

Endothelial Cell-to-Cell Junctions: Molecular Organization and Role in Vascular Homeostasis

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Bazzoni, Gianfranco, and Elisabetta Dejana. Endothelial Cell-to-Cell Junctions: Molecular Organization and Role in Vascular Homeostasis. *Physiol Rev* 84: 869–901, 2004; 10.1152/physrev.00035.2003.—Intercellular junctions mediate adhesion and communication between adjoining endothelial and epithelial cells. In the endothelium, junctional complexes comprise tight junctions, adherens junctions, and gap junctions. The expression and organization of these complexes depend on the type of vessels and the permeability requirements of perfused organs. Gap junctions are communication structures, which allow the passage of small molecular weight solutes between neighboring cells. Tight junctions serve the major functional purpose of providing a “barrier” and a “fence” within the membrane, by regulating paracellular permeability and maintaining cell polarity. Adherens junctions play an important role in contact inhibition of endothelial cell growth, paracellular permeability to circulating leukocytes and solutes. In addition, they are required for a correct organization of new vessels in angiogenesis. Extensive research in the past decade has identified several molecular components of the tight and adherens junctions, including integral membrane and intracellular proteins. These proteins interact both among themselves and with other molecules. Here, we review the individual molecules of junctions and their complex network of interactions. We also emphasize how the molecular architectures and interactions may represent a mechanistic basis for the function and regulation of junctions, focusing on junction assembly and permeability regulation. Finally, we analyze in vivo studies and highlight information that specifically relates to the role of junctions in vascular endothelial cells.

I. GENERAL FEATURES OF ENDOTHELIAL JUNCTIONS

Endothelial cells adhere to one another through junctional structures formed by transmembrane adhesive proteins that are responsible for homophilic cell-to-cell adhesion. In turn, the transmembrane proteins are linked to

specific intracellular partners, which mediate anchorage to the actin cytoskeleton and, as a consequence, stabilize junctions (for a review, see Ref. 76). Junctions in endothelial and epithelial cells share common features. In addition, in both cell types, two major types of junctions have been described and named adherens junctions (AJ) and tight junctions (TJ). However, epithelial cells also

form desmosomes, which are absent in the endothelium (for a review, see Refs. 30, 76, 179, 273, 290). In addition to cell-to-cell adhesion, another type of junction, the gap junction, mediates cell-to-cell communication. This structure is formed by connexins (Cx), and three of these proteins (i.e., Cx43, Cx40, and Cx37) are expressed in the endothelium. In turn, connexins are organized in connexons, which act as channels for the intercellular passage of ions and small-molecular-weight molecules (292).

In epithelial cells, junctions are better organized, with TJ and AJ following a well-defined spatial distribution along the intercellular cleft. TJ (or *zonula occludens*) are concentrated at the apical side of the rim, while AJ (or *zonula adherens*) are located below the TJ. In contrast, in endothelial cells, the junctional architecture is less defined and, along the cleft, AJ are intermingled with TJ (290).

As to the function, junctions in the endothelium control different features of vascular homeostasis. For instance, permeability to plasma solutes is controlled, to a considerable extent, by junction permeation. In addition, leukocyte extravasation and infiltration into inflamed areas require finely regulated opening and closing of cell-to-cell contacts (159, 221). Notably, junctional proteins can also transfer intracellular signals, which modulate endothelial cell growth and apoptosis (179). We may expect that the transduced information communicates to the cells their own position, mediates contact-dependent inhibition of growth, and establishes cell polarity.

The organization of endothelial junctions varies along the vascular tree in function of organ-specific requirements (290). For instance, in the brain, where a strict control of permeability between blood and the nervous system is required, junctions are well developed and rich in TJ (274, 352). In contrast, postcapillary venules, which allow dynamic trafficking of circulating cells and plasma proteins, display poorly organized TJ. These morphological features may also account for the high sensitivity of postcapillary venules to permeability-increasing agents, such as histamine and bradikinin. In contrast, the endothelium of large arteries, which tightly controls permeability, has a well-developed system of TJ. Finally, lymphatic endothelium displays specific junctional structures, the complexus adhaerentes (283, 284), which are likely to be specialized in controlling the passage of lymphocytes from and to different compartments.

The development of fully mature endothelial junctions is reached only late in development and, in general, junctions are not completely differentiated in the embryo. A typical example is the blood-brain barrier, which reaches full functional differentiation only after birth (274, 304). This conclusion is supported by data obtained in the *in vitro* system of embryonic stem cells' differentiation to endothelial cells, where junctional proteins (i.e.,

VE-cadherin and PECAM) become detectable after a few days of differentiation (187, 336).

In general, junctions are dynamic structures. During the organization of an endothelial monolayer, cell-to-cell contacts follow different steps of maturation. Evidence in epithelial cells indicates that adhesive membrane proteins of AJ and TJ first form adhesion complexes at sites of cell-to-cell contacts and then tend to organize in zipper-like structures by lateral adhesion along the cell border (57, 230, 361, 362). The intracellular partners of transmembrane adhesive proteins also vary during junction maturation and stabilization (18, 177, 179). In addition, even after contacts have been stably formed, adhesion proteins are still in a dynamic equilibrium and recycle continuously between plasma membrane and intracellular compartments.

In this review, we focus on AJ and TJ, as well as on their molecular organization and functional changes. In some cases, considering the high homology of the two systems and the paucity of information on endothelial cells, we will draw a parallelism between epithelial and endothelial cells, taking into account cell-specific differences, when they are present. We also focus on the role of junctions in the organization of vessels and in the control of permeability to plasma solutes. We consider only marginally the role of junctions in leukocyte extravasation, since several excellent and recent reviews on this specific topic are already available in the literature (see, for instance, Refs. 159, 221).

II. ADHERENS JUNCTIONS

A. Molecular Components of AJ

1. The cadherin-catenin complex

A) CADHERINS. AJ are ubiquitously distributed along the vascular tree and are expressed in both blood and lymphatic vessels. These structures are formed by transmembrane adhesion proteins of the cadherin family, which mediate homophilic adhesion and are able to organize in multimeric complexes at the cell borders (1, 10, 11, 122, 358). Endothelial cells express a specific cadherin called vascular endothelial (VE)-cadherin (77). This protein cannot be found in any other cell type, including blood cells or hemopoietic stem cells (178), and, like a signature for the endothelium, is expressed during development, when cells become committed to the endothelial lineage (46). VE-cadherin is present in all endothelial cells of all types of vessels.

Extrapolation of data obtained from the crystal structure of other cadherins (10) suggests that VE-cadherin dimerizes laterally *in-cis* and makes head-to-head contacts *in-trans*, via the most amino-terminal repeats, thus

promoting cell-to-cell adhesion. Other data, however (54, 70), point to deep interdigitations among cadherins on two contiguous cells and strongly suggest that there are multiple binding domains along the extracellular domain. The transmembrane and intracellular domains of cadherins may also play a role in dimerization and possibly in multimerization (141).

The remarkable cell specificity of expression prompted investigators to study the VE-cadherin promoter (113). The promoter contains three domains important for transcriptional regulation: a proximal domain, which promotes transcription in a cell type-independent manner, and two negative control regions, which abolish transcription in nonendothelial cells. Such regulation, with positively acting proximal domains and cell type-specific silencing domains, is a rather unique feature among endothelial promoters but is reminiscent of promoters that confer tissue-specific expression. Transgenics expressing chloramphenicol acetyltransferase (CAT) under the VE-cadherin promoter confirmed the endothelial specificity of promoter expression *in vivo* (113).

The tissue specificity of VE-cadherin has an exception, which is the cytotrophoblast. During the establishment of human placenta, cytotrophoblasts invade the uterine interstitium and vasculature, thus anchoring the fetus to the mother and establishing blood flow to the placenta (369). Cytotrophoblasts invading spiral arterioles replace the maternal endothelium and express adhesion molecules that are typical of endothelial cells, such as PECAM, $\alpha_v\beta_3$ -integrin, and VE-cadherin (369).

Even if VE-cadherin is the most prominent cadherin at the AJ, it is not the only cadherin expressed in endothelial cells. N-cadherin can be found at comparable levels in most of the endothelial cells examined so far (228). P-cadherin expression was noticed by PCR analysis but could not be seen by antibody staining (273). T-cadherin was also found in the vasculature in tissue sections (154), but its biological function in this cell type has not been clarified yet.

Despite high expression levels, in the presence of VE-cadherin, N-cadherin does not localize to AJ and remains diffusely distributed on the cell membrane (228). This peculiar behavior is not cell specific but is related to functional or structural features of these two cadherins. Cotransfection of VE- and N-cadherin in Chinese hamster ovary (CHO) cells prevented N-cadherin localization at the junctions. A similar result was obtained upon transfection of a VE-cadherin mutant (truncated in the cytoplasmic domain), suggesting that the extracellular region of VE-cadherin (or the residual cytoplasmic tail) may be responsible for N-cadherin exclusion from intercellular junctions. Regardless of the mechanism, these observations suggest that VE- and N-cadherin may exert different functions in endothelial cells. An interesting possibility is that N-cadherin promotes endothelial cell adhesion and

communication with mesenchymal cells expressing N-cadherin, such as pericytes, smooth muscle cells, and astrocytes. Direct contacts among endothelial cells and the underlying smooth muscle cells, the myoendothelial junctions, have been described by electron microscopy, suggesting that they may be required for a coordinated response of the vessel wall to stimuli (290). N-cadherin is indeed clustered at the basal side of endothelial cells in contact with pericytes and astrocytes in the brain (194), suggesting that this interaction may be important in the elongation of vascular sprouts and in the protection of endothelial cells from apoptosis. Finally, Williams et al. (343) identified a region in the N-cadherin extracellular domain that is responsible for the activation of fibroblast growth factor receptors. This receptor is present in endothelial cells and is able to induce cell growth. It is possible that N-cadherin modulates angiogenesis in this way.

In the endothelium, another cadherin-like protein was described and named VE-cadherin-2 (314). This protein displays homology with other members of the cadherin family, but has a completely unrelated cytoplasmic tail. For its structural characteristics, it was included in the protocadherin family (98).

B) CATENINS AND OTHER INTRACELLULAR PARTNERS OF CADHERINS. The cytoplasmic tail of VE-cadherin is highly homologous to that of other classic cadherins (10, 313) and, through its carboxy-terminal region, it binds β -catenin and plakoglobin (also called γ -catenin). These two proteins are homologous and contain 10–13 so-called armadillo repeats, which are also present in many other signaling proteins. Both β -catenin and plakoglobin link α -catenin, which is homologous to vinculin and anchors the complex to actin (38, 337). α -Catenin can also bind α -actinin (168, 234) and vinculin (340), which may further stabilize AJ anchorage to actin (Fig. 1).

ZO-1, which is commonly considered a cytoplasmic component of TJ (see below), could be found at the AJ at early steps of their organization (151). ZO-1 localization at AJ is due to its binding to α -catenin and is transient, since the protein subsequently moves away and concentrates to the TJ (see below).

