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Biochimica et Biophysica Acta 1778 (2008) 601–613

Biochimica et Biophysica Acta BBA

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Review

The cytoplasmic plaque of tight junctions: A scaffolding and signalling center

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Received 2 July 2007; received in revised form 25 September 2007; accepted 26 September 2007
Available online 9 October 2007

Abstract

The region of cytoplasm underlying the tight junction (TJ) contains several multimolecular protein complexes, which are involved in scaffolding of membrane proteins, regulation of cytoskeletal organization, establishment of polarity, and signalling to and from the nucleus. In this review, we summarize some of the most recent advances in understanding the identity of these proteins, their domain organization, their protein interactions, and their functions in vertebrate organisms. Analysis of knockdown and knockout model systems shows that several TJ proteins are essential for the formation of epithelial tissues and early embryonic development, whereas others appear to have redundant functions.

Keywords: Tight junctions; Cytoplasmic proteins; ZO-1; ZO-2; ZO-3; MAGI; Afadin; MUPP1; Angiomotin; Cingulin; JACOP; Symplekin; ASH1; Ubinuclein; GEF-H1; Rich1; Tuba; Rab13; Rab3B; PDZ; PAR-3; PAR-6; PALS-1; PATJ

Contents

1. Introduction

TJs are of fundamental importance in the physiology of epithelial cells and tissues. First, they constitute a semi-permeable barrier that regulates the flow of ions, solutes and cells across paracellular spaces. Second, they contribute to the

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doi:10.1016/j.bbamem.2007.09.032

establishment and maintenance of apico-basal polarity. Third, they are targets and effectors of signalling pathways that control gene expression, cell differentiation and proliferation. In all these functions, a key role is played by proteins present in the cytoplasm underlying the TJ membrane (the TJ "plaque").

The structural backbone of the TJ plaque is formed by proteins that contain PDZ domains. The PDZ domain is an evolutionarily conserved 80–90 residue domain, which was originally identified in *P*sd-95 (post-synaptic density protein 95), *D*lg (Discs-large, a protein in *Drosophila*, mutation of which leads to overgrowth of wing imaginal discs) and *Z*O-1 (the TJ protein Zonula Occludens 1). PDZ-domain-containing proteins interact with other PDZ proteins through their PDZ domains, anchor TJ membrane proteins to the cytoskeleton, and are implicated in a variety of signalling mechanisms. The second, heterogeneous group of TJ proteins is formed by TJ proteins that lack PDZ domains. These proteins appear to be primarily involved in the regulation of signalling at TJ, rather than provide the basic structural elements of TJ. The current review will focus on some of the most recent advances in the characterization of proteins belonging to these groups, with the exception of polarity complex proteins of TJ (including aPKC, PAR-3, PAR-6, PATJ and PALS-1), that are discussed in the article by Assemat et al., in this issue. The reader is also referred to other articles in this issue, and to past reviews [1–5] for additional information on cytoplasmic TJ proteins that are not mentioned here.

Interestingly, a proteomic approach to analyze the protein composition of TJ has revealed that the TJ plaque may contain over 912 protein components, which are found associated with aPKC in immunoprecipitates. Clusters of proteins related to the synapse, signalling, cytoskeleton, cell adhesion, and vesicular

