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REVIEW

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Epithelial integrity, junctional complexes, and biomarkers associated with intestinal functions

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ABSTRACT
An intact intestinal barrier is crucial for immune homeostasis and its impairment activates the immune system and may result in chronic inflammation. The epithelial cells of the intestinal barrier are connected by tight junctions, which form an anastomosing network sealing adjacent epithelial cells. Tight junctions are composed of transmembrane and cytoplasmic scaffolding proteins. Transmembrane tight junction proteins at the apical-lateral membrane of the cell consist of occludin, claudins, junctional adhesion molecules, and tricellulin. Cytoplasmic scaffolding proteins, including zonula occludens, cingulin and afadin, provide a direct link between transmembrane tight junction proteins and the intracellular cytoskeleton. Each individual component of the tight junction network closely interacts with each other to form an efficient intestinal barrier. This review aims to describe the molecular structure of intestinal epithelial tight junction proteins and to characterize their organization and interaction. Moreover, clinically important biomarkers associated with impairment of gastrointestinal integrity are discussed.

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Introduction

The interplay between the host and environment holds the key to health and well-being. A major unit of this interaction is the gastrointestinal tract due to I) the large surface area of approximately 400 m^2, which may be exposed to foreign antigens and toxic molecules, II) its diverse microbial community, and III) the adherent complex system of mucosal immune cells that facilitate the major innate and adaptive defense mechanisms.[1–5] Key to the maintenance of gut immune homeostasis is the capacity of the intestinal barrier to separate luminal antigens, including microbial communities, from mucosal immune cells.

Increased epithelial permeability to foodborne allergens, pathogens and toxins, local inflammatory reactions, and nutrient malabsorption are considered not only in terms of immediate disease conditions, but more importantly as factors increasing the prevalence of many chronic inflammatory conditions, autoimmune diseases as well as allergies.[6–12]

Maintenance of the intestinal barrier is acquired by a variety of specific and nonspecific mechanisms, but intestinal epithelial cells form a first line of host defense against potentially harmful stressors reaching the luminal environment.[1,5,7,11]

The lining epithelial cells are connected by multiple protein structures denoted as apical junctional complexes, including tight junctions (TJs), adherens junctions, and desmosomes (Figure 1).[13–15] Adherens junctions play a role in initiation and stabilization of cell-cell contacts through a family of intercellular adhesion molecules and consist of transmembrane proteins, including E-cadherin and nectin as well as associated cytoplasmic proteins catenins, which are directly connected to the actin cytoskeleton.[16] Desmosomes are patch-like intercellular junctions at the lateral sides of plasma membranes that join adjacent cells together and provide anchoring sites for intermediate filaments.[15,17,18] TJ proteins are thought to be the most essential components of these multiple structures and it is

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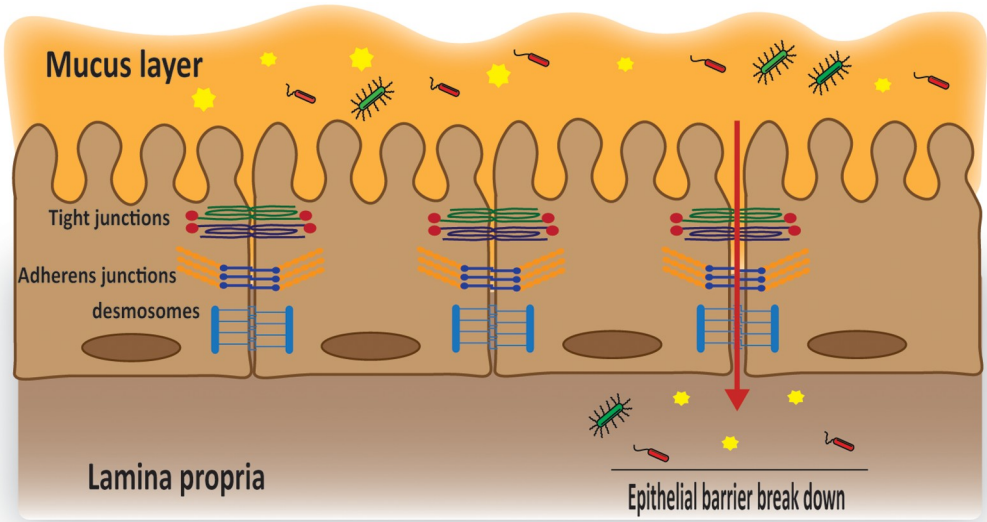

Figure 1. Schematic illustration of the epithelial junctional complexes. Note: The intestinal epithelial barrier is primarily regulated by the junctional complexes consisting of tight junctions (TJs), adherens junctions, and desmosomes. TJs are the most apical structures, followed by adherens junctions and desmosomes that are located along the lateral cell membrane. Damage to the intestinal epithelial barrier facilitates the transfer of luminal antigens and proinflammatory stimuli (yellow heptagrams) and even different pathogens (red and green rod-shaped bodies) through the epithelial layer into the lamina propria resulting in immunological and inflammatory reactions, which lead to further intestinal barrier damage.

now well-established that the mucosal barrier function cannot be maintained without a well-organized pattern of this anastomosing network of sealing strands comprising over 50 proteins. The intercellular space completely disappears at the level of TJs, whereas in adherens junctions and desmosomes, the adjacent membranes are 15–20 nm apart (Figure 1).[13,16,17,19,20] Each individual component of the TJ network is structurally and functionally different, but closely interacts with each other to form an efficient and functional barrier.

This review aims to describe the molecular structure and expression of intestinal epithelial TJ proteins and to characterize the detailed architecture, organization, and interaction of some of the key TJ proteins. It is important to highlight that the information presented in this review focused on the evidence from *in vivo* animal (mostly murine) studies and strengthen the discussion of the clinical relevance for human patients.