An additional VE-cadherin partner is p120, which is a src substrate and is homologous to β -catenin and plakoglobin (4, 5). However, p120 binds to a membrane-proximal domain of VE-cadherin (316) and does not associate to α -catenin or other actin binding proteins. The functional role of p120 is complex. In some cell types, p120 increases cadherin-based adhesion, but in others it has an inhibitory role (13, 244, 316, 362). Tyrosine phosphorylation of VE-cadherin correlates with higher association to p120 (177). N-cadherin in endothelial cells presents lower phosphorylation in tyrosine and lower binding to p120 (227). Whether this may explain some of the functional differences of these two cadherins in the endothelium is unknown. It is intriguing that classic cadherins bind es-

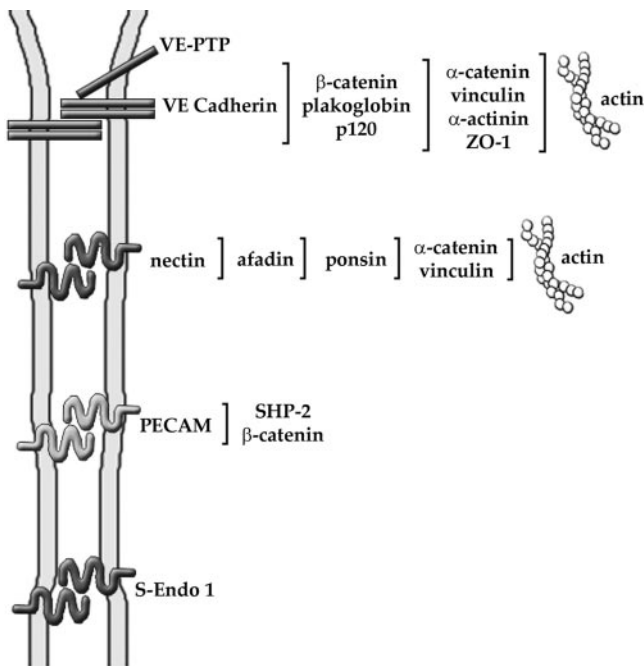


FIG. 1. Molecular organization of adherens junctions (AJ) and other junctional transmembrane proteins. VE-cadherin is a transmembrane protein at AJ. Through its cytoplasmic tail it binds β -catenin, plakoglobin, and p120. β -Catenin and plakoglobin in turn link α -catenin, which binds vinculin, α -actinin, and ZO-1. These interactions mediate the anchorage to actin microfilaments. Nectin is a transmembrane protein of the IgG superfamily, which promotes homophilic cell-cell adhesion. At the intracellular level, nectin binds afadin, which binds ponsin, which in turn associates to α -catenin and vinculin and through them to actin. Outside AJ, PECAM, another member of the IgG family, induces intercellular adhesion and binds the phosphatase SHP-2 and β -catenin. S-endo-1, also called CD146 and Muc 18, promotes homophilic adhesion, but its intracellular signaling and cytoskeletal partners are unknown.

essentially the same molecules at their cytoplasmic domain but may exert different biological activities (166, 233). There should be additional and specific intracellular partners that are able to transfer cadherin- or cell-specific signals.

VE-cadherin interacts with endothelial-specific signaling proteins. For instance, it can bind to vascular endothelial growth factor (VEGF) receptor 2 (49, 286, 367) and modulate signaling through phosphatidylinositol (PI) 3-kinase. In addition, it was recently found that VE-cadherin interacts with an endothelial-specific receptor-protein tyrosine phosphatase called VE-PTP. This interaction is unusual, as it is mediated by the extracellular domains of the two proteins. VE-PTP reduces the VEGF-induced phosphorylation of VE-cadherin and strengthens cell-to-cell adhesion (229).

A specific feature of VE-cadherin is that it may behave as a desmosomal-like cadherin. As mentioned above, endothelial cells do not have classic desmosomes and do not express desmosomal cadherins (111). However, in lymphatics and in some blood endothelial cells, VE-cadherin, through plakoglobin or p0071 (48), can recruit the

desmosomal proteins desmoplakin and vimentin at the membrane (172, 324). This novel desmosomal-like structure (complexus adhaerentes; Refs. 283, 284) is specific for endothelial cells, since, in epithelial cells, plakoglobin and desmoplakin are mostly concentrated in desmosomes in association with desmocollins and desmoglins, which are absent in the endothelium.

Interestingly, the *in vivo* inactivation of the *desmoplakin* gene leads to a complex phenotype, which includes vascular defects, such as a reduced number of capillaries and the presence of discontinuities among endothelial cells in these types of vessels (109). These data suggest that the lack of a correct organization of complexus adhaerentes may be the reason for these abnormalities.

2. The nectin-afadin complex

This complex has been described in epithelial cells and, although its components are present in the endothelium, little is known about its functional role in this cell type. The complex consists of at least three components, i.e., nectin, afadin, and ponsin. The nectin family of calcium-independent cell adhesion molecules comprises four members. Through the cytoplasmic tails, nectins bind to the PDZ-containing protein afadin (also called AF-6), which in turn connects nectins to the actin cytoskeleton (310, 312). Ponsin binds afadin, vinculin, and α -catenin. Nectins and afadin colocalize with cadherins and can reciprocally interact through α -catenin. Therefore, it has been suggested that nectin/afadin may be involved in AJ organization. However, afadin was also found in association with TJ proteins, such as JAM-A (88), and nectins can interact with ZO-1. So, the specific localization of these proteins at AJ may depend on the cell type and/or the complex may play a more general role in the organization of different junctional structures.

B. Outside AJ

Endothelial cells express other adhesive proteins, which are concentrated to the intercellular clefts but are not specifically confined to the AJ and TJ. Among these molecules, PECAM is one of the most extensively studied. It is a transmembrane immunoglobulin concentrated at intercellular contacts in the endothelium and is also expressed in leukocytes and platelets (for a review, see Refs. 79, 159, 221, 231, 232). PECAM promotes either homophilic or heterophilic adhesion (221). Heterophilic ligands include the $\alpha_v\beta_3$ -integrin (259). Using blocking antibodies, PECAM was found to participate in vascular angiogenesis in the adult (78), even if the null mutation of the *PECAM* gene did not cause detectable changes in vascular development in the embryo (86, 315). Several *in vitro* and *in vivo* data indicate that PECAM may modulate

leukocyte infiltration (for a review, see Refs. 159, 221, 231, 334). Inactivation of the *PECAM* gene in mouse increases vascular permeability and sensitivity to experimental autoimmune encephalomyelitis (117). In other models of inflammation, however, the role of PECAM was less apparent, thus suggesting compensation with other adhesive proteins (86, 315). It has been recently reported that neutrophils of *PECAM*-null mice can traverse endothelial cells but remain entrapped between endothelial monolayer and basement membrane (315). This effect seems to be due to defective expression of $\alpha_6\beta_1$ on the neutrophil membrane upon activation (75). More recent data show those leukocytes need to interact sequentially with PECAM and another junctional protein, i.e., CD99, for efficient transmigration (282).

The way through which PECAM transfers intracellular signals is not yet fully clear. It can bind intracellular partners mainly through phosphorylation-related kinases (for review, see Ref. 145). The tyrosine residues are located in an immunoreceptor tyrosine based activation motif (ITAM) (198). Among the intracellular partners of PECAM, the best studied are SHP-2 and β -catenin (144). SHP2 may play a role in the Ras-mitogen-activated protein kinase (MAPK) activation cascade (for review, see Ref. 145). Tyrosine phosphorylated β -catenin associates with PECAM, even if PECAM itself does not need to be phosphorylated. PECAM may exert similar biological activities like the cadherins through its binding to β -catenin (see below). In addition, it has been suggested that PECAM can modulate cadherin-mediated cell-cell interactions through the interaction with different intracellular proteins and activation of the MAPK pathway (287). Finally, PECAM can modulate cell adhesion and migration possibly through an interaction with matrix adhesion receptors, such as integrins (156, 306, 308).

Another endothelial junctional protein is S-endo 1, which is also called CD146 and Muc 18. This immunoglobulin-like protein was originally described in melanoma, but is also expressed in smooth muscle and endothelial cells and not in hemopoietic stem cells and blood cells. S-endo 1 is located at endothelial junctions (27) and induces homophilic cell adhesion.

Endoglin is expressed at high levels on vascular endothelial cells and is concentrated at interendothelial junctions. Its role as an accessory component of the receptor system of members of the transforming growth factor- β superfamily has been investigated in detail (256). Mutations of the *endoglin* gene in humans determine a vascular disorder called hereditary hemorrhagic telangiectasia type 1 that is characterized by arteriovenous malformations. In mice, inactivation of the *endoglin* gene leads to a vascular phenotype that is reminiscent of *VE-cadherin*-null animals (42, 212). Whether the junctional localization of endoglin has functional consequences for its interaction with transforming growth factor- β receptor

and/or for cell interaction and vascular remodeling is still an open issue.

C. Intracellular Signaling Through AJ

1. Transcriptional activity of catenins

A prominent feature of β -catenin, plakoglobin, and p120 is the ability to translocate to the nucleus and, in association with other transcription factors, to modulate gene expression. β -Catenin is a key member of the Wnt signaling pathway (38, 39, 258). This family of growth and differentiation factors plays a crucial role in cell specification during embryonic development. Many members of the Wnt family act through β -catenin transcriptional activity (for a review, see Ref. 39). When β -catenin is released in the cytosol, it can be quickly inactivated through phosphorylation in amino-terminal serine and threonine by the action of casein I α and glycogen synthase kinase-3 (GSK-3) in complex with the tumor suppressor axin and adenomatous polyposis coli (APC) (39, 196). Upon phosphorylation, β -catenin is ubiquitinated and degraded in proteasomes. If β -catenin phosphorylation is inhibited (as by Wnt signaling), it can translocate to the nucleus and bind the Tcf/LEF transcription factors complex and influence gene transcription. Mutations of either *β -catenin* or members of its degrading machinery have been associated with malignant cell transformation (see for a review Ref. 261). In general, overexpression of β -catenin in different cell systems leads to increased cell proliferation and reduced sensitivity to apoptosis, so that β -catenin stabilization or permanent signaling may facilitate tumor progression. There is a long list of β -catenin target genes, which will likely increase in the future and includes genes important in cell division and apoptosis (38, 39, 261).

When β -catenin is bound to cadherins, it is stabilized and retained at the cell membrane. Therefore, absence or mutation of cadherins may increase the pool of free β -catenin in the cytosol and its signaling activity (115, 297). An extensive literature is available showing an inverse correlation between cadherin expression and tumor progression (for a review, see Ref. 313).