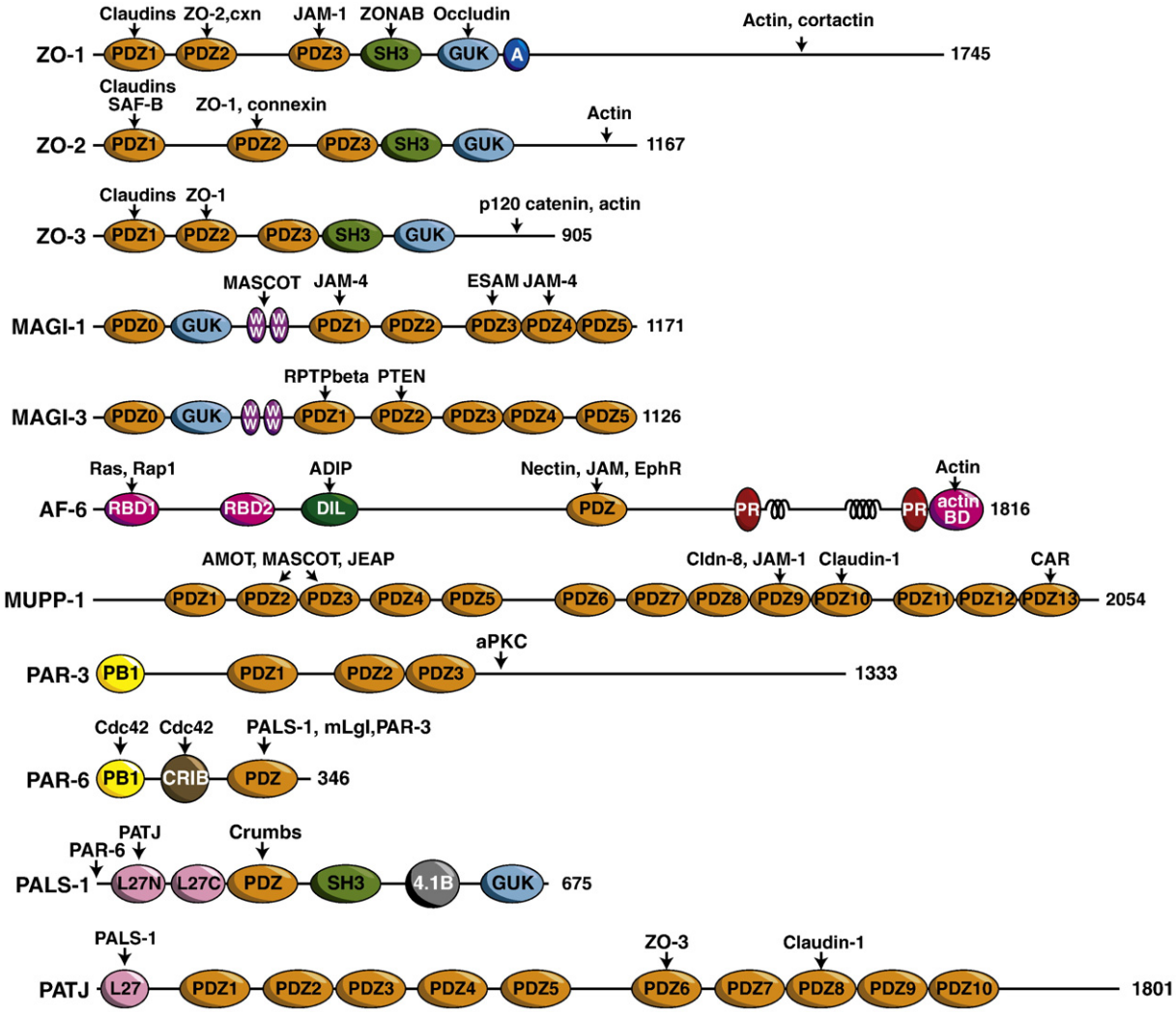

Fig. 1. Schematic representation of cytoplasmic TJ proteins with PDZ domains (PDZ TJ proteins). The major interacting protein partners are indicated. Protein domains are outlined as colored shapes, or as coiled lines (coiled-coil domains). Numbers on the right indicate amino acid number (in the human protein). SH3, Src homology region 3 domain; GUK, guanylate kinase domain; A, acidic domain; WW, domains with conserved tryptophans; RBD, Rap1 binding domain; DIL, dilute domain (first identified in an unconventional myosin family in the mouse mutant "dilute" strain); PR, proline-rich domain; actinBD, actin-binding domain; PB1, PC-binding motif (scaffold module that mediates the formation of heterodimers or homo-oligomers); CRIB, Cdc42/Rac interactive binding region; L27, LIN-2,-7 homology domain (scaffold module found in several proteins involved in the assembly of polarized cell-signalling structures); 4.1B, homology domain to protein 4.1B. Please note that polarity complex proteins (PAR-3, PAR-6, PALS-1, PATJ) are discussed in the chapter by Le Bivic et al., in this issue.

traffic, and weaker clusters, related to cell growth, cell migration, translation and transcription were identified [6]. Although some of these proteins may associate with aPKC independently of TJ, these results indicate that our understanding of the molecular complexity of TJ may still be in its infancy.

2. PDZ proteins of the TJ plaque

A large group of cytoplasmic TJ proteins contains one or multiple PDZ domains: 1) The ZO proteins (ZO-1, ZO-2, ZO-3); 2) The membrane-associated guanylate kinase inverted proteins (MAGIs): MAGI-1 and MAGI-3; 3) The multi-PDZ protein MUPP1; 4) The Ras target protein AF-6/afadin; 5) PAR-3, PAR-6, PALS-1, and PATJ, which form multiprotein complexes involved in the establishment of apico-basal epithelial polarity (see Assemat et al., in this issue) (Fig. 1). PDZ domains have two major functions: A) to anchor integral membrane proteins (receptors, adhesion proteins, etc), through the interaction with their cytoplasmic tails, which contain a specific COOH-terminal sequence; B) to bind to the PDZ domains of other PDZ proteins, thus leading to the formation of dimers and scaffolding networks [7]. Multiple functions of PDZ domains are possible because subtle changes in PDZ domain structure can have a profound impact on ligand specificities and biological functions, and the intrinsic selectivity of PDZ domains is tuned to minimize cross-reactivity [8,9]. PDZ-containing proteins of TJ contain additional structural domains, which function as scaffolds for signalling proteins, such as small GTPases and their regulators, kinases and transcription factors.

2.1. ZO proteins

ZO-1 (220 kDa) was the first TJ-associated protein to be identified, through the production of a specific monoclonal antibody raised against a preparation of liver membranes [10]. Subsequently ZO-2 (160 kDa) and ZO-3 (130 kDa) were identified as proteins that co-immunoprecipitated with ZO-1 [11,12]. ZO-1, ZO-2 and ZO-3 share a similar structural organization, with a N-terminal region containing 3 PDZ domains, followed by one SH3 (Src homology 3) domain, and a GUK (guanylate kinase) domain (Fig. 1), and thus belong to the MAGUK (*M*embrane *A*ssociated *Gu*anylate *K*inase) family of proteins [13]. The C-terminal region of ZO-1 and ZO-2 includes an acidic domain, located just after the GUK domain, and a proline-rich region containing alternatively spliced regions (α domain in ZO-1, β domain in ZO-2). The α^- isoform of ZO-1, lacking the α domain, is expressed prior to the α^+ isoform in early mouse development, suggesting that it may be involved in the initial stages of junction formation [14].

ZO proteins are at the center of a network of protein interactions. The first PDZ domain of ZO-1, ZO-2 and ZO-3 binds directly to the C-termini of claudins [15] (Fig. 1). Recent experiments showed that the interaction between the PDZ domains of ZO-1 and ZO-2 and claudins plays a fundamental role in the assembly of TJ strands [16]. In fact, mammary epithelial cells in which ZO-1 expression has been abolished by targeted deletion, and ZO-2 expression has been reduced by RNAi-mediated depletion, fail to assemble the TJ strands and establish a TJ permeability barrier [16] (Table 1). This phenotype can be rescued by exogeneous expression of either ZO-1 or ZO-2, indicating a functional redundancy between these two proteins in building a scaffold for claudins recruitment. When ZO-1 mutants that fail to localize to junctions, but target to the lateral membrane, were expressed in ZO-1 knockout/ZO-2-depleted cells, ectopic formation of TJ strands was observed [16]. Taken together, these data demonstrate that an assembly of a cytoplasmic scaffold of ZO proteins is necessary and sufficient for claudin polymerization into TJ strands. Interestingly, expression of exogeneous ZO-3 (which is not expressed in this epithelial cell line) does not rescue the phenotype, and junctional recruitment of ZO-3 requires either ZO-1 or ZO-2, indicating that ZO-3 function is not redundant with ZO-1 and ZO-2, at least in mammary epithelial cells [16]. It is also noteworthy that cells lacking ZO-1, ZO-2 and ZO-3 show an apparently normal apico-basal polarity [16], suggesting that the multimolecular complexes that are involved in the establishment of apico-basal polarity (PAR-3–PAR-6–aPKC, PALS-1–PATJ-Crumbs, Dlg-Scribble) (see article by Assemat et al., in this issue) can still assemble and function normally in the absence of TJ strands and ZO proteins.