Molecular architecture of TJs

TJs are dynamic structures with a complex architecture that seal adjacent epithelial cells near the luminal surface and thus prevent a paracellular transport of luminal antigens.[15,21,22] TJ proteins are composed of: I) transmembrane proteins whose extracellular domains cross the plasma membrane and interact with their partners on the adjacent cells and II) cytoplasmic scaffolding proteins, which are entirely located on the intracellular side of the plasma membrane. Transmembrane TJ proteins form a linear barrier at the apical-lateral membrane of the cell and consist of occludin (OCLN), claudins (CLDNs), junctional adhesion molecules (JAMs) and tricellulin. The cytoplasmic scaffolding proteins, including zonula occludens (ZOs) proteins, cingulin, and afadin, provide a direct link between transmembrane TJ proteins and the intracellular cytoskeleton. The cytoskeleton plays a crucial role in the regulation of TJ-based integrity.[17,23–25] TJ proteins are differentially

expressed along the gastrointestinal tract, and this expression pattern is probably related to the differences in exposure to luminal substances between individual intestinal segments. As an example, it has been shown that TJ proteins are less expressed in crypts compared to villi tips; hence, intercellular tightness is increasing from the crypt to the apical parts of a villus.[24,26–29]

Occludin (OCLN)

Molecular structure

OCLN (~65 kDa) is the first discovered TJ protein with a critical role in sealing the adjacent cells. It contains one intracellular and two neutral extracellular loops, as well as four transmembrane domains, a short NH_2-terminal cytoplasmic domain and a long COOH-terminal cytoplasmic domain (Figure 2).[27]

Expression

It has been shown that OCLN has a consistent expression throughout the entire intestine and appears as distinct dot-like or line-forming structures at the apical region of the lateral plasma membrane of the epithelial cells of a villus.[30–33] However, only the phosphorylated form of OCLN is incorporated into the intracellular junctional complexes, whereas the non-phosphorylated OCLN is distributed in an erratic expression pattern along the basolateral plasma membrane and therefore this form does not contribute to barrier integrity.[19,25,34]

Intestinal barrier function

Homophilic interaction between the extracellular loops of OCLN with the identical loops originating from OCLN in the adjacent cells seems to form a barrier against macromolecules, but not against

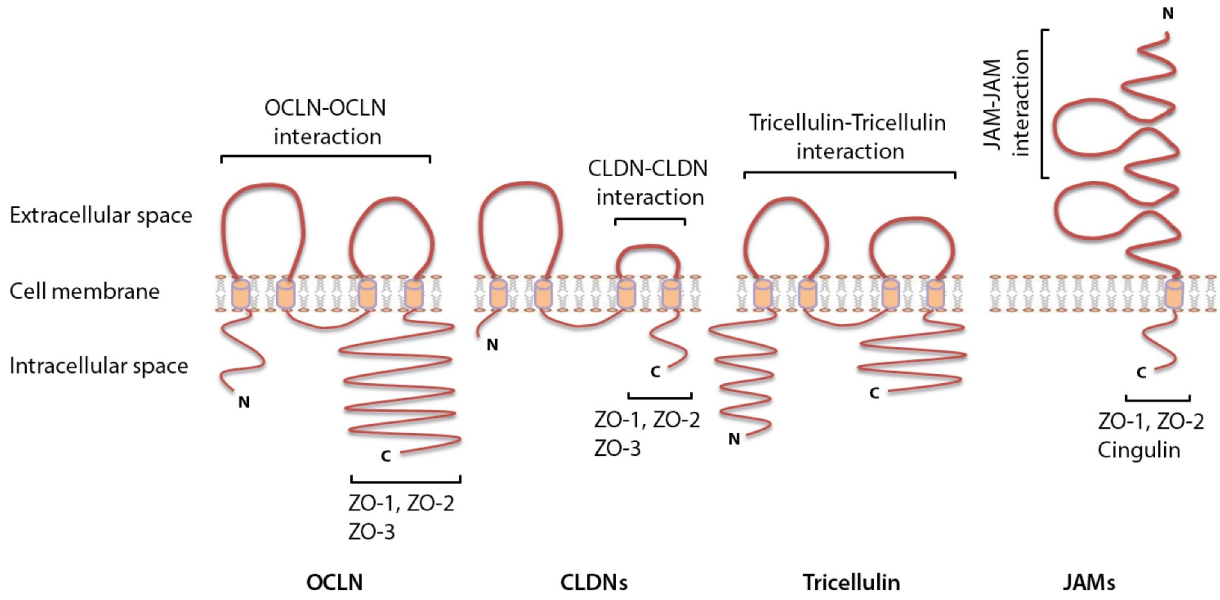

Figure 2. Schematic representation of the molecular structure of different transmembrane tight junction (TJ) proteins and the related direct interaction with different transmembrane as well as cytoplasmic scaffolding proteins. OCLN, CLDNs and tricellulin have a similar topography, with one intracellular, two extracellular loops, four transmembrane domains, and cytoplasmic N- and C-terminal domains. JAMs are characterized by two extracellular Ig-like domains, a transmembrane domain and a C-terminal cytoplasmic domain. The C-terminal cytoplasmic domain has been shown to be crucial in membrane targeting of OCLN, CLDNs and JAMs to the TJ network, whereas, both N- and C-terminal domains of tricellulin seem to play a relevant role in tricellulin localization at the TJ network. Homophilic interactions of different transmembrane TJ proteins from adjacent cells (between proteins of the same kind) are mediated through the two extracellular loops (OCLN and tricellulin), a second extracellular loop (CLDNs) or membrane-distal extracellular Ig-like domains (JAMs). The C-terminal domain of OCLN, CLDNs and JAMs interacts with different cytoplasmic scaffolding TJ proteins, including ZOs and cingulin, by which they are connected to the actin cytoskeleton. C, COOH-terminal domain; CLDNs, claudins; JAMs, junctional adhesion molecules; N, NH2-terminal domain; OCLN, occludin; ZOs, zonula occludens.