An important question is how β -catenin may detach from the cadherin tail. Tyrosine phosphorylation of β -catenin and, in particular, phosphorylation of residue Tyr-654, causes a sixfold reduction in affinity of β -catenin for the cytoplasmic tail of E-cadherin (139, 272). The reduction in affinity likely increases β -catenin release in the cytosol and eventually promotes its nuclear translocation. In contrast, serine phosphorylation of specific residues in the cadherin tail leads to specific phosphoserine interactions with β -catenin (139). Both casein kinase II and GSK-3 β can induce phosphorylation of these sites and strongly increase β -catenin binding to cadherins, thus

reducing β -catenin signaling and increasing the strength of cell-to-cell adhesion (192).

Little is known about the transcriptional role of β -catenin in endothelial cells. As other cadherins in other cell types (297), VE-cadherin may contribute to contact inhibition of growth (50) by limiting β -catenin transcriptional activity (333). Interestingly, similarly to other malignant tumors, angiosarcomas (while expressing several endothelial markers) are negative for VE-cadherin staining (204), thus suggesting that the absence of the cadherin might increase the levels of free β -catenin and contribute to endothelial cell growth and transformation.

Plakoglobin was also shown to bind to Tcf/LEF, although to a site different than β -catenin (215), and influence cell transcription in both a positive and a negative way (for review, see Ref. 38). Similarly to β -catenin, overexpression of plakoglobin in different tissues induced cell proliferation and transformation (128, 370). p120 also binds to specific transcriptional partners, including Kaiso (5), and can transcriptionally upregulate different genes, including E-cadherin (147).

A general consideration emerging from this complex picture is that several cytoplasmic members of AJ (but also TJ; see below), in addition to promoting anchorage of junctional transmembrane proteins to actin, may also translocate to the nucleus and influence transcription. This finding, together with the observation that junctions are frequently altered during tumor transformation, supports the concept that these structures may have a more complex role, and that, in addition to simply promoting cell-to-cell adhesion, they may also modulate cell growth and differentiation.

2. Other signaling pathways at the junctions

In addition to maintaining β -catenin at the membrane and preventing its nuclear translocation, cadherins may also signal through other pathways. In endothelial cells, null mutation of *VE-cadherin* has lethal effects, and embryos die in utero at early stages of development (49). Although endothelial cells are able to form the primitive vascular plexus, vascular remodeling is missing and, already at embryonic day 8.75–9.00, endothelial cells appear disconnected from each other, detach from the underlying basement membrane, and lay scattered inside the vascular lumen. In the heart, the endocardium is highly disorganized, trabeculation is impaired, and the myocardium is loosely assembled.

To gain a mechanistic view of the defects linked to the absence of *VE-cadherin*, we cultured endothelial cells from *VE-cadherin*-null animals. By comparing isogenic endothelial cell lines that differ for VE-cadherin expression, we found that several vascular responses are severely impaired by the absence of the protein.

First, *VE-cadherin* $-/-$ cells are more prone to apoptosis and are unable to respond to VEGF protective signals (49). We found that, similarly to other cadherins (360), VE-cadherin clustering at the junctions can activate PI 3-kinase and Akt phosphorylation. Upon activation with VEGF, the VEGFR2 and PI 3-kinase subunits associate to the VE-cadherin/catenin complex, leading to more efficient activation of Akt (49). Mutants of VE-cadherin missing β -catenin or plakoglobin binding domain cannot form this multiprotein complex (49). In contrast, mutants lacking the p120 binding domain can still associate to VEGFR2, albeit less efficiently.

Dismantling VE-cadherin from cell-to-cell contacts with blocking antibodies reduced both Akt phosphorylation by VEGF and its protective effect on apoptosis (49). This finding suggests that not only VE-cadherin expression but also its clustering at junctions is required for a correct interaction with intracellular partners. Others have found that cadherins may interact with growth factor receptors, such as epidermal growth factor (EGF) receptor (85, 256).

Both N- and VE-cadherins can directly bind to shc, an adaptor protein that participates in the Ras signaling pathway (357, 367). We found that shc binding to VE-cadherin requires cell activation by VEGF and tyrosine phosphorylation of VE-cadherin cytoplasmic tail. The interaction decreased shc phosphorylation after VEGF and likely reduced the persistence of the mitogenic stimulus (367).

Similarly to E-cadherin (224, 242), VE-cadherin expression and clustering at the junctions influence the activation of small GTPases by inducing Rac and reducing RhoA activation (181). The Rac guanine exchange factor Tiam (123, 201) codistributes with VE-cadherin at AJ, but remains diffuse on the cell membrane in VE-cadherin negative cells (181). It is likely that VE cadherin activates and recruits Tiam through the induction of the PI 3-kinase pathway and that Tiam, in turn, activates Rac (Fig. 2). The biological consequences of inducing this pathway are multiple. First, Rac induces actin redistribution in endothelial cells and acquisition of the typical cobblestone morphology (181). In addition, Rac may be also involved in cell migration, growth, and differentiation (265).

An interesting observation is that Rac activation and Rho inhibition are typical of confluent epithelioid cells (371) and are induced by expression and clustering of E- and VE-cadherins, which are typical of epithelial and endothelial cells, respectively. At odds, N-cadherin, which seems to be associated with a migratory phenotype (233), induces Rho activation and Rac inhibition (55).

Also p120 may influence small GTPases. When it is released in the cytosol, it binds and inhibits Rho with complex consequences for cell adhesion and motility (3, 120, 239).

Several phosphatases (PTP- μ , PTP-K, SHP-1, SHP-2, PTP-LAR, and PTP-B) have been found to associate with

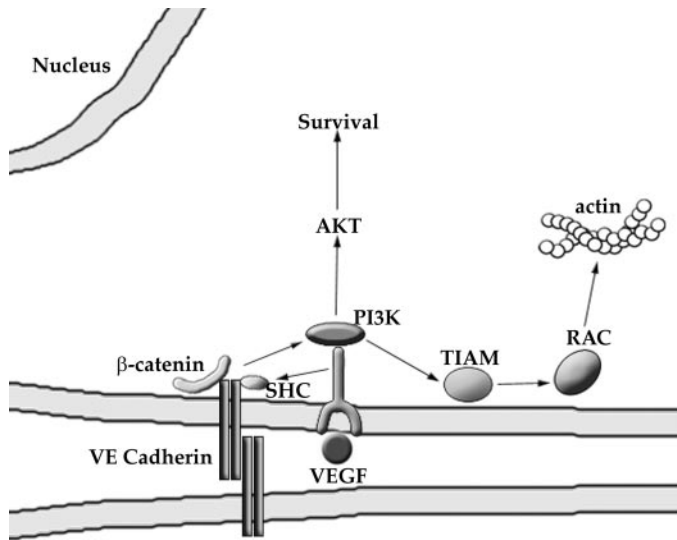


FIG. 2. Schematic representation of VE-cadherin signaling through phosphatidylinositol 3-kinase (PI3K). VE-cadherin can associate to vascular endothelial growth factor receptor 2 (VEGFR2) and induce PI3K activation. The enzyme inhibits cell apoptosis through phosphorylation of Akt. In addition, PI3K activation brings Tiam at the membrane. Tiam is a guanine exchange factor, which activates Rac and, as a consequence, induces actin rearrangement. VE-cadherin can bind shc when cells are stimulated by vascular endothelial growth factor (VEGF), but the functional consequences of this interaction are still unknown.

the cadherin/catenin complex (25, 43, 99, 176, 317, 323) and are likely to modulate phosphorylation of the complex and/or its intracellular partners. As mentioned above, VE-PTP is an endothelial-specific phosphatase, which associates with VE-cadherin (229). Another interesting phosphatase is DEP1 or CD148 (250), which, although not endothelial specific, is upregulated by cell confluency and localizes at intercellular junctions in confluent endothelial cells (311). In other cells, it can bind p120 and β -catenin (135). In a recent paper, we have reported that VE-cadherin is important in contact inhibition of cell growth. More specifically, VE-cadherin binds VEGFR2 and induces its dephosphorylation through the action of DEP-1 (182).

Other unexpected activities of VE cadherin have been reported. It was found that VE-cadherin may act as a transducer of shear stress signals acting in concert with VEGFR2 (286). When endothelial cells are exposed to shear, VEGFR2 associates to the VE-cadherin- β -catenin complex and translocates to the nucleus. This phenomenon does not occur in absence of VE-cadherin and, consistently, VE-cadherin expression is required for induction of shear-responsive promoters. Similarly, also PECAM was reported to transduce shear-dependent cell activation (248). In endothelial cells exposed to shear, PECAM is phosphorylated in tyrosine, associates with SHP-2, and regulates extracellular signal-regulated kinase (ERK). It is of interest that two endothelial-specific junc-

tional proteins, such as PECAM and VE-cadherin, may act as “mechanosensors” for shear changes.

VE-cadherin may also act as fibrin receptor, and this interaction induces endothelial cells to form capillary-like structures in three-dimensional gels (18). The intracellular signals transferred by fibrin through VE-cadherin are still unknown but suggest a “nonjunctional” role of this protein.

From all these observations, it appears that cadherins, similarly to integrins, may promote the formation of multiprotein complexes, by binding different effectors and facilitating their reciprocal interaction. Cadherin ability to cluster at junctions may further amplify this process by creating multimeric complexes, where protein-protein interaction is optimized.

Cadherins may trigger rapid and short-lasting responses (257), such as PI 3-kinase or MAPK activation at early moments of contact formation, which may be important for quick signaling of cell position. However, cadherins and, in particular, VE-cadherin may also transfer lasting signals, which contribute to the maintenance of cell homeostasis. Comparing long-term confluent cultures of *VE-cadherin* $+/+$ and $-/-$ cells, we found that this protein protects endothelial cells from apoptotic stimuli and attenuates cell growth. These are lasting effects, which require constitutive expression and clustering of VE-cadherin at junctions and likely continuous intracellular signaling.

D. Modulation of AJ Organization and Vascular Permeability

Endothelial cells control the passage of plasma proteins and circulating cells from blood to tissues. This function is finely regulated by the so-called transcellular and paracellular pathways (87, 97, 214, 268, 290, 298). The transcellular pathway defines the passage of plasma components through the endothelial cytoplasm by the action of vesicular systems and fenestrae. The paracellular pathway is regulated by opening of cell-to-cell junctions and/or by rearrangement of their architecture. Although both TJ and AJ can play an important role in the control of endothelial permeability, TJ have been always considered as the key regulators of this function (see sect. III).

A clear role for VE-cadherin in maintaining permeability was demonstrated by experiments *in vivo*, where anti-VE-cadherin antibodies were injected in mice (69). This treatment induced a marked increase in vascular permeability within a few hours. *In vitro* and *in vivo* staining of VE-cadherin showed that the antibodies were selective, as they were able to disrupt VE-cadherin clustering at the junctions, while leaving the distribution of other junctional components unchanged. Notably, the effect of VE-cadherin inhibition was strong in lungs and

heart but undetectable in other organs, such as brain, muscles, and skin. This observation suggests that the relevance of VE-cadherin in controlling vascular permeability is different along the vascular tree and is likely dependent on the type and organization of other junctional structures.