Table 1
Phenotypes of KO mice/cells for cytoplasmic TJ proteins

Protein	Model	Phenotype	References
PTEN	Mouse and ES cells	Embryonic lethality for mice. Heterozygous mice show hyperplasia, displasia and tumor development in epithelia. Aberrant embryoid bodies, cells unable to differentiate.	[155]
AF-6/l-afadin	Mouse	Embryonic lethality at 10 days p.c. In cells of embryo and embryoid bodies ectoderm, reduction in length of apical junctions, loss of polarity, disorganization of AJ and TJ.	[72,73]
Angiomotin	Mouse	Embryonic lethality following gastrulation.	[102]
Cingulin	ES cells	Up-regulation of expression of several genes, including claudin-2, claudin-6, claudin-7, and occludin. Normal TJ.	[92]
ZO-1	Mammary epithelial cells (EpH4)	TJ structurally and functionally normal, apico-basal polarity normal. Cingulin not recruited to junctions. Up-regulation of ZO-2. In combination with ZO-2 knockdown, failure to assemble TJ strands, no TJ barrier, delay in the formation of belt-like adherens junctions.	[16,36]
ZO-3	Mouse, F9 teratocarcinoma cells	Mice viable and fertile, no detected phenotype. Normal differentiation of F9 cells, TJ normal except for an increased ZO-2 junctional immunolocalization.	[47]
Par-3	Mouse	Midgestational embryonic lethality with defective establishment of apical cortical membrane in epicardial cells.	[156]

The second PDZ domain of ZO-1 is responsible for a homo- and heterodimerization, through interaction with another ZO protein [17], and for binding to connexins [18,19]. Dimerization appears crucially important in ZO proteins scaffolding function, since junctional recruitment of claudins in cells transfected with a ZO-1 mutant construct occurred only upon chemically-induced dimerization [16]. The third PDZ domain of ZO-1 interacts in vitro with JAM-1, an Ig-like TJ membrane protein which is not involved in TJ strands formation, but interacts with the PDZ domain of PAR-3, providing a link between ZO-1/claudin-based TJ strands and the PAR-3–PAR-6–aPKC polarity complex [20]. Surprisingly, the three PDZ domains of ZO-1 are not sufficient to ensure junctional recruitment [16,21,22], despite their interaction with different TJ membrane proteins. An additional region, comprising the SH3, GUK and acidic domains, is required to recruit ZO-1 to junctions [16,22]. The GUK region is responsible for occludin interaction [22,23], suggesting that occludin may be responsible for recruiting ZO-1 to TJ. However, since occludin-deficient cells contain apparently normal TJ, where ZO-1 is correctly localized [24], alternative interactions through this region must be involved. One such interaction could be the one with α-catenin, a cytoplasmic actin-binding protein that associates with the β-catenin/E-cadherin complex at adherens junctions, and which interacts with ZO-1 [25,26]. Since constructs lacking the SH3-GUK region of ZO-1 failed to rescue the phenotype of ZO-1 knockout/ZO-2 depleted cells, it was proposed that this region is involved both in recruitment of ZO-1 to adherens-type junctions, through α-catenin interaction, and dimerization [16]. However, ZO-1 can be recruited to the membrane in cells deficient in α-catenin [27,28], suggesting that the interaction with α-catenin is not critical in ZO-1 recruitment to junctions. Other interactions of ZO-1, for example with the nectin-afadin complex, may be critical to target ZO-1 to the membrane [29]. In another recent study, it was shown that ZO-1 depletion causes a delay in junction assembly, and the isolated SH3 domain of ZO-1 can rescue this phenotype, although it does not localize correctly to junctions, suggesting that the SH3 domain may act by titrating out a yet unidentified factor, which has an inhibitory role in junction assembly [30] (Table 2). The SH3 domain of ZO-1 is known to interact in vitro with an unknown kinase [31], the transcription factor ZONAB [32] and a heat-shock protein [33], but the role of these proteins in TJ assembly is unclear. In summary, although it remains to be determined how ZO-1 and ZO-2 are recruited to nascent junctions, their presence is necessary and sufficient to assemble claudin-based TJ strands.

The C-terminal, proline-rich domain of ZO-1 interacts with actin and cortactin [26,34,35], suggesting that ZO-1 functionally links TJ membrane proteins to the actin cytoskeleton, by binding to membrane proteins through its N-terminal half, and to actin through its C-terminal half. The C-terminal region of ZO-1 (lacking the GUK domain, the acidic region and the α domain) is sufficient for actin interaction, based on colocalization and actin co-pelleting experiments from cell lysates [34]. However, recent rescue experiments using ZO-1 knockout/ZO-2 knockdown cell lines indicate that the acidic domain, located just after the GUK domain, is required in order for ZO-1 to contribute to the formation of belt-like adherens junction and linear actin cables in vivo [36]. In addition, activation of the small GTPase Rac1 appears to be impaired during epithelial polarization in cells lacking ZO-1 and ZO-2 [36], indicating that ZO-1 and ZO-2 may affect cytoskeletal organization both directly, through interaction with actin, and indirectly, via interaction with other actin-binding proteins, and with regulators of Rac1 activity.