small ions.[19,25,34,35] The COOH-terminal cytoplasmic domain has been shown to be required for correct integration of OCLN into the TJ network. The link between OCLN and the intracellular cytoskeleton is also documented through the interaction of the -COOH terminus with several cytoplasmic scaffolding TJ proteins, such as ZO proteins (Figures 2 and 4).[27] Controversial findings have been reported regarding the role of OCLN in the regulation of paracellular permeability, since OCLN knockout mice showed a well-organized TJ architecture and a normal barrier function comparable to those observed in wild-type mice. On the other hand, different *in vivo* studies support the concept that OCLN is a functional component of the TJ network and has a significant role in the regulation of paracellular permeability.[19,25,36–38] It is further known that OCLN plays a role in TJs reassembly since the absence of OCLN in canine MDCK epithelial cells causes a pronounced decrease in expression of CLDNs and functional recovery as assessed by measurement of the transepithelial electrical resistance (TEER). However, the expression of ZOs and JAM-A are not affected by OCLN as demonstrated in knockdown MDCK cells.[39] The restoration of OCLN (higher expression) together with higher TEER values have been reported to improve intestinal barrier function in intestinal porcine epithelial cells (IPEC-J2).[40]

Clinical relevance

Decreased protein expression and redistribution of OCLN have been observed in the intestines of patients with Crohn's disease, ulcerative colitis, celiac disease and irritable bowel syndrome.[7,41–46] In addition, small intestinal biopsy specimens of patients with a known food allergy showed a lower protein expression of OCLN compared to those from normal subjects when re-exposed to the food allergen.[47]

Claudins (CLDNs)

Molecular structure

CLDNs (~20–27 kDa) consist of one intracellular loop, two extracellular loops, four transmembrane domains, a short NH2-terminal cytoplasmic domain together with a longer COOH-terminal cytoplasmic domain (Figure 2); however, a few members of the CLDN family exhibit a different structure.[27,48] CLDN5, −16 and −25, for example, have a higher molecular mass owing to the long NH2 terminus.[48] It is well known that the half-life of CLDNs mainly depends on length and relevant amino acid sequences of the COOH-terminal cytoplasmic domain.[24,49] A strong correlation has been previously reported between length of the cytoplasmic domains of CLDNs and their half-lives; for example, the cytoplasmic domain of CLDN2 is two times as long as the cytoplasmic domain of CLDN4, whereas the half-life of CLDN2 is more than 3-fold higher compared to CLDN4.[49] The first extracellular loop of CLDNs is recognized by well-conserved charged amino acids, although the number, nature and orientation of charges are different depending on the CLDN type.[48,50,51] In this respect, CLDN10b, −13 and −17 possess positively charged residues and CLDN2, −7, −10a, −12 and −15 carry negatively charged residues. The charge selective properties of the first extracellular loop enable CLDNs to create paracellular pores for small ions. It is shown that positively and negatively charged CLDNs act as anion and cation pores, respectively.[27,34,48,52,53]

Expression

Twenty-seven members of the CLDN family are recognized so far in rodents, however their expression in humans is not identical. Twenty-six CLDNs have been discovered by genomic cloning in humans, but the rodent CLDN13 does not have a human homolog.[48,54] Gene expression of CLDN1-27 is profiled and all members, except CLDN6, −9, −11, −16, −19 and −22, have also been distinguished throughout the mouse intestine.[30,48,54–56] However, the majority of the

Table 1. The expression pattern of different CLDNs along the mouse intestine[24,30,58,59].

	Duodenum	Jejunum	Ileum	Colon
CLDN3	+	++	+++	++++
CLDN4	++++	+++	++	+
CLDN7	++++	++++	++++	++++
CLDN8	-	-	+	++
CLDN12	+	+++	++++	++
CLDN13	-	-	-	++
CLDN15	+++	++++	++	+

CLDNs show an inconstant expression pattern throughout the different segments of the gastrointestinal tract.[57] For example, the expression pattern of the different CLDNs along the entire mouse intestine is summarized in Table 1. In addition, CLDNs show a different subcellular distribution alongside the intestinal epithelium. For example, CLDN1 and CLDN3 are expressed laterally between adjacent cells along the crypt-to-villus axis, whereas CLDN2 distribution is mainly restricted to the crypts of the entire intestine. CLDN4 is predominantly expressed in the upper part of the villi alongside the entire intestine. On the contrary, CLDN7 is more expressed on the basolateral surfaces of the intestinal epithelium compared to the apical surfaces.[24,30,55,58–60]

Intestinal barrier function

CLDNs appear to be the major structural components of the TJ proteins and are known as the backbone of the TJ network.[61–63] It has been previously discovered that the second extracellular loop of CLDNs is critically involved in homo- and heterophilic interactions between CLDNs. These interactions among CLDN members are previously claimed to be much stronger than those of the other TJ proteins, including OCLN–OCLN interaction or tricellulin–tricellulin interaction (Figure 2).[48,64] The COOH-terminal cytoplasmic domain has been shown to be crucial in membrane targeting of CLDNs to the TJ network and mutations in this domain result in internalization and subsequent degradation of CLDNs.[24,48] In addition, the COOH-terminus of all CLDNs (with the exception of CLDN12, −16, −19a, −21 and −24 to −27) ends in PDZ-binding motifs interacting with PDZ domains of scaffolding protein, including ZO-1, ZO-2 and ZO-3, which are subsequently connected to the intracellular cytoskeleton (Figures 2 and 4).[48,65,66] The phenotypes of CLDN knockout mice reveal the importance of specific CLDNs for development, viability and intestinal integrity. For example, CLDN1 and CLDN5 knockout mice die shortly after birth; whereas CLDN7 knockout mice have severe intestinal defects, including mucosal ulcerations, epithelial cell sloughing, villi disruption, significant intercellular gaps between adjacent epithelial cells, enhanced paracellular permeability and colonic inflammation.[48,59,67]