AJ organization displays different characteristics in growing vessels compared with resting vasculature. It was found that tumor microvasculature is particularly sensitive to VE-cadherin blocking antibodies. Some monoclonals were able to dismantle VE-cadherin junctions in tumors without affecting permeability of constitutive vessels (70, 191). In addition, VE-cadherin presents epitopes, which are only exposed in tumor vasculature and not in systemic vessels (191).

These *in vivo* data are supported by *in vitro* observations. AJ organization is different at different stages of cell confluency. In sparse cells, VE-cadherin is highly phosphorylated in tyrosine and preferentially binds β -catenin and p120 (177). In contrast, in long confluent cells, the amount of plakoglobin linked to VE-cadherin is higher (178). These changes correlate with a lower control of permeability in subconfluent cells, compared with stabilized long-confluent cultures (9).

It is conceivable that the response to permeability-increasing agents is influenced by the degree of junction organization and strength. For instance, the effect of histamine is more marked in loosely confluent cells, where this agent induces cell retraction and gap formation. This was accompanied by stronger increase in VE-cadherin tyrosine phosphorylation compared with long-confluent cultures (9). Hence, as suggested by *in vivo* data, agents able to disrupt junctions in subconfluent and growing endothelium may be inactive in stabilized long-confluent cells.

An important issue is how permeability-increasing agents modify junctional architecture and eventually create intercellular gaps. Cell retraction is certainly an intuitive mechanism, which has been observed in cultured cells upon exposure to thrombin, histamine, plasmin, and other permeability-increasing agents (9, 68, 84, 92, 130, 288, 298, 338) or *in vivo* in inflamed vessels (26). More subtle changes, such as phosphorylation of junctional proteins and their dissociation from the actin cytoskeleton, may result in increased permeability without frank appearance of intercellular gaps (9). *VE-cadherin* $-/-$ endothelial cells present increase in permeability but no intercellular gaps (70, 227). This may explain why *in vivo* conditions of apparent increase in permeability are not always accompanied by morphological evidence of junction disruption. In general, however, it is likely that the combination of both cell retraction and junctional changes leads to marked increase in permeability. Endothelial cell contraction has many characteristics in common with smooth muscle cell retraction and depends on

calcium/calmodulin-dependent myosin light-chain kinase (for a review, see Refs. 84, 298).

Small GTPases may regulate the organization of AJ and TJ (44, 351). In keratinocytes, the activity of Rho and Rac is required for successful assembly of cadherins and AJ formation (44). This, however, is not the case for endothelial cells, where VE-cadherin remains unperturbed, even in the presence of dominant negative mutants of these small GTPases (45). The lack of response is related to the cell-specific context, since the function of VE-cadherin in transfected CHO cells was susceptible to Rho and Rac inhibition. It is possible that different targets of the small GTPases are expressed in different cell types and that endothelial and epithelial cells regulate permeability in different and cell-specific ways.

Although apparently dispensable for the formation and maintenance of VE-cadherin at AJ in the endothelium, small GTPases play an important role in the action of permeability-increasing agents. Thrombin strongly increases RhoA and Rac activation (329, 330, 338, 351), and this causes endothelial cell rounding and retraction, as well as increase in permeability (92, 130, 338). However, Rho activation alone is not sufficient to increase endothelial permeability. When the *Escherichia coli* cytotoxic necrotizing factor-1 was added to endothelial cells, it caused strong induction of Rho and stress fibers reorganization (339). VE-cadherin, however, remained at junctions, and no sign of cell retraction was reported. These observations underline that both cytoskeletal rearrangements and junctional protein modifications are needed for clear appearance of intercellular gaps and eventually increase in permeability.

Phosphorylation in tyrosine of junctional components has been described after thrombin (323), VEGF (91), or histamine (9). Thrombin would increase phosphorylation by inducing dissociation of the phosphatase SHP2 from the VE-cadherin complex. Thrombin may also change VE-cadherin-catenin organization at junctions and reduce the amount of plakoglobin associated with the complex (262). Inhibitors of protein kinase C (PKC) inhibit junction reorganization and the increase in permeability induced by thrombin.

VEGF induces a rapid phosphorylation of VE-cadherin and catenins, which is followed by increase in junction permeation (91) and in some cases VE-cadherin redistribution from intercellular junctions (355). This effect may be inhibited by the phosphatase VE-PTP (see above) (229).

Overall, these data support the concept that tyrosine phosphorylation of VE-cadherin and/or its intracellular partners is involved in regulating the strength of cell-to-cell contacts. In general, however, it cannot be excluded that cadherin and catenin phosphorylation may represent a general reaction to stressful agents, which results in

increased permeability but may also trigger more complex cell responses and interactions.

Association of the cadherin-catenin complex to actin microfilaments stabilizes junctions. Truncated VE-cadherin, which is unable to bind β -catenin and to link actin, exerts a lower control of paracellular permeability. Mobilization of VE-cadherin and catenin in the detergent-soluble fraction, which is considered an indirect measure of dissociation from the cytoskeleton, is always increased in conditions of high permeability (9, 177). Similar findings were obtained using E-cadherin-expressing epithelial cells (175, 331). Therefore, part of the mechanism of action of permeability-increasing agents may consist of dissociating VE-cadherin from the actin cytoskeleton.

Activation of Rho and increase in intracellular Ca^{2+} levels may act in this direction. More specifically, it has been found that IQGAP1 (100), an effector of small GTPases, such as Rho, Cdc42 and Rac1, negatively regulates cadherin-mediated cell-to-cell adhesion by displacing α -catenin from its binding to β -catenin. This would in turn cause cadherin-catenin complex dissociation from the actin cytoskeleton and cell-cell dissociation (134). These effects have been described in epithelial cells, but IQGAP is also expressed in endothelial cells and thus may modulate permeability in this cell system as well.

Other ways for rapid cadherin regulation is recycling (185) and digestion by proteases (68, 148, 203, 238, 267). Classic cadherin and, in particular, VE-cadherin extracellular domain is highly susceptible to lysis (77). Induction of metalloproteases may induce cleavage and secretion of the extracellular domain of E-cadherin (58, 238) and promote junction disruption. However, cadherins may be cleaved also at the membrane-cytoplasm interface by proteases activated during apoptosis or Ca^{2+} imbalance (129). It was found that this process might be mediated by the presenilin 1/ γ -secretase system, which dissociates E-cadherin and β -catenin from the cytoskeleton (203). Furthermore, the cleavage induces the release of E-cadherin cytoplasmic domain and increases the levels of cytosolic β -catenin and α -catenin. This system works also for VE-cadherin, thus suggesting that it may promote disassembly of junctions and increase in permeability. Finally, ADAM15, which belongs to a family of proteins containing both a disintegrin and a protease domain, codistributes and associates with VE-cadherin at AJ. The biological role of this interaction is not yet clear, but it is possible that ADAM15 contributes to the local cleavage of VE-cadherin (124).

Finally, other proteins outside junctions may contribute to the control of permeability. For instance, PECAM can be phosphorylated by permeability-increasing agents, such as VEGF (91), shear stress, or can be displaced and internalized upon activation with inflammatory cytokines (266). In PECAM-null animals, the absence of this protein

leads to increase in vascular permeability, but only in pathological conditions (117).

E. Conclusions

AJ in endothelial cells are key structures for the maintenance of cell-specific properties, such as permeability to solutes or inflammatory cells, contact inhibition of cell growth, and apoptosis. The molecular organization of AJ, although not fully elucidated yet, is complex and includes both adhesive and signaling molecules. It is likely that additional membrane and cytoplasmic junctional proteins will be found in the future, which may interact, modulate AJ organization, and promote the transfer of intracellular signals. Manipulation of AJ and other junctional adhesive proteins may be important in the therapy of vascular inflammatory reactions, edema, and angiogenesis.

III. TIGHT JUNCTIONS

A. Introduction to TJ

1. Intercellular strands as morphological and functional units of tight junctions

Forty years ago, in 1963, TJ were defined by electron microscopy as a specialization of the plasma membrane and were named *zonula occludens*. In thin sections, TJ appear like a sequence of fusions (or “kisses”), which are formed between two adjacent cells by the outer leaflets of the plasma membrane. At higher magnification, however, it becomes clear that the membranes are not fused, but in tight contact to each other. In freeze-fracture preparations, TJ look like anastomosis of fibrillar strands within the plasma membrane. In turn, the strands are composed of particles that, with some exceptions, are preferentially localized to the internal leaflet (P-face). In correspondence of the strands, mostly on the external leaflet (E-face), there are complementary grooves (96). TJ are the most apical component of the junctional complex. Together with AJ, desmosomes, and gap junctions, TJ mediate adhesion and communication between adjoining cells. Specifically, TJ are responsible for regulating paracellular permeability and maintaining cell polarity, which are often referred to as “barrier” and “fence” function, respectively.

The appearance of TJ as fusions between adjacent membranes, right at the boundary between apical and basolateral domains, suggested that TJ might be instrumental in occluding the lateral intercellular space (LIS). In addition, it was hypothesized that the function of TJ might consist of restricting both the diffusion of solutes across intercellular spaces (barrier function) and the

movement of membrane molecules between the apical and basolateral domains of the plasma membrane (fence function). Subsequent studies lent further support to this hypothesis, by correlating number and complexity of the TJ strands with the ability to restrict fluxes of electrolytes. In this conceptual framework, it was proposed that cohesive interactions between TJ molecules localized on apposing strands might provide the mechanistic basis for the barrier and fence function of TJ. However, at present, demonstration that strands are indeed the morphological and functional units of TJ is far from being proven (320). In the past, even the biochemical nature of the strands has been debated. Some researchers proposed that the strands were made of lipids forming cylindrical micelles within the membrane bilayer (161). Now, it is commonly accepted that the strands are primarily (but perhaps not exclusively, Ref. 118) composed of proteins. However, important questions have not been exhaustively answered yet. Specifically, which proteins are the essential building blocks of TJ? How do they mutually interact during TJ assembly? Finally, how do these proteins mediate the barrier and fence functions?

As in some other areas of cell biology, appreciation of the functional role of TJ predated description of their molecular architecture. Only in the past decade, a powerful combination of biochemical, immunochemical, and recombinant techniques has allowed the discovery and characterization of several TJ proteins. In parallel, researchers have been realizing that these proteins are capable of multiple interactions, both among themselves and with other molecules, including signaling mediators and transcription factors. We provide here a description of the individual TJ molecules (see sect. III B) and an overview of their complex network of interactions (see sect. III C). The challenging task to understand how interactions among TJ components bring about TJ assembly and function is just at the beginning. In this review, we emphasize the molecular basis of TJ function and regulation, with reference to TJ assembly (see sect. III D), as well as TJ-mediated regulation of paracellular permeability (see sect. III E) and cell polarity (see sect. III F). Finally, we survey *in vivo* studies on the physiological role of TJ molecules (see sect. III G).