ZO-2 has a domain organization similar to ZO-1, but contains a shorter C-terminal region, which shows only 25% sequence homology to ZO-1 [37,38]. Like ZO-1, ZO-2 binds claudins through its first PDZ domain, ZO proteins and connexins through the second PDZ domain, actin through its C-terminal region, and occludin and α-catenin, presumably through the central region, comprising SH3 and GUK domains [15,39,40]. These shared protein interactions underline the concept that ZO-2 and ZO-1 have redundant functions in promoting claudin assembly into TJ strands [16]. It was recently shown that silencing of ZO-2 expression leads to changes in the development and function of the paracellular permeability barrier of TJ [41] (Table 2). In contrast to these results, however, no effect of ZO-2 silencing on the barrier properties of cultured

Table 2
Phenotypes of knockdown cells for cytoplasmic TJ proteins

Protein	Model	Phenotype	References
ZO-1	MDCK cells	Delay in TJ assembly.	[30]
	MCF-10A cells and MDCK cells	Increased cell density of MCF-10A cells. Abnormal morphology of cysts derived from MDCK cells grown in 3-D matrigel.	[149]
ZO-2	Mammary epithelial cells (EpH4)	In conjunction with KO of ZO-1, failure to assemble TJ strands, no TJ barrier, delay in the formation of belt-like adherens junctions.	[16,47]
	MDCK cells	Normal TJ assembly.	[30]
	MDCK cells	Increased paracellular permeability, loss of polarity, delay in junction assembly, atypical monolayer architecture.	[41]
Cingulin	MDCK cells	RhoA-dependent increase in claudin-2 expression and cell proliferation and density. Up-regulation of ZO-3. No effect on organization and assembly of TJ.	[93]
MAGI-1	Endothelial cells	Suppression of cell–cell contact-induced Rap1 activation and VE-cadherin-mediated cell–cell adhesion after Ca^{++} switch.	[57]
Symplekin	Intestinal adenocarcinoma cell line HT29-16E	Loss of immunofluorescent staining for ZONAB, decreased ZONAB protein levels, decreased ZONAB activity in reporter assay.	[132]
JEAP/Angiomotin-like-1	Zebrafish embryos	Epiboly arrest and aberrant convergent extension. Disruption of juxtamembrane actin fibers and loss of filopodia and lamellipodia.	[103]

epithelial cells was observed in another study [30] (Table 2). These discrepancies may be due to different levels of silencing or target sequences, and should be resolved in the literature in the coming years.

The domain organization of ZO-3 (130 kDa) is similar to that of ZO-1 and ZO-2, except that the C-terminal region is the shortest [42] (Fig. 1). Like ZO-1 and ZO-2, ZO-3 interacts with claudins, occludin, and actin [40,43]. In addition, an interaction with the E-cadherin associated protein p120-catenin has been described [43]. Overexpression of the N-terminal half of ZO-3 (NZO3) delays TJ assembly in cultured cells [44]. It was proposed that excess NZO3 could prevent the intramolecular interaction between the COOH-terminal and N-terminal half of endogeneous ZO-3, and would thus affect interaction with p120 catenin, and RhoA signalling. Interestingly, ZO-3 is the only one among ZO proteins to be specifically expressed in TJ-bearing epithelia, whereas ZO-1 and ZO-2 are associated with cadherin-based junctions in non-epithelial cells and tissues [45,46]. The targeted deletion of ZO-3 from mouse tissues and cultured cells does not result in any detectable phenotypic effects [47] (Table 1). Thus, the physiological function of ZO-3 has not yet been substantially defined, and it may be redundant with that of other cytoplasmic TJ proteins.

2.2. MAGI proteins

MAGI proteins (MAGUKs with inverted domain structure) are characterized by two WW domains (instead of the SH3 domain present in ZO proteins), and an inverted arrangement of protein-interacting domains, with the GUK-WW domains preceding five PDZ domains (Fig. 1). Among several differentially spliced members of the MAGI family, only MAGI-1 and MAGI-3 are associated with TJ [48,49], whereas MAGI-2 is a neural-specific isoform [50].

The PDZ domains of MAGI proteins have been involved in the interaction with three different classes of proteins (Fig. 1): a) integral membrane proteins, including different types of receptors (reviewed in [49]), and two putative TJ-associated Ig-like adhesion molecules: JAM-4 [51,52], and ESAM [53]; b) junction-associated signalling molecules, including K-RAS, the Rho GEF mNEt1, the Rap1 GEF PDZ-GEF1, β-catenin, a receptor tyrosine phosphatase, and the tumor suppressor PTEN [49,54–57]; c) the adenoviral oncoprotein E4-ORF1 [58].

Depletion of MAGI-1 from endothelial cells results in the impairment of VE-cadherin-based cell adhesion, indicating that MAGI-1 is required for adhesion, via its binding to the Rap1 GEF [57] (Table 2). However, MAGI-1 depletion does not affect the localization of ZO-1 [57]. Thus, although the specific role of MAGI proteins in the assembly and function of TJ remains unclear, the present data suggest that they could have both scaffolding and signalling functions.

2.3. AF-6/afadin

AF-6/afadin was originally identified as the fusion partner of the acute lymphoblastic leukemia-1 (ALL-1) protein [59], and has been immunolocalized both at TJ and at cadherin-based junctions [60,61]. AF-6/afadin exists in differentially spliced variants, distinguishable on the basis of the presence of actin-binding domains [61]. Afadin contains one PDZ domain, which interacts with the C-termini of nectin and JAM Ig-like adhesion molecules, and the Eph subfamily of receptor tyrosine kinases [62–64]. The first Ras-binding domain within the N-terminus of afadin regulates the activity of the small GTPase Rap1 and interacts with Ras [65–67] (Fig. 1). The invertebrate homolog of afadin, Canoe, is thought to be an effector of Rap1 and both an effector and target of Ras in vivo [68–70]. ZO-1 also interacts with the N-terminal region of afadin [60]. The dilute (DIL) domain of afadin interacts with ADIP (afadin DIL domain-interacting protein), which in turn binds to α-actinin [71]. In addition, the C-terminus of l-afadin (the epithelial afadin isoform) interacts with ponsin and profilin, thus providing different molecular interactions through which afadin can link transmembrane adhesion proteins to the actin cytoskeleton [61,63,66].