Clinical relevance

Clinical studies show decreased protein expression and redistribution of CLDN3, CLDN5 and CLDN8 in the intestine of Crohn's disease patients, whereas in the intestine of ulcerative colitis patients decreased protein expression and redistribution of CLDN1 and CLDN4 has been reported. Furthermore, protein expression of CLDN2 is consistently increased in the intestine of patients with Crohn's disease as well as in patients suffering from ulcerative colitis. The protein expression of CLDN1 is decreased in irritable bowel syndrome patients and is irregularly distributed in the colonic mucosa.[7,41–45,68–70] Increased protein expression of CLDN2 and CLDN3 is observed in the intestine of patients with celiac disease.[43,71] In addition, small intestinal biopsy specimens of patients with food allergy exposed to food allergens showed a decreased protein expression of CLDN1 compared to those from normal subjects.[47]

Junctional adhesion molecules (JAMs)

Molecular structure

JAMs (~30–40 kDa) belong to the immunoglobulin (Ig) superfamily of proteins characterized by two extracellular Ig-like domains, a transmembrane domain and a short COOH-terminal cytoplasmic domain (Figure 2).[72] Based on sequence similarities in the cytoplasmic domain, JAMs are classified into two subfamilies. The first subfamily consists of JAM-A, JAM-B and JAM-C, with a class II PDZ binding motifs at their COOH-terminal ends, that directly interact with TJ scaffolding proteins, including ZO-1, ZO-2 and cingulin (Figure 2).[29,73–75] The second subfamily consists of coxsackie and adenovirus receptor (CAR), endothelial selective adhesion molecule (ESAM) and JAM-4 containing class I PDZ binding motifs.[24,34,76] Within the JAM family, the role of JAM-A is of particular interest in the regulation of barrier function, which will be discussed in more detail below.

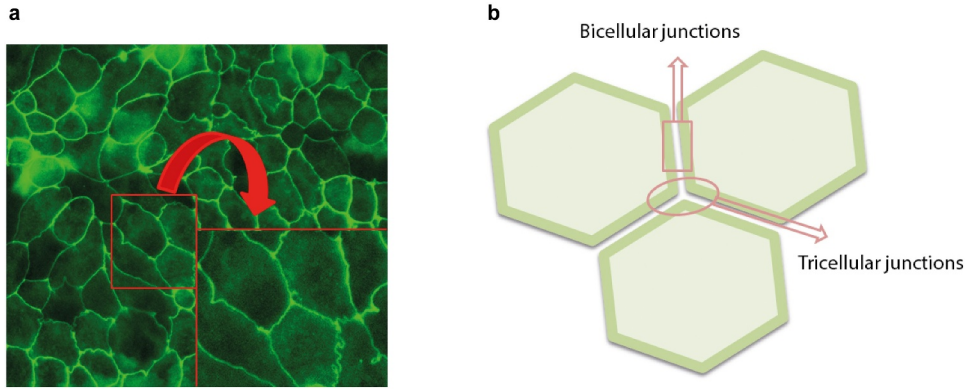

Figure 3. Immunofluorescence picture Magnification 400X (a) and schematic drawing (b) of the bicellular junctions (contact sites between two adjacent cells) and tricellular junctions (contact sites between three adjacent cells). The insert is a (non-defined) enlargement of the area marked in the original picture to visualize the bicellular junctions in more detail.

Expression

Like OCLN, JAM-A is also constantly expressed throughout the mouse intestine and is localized laterally between adjacent epithelial cells.[30,31,77]

Intestinal barrier function

JAM-A is generally believed to have dual functions. Within the immune system, it has been implicated to control the recruitment of leukocytes to the site of inflammation, whereas along intestinal epithelial cells, dominant expression of JAM-A has been observed in the region of TJs, suggesting a critical role in barrier function.[64,72,78,79] Homophilic interaction of JAM-A between adjacent cells is mediated through membrane-distal extracellular Ig-like domains (Figure 2) forming a barrier against luminal substances. The short cytoplasmic domain of JAM-A terminates with PDZ-binding motifs that interact with different cytoplasmic scaffolding proteins, including ZO-1, ZO-2 and cingulin (Figures 2 and 4) by which it is linked to the intracellular cytoskeleton.[64,72,80] Although JAM-A knockout mice display a normal epithelial architecture, intestinal integrity tests confirm an increased gut permeability to different paracellular markers and a decreased TEER in mucosal tissue samples obtained from these animals.[78,81] In addition, it has been shown that JAM-A is involved in the recovery of epithelial barrier function after disruption of TJs by transient calcium depletion, since inhibition of JAM-A leads to retarded TJs reassembly in human T84 epithelial cells shown by disrupted redistribution of OCLN and decreased TEER recovery.[79]

Clinical relevance

Decreased protein expression of JAM-A is observed in the intestines of patients with Crohn's disease and in patients suffering from ulcerative colitis.[81,82]

Tricellulin

Molecular structure

Tricellulin (~64 kDa) shares a few structural characteristics with OCLN, including one intracellular, two extracellular loops, four transmembrane domains, a cytoplasmic COOH-terminal domain and a cytoplasmic NH2-terminal domain, which is longer compared to the NH2-terminal domain of OCLN (Figure 2).[29,83,84]

Expression

Although the above mentioned transmembrane TJ proteins are mainly involved in sealing the intercellular space between two adjacent cells (known as bicellular junctions), tricellulin is the first TJ protein identified at tricellular junctions between three adjacent epithelial cells (Figure 3a,b).[83–85] Tricellulin is differentially expressed throughout the mouse intestine (strongly expressed in the