TJ are expressed in both epithelial and endothelial cells. In columnar epithelial cells, TJ clearly subdivide the plasma membrane into apical and basolateral domains (which face lumen and connective tissue, respectively). In addition, some epithelial cell lines have been widely used as *in vitro* systems for the study of TJ structure, function, and regulation. Hence, research on TJ has focused on epithelial cells, and most information reviewed here of necessity refers to epithelial biology. At variance, the role of TJ in endothelial cells has been analyzed less extensively. Yet, in these cells too, TJ restrict permeability and separate the membrane into apical and basolateral re-

gions (which face blood and perivascular spaces, respectively). Hence, several conclusions obtained from epithelial systems can be reasonably applied to endothelial cells. However, some important caveats should be taken into account when making such extrapolations. First, in most endothelial cells, the precise localization of TJ and their separation from other junctional organelles is not so clear-cut as in epithelial cells. Second, even if endothelial and epithelial cells share numerous TJ components, the same molecules might be differentially assembled and regulated in the two cell types. Third, there is considerable variability among different segments of the vascular tree. Specifically, in large vessels, TJ are well developed in arteries and less elaborate in veins. Similarly, in small vessels, TJ are well organized in arterioles, but loosely organized (even with some gaps) in postcapillary venules, which are a preferential site for the extravasation of plasma proteins and circulating leukocytes. Finally, TJ are well developed in brain vessels, where they contribute to the blood-brain barrier, and less organized in other organs, which are characterized by high rate trafficking (291). When available, we highlight information that specifically relates to endothelial TJ and their unique role in vascular function.

B. Molecular Components of TJ

Like other junctional organelles, TJ are composed of both transmembrane and intracellular molecules (Fig. 3). In 1986, zonula occludens-1 (ZO-1) was discovered as the first intracellular component of TJ. In subsequent years, extensive effort aimed to identify integral membrane proteins that might act as partners for ZO-1. Occludin, the first transmembrane protein identified at the TJ, was discovered 7 years later, in 1993. The last few years have been witnessing the identification of novel TJ components at an ever-increasing pace. The long list of TJ proteins in itself speaks for the complexity of the molecular architecture of TJ. Our aim in this section consists of providing a brief survey of individual TJ molecules as an introduction to subsequent sections on molecular interactions and the molecular basis for TJ function. For a more detailed description, the reader is referred to other reviews (51, 216, 303).

1. Integral membrane proteins

A) OCCLUDIN. Occludin was identified using monoclonal antibodies raised against the junctional fraction of chick liver. Occludin, which has a molecular mass of 65 kDa, predictably contains two extracellular loops and four membrane-spanning regions. Both the amino and carboxy termini are localized in the cytoplasm (104). Transcripts of occludin from different vertebrate species only display a high degree of identity in the first extracel-

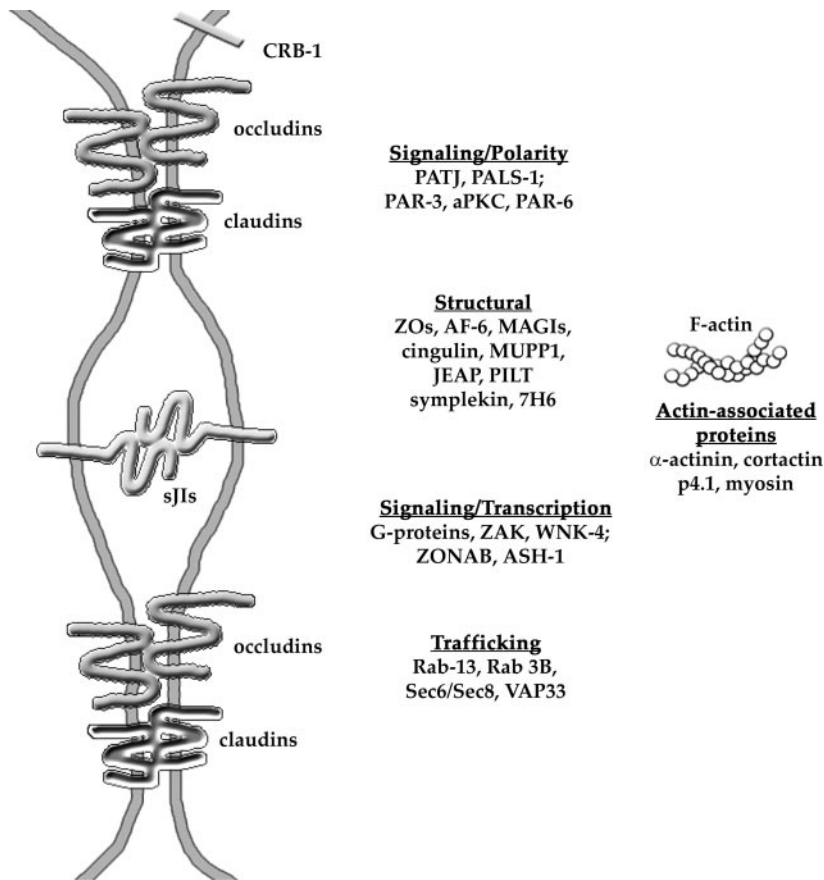


FIG. 3. Schematic representation of tight junctions (TJ). The apparent fusions of adjoining plasma membranes are represented here as the preferential site for TJ strands. Occludin, claudins, and small junctional immunoglobulins (sJI; e.g., JAM-A) are transmembrane proteins at the TJ. Through their cytoplasmic tails, they bind a vast array of cytoplasmic and cytoskeletal proteins, which in turn are involved in functions as diverse as cell signaling, cell polarity, transcriptional regulation, and membrane trafficking. A prominent role is represented by the structural assembly of protein complexes, which link the transmembrane proteins to cortical actin and to actin microfilaments.

lular loop (which contains several glycine and tyrosine residues) and in the carboxy-terminal tail (8). The functional importance of these domains in cell adhesion and molecular associations is discussed below. Occludin-1B is an alternatively spliced variant that contains an insertion of 56 amino acids. Its subcellular localization and tissue distribution are indistinguishable from those of occludin (223). Occludin is exclusively localized at the TJ of epithelial and endothelial cells. Interestingly, expression of occludin in the endothelium correlates with the permeability of different segments in the vascular tree. Occludin is expressed at high levels (with a continuous distribution) in brain endothelial cells and at much lower levels (with a discontinuous pattern) in endothelial cells of non-neural tissues (131).

Like other transmembrane components of TJ, occludin might contribute to intercellular adhesion. Upon expression in fibroblasts, exogenous occludin localizes to points of cell-cell contact (in confluent cells) and induces aggregation (in cells kept in suspension). Surface expression and adhesion, however, are not intrinsic properties of occludin, but require that endogenous ZO-1 be organized at the membrane, probably at the AJ. Adhesion is likely dependent on the conserved first extracellular loop of occludin, as synthetic peptides corresponding to this domain inhibit adhesion (326). At variance, a peptide that

encompasses the second loop removes occludin from the TJ of *Xenopus* kidney epithelial cells and affects transepithelial electrical resistance (TER; Ref. 354). In Madin-Darby canine kidney (MDCK) cells, expression of a deletion mutant of occludin lacking the cytoplasmic tail removes endogenous occludin from TJ and affects both the flux of nonelectrolyte solutes and the fence function of TJ, even if it leaves TER unaffected (24). Finally, occludin may also play a role in the transepithelial migration of leukocytes (140). Thus, in addition to contributing to intercellular adhesion, occludin is involved in multiple TJ functions.

B) CLAUDINS. The family of claudins comprises more than 20 members. Like occludin, claudins have four membrane-spanning regions, two extracellular loops, and two cytoplasmic termini. However, they are smaller (with a molecular mass of 22 kDa) and display no sequence similarity to occludin (218). Claudins-1 and -2 were discovered in the same fraction where occludin had been identified. Upon expression in L fibroblasts, claudins-1 and -2 concentrate at sites of cell contact, reconstitute TJ-like strands, and recruit occludin to the strands. Interestingly, claudin-1-based strands are continuous and associated with the P-face, whereas claudin-2-based strands are fragmented and associated with the E-face (106), thus suggesting that claudin-2 forms more leaky TJ than claudin-1.

In addition, claudin-2 is differentially expressed in two MDCK strains that are characterized by different TER. Specifically, claudin-2 is absent in MDCK I but present in MDCK II (the strain with lower TER), and transfection of claudin-2 in MDCK I decreases TER to the levels observed in MDCK II (102). In vivo, claudin-2 is expressed in the proximal tubules of the kidney, the site of passive resorption of sodium and water (90).

Some claudins have a limited distribution. For instance, claudin-5 is restricted to endothelial cells. Upon transfection in L fibroblasts, claudin-5 forms TJ-like strands that associate with the E-face (220), as it is commonly found in most endothelial cells. In contrast, strands associate with the P face in endothelial cells of the blood-brain barrier, which express both claudins-1 and -5. Interestingly, small vessels of glioblastoma do not express claudin-1, and the remaining claudin-5-based TJ strands are only detectable on the E-face (193). These molecular alterations might contribute to the formation of edema, which is a clinical feature of brain tumors. The involvement of claudins in paracellular permeability is discussed in detail in section III E1.

C) JUNCTIONAL ADHESION MOLECULE-A AND RELATED MOLECULES. Junctional adhesion molecule-A (JAM-A, which was previously called JAM, JAM-1, and F11R) is a 32-kDa glycoprotein that is composed of an extracellular region, a transmembrane segment, and a short cytoplasmic tail. Upon transfection in CHO cells, JAM-A localizes to sites of cell contact and reduces paracellular fluxes, possibly by favoring intercellular adhesion. The molecule was named after its subcellular localization and predicted function in adhesion (205) (see for review, Refs. 29, 31, 159, 221). In addition, a blocking antibody and a recombinant fragment (190, 197) inhibit the establishment of TER, thus further supporting a role for JAM-A in TJ function. Finally, JAM-A contributes to the transendothelial migration of leukocytes, as determined using in vitro assays and in vivo models of inflammation in mice, such as accumulation of leukocytes in subcutaneous spaces (205) and in cerebrospinal fluid (80).

The extracellular segment of JAM-A comprises two Ig-like domains, an amino-terminal (V_H -type) and a carboxy-terminal (C_2 -type) fold, respectively. In solution, a recombinant soluble protein, which corresponds to the whole extracellular domain of JAM-A, binds in a homophilic manner, thus suggesting that JAM-A may mediate homotypic cell adhesion (32). In addition, JAM-A forms parallel and noncovalent homodimers, which might expose at the cell surface an adhesive interface for the homophilic interactions (171). Interestingly, JAM-A may also mediate heterophilic adhesion to ligands as diverse as the leukocyte $\alpha_L\beta_2$ -integrin (249) and the attachment protein signal 1 of Reovirus (28).