Afadin is crucially important for the establishment of polarized epithelia during embryogenesis, since targeted deletion of l-afadin results in early lethality of mouse embryos, with a loss of apico-basal epithelial polarity, disorganization of the ectoderm, impaired migration of the mesoderm and loss of both ectodermal- and mesodermal-derived structures [72,73] (Table 1). In summary, AF-6 is essential for epithelial morphogenesis, through its ability to function as a scaffold, link to the actin cytoskeleton and modulate the Ras/Rap1 signalling pathway.

2.4. MUPP1

MUPP1 was originally identified by a yeast 2-hybrid screen, as a protein interacting with the serotonin receptor [74]. MUPP1 contains a N-terminal MRE domain, and 13 PDZ domains (Fig. 1) and thus shows a domain organization similar to the polarity protein PATJ (Le Bivic et al., this issue). In epithelial cells, MUPP1 is localized at TJ, where it interacts with claudins, JAM-1, CAR, Crumbs1, through different PDZ domains [75–78] (Fig. 1). In addition, recent data indicate an interaction of MUPP1 with the angiomotin/JEAP family of proteins, through its PDZ2/3 domains [79]. Like other PDZ-containing TJ proteins, MUPP1 is also a target for the adenoviral oncoprotein E4-ORF1 [80]. The specific role of MUPP1 in TJ assembly and regulation is not known, although its domain organization and protein interactions suggest that it has a scaffolding function, and its function may be redundant with that of PATJ.

3. Non-PDZ proteins of the TJ plaque

3.1. Cingulin and JACOP/paracingulin

Cingulin and JACOP/paracingulin are two vertebrate-specific, junction-associated proteins characterized by a globular head domain, a coiled-coil rod domain, and a small globular tail (Fig. 2).

Cingulin (Mr 140 kDa) was identified as a TJ-specific protein that co-purified with myosin, and was specifically expressed in tissues containing epithelial TJ [81,82]. Cingulin

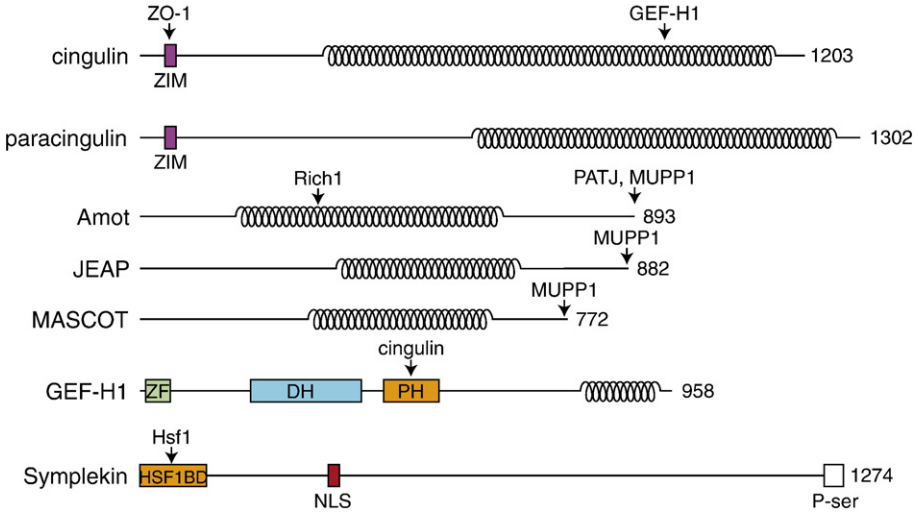

Fig. 2. Schematic representation of cytoplasmic non-PDZ TJ proteins. The major interacting protein partners are indicated. Protein domains are outlined as colored shapes, or as coiled lines (coiled-coil domains). Numbers on the right indicate amino acid number (in the human protein). ZIM, ZO-1 Interacting Motif; ZF, Zinc Finger; DH, Dbl homology domain; PH, pleckstrin homology domain; HSF1BD, Hsf1 binding domain; NLS, nuclear localization signals; P-Ser, phosphoserine.

forms a parallel homodimer of two subunits [83] (Fig. 3), and its head domain interacts with several TJ proteins [84–86], and with actin [87]. However, the key functional interaction is with ZO-1, through the conserved "ZIM" (*Z*O-1 *I*nteraction *M*otif) (Fig. 2), which is required both for ZO-1 binding, and for junctional recruitment of cingulin in transfected cells [88]. Indeed, ZO-1

