junction disruption, most findings referenced in this review are necessarily simplistic. However, these studies at a minimum identify key areas and relevant pathways that should allow us to ask increasingly sophisticated questions about the importance of tight junction protein phosphorylation. For example, the growing availability of structural information (as in[34,89]) can be used to guide mutational analyses and interpretations about phosphorylation in well-ordered protein domains. In addition, the recognition that intrinsically disordered protein domains are enriched in phosphorylation[101] and are particularly important in trafficking and protein localization[102] is highly relevant. The cytoplasmic domains of cldns and JAM-A, as well as regions within the ocln tail are predicted to be disordered by several algorithms;[103,104] phosphorylation in these regions has different implications than that in highly structured domains.[12] Further, it is important to remember that all of the tight junction integral membrane proteins have multiple phosphorylation sites

that in combination are likely to have complex and interacting contributions to their behavior. In many cases the domains in other proteins with which these phosphorylated proteins interact are themselves likely to be variably phosphorylated; for example, it has recently been shown that threonine phosphorylation on T770/T772 in the GUK (guanylate kinase) domain of ZO-1 by PKCε disrupts interaction with ocln.[105] Phosphorylation can be difficult to detect, is dynamic and reversible and can have subtle long range as well as large local effects. As algorithms improve, molecular modeling is likely to be increasingly useful in predicting how phosphorylation might alter protein interactions.[106]

Dynamic phosphorylation changes are clearly relevant to regulation of the interactions and stability of tight junction proteins, and better understanding of the role of this post-translational modification will provide important biologic and potentially therapeutic insights in the regulation of the paracellular barrier.

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