More recently, independent groups identified two molecules that are homologous to JAM-A, namely, JAM-C

and JAM-B. Similarly to JAM-A, these novel members of an emerging family of small junctional proteins all display an amino-terminal V_H -type and a carboxy-terminal C_2 -type Ig-like fold, a single transmembrane region, and a short cytoplasmic tail ending with a putative PDZ-binding motif. JAM-C, which has been described in mouse as JAM-2 (17) and in humans as JAM-3 (14), has been localized in high endothelial venules and lymphatic vessels of lymphoid organs, as well as in vascular structures of the kidney. Ectopically expressed JAM-C localizes closely to the TJ of transfected epithelial cells and increases paracellular permeability (16). Similarly, lymphocyte migration is increased in JAM-C-transfected endothelial cells (158). Hence, it is intriguing to speculate that different JAMs at the TJ may differentially regulate junction patency. JAM-B has also been described in mouse and humans and was originally named VE-JAM (253) and JAM-2 (73). It has a broad tissue distribution and is capable of both homophilic and heterophilic interactions with JAM-C. However, albeit localized to intercellular contacts, its precise localization to the TJ remains unclear.

Finally, two additional small junctional Ig-like proteins with a similar V_H - C_2 tandem arrangement have been described at the TJ. The former is ESAM, an acronym for endothelial cell-selective adhesion molecule (132). The localization of ESAM at the TJ is supported by its colocalization with ZO-1 in brain and muscle capillaries (226). The latter molecule is CAR (Coxsackie- and adeno-virus receptor), which also participates in TJ assembly and in the regulation of paracellular permeability (65).

2. Intracellular proteins

Among intracellular TJ components, ZO-1 (together with the related proteins ZO-2 and ZO-3) is perhaps the most extensively studied molecule (Fig. 4). The ZO proteins belong to the family of membrane-associated guanylate kinases (MAGUK), which possess a distinct modular organization, associate peripherally with the membrane, and assemble molecular complexes at junctional structures, such as TJ and synapses (for a review, see Ref. 93). In addition to the ZO proteins, TJ comprise other MAGUKs, as well as a heterogeneous class of cytoplasmic molecules that mediate functions as diverse as anchorage to actin, establishment of cell polarity, membrane trafficking, cell signaling, and control of gene expression.

A) ZO PROTEINS. The monoclonal antibody R26.4C, which was raised against the junctional fraction of the liver, recognizes an antigen of 220–225 kDa. The protein was named ZO-1 after its localization at the *zonula occludens* of several epithelial and endothelial cell types (299). ZO-1 distribution, however, is variable depending on the cell type. In particular, ZO-1 is restricted to the TJ in those epithelia (e.g., in intestine) and endothelia (e.g., in brain and arteries) that form distinct junctional complexes. At

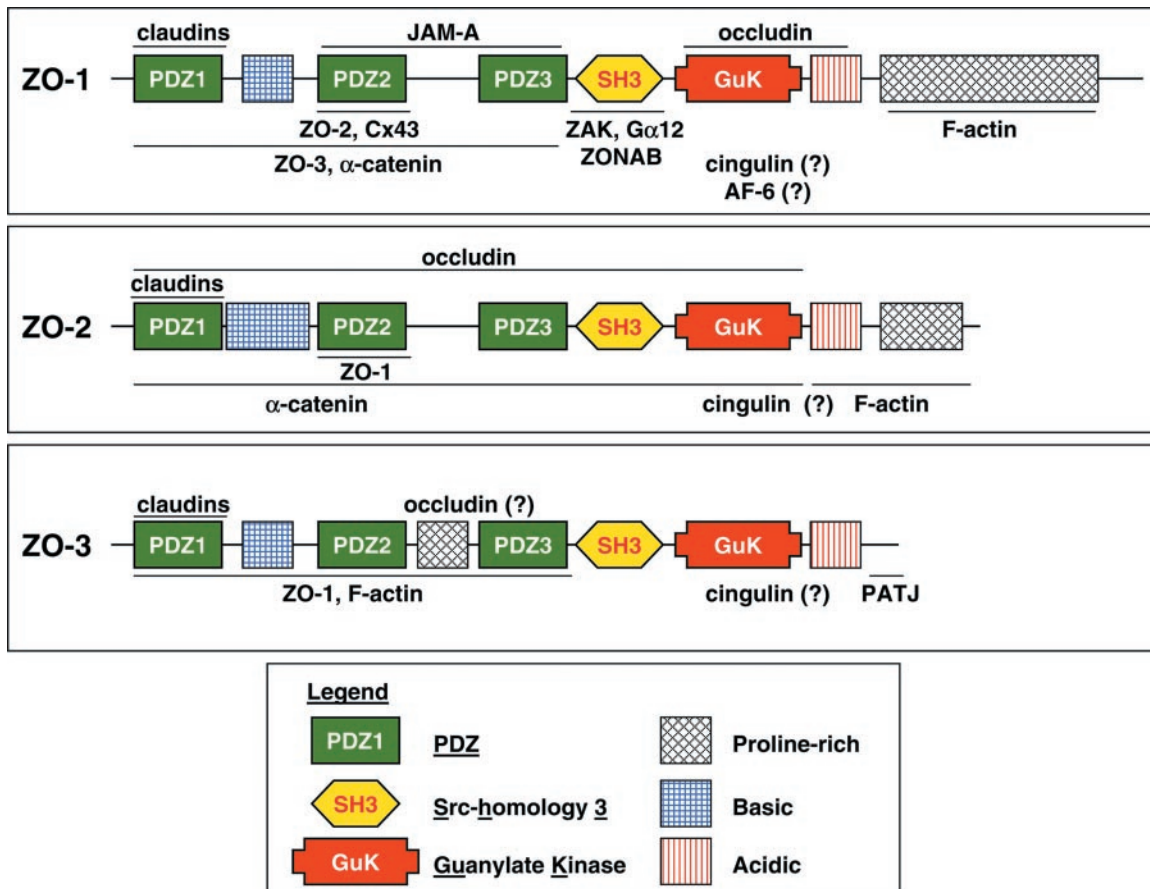


FIG. 4. Molecular complexes at the TJ. Modular structure of the three ZO proteins. The binding regions for most of the ZO-interacting proteins are indicated.

variance, ZO-1 localizes to both TJ and AJ in other epithelial cells (e.g., hepatocytes) and in nonneural endothelial cells, in which TJ are not so well developed (152, 188). Finally, ZO-1 is also expressed in cells that do not form TJ at all, such as astrocytes and dermal fibroblasts (137).

ZO-1 contains three PDZ, an SH3, and a guanylate kinase domain, as well as an acidic and a proline-rich region. Between these two regions, alternative splicing inserts a stretch of 80 amino acids, which is termed motif α (345). The ZO-1- α^+ isoform is expressed in most epithelia, while the α^- isoform is only detectable in endothelial cells, Sertoli cells, and slit diaphragms of kidney glomeruli. As these cells have dynamic junctions, absence of the motif α might correlate with TJ plasticity (20, 173). As to ZO-1 regulation, calcium-dependent intercellular adhesion is required for ZO-1 localization to the TJ (7, 289). Once localized to the TJ, ZO-1 becomes insoluble in nonionic detergents, suggesting strong association with cortical actin (6).

ZO-2 and ZO-3 share sequence homology and domain organization with ZO-1 (157). ZO-2 has a molecular mass of 160 kDa and may contain the motif β , an alternatively spliced segment of 32 amino acids (34). Although initially

regarded as a TJ-specific molecule (121), ZO-2 can also localize to spotlike AJ in nonepithelial cells (150). ZO-3 has a molecular mass of 130 kDa. At variance with ZO-1 and -2, the proline-rich motif is located between the second and third PDZ domain (127). Expression of the amino-terminal half of ZO-3 perturbs junction assembly (348) and, as more recently reported, produces global effects on the actin cytoskeleton, with decreased formation of stress fibers and focal adhesions, which is likely related to reduced RhoA activity (349). Finally, in addition to ZO proteins, other MAGUK (i.e., MAGI and CASK) have been reported at the TJ (see sect. III C1).

B) OTHER INTRACELLULAR PROTEINS. The remaining class of intracellular proteins comprises three non-MAGUK that contain PDZ domains (i.e., AF-6/Afadin, PAR-3/ASIP, and MUPP-1). AF-6 contains a Ras-binding, a PDZ, and a myosin V-like domain. The subcellular distribution of AF6 is still controversial, as it has been reported either at the TJ (359) or at the AJ (202) (see also above). PAR-3/ASIP has been localized at the TJ of MDCK cells and enterocytes (155). The analogy with its *Caenorhabditis elegans* homolog, which controls axis formation during early embryonic development, suggests that PAR-3 might play a role

during the establishment of cell polarity in vertebrate cells (245). Its involvement in cell polarity is discussed in section III F. Finally, multi-PDZ domain protein-1 (MUPP-1) contains 13 PDZ domains and has been detected exclusively at the TJ of epithelial cells (125).

Additional molecules that lack PDZ domains localize at the TJ. Cingulin is a 140- to 160-kDa component of endothelial and epithelial TJ (60). It contains a globular head, an α -helical rod domain, and a carboxy-terminal tail. The central rod domain mediates the formation of coiled-coil parallel dimers, which can further aggregate (71). Albeit colocalized with ZO-1, cingulin is more distant from the TJ than ZO-1, as evaluated by electron microscopy (302). Symplekin is a widely distributed 125-kDa protein. In epithelial and Sertoli cells, it localizes both at the TJ and in the nucleus. In other cell types, symplekin is only detectable in the nucleus, which suggests a possible role in the regulation of gene expression. It is not expressed in endothelial cells (165). Finally, 7H6 is a 155-kDa antigen localized at the TJ of hepatocytes and other polarized cells (368). It likely plays a role in the barrier function of TJ in epithelial and endothelial cells (281).

PILT and JEAP have been identified recently at the TJ. PILT (protein incorporated later into TJ) is a 61-kDa protein containing a proline-rich domain. As the acronym indicates, the molecule is only recruited to TJ following the formation of claudin-based strands, even if it does not directly interact with claudin (164). JEAP (junction-enriched and -associated protein) is a 98-kDa protein that contains a polyglutamic acid repeat, a coiled-coil domain, and a carboxy-terminal consensus motif for binding PDZ domains. It is specifically expressed in epithelia of exocrine glands, such as pancreas, lachrymal, and salivary glands (235). An intriguing finding is the localization at the TJ of the serine-threonine kinase WNK-4, whose mutations cause a form of secondary hypertension in humans (346). Finally, the monomeric G proteins Rab3b and Rab13 (341, 366), as well as VAP-33 and the Sec6/Sec8 complex (119, 183), localize to the TJ. The role of these proteins in membrane and vesicle trafficking suggests that TJ may act as target sites for vesicular targeting and docking, during cell polarization and TJ formation.