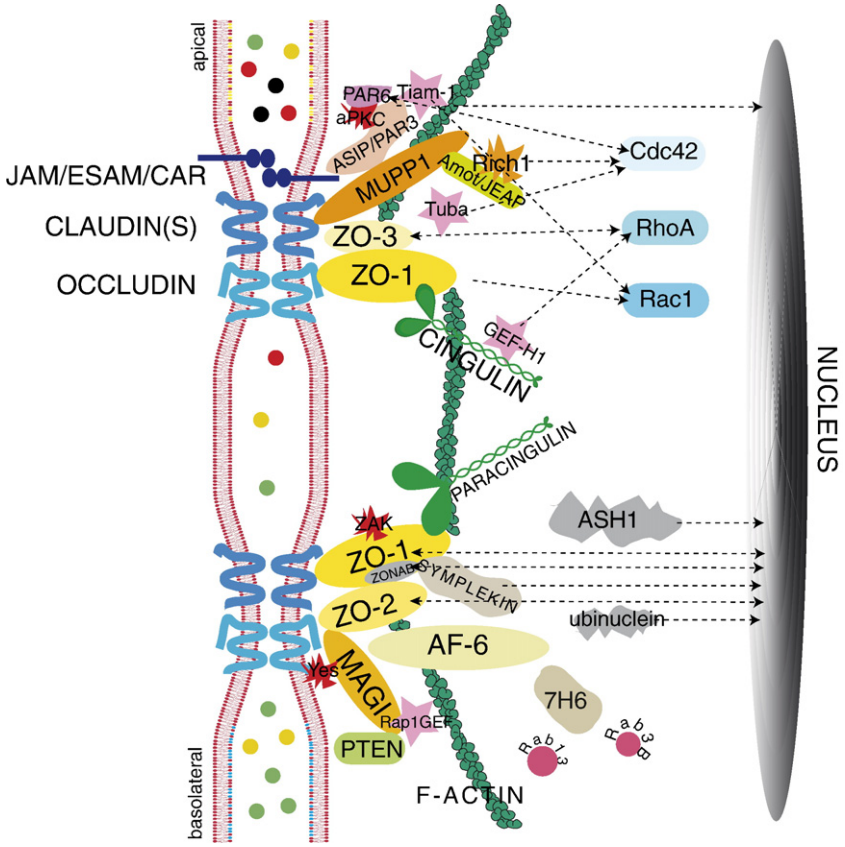

Fig. 3. Schematic representation of the different proteins present in the cytoplasmic region of TJ, some of which interact with TJ membrane proteins (JAM/ESAM/CAR, claudins and occludin), which are depicted crossing the membrane bilayers of two adjacent cells. The major PDZ proteins represented here are ZO-1, ZO-2, ZO-3, PAR-3, PAR-6, MUPP1, MAGIs and AF-6 (PATJ and PALS-1 have been omitted). Kinases (aPKC, ZAK, c-Yes) are represented by red irregular shapes. GEFs (Tiam1, GEF-H1, Tuba, Rap1GEF) are represented by pink stars. Membrane traffic regulators (Rab3B and Rab13) are represented by circles. The structural and/or functional interaction of cytoplasmic TJ proteins with signalling proteins (Cdc42, RhoA and Rac1) is indicated by dotted arrows. Note that the interaction of ZO-3 with RhoA is indirect, through p120 catenin [43]. It is not clear whether the interaction of ZO-1 with Rac1 is direct or indirect [16]. Arrows directed to the nucleus are linked to proteins that have been shown to have dual nuclear/junctional localization (PAR-3, PAR-6, ASH1, ZO-1, ZO-2, ZONAB, symplekin, ubinuclein).

knockout cells lose cingulin junctional staining, confirming that cingulin is recruited to junctions via its interaction with ZO-1 [89]. The coiled-coil rod domain of cingulin is responsible for dimerization [84], however its heptad repeat pattern indicates that cingulin does not have the tendency to form filaments or supramolecular aggregates, like myosins [83]. The rod domain sequence is particularly well conserved across species [90], and interacts with the RhoA regulator GEF-H1, resulting in its inactivation [91].

Cingulin knockout and knockdown epithelial cells contain normal TJ, based on morphological and functional assays [92,93] (Tables 1 and 2). In addition, TJ organization and barrier function is unaffected by inducible up-regulation of full-length cingulin or its head and rod/tail domains [90]. Thus, cingulin does not play a direct role in the structure and function of TJ. However, cingulin mutation or down-regulation alters the expression of a large number of genes in embryoid bodies, among which four TJ protein genes (claudin-2, occludin, claudin-6 and claudin-7) [92]. Cingulin knockdown in MDCK cells up-regulates claudin-2 and ZO-3 expression, and increases cell proliferation and monolayer density at confluence [93]. All these phenotypic effects, except for ZO-3 up-regulation, are inhibited by expression of dominant-negative RhoA, indicating that regulation of gene expression and cell proliferation by cingulin is largely dependent on RhoA [93]. Interestingly, mammary epithelial cells lacking ZO-1, where cingulin is no longer associated with junctions, have the same proliferation rate as parental cells [89], suggesting that either the function of cingulin in regulating proliferation through RhoA is independent of its association with junctions, or that this function is cell-context-dependent.

JACOP/paracingulin was identified as a novel antigen in a junction-enriched fraction isolated from mouse liver [94] and as a protein with sequence and domain organization similar to cingulin [94,95]. Unlike cingulin, JACOP/paracingulin is present at both TJ and adherens-type junctions, and is absent from junctions of the small intestine, whereas it is detected in endothelial cells [94]. In transfected fibroblasts, JACOP/paracingulin is recruited to actin stress fibers, and both head and rod domains are recruited to junctions in transfected epithelial cells [94]. Whether cingulin and JACOP/paracingulin have redundant functions, and what proteins JACOP/paracingulin interacts with, remain to be investigated. However, since JACOP/paracingulin contains the ZIM domain (Fig. 2), it is likely that ZO-1 is involved in its junctional recruitment.

3.2. Angiomotin family proteins

Angiomotin (Amot), JEAP (Angiomotin-like-protein1) and MASCOT (Angiomotin-like-protein2) are three members of a family of proteins characterized by the presence of a central coiled-coil domain, and a C-terminal PDZ interaction sequence motif (Fig. 2). The first member of this family, Amot, was identified by a yeast two-hybrid screen, as a protein interacting with and mediating the activity of angiostatin, a circulating inhibitor of angiogenesis, in endothelial cells [96,97]. JEAP (Junction Enriched and Associated Protein) was identified as a TJ-associated protein by a fluorescence localization-based expression cloning method [98]. JEAP was found to be specifically expressed in epithelial cells of exocrine glands, but not in other epithelial tissues [98]. MASCOT (MAGI-associated coiled-coil tight junction protein) was identified as a protein interacting with the first WW domain of MAGI-1, and showing sequence similarity to Amot [99] (Fig. 2).

There is some disagreement about the intracellular localization of Amot and related proteins. Bratt et al. provided evidence, based on cross-linking and immunofluorescence experiments, that Amot is a transmembrane protein, with the angiostatin-binding domain exposed on the cell surface [100]. On the other hand, biochemical fractionation experiments suggest that the Amot family members are peripherally associated to the membrane [79].