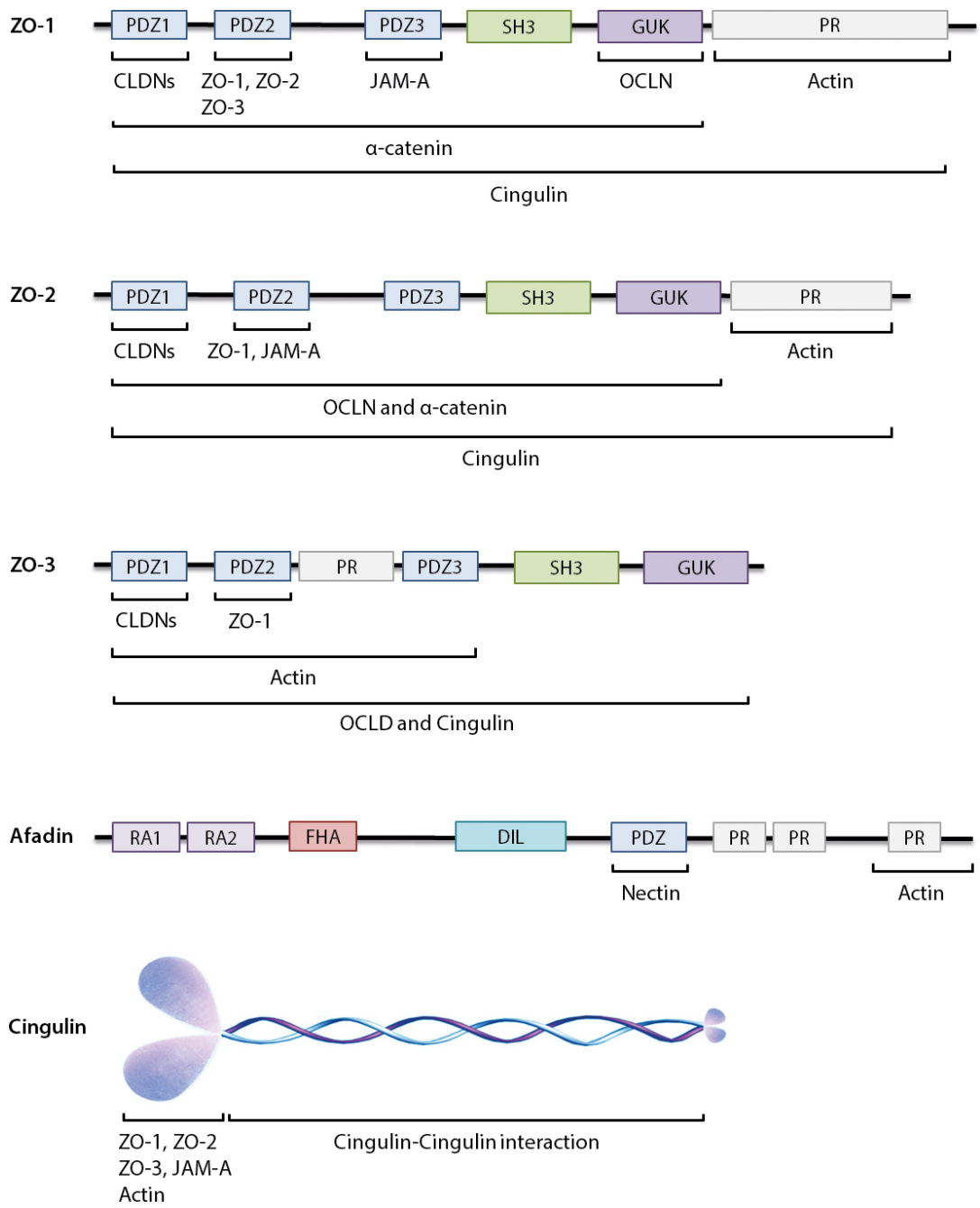

Figure 4. Schematic diagrams of the structural organization of individual cytoplasmic scaffolding proteins and the related direct interaction with different tight junctions (TJs) as well as adherens junctions. ZO proteins carry three PDZ domains, a Src homology 3 (SH3) domain, a guanylate-kinase homology (GUK) domain and proline-rich (PR) region. Afadin consists of two Ras-binding domains (RA1 and RA2), a forkhead-associated (FHA) domain, a diluted (DIL) domain, a PDZ domain and three proline-rich (PR) domains. Cingulin exists as a parallel homodimer of two subunits, each comprised of a N-terminal globular head region, a long α-helical coiled-coin rod region and a small globular tail. CLDNs, claudins; JAM-A, junctional adhesion molecule-A; OCLN, occludin; ZOs, zonula occludens.

small intestine, but weakly expressed in the large intestine) and appears as dot-like structures in the corners where three adjacent epithelial cells meet.[85,86] However, it has been shown that tricellulin overexpression directs this protein into both bicellular as well as tricellular junctions.[87] The tricellular location of tricellulin is adjusted by angulins. Angulin-1, −2 and −3 as transmembrane proteins also contribute to barrier function related to tricellulin recruitment to tricellular junctions as

an essential protein for integrity of tight junction network and barrier formation.[63,88] Although, there is no evidence showing direct and physical interaction between tricellulin and OCLN, it is proposed that these two proteins may affect each other's cellular distribution.[86] For example, *in vitro* studies using MDCK cells showed that knockdown of OCLN results in mislocalization of tricellulin from tricellular to bicellular junctions, whereas tricellulin deficiency causes thinner and disrupted continuity of OCLN distribution at bicellular junctions with teardrop-shaped accumulation of OCLN at tricellular junctions.[85,89,90]