C. Molecular Interactions at the TJ

Numerous molecular interactions involving TJ molecules have been reported in the literature. To provide an overview of this complex issue, in Figure 5 we present the available information in the style of interaction maps, as proposed by Kohn (170). By analogy to electric circuit diagrams, each molecule appears only once in the map, and its interactions are indicated by connecting lines that originate from the molecule itself. In particular, solid lines terminated at both ends by arrows indicate noncovalent

binding. Similarly to nodes in circuits, a small filled circle on each line represents a binary complex that is composed of a pair of interacting molecules. Also, dashed lines starting from a kinase and pointing toward a substrate represent phosphorylation. When novel information becomes available, other types of molecular interactions (e.g., dephosphorylation, proteolysis, inhibition, and stimulation) can be conveniently annotated in the map using additional symbols. Molecular maps offer the advantage of visually grouping into subsystems molecules that are possibly involved in the same biological function (e.g., linkage to the cytoskeleton, cell signaling, cell polarity, and control of gene expression). We have highlighted such subsystems with a color code and with curved boxes demarcated by dashed lines. Unfortunately, in spite of the wealth of reports on interactions at the TJ, information on function is far from being complete. In addition, with few exceptions, the kinetics of these interactions have not been determined. Finally, it is still unclear whether the molecular partners bind in a mutually exclusive manner or, alternatively, whether distinct complexes may coexist at the TJ. It is expected that future research will help us fill the gaps in the interaction map and (more importantly) in our understanding of TJ function. Hereafter, the text describes the interactions as annotations to the map and provides grid coordinates (in bold characters) to facilitate the location of each "node" in the map.

1. Molecular interactions among TJ components

A) INTERACTIONS OF OCCLUDIN. Occludin binds directly the three ZO proteins (Fig. 5, **B3**). The interaction of occludin with ZO-1 requires the carboxy-terminal domain of occludin (105), which contains a coiled-coil motif (241), and the region encompassing the SH3 and guanylate kinase domains of ZO-1 (94). As assessed by surface resonance spectroscopy, the interaction is saturable, with dissociation constant in the micromolar range (285). The biological relevance of the association is underscored by the following observation. Replacing the cytoplasmic domain of the gap junction protein connexin-32 with the ZO-1-binding region of occludin results in recruitment of the mutated connexin to TJ strands (217). The cytoplasmic domain of occludin also binds ZO-2 (150) and ZO-3 (127), with similar molecular requirements.

B) INTERACTIONS OF CLAUDINS. Claudin-1 to -8 bind directly ZO-1, ZO-2, and ZO-3 (Fig. 5, **B2**). The interaction is mediated by the cytoplasmic domain of claudins and the first PDZ domain of the ZO proteins (149). Claudins contain a putative motif for binding PDZ domains (294). Starting from the carboxy terminus, claudins have a valine (at position 0) and a tyrosine (at position -1). The residue at position -2 is either charged (aspartate in claudin-1 and -3, and glutamate in claudin-7), polar (as-

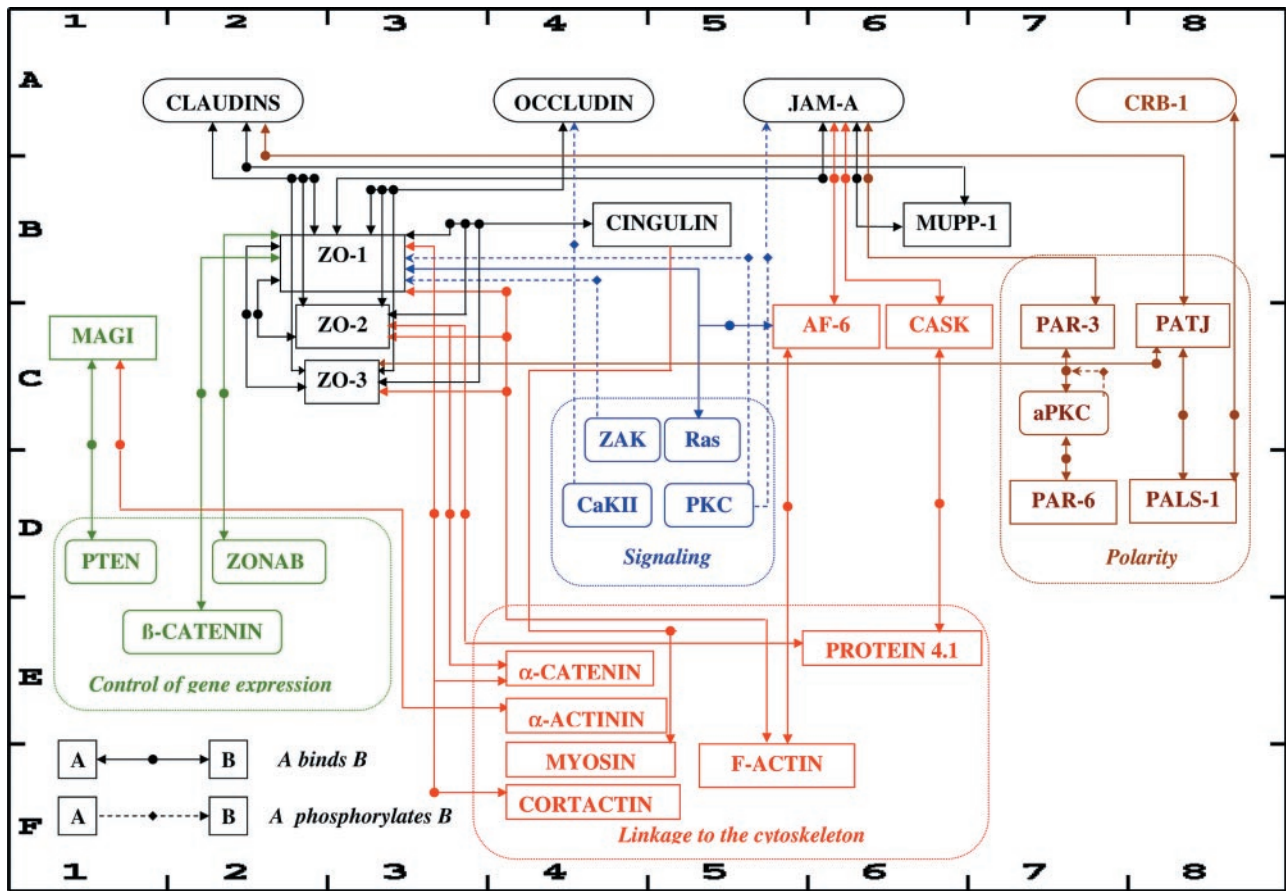


FIG. 5. Molecular interactions at the TJ. All the molecular interactions discussed in the text are schematically represented here in the style of “molecular interaction maps.” Subsets of junctional components that are possibly involved in the same function are color-coded and demarcated by curved boxes. See text (sect. III C) for details.

paragine in claudin-4, -5, and -6, and glutamine in claudin-8), or a glycine (in claudin-2). It should also be taken into account that the ZO proteins interact with each other (see sect. III C1D). Hence, besides binding directly the individual ZO molecules, claudins (and occludin as well) might also bind them indirectly, via another interposed ZO protein.

C) INTERACTIONS OF JAM-A. The conserved residues phenylalanine, leucine, and valine at the carboxy terminus of JAM-A represent a consensus motif for binding type II PDZ domains (Fig. 5, B6). JAM-A interacts with at least five proteins containing this domain, i.e., ZO-1 (33), AF-6/Afadin (88), PAR-3/ASIP (89, 153), CASK/Lin-2 (206), and MUPP-1 (125). The interaction between the PDZ domains in these proteins and the PDZ-binding motif of JAM-A is direct, as evaluated by *in vitro* binding assays. Some of these interactions may be functionally relevant for linking JAM-A to the cytoskeleton, as both ZO-1 (B4) and AF-6 (D5) associate directly with F-actin, while CASK associates indirectly (D6), via the actin/spectrin binding protein 4.1 (64). Other interactions are likely involved in cell signaling, as AF-6 binds Ras (see sect. III C1D; C5). Finally,

the association with PAR-3 might play a role in cell polarity, as PAR-3 interacts with the λ - and ζ -isoforms of atypical PKC (C7), as well as with the PDZ protein PAR-6 (D7). In addition, the kinase activity of atypical PKC may regulate its own interaction with PAR-3. As mentioned above, the PAR-3/atypical PKC/PAR-6 complex plays a central role in the polarization of *C. elegans* embryo (155). More recent evidence supports a direct role for individual members of the complex in the assembly of epithelial TJ. Specifically, PAR-6 negatively regulates TJ formation (110). At variance, PAR-3 exerts a positive effect, possibly as a consequence of its interaction with aPKC (133). Finally, the function of aPKC is more likely related to the ability of promoting the maturation of initial junctional contacts (307). For a schematic representation of the complex, see Figure 6.

D) INTERACTIONS OF INTRACELLULAR TJ PROTEINS. In addition to binding transmembrane proteins, ZO-1, ZO-2, and ZO-3 interact with several cytoplasmic molecules. First, the ZO proteins bind each other (Fig. 5, C2). Specifically, ZO-1 binds ZO-2, and the interaction requires the second PDZ domain of each partner (94, 150). In addition, ZO-1 binds

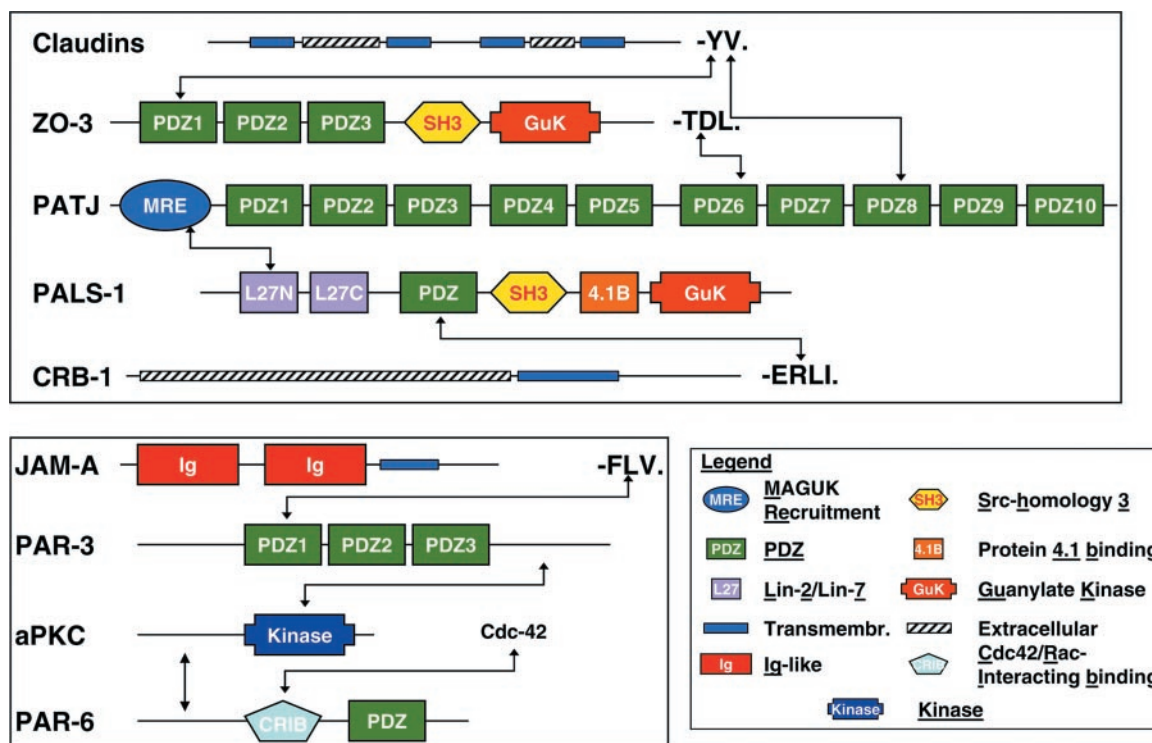


FIG. 6. TJ and cell polarity. Modular structure and binding sites of two TJ complexes, which are involved in the regulation of cell polarity, such as the PATJ/PALS-1/CRB-1 complex (*top panel*) and the PAR-3/aPKC/PAR-6 complex (*bottom panel*).