Several protein interactions have been described for Amot family members. MASCOT and JEAP interact with the first WW domain of MAGI-1, through a LPXY motif localized in the N-terminus [99]. In addition, exogeneously expressed Amot forms a complex with MAGI-1 [100]. The coiled-coil domains of MASCOT and JEAP mediate self–self interaction, which could potentially result in the formation of homo- and hetero-polymers at TJ [99]. On the other hand, the coiled-coil domain of Amot binds to and recruits to TJ the Cdc42 GAP Rich1, which is necessary to maintain TJ integrity, by regulating Cdc42 activity [101]. The PDZ domain-binding motif of Amot interacts with PATJ and thus targets Amot to TJ [101]. In addition, it interacts, as MASCOT, with the PDZ2 and PDZ3 domains of MUPP1, whereas JEAP only binds to the PDZ3 domain of MUPP1 [79]. However, the physiological relevance of these latter interactions remains to be established, since the expression of dominant-negative mutants of PATJ and MUPP1 does not affect the localization of Amot family members [79].

Targeted deletion of Amot in mice results in an early embryonic lethal phenotype, with defects in the migration of visceral endoderm cells in specific regions of the embryo [102] (Table 1). In addition, knockdown of angiomotin-like-1 (JEAP) in zebrafish embryos delays epiboly and impairs convergence and extension movements, coincident with loss of membrane protrusions and disorder of F-actin [103] (Table 2). Thus, Amot and related proteins appear to be of crucial importance in the regulation of morphogenetic cell movements.

3.3. Signalling proteins and regulators of small GTPases

Different lines of evidence indicate that the cytoplasmic plaque of TJ contains a large variety of signalling molecules (see also [6]): 1) kinases such as ZAK [31], WNK4 [104], and c-Yes [105]; 2) phosphatases, such as PTEN [55] and PP2A [106,107]; 3) regulators of membrane traffic, such as Rab13 and the associated protein JRAB/MICAL-L2 [108–110], Rab3B [111], and VAP-33 [112]; and 4) regulators of the activity of small GTPases (see below). Additional cytoplasmic proteins have been localized at TJ, for which little is known about functional interactions with other TJ proteins, and specific roles at TJ: 7H6/barmotin [113,114], LYRIC [115], 4.1 and spectrin [116]. Finally, the role of the actomyosin cytoskeleton in regulating TJ structure and

function has been extensively investigated (see article by Turner in this issue).

Recent studies have provided new information about the functional interaction of guanine nucleotide exchange factors (GEFs), and GTPase activating proteins (GAPs) with cytoplasmic proteins of TJ. GEFs and GAPs act as activators and inhibitors, respectively, of small GTPases (Rho, Rac, Cdc42), which in turn control signal transduction pathways linking membrane receptors to the dynamic assembly and disassembly of the actin cytoskeleton, cell–cell and cell–substrate adhesions. The molecular organization and function of TJ is severely affected when the activity of small GTPases is experimentally altered [117,118], suggesting that GTPase activity must be finely tuned during each step of junction assembly, from the initial cell–cell contact, to junction stabilization. Several adaptor proteins of TJ have been implicated in this regulation, either by recruiting (and affecting the activity of) GEFs and GAPs, and/or by restricting their localization, and/or by being targets of activated GTPases. Initial cell–cell contact and formation of cadherin- and nectin-based primordial adhesions lead to activation of Rac1 and Cdc42, and inhibition of RhoA activity [119]. Active Cdc42 binds to PAR-6, thus activating the polarity complex, with PAR-3 and atypical protein kinase C [120,121]. PAR-3 also acts as a scaffold for the Rac1 activator (GEF) Tiam1 in epithelial and neuronal cells [122–124]. In MDCK cells, loss of PAR-3 results in increased Rac1 activity and delayed TJ assembly, suggesting that PAR-3 functions to inhibit Tiam1 function and/or to restrict its localization at TJ [122]. The notion that Tiam1 must be present at initial sites of junction assembly to activate Rac1 is also supported by the observation that Tiam1-deficient keratinocytes fail to mature cadherin-based junctions and form TJ [123]. Interestingly, Tiam1 activity is also important in regulating invasiveness of lymphoma cells and Ras-transformed epithelial cells [125,126].

The second GEF that interacts with a cytoplasmic TJ protein is GEF-H1, which interacts with cingulin [91] (Fig. 2). The inactivation of GEF-H1 following its binding to cingulin in confluent cultures has been proposed as a mechanism through which RhoA activity is down-regulated upon junction formation in confluent cells [91]. Down-regulation of RhoA activity correlates with inhibition of G1/S phase transition [119]. Indeed, cingulin depletion leads to increased cell proliferation and claudin-2 expression, in a RhoA-dependent manner [93], consistent with the idea that cingulin contributes to down-regulating RhoA activity, and hence cell proliferation, via its interaction with GEF-H1.

The third GEF which is concentrated at apical junctions is the Cdc42 GEF Tuba [127]. Although no interaction with TJ proteins has been identified for this GEF, Tuba depletion impairs both adherens junction and TJ organization, strengthening the notion that Cdc42 activity is required for TJ formation, and that multiple GEFs may be sequentially involved in cell junction assembly, in a redundant or independent manner [127].

The activity of Cdc42 is also regulated by GAPs. The Cdc42 GAP protein Rich1 is targeted to TJ through its interaction with Amot, which in turn is recruited to TJ by virtue of its interaction with the polarity complex protein PATJ [101] (Fig. 2). Rich1 is required to maintain the integrity and stability of TJ, since TJs are disassembled more rapidly following calcium depletion in cells depleted of Rich1 [101]. Overexpression of Amot has the same effects on TJ as depletion of Rich1, indicating that Amot inhibits Rich1 GAP activity [101].

In summary, multiple GEFs and GAPs appear to be recruited into TJ by their interactions with cytoplasmic adaptor proteins, resulting in a spatially restricted control of small GTPases activity during the different phases of junction assembly and stabilization.

3.4. Symplekin

Symplekin is an ubiquitously expressed protein that is enriched in the nucleus and associates with TJ in polarized epithelial cells [128]. Symplekin interacts with the cleavage and polyadenylation specificity factor CPSF, and is believed to be involved both in 3′-end processing of pre-mRNA and regulated RNA polyadenylation in the cytoplasm [129,130]. In addition, symplekin forms a complex with the heat-shock factor HSF-1 [131], and the transcription factor ZONAB [132]. Reporter assay experiments indicate that symplekin may regulate the stability and intracellular levels of ZONAB, with effects on its transcriptional activity and downstream events, such as expression of cyclin D1 and cell proliferation [132]. The molecular mechanisms through which symplekin becomes recruited to TJ, and its specific role in canonical TJ functions are not known.