Intestinal barrier function

Homophilic interaction of extracellular loops with the identical loops originating from tricellulin in the adjacent cells form a barrier against macromolecules (Figure 2). Unlike OCLN, both NH2- and COOH-terminal domains of tricellulin seem to play a relevant role in tricellulin localization at the TJ network.[83,84] Tricellulin appears to be directly involved in the establishment of a barrier at the tricellular junctions, since strands of bicellular TJs are not continuous at tricellular junctions. Although tricellulin knockout mice have not been described yet, a few *in vitro* studies have highlighted the importance of tricellulin. For example, the inhibition of tricellulin in mouse Eph4 epithelial cells severely compromise the epithelial barrier function observed by a decrease in TEER and increase in fluorescein isothiocyanate-dextran permeability.[83,85] HT-29/B6 monolayers, a mouse model, and human biopsies showed that IL-13 induced down regulation in tricellulin expression resulted in enhanced permeability to macromolecules.[91] A study by Caco-2 monolayer demonstrated that enteropathogenic Escherichia coli infection has the potency to decrease tricellulin levels, which is accompanied by an increase in paracellular permeability.[92] In addition, tricellulin has been shown to play a role in TJs reassembly and its role is not restituted by other TJ proteins, since tricellulin knockdown Caco-2 cells show a delay in TJs reassembly and barrier development.[86]

Clinical relevance

Decreased protein expression of tricellulin is observed in the intestines of patients with ulcerative colitis.[91,93]

Zonula occludens (ZOs)

Molecular structure

The family of ZOs are comprised of three closely related isomers known as ZO-1 (~220 kDa), ZO-2 (~160 kDa) and ZO-3 (~130 kDa), belonging to the membrane associated guanylate kinase-like homologs (MAGUK) family.[94] The MAGUK family is mainly referred to a group of proteins found at the sites of cell–cell contacts and they function as molecular adaptors in different cellular processes, including cell–cell communications, epithelial polarization and signal transduction.[95,96] Similar to the other MAGUK members, ZO proteins carry a Src homology 3 (SH3) domain, a guanylate-kinase homology (GUK) domain, proline-rich (PR) region and in this case three PDZ domains (Figure 4).[94,95]

Expression

Consistent expression of ZO-1 has been shown throughout the mouse intestine and appears as distinct line-forming structures at the apical region of the plasma membrane of the epithelial cells along the crypt-to-villus axis.[31,32,97] Further studies are needed to compare the level of expression of ZO-2 and ZO-3 along the mouse intestine.

Intestinal barrier function

ZO proteins directly interact with both transmembrane TJ proteins (such as OCLN, CLDNs and JAM-A) as well as with adherens junctions (such as α-catenin and E-cadherin), thus provide the structural basis for the formation of apical junctional complexes and link them to the actin cytoskeleton.[94,95,98] The PDZ2 domain of ZO-1 appears to be crucial for both homodimerization (ZO-1/ZO-1 dimer) and hetrodimerization (ZO-1/ZO-2 dimer or ZO-1/ZO-3 dimer) of this protein, while no direct binding has been reported between ZO-2 and ZO-3 (Figure 4).[94,99] The GUK domain and the PDZ3 domain of ZO-1 have been proved to interact with OCLN and JAM-A, respectively, whereas PDZ1 of all three ZO proteins are able to bind to the COOH-terminus of CLDNs and induce independent regulation of both polymerization and localization of CLDNs in epithelial cells. The PDZ2 domain of

ZO-2 interacts with JAM-A, while no binding has been reported between ZO-3 and JAM-A (Figure 4).[19,72,94,95,100]

Both ZO-1 and ZO-2 proteins appear to play a crucial role in maintaining the epithelial barrier function. Early embryonic lethality of mice lacking ZO-1 and ZO-2 reveals the importance of these proteins for development and viability. *In vitro* data using MDCK and Eph4 cells confirm the functional role of ZO-1 and ZO-2 in the intestinal integrity, since inhibition of either ZO-1 or ZO-2 triggers atypical monolayer architecture and alters expression of apical junctional complexes, such as decreasing the amount of OCLN and E-cadherin.[101–106] In addition, ZO-1 is shown to be essential for TJs reassembly, since knockout of ZO-1 in MDCK and Eph4 cells causes a pronounced delay in the recruitment of CLDNs and OCLN, after calcium switch assay, to the newly formed apical junctional complexes.[107,108] ZO-2 functions as an important regulator for barrier function via Rho protein activity where the ZO-2 silencing can resulted in basal stress fibers formation through RhoA signaling activation.[109] ZO-3 seems to be unessential for the regulation of intestinal integrity and viability, since ZO-3 deficiency displays no apparent phenotype neither in mice nor in epithelial cells.[106,110]

Clinical relevance

Decreased protein expression of ZO-1 is observed in the intestines of patients with celiac disease.[43] A literature review by Sugita and Kabashima illustrated that dysregulation and decrease in ZO-1 expression can be detected in chronic rhino sinusitis, zinc deficiency, atopic dermatitis and asthma.[111]

Afadin

Molecular structure

Afadin (~206 kDa) is a multi-domain protein and consists of two Ras-binding domains (RA1 and RA2), a forkhead-associated (FHA) domain, a diluted (DIL) domain, a PDZ domain and three proline-rich (PR) domains (Figure 4).[29,112–114]

Expression

Afadin is expressed throughout the mouse intestine and is localized at both TJs and adherens junctions. Afadin appears as distinct dot-like or line-forming structures at the apical region of the plasma membrane of the epithelial cells along the crypt-to-villus axis.[113,115]

Intestinal barrier function

The PDZ and proline-rich domains of afadin have been associated with either direct or indirect interaction of afadin with different cell adhesion proteins, including nectin, E-cadherin, JAM-A, ZOs and CLDNs (Figure 4).[64,75,112,116–118] It is already known that afadin plays a crucial role in establishment and proper organization of the apical junctional complexes as well as providing a physical link between different components of apical junctional complexes and the intracellular cytoskeleton.[112,113,119] It has been reported that the architecture of epithelial apical junctions in both the small and large intestines are preserved in afadin-knockout mice; however, this lack of afadin results in impaired intestinal homeostasis and increased intestinal permeability.[115] A study with T84 intestinal epithelial cells demonstrated that the consequence of *Aeromonas sobria* proteases induced decomposition in nection-2 and afadin leading to as alterations in intestinal barrier function.[120] *Aeromonas* species are known to cause human gastrointestinal infections.[121] In addition, it is believed that afadin has a crucial role in recruitment of different TJ proteins to the apical side of the cell–cell adherens junctions, since afadin-depleted MDCK cells show a significant delay in the reassembly of TJs and it subsequently enhances epithelial permeability.[64,116,117,122,123]

Clinical relevance

Nothing is known about the clinical relevance of afadin-related barrier defects in human diseases.

Cingulin

Molecular structure

Cingulin (~140 kDa) is a non-PDZ cytoplasmic scaffolding TJ protein and exists as a parallel homodimer of two subunits, each comprised of a N-terminal globular head region, a long α-helical coiled-coin rod region and a small globular tail (Figure 4).[124]

Expression

Cingulin is expressed throughout the mouse intestine; however, subcellular distribution and differences in level of expression alongside the mouse intestine has not been fully investigated.[125,126]

Intestinal barrier function

The head domain of cingulin appears to control the efficient recruitment of cingulin into cell-cell junctions through its interaction with ZOs, JAM-A and actin, whereas the rod domain is essential for dimerization of cingulin.[73,74,124,127,128] It is still not clear whether cingulin plays a fundamental role in barrier function, since different in vitro and in vivo studies showed that neither depletion nor overexpression of cingulin (full-length or the head domain of cingulin) cause obvious changes in the molecular organization of the TJ network. In addition, cingulin knockdown MDCK cells show normal TJs reassembly and barrier development.[74,125,129,130] In addition, the in vivo functional intestinal barrier can be formed in the absence of cingulin. A study by Guillemot *etal.*[125] showed that cingulin is involved in signaling networks that regulate the CLDN2 expression and cingulin knockout mice show a two-fold increase in the level of CLDN2 proteins in the duodenum. However, this CLDN2 effect is not sufficient to induce intestinal barrier breakdown.

Clinical relevance

A down-regulation in cingulin gene expression was detected in intestinal specimens of necrotizing enterocolitis patients compared to respective controls.[131] UC patients' colonic biopsies revealed that cingulin levels of these patients were lower compared to healthy controls, which could be related to impaired barrier formation.[132]

Biomarkers for assessment of intestinal barrier function and related clinical relevance for diagnosis and treatment of different diseases

A healthy and functional gastrointestinal barrier is of major importance for animal and human well-beings, since the compromised barrier can be recognized in the main etiology of various inflammatory, autoimmune, allergic situations. Crucial factors related to a stable gastrointestinal barrier are: a tight structure, including vascular endothelium, the epithelial cells and mucus layer together with strong host immune activity (since more than 70% of immune system cells could be found in gastrointestinal tract) and neuroendocrine activity of the gut as well as proper interactions between host body and gastrointestinal microbiota, diet, feed and food digestion and absorption.[133] The pivotal role for gastrointestinal barrier can be emphasized by the fact that the barrier is a dynamic entity and a location of interaction between host body and the gut content. However, identification of valid and applicable diverse biomarkers is crucial for the assessment of the intestinal barrier function. Therefore, this review demonstrates a summary of potential biomarker candidates for evaluation of the intestinal barrier function, which could contribute to the early detection, diagnosis and treatment of impaired intestinal barrier function and associated diseases.

Fatty acid binding proteins (FABP)

FABP as cytosolic, small, lipophilic proteins are important parts of the mature enterocytes. The FABP proteins play an important role in the biosynthesis of lipids, in a way that the transportation of fatty acids by FABPs through apical membrane of enterocytes toward the endoplasmic reticulum is necessary for lipid formation. Three different FABP could be detected in GI tract: intestinal FABP (I-FABP), mainly in jejunum and to some extent in colon, liver FABP (L-FABP) in liver, kidney and intestine, and ileal bile acid-binding

protein (I-BABP).[134] IFAB is recognized as useful marker of intestinal ischemia due to the surgical procedures and obstruction in mesenteric blood flow or other vascular flow. Longer period of intestinal ischemia-reperfusion has significant consequences, such as intestinal barrier alterations.[135] Furthermore, the measured elevated levels of FABPs are recognized as indicators for intestinal epithelial cell disturbance.[134] For example, I-FABP could be nominated as the potential marker for follow-up procedure in celiac diseases.[136] Additional studies showed that I-FABP could be evaluated as the biomarker for the progression of Crohn's disease.[137]

Citruline

Small intestinal enterocytes are able to produce citruline as a non-protein amino acid. The plasma levels of citruline are recognized as a biomarker of functional enterocytes.[138] A meta-analysis showed that there is a positive correlation between citruline levels (in plasma and serum) and the length of the small bowel in short bowel syndrome patients, whereas lower citruline levels in samples of patients are indicative of intestinal insufficiency.[139] A study by Papadia *et al.* provided evidence that the plasma citruline concentration could be encountered as quantitative biomarker for small bowel integrity in HIV-positive patients.[140]

Glutathione S-transferases (GSTs)

GST plays a key role in cell protection, antioxidant activities and defense of the body against toxic and hazardous compounds via conjugation of harmful substances with glutathione.[141] αGST, as a member of the GST family, is recognized as a potential marker for barrier integrity, since the intestine, liver and kidney are the main sites for the expression of the alpha subgroup of the GST family. Previous studies demonstrated that there is a reasonable link between mesenteric ischemia and plasma levels of αGST.[142,143] It should be noted that increased plasma levels of αGST are not limited to the intestinal damage, since it could also be related to the kidney and liver functions. Therefore, αGST is only useful biomarker for intestinal barrier integrity when there is clear evidence for intestinal damage (and no evidence for kidney and liver damage).