4. Regulation of gene expression by TJ plaque proteins

The first indication that TJ plaque proteins may be involved in the regulation of gene expression was the observation that transcription factors, and proteins involved in nucleic acid metabolism and function, as well as several TJ plaque proteins can have dual localization, both at TJ and in the nucleus. The nuclear localization was typically detected when cells are actively proliferating and sub-confluent, whereas the TJ localization is detected when cells have reached confluence, suggesting that TJs act to sequester these factors away from the nucleus when cells are fully polarized and become confluent [133,134].

ZO-1 was first detected in the nucleus of sub-confluent cultured cells and of intestinal cells undergoing dissociation of cell–cell contacts [135]. Living cells expressing a GFP-tagged form of ZO-1 also exhibited nuclear localization of ZO-1 when at low confluence [136], consistent with the idea that the nuclear localization of ZO-1 is inversely related to the extent and/or maturity of cell–cell contacts [135]. ZO-1 was subsequently found to interact with ZONAB, e.g. the DNA-binding Y-box transcription factor DbpA [137], which exhibits dual junctional/nuclear localization [32]. The TJ localization of other transcription factors, such as ubinuclein [138] and human ASH1 have been reported [139] (see also [2,133]). However, it is still unclear whether these transcription factors interact with any specific TJ protein, and how TJ assembly may regulate their activity. ZO-2 is also detected in the nucleus in sparse cultured cells, or under conditions of stress [140,141]. Nuclear immunolocalizations, nuclear localization signals, and nuclear export

signals have been described for ZO-2, ZO-3, PALS-1, MAGIs, PAR-6, PAR-3, and cingulin [133,139,142–146].

There are at least four mechanisms through which TJ proteins may regulate gene expression and cell proliferation. First, direct interaction with and sequestration of transcription factors at TJ. This is what has been shown for ZONAB, whose activity and nuclear versus junctional localization is dependent on its interaction with ZO-1 and RalA, once it accumulates at junctions in confluent epithelial monolayers [32,132,147–149]. Interestingly, high levels of expression of ZONAB/DbpA correlate with advanced stages of human hepatocellular carcinoma [150]. However, transgenic mice expressing DbpA in hepatocytes do not develop hepatocellular tumors, despite the up-regulation of 11 genes, and the down-regulation of 9 genes [151]. Thus, it is unclear whether ZONAB levels/activity actually controls the proliferative rate of hepatic epithelial cells in vivo. It should also be noted that although ZO-1 has been implicated as a regulator of gene expression and cell proliferation, through its interaction with ZONAB, no increase in cell growth and proliferation was seen in cells lacking either ZO-1, or ZO-1 and ZO-2 [16,89]. Thus, the ability of ZO-1 and ZONAB to regulate cell proliferation may be context- or cell-type-dependent. The mechanism of junctional sequestration may also be applied to ZO-2, which associates and/or colocalizes with several nucleic-acid-binding proteins: the pre-mRNA splicing protein SC35 [140], the scaffold attachment factor B (SAF-B) [141] (Fig. 1), the transcription factors Jun, Fos, AP-1 and CCAAT/enhancer-binding protein (C/EBP) [152].

The second mechanism through which cytoplasmic TJ protein may control gene expression and cell proliferation is by forming a scaffold for (and possibly regulating the activity of) transcription factors in the nucleus. This type of regulation could in principle be hypothesized for all TJ plaque proteins that have been shown to have dual nuclear-junctional localizations, including ZO-1, ZO-2, and other PDZ proteins [133]. For example, PAR-6 has recently been shown to be a constitutive nuclear protein, localized at nuclear "speckles", where it interacts with Tax, a transcriptional activator of the human T-cell leukemia virus-1 (HTLV-1) long terminal repeat [144]. Furthermore, PAR-3 interacts with components of the DNA-dependent protein kinase complex, and may be involved in double-strand DNA break repair [146].

The third possible mechanism of control of gene expression by TJ plaque proteins is through the regulation of β-catenin signalling. Such mechanism could explain the epithelia-mesenchymal transition induced by the expression of ZO-1 mutants which fail to localize to the membrane, in different types of cultured cells [21,153,154].

Finally, the fourth mechanism through which TJ plaque proteins may control gene expression and cell proliferation is by modulating the activity of small GTPases, which in turn control the activity of transcription factors. This mechanism has been shown for cingulin, which controls claudin-2 expression and cell proliferation in MDCK cells by regulating the activity of RhoA [93].

In summary, several lines of evidence point to TJ proteins as multifunctional platforms, that both participate in the architecture of TJ, and recruit and regulate signalling molecules and transcription factors, according to the dynamic changes in the proliferative and differentiative state of epithelial cells.

5. Conclusions and perspectives

A remarkable progress has been made in recent years in the identification of cytoplasmic TJ proteins, interacting partner proteins, and signalling pathways involving these proteins. Fig. 3 provides a schematic representation of some of the proteins present at TJ, their interactions with membrane proteins, GEFs and GAPs, and their shuttling to and from the nucleus.

The picture that emerges is the one where cytoplasmic proteins of TJ are dynamic targets and effectors of cellular processes that coordinate development of apico-basal polarity, junction assembly, establishment of tissue barriers, and proliferation. However, the details of this picture remain to be clarified by additional knockout and knockdown experiments. One take-home message from studies on mouse models is that many of the putative roles of TJ proteins, as hypothesized on the basis of studies on cultured cells, do not always have an equally significant impact in whole organisms, at least under normal laboratory conditions. This could be due to functional redundancy between different proteins, but also to context-dependent differences, and compensatory effects. The phenotypic characterization of knockout mice for TJ proteins will be complicated by the fact that several of these proteins are also found in other structures, such as gap junctions. Thus, it will become increasingly important to use a combination of experimental approaches in multiple model systems to clarify the physiological roles of cytoplasmic TJ proteins.

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