Zonulin

Zonulin is identified as a protein that modulates intestinal permeability by disassembling the intercellular tight junctions and appears to play a key role in the pathogenesis of autoimmune diseases.[144] Zonulin has been nominated as the potential biomarker in different pathological conditions, including autoimmune (ankylosing spondylitis, celiac, Crohn's diseases, Systemic lupus erythematosus, type 1 diabetes) diseases, nervous system diseases (schizophrenia, multiple sclerosis) and neoplastic circumstances (oral squamous cell carcinoma, lung, ovarian, breast and pancreatic cancers).144–150 Watts *et al.* reported that the loss of intestinal barrier function induced by zonulin plays a role in pathogenesis of type 1 diabetes, and zonulin upregulation was associated with increased gut permeability in subjects with type 1 diabetes. In this way zonulin measurement can precede the onset of diabetes and a zonulin inhibitor might preserve beta cell function at an early state.[151]

Diamine oxidase and $_{D}$-lactate

The diamine oxidase enzyme could be detected in the apical part of villus cells and is continuously released from the intestinal mucosa, and has the ability to catalyze the deamination of histamines. Diamine oxidase enzyme levels in the blood can be used as a biomarker of intestinal permeability. Small intestinal mucosal damage may decrease the diamine oxidase activity. In addition, $_{D}$-lactate, produced by many of the bacteria found in the GIT, showed enhanced levels in the circulation following intestinal damage.[152–155] Recently, it has been demonstrated that $_{D}$-lactate and diamine oxidase serum levels appear to be biomarkers in patients with Crohn's disease.[156] Więcek *et al* reported that $_{D}$-lactate serum levels in patients who suffering from cystic fibrosis might be a good indicator for exocrine pancreatic insufficiency in relation to dysbiosis or overgrowth in the intestines.[157]

Secretary immunoglobulin A

Secretary IgA (SIgA) is known as the most abundant antibody in the intestinal lumen and the first line of antigen-specific defense against enteric toxins and hazardous microorganisms.[158] The amount of SIgA secreted daily into the human intestinal lumen is almost 3 g, reflecting the crucial function of the antibody for intestinal health. Additionally, it has been shown that a high percentage of SIgA binds to the bacterial cell wall, resulting in decreased bacterial access to the host targets and changes in intestinal microbial composition, therefore preventing colonization and toxicity as well as damage to epithelial cells.[159–161] The aforementioned information suggests that the quantification of fecal SIgA may provide useful information for intestinal epithelial health and barrier function.

Intestinal alkaline phosphatase

Alkaline phosphatase (AP) is a member of hydroxylase enzymes, which is capable of removing phosphate groups from nucleotides, proteins and lipids. There are four different isoforms of AP including intestinal AP (IAP). The regulation of duodenal pH, inhibition of bacterial translocation through intestinal epithelium and regulation of dietary lipids transport are the key roles of IAP.[162] Previous studies demonstrated that fecal IAP levels are decreased in patients with type 2 diabetes, inflammatory bowel disease and celiac disease.[162–165] It is also known that IAP has the potency to detoxify and attenuate LPS-induced adverse effects.[166,167]

Calprotectin

Calprotectin is recognized as the calcium- and zinc-binding, neutrophil-derived protein serves as an antimicrobial protein in innate immune responses. The calprotectin can be detected in feces and plasma, of note that the feces concentration is higher than plasma.[168] Concerning the mode of activity, the recruitment of monocytes and macrophages could be facilitated by calprotectin additionally, the zinc-dependent metalloproteinases as an activating agent for proinflammatory cytokines (such as TNF) can interact with calprotectin.[169]

It has been suggested that the blood and feces levels of calprotectin is enhanced in IBD patients.[168,170,171] The concentration in feces correlated with the intestinal mucosa infiltration, which is mediated by polynuclear neutrophils.[169]

Conclusions

It is well accepted that regulation of TJ proteins are indispensable to maintain the healthy intestinal barrier function. Here, we have summarized the relevant knowledge about the molecular structure and the expression patterns of different transmembrane (discriminating between bicellular and tricellular TJs) and cytoplasmic scaffolding TJ proteins as well as useful biomarkers for assessment of gastrointestinal functionality. TJs are dynamic structures with a complex architecture and are composed of series of different proteins. The direct interaction among different TJ proteins is essential for the establishment of an efficient paracellular barrier against luminal harmful substances. The molecular structure and the expression pattern and function of different transmembrane TJs can be related to various clinical conditions, particularly to local chronic inflammatory processes such as ulcerative colitis and Crohn's disease. Subsequently there is an increasing need for validated and reliable biomarkers of intestinal integrity to improve the quality of animal experiments seeking for new diagnostic tools and enabling the monitoring of therapeutic interventions both in preclinical research and human health care. A compromised function of the TJ will lead to a leaky gut syndrome with even in the absence of specific pathogens is followed by the translocation of commensal microbiota colonizing the intestinal tract and an increased permeability of microbial metabolites.[172] Prominent examples associated with a leaky gut syndrome are inflammatory reactions in the pancreatic tissue, leading to diabetes mellitus type 2, obesity, and chronic liver diseases.[173,174] Chronic inflammatory processes at the intestinal mucosal barrier also affect the balance and diversity of intestinal microbiota, promoting the persistence of these disease conditions. Common indicators of destabilized microbiota are the increased prevalence of *Clostridium difficile* and the rapid proliferation of Gram-negative bacteria (*Escherichia* and *Klebsiella* spp) resulting in the

release of bacterial endotoxins, which reach the liver and even the systemic circulation, thereby inducing a multi-organ inflammatory syndrome.[175] Recent research identified the broad impact of these mechanisms on multiple diseases conditions, such as cardiovascular diseases and major neurological and psychiatric disorders, such as Parkinson's disease, multiple sclerosis, autism spectrum disorder and depression.[176–178] These few examples may highlight again the overall importance of epithelial integrity and the crucial role of the junctional complexes in the maintenance of intestinal integrity and pathogenesis of important chronic diseases.

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