ZO-3 (127). However, not all the possible combinations are allowed, as ZO-2 does not bind ZO-3. Consequently, the three proteins do not form a ZO-1/ZO-2/ZO-3 trimer, but rather independent ZO-1/ZO-2 and ZO-1/ZO-3 complexes (347).

Second, the ZO proteins associate with the cortical cytoskeleton. ZO-1 (94, 151), ZO-2 (150), and ZO-3 (347) coprecipitate with F-actin (B4 and C4). The association requires the proline-rich region in the carboxy-terminal half of the ZO proteins. Recently, the actin-binding region of ZO-1 has been mapped more precisely to a 220-amino acid region. Interestingly, this region is required for accumulation of ZO-1 to an actin pool localized at the puncta of free cell edges before cell-cell adhesion takes place (95). Interestingly, deletion mutants of the ZO proteins that encompass the carboxy-terminal half distribute to the lateral surface and other actin-rich structures of the plasma membrane. In contrast, deletion mutants that encompass the amino-terminal half localize to the TJ. In addition, both ZO-1 (151) and ZO-2 (150) bind α -catenin (D3). The interaction involves the amino-terminal half of the ZO proteins and carboxy-terminal residues of the catenin (146). Besides associating with cadherins at the AJ, α -catenin binds the actin-associated proteins vinculin and α -actinin. It is unclear, however, whether the interaction with α -catenin only involves the pool of ZO-1 and ZO-2 that localizes, in some cell types, to the AJ. Similarly,

ZO-1 (as well as its *Drosophila* homolog Tam-A) binds cortactin (F3, Ref. 163), another F-actin-associated protein (138). In addition, ZO proteins also bind cingulin (B3), which in turn binds myosin II heavy chain (E5), which represents a plausible molecular basis for the contraction of the perijunctional actomyosin ring in the control of permeability (71). Finally, the MAGUK protein MAGI-1 (membrane-associated guanylate kinase inverted-1) has been recently shown to bind α -actinin-4 (C1) (255), while ZO-2 binds protein 4.1R (D3) at the TJ (208). More in general, it is conceivable that the ability of ZO proteins to link integral membrane proteins with cortical actin might be primarily instrumental in assembling TJ.

Third, ZO-1 engages in molecular interactions with signaling molecules (see sects. III C2B and III C2C) and transcription factors (see sect. III C3). Here, we mention that ZO-1 associates with AF-6 (359), which in turn binds active Ras (174). Interestingly, the interaction of ZO-1 with AF-6 is mediated by the Ras-binding domain of AF-6 and is inhibited by Ras (C5), which might explain (at least in part) why overexpression of active Ras perturbs cell-cell contacts.

Fourth, individual MAGUK molecules may bind more than one integral membrane partner at the same time. For instance, ZO-1 binds both occludin (B3) and JAM-A (B6), via the guanylate kinase and the second PDZ domain, respectively. In addition, MUPP-1 binds both claudin-1

(B2) and JAM-A (B6), via the 9th and 10th PDZ domain, respectively. This feature might be instrumental in assembling and stabilizing the arrays of transmembrane proteins at the TJ strands.

Fifth, in addition to the Par-3/Par-6/aPKC complex, which has been described in section III C1c, another TJ complex is involved in the establishment and/or maintenance of cell polarity. The complex is composed of Pals1, Pals1-associated TJ protein (PATJ), and CRB1, which are the mammalian homologs of *Drosophila* Stardust, Disc lost, and Crumbs, respectively. This ternary complex is detectable at the TJ (271). The L27N domain of Pals1 binds the MAGUK recruitment domain of PATJ (C8), while the unique PDZ domain of Pals1 binds the carboxy terminus of the apical polarity determinant CRB-1. In addition, targeting of the Pals1/PATJ/CRB-1 complex to the TJ requires the interaction of the sixth and eighth PDZ domains of PATJ with the carboxy termini of ZO-3 (C8) and claudins (B2), respectively (270). These interactions are summarized in Figure 6. A recent report has identified a molecular link between the two polarity-related complexes of the TJ. Specifically, the amino terminus of PALS-1 interacts directly with the PDZ domain of Par-6. In addition, either a dominant negative PATJ or Par-6 overexpression affects TJ assembly (142).

2. Interactions of TJ components with signaling molecules

TJ components interact with several signal transduction molecules, such as G proteins (both trimeric and monomeric) and protein kinases (both A and C). An in-depth analysis is beyond the scope of this manuscript, and the reader is referred to more detailed reviews (36, 162). Here, we provide a brief summary of these molecular interactions and their possible functional consequences for TJ regulation.

A) INTERACTIONS WITH G PROTEINS. Among trimeric G proteins, the inhibitory and pertussis toxin-sensitive $G_{i-2}\alpha$ subunit colocalizes with ZO-1 at cell contacts in MDCK cells. In addition, it can be coprecipitated with ZO-1, even if it is not known whether the two molecules are directly linked to each other (82). Overexpression of $G_{i-2}\alpha$ (as well as expression of the related $G_{i-0}\alpha$ subunit) accelerates TJ assembly and TER establishment upon addition of extracellular calcium (276). To date, the ligand and the receptor that activate the G protein remain unknown.

Among monomeric G proteins, GTPases of the Rab and Ras families have been identified at the TJ and their possible role is discussed in sections III B2B and III C3. GTPases of the Rho family may also affect TJ and other junctional complexes. Evidence, however, has been obtained mostly using exogenous inhibitors and overexpression systems. While these approaches confirm a role for Rho proteins in TJ regulation, they do not allow concluding

whether the overall effect is either positive or negative. On one side, some data support a positive role. For instance, Rho inhibition upon C3 transferase microinjection affects TJ assembly (242), and Rho activation upon RhoA-V14 expression prevents TJ disassembly (112). On the other side, however, both constitutively active and dominant negative forms of RhoA (and Rac1) perturb TJ morphology, TER, and permeability (160). Along the same line, overexpression in MDCK cells of the canine homolog of human GEF-H1, a guanine nucleotide exchange factor for Rho, which normally localizes to the TJ, results in increased paracellular permeability (37). In addition, no information is available about physical associations of Rho GTPases with TJ proteins. Finally, it is unknown whether these mediators may indirectly affect TJ function by interfering with the cytoskeleton.

B) INTERACTIONS WITH PROTEIN KINASES. In spite of numerous studies on the issue, the role of protein kinase A (PKA) in TJ regulation remains controversial, as both stimulatory and inhibitory effects have been reported. The effect of agents that either affect (e.g., forskolin) or mimic PKA (e.g., dibutyryl cAMP) is influenced by experimental variables, such as concentration, incubation time, and cell type (for a review, see Ref. 36).

Similarly, experimental variables do not allow drawing unambiguous conclusions about the role of PKC in TJ function. For instance, PKC inhibition stabilizes TJ upon removal of extracellular calcium (61). Correspondingly, PKC stimulation with phorbol ester causes TJ disassembly (247). In contrast, however, PKC activation with diacylglycerol analogs induces TJ assembly (22). A major limitation of the available agents is the lack of selectivity for different isoforms of PKC. Thus only the development of isoform-selective reagents will provide a definitive answer. Along this line, it is worth recalling that two PKC isoforms (i.e., the calcium and phorbol ester-insensitive λ and ζ atypical PKC) associate with the PDZ-protein PAR-3 at the TJ (155).

C) PHOSPHORYLATION OF TJ MOLECULES. Some TJ components are phosphorylated on serine and threonine. For instance, ZAK (ZO-1-associated kinase), a serine/threonine kinase that binds the SH3 domain of ZO-1, phosphorylates the carboxy terminus of ZO-1 (21; Fig. 5; B4). In addition, PKC phosphorylates ZO-1 in vitro (B5). Finally, a PKC inhibitor concomitantly affects ZO-1 phosphorylation and TJ assembly (305). ZO-1 phosphorylation, however, does not correlate always with TJ function (301). Similarly, cingulin is phosphorylated in vivo, but the correlation with TJ function remains unclear (59).

Casein kinase II, another serine/threonine kinase, phosphorylates the carboxy terminus of occludin in vitro (72; B4). Phosphorylation of occludin correlates with decreased solubility in nonionic detergents and incorporation into nascent TJ strands (279). Nonetheless, a fusion protein containing the carboxy terminus of occludin is

phosphorylated *in vivo*, but is not incorporated into TJ, suggesting that phosphorylation *per se* is not sufficient for recruiting occludin to the TJ (209). In platelets, JAM-A is another target for serine/threonine phosphorylation, possibly by classical PKC (251; **B5**). The biological relevance of JAM-A phosphorylation, however, is unknown. Finally, it should be stressed once again that PKC phosphorylates AJ and cytoskeleton components too, thus possibly affecting TJ in indirect ways.

Phosphorylation of ZO-1 on tyrosine has also been reported, even if neither the kinase responsible nor the functional consequences have been established. In endothelial cells, tyrosine phosphatase inhibitors cause junction disruption (296). Similarly, vascular endothelial growth factor induces tyrosine phosphorylation of ZO-1 and occludin, as well as enhanced paracellular permeability (12). At variance, in epithelial cells, epidermal growth factor-induced tyrosine phosphorylation correlates with junction formation (327).

3. Interactions of TJ components with regulators of cell proliferation

ZO-1 and other TJ components interact with molecules that regulate cell growth and survival. First, ZO-1 binds the Y-box transcription factor ZONAB (Fig. 5, **C2**), which in turn inhibits expression of the *ErbB2* gene product (23). Second, in the early stages of junction formation, ZO-1 binds the AJ component β -catenin (263; **C2**), which is also a potential oncogene. Interestingly, an antioccludin peptide affects β -catenin-induced transcription of *c-myc* (335). Additional evidence suggests a role for ZO-1 in proliferation. For instance, ZO-1 localization at the TJ is altered in human breast cancers (136), and expression of ZO-1 deletion mutants in epithelial cells causes transition to a mesenchymal and tumorigenic phenotype (264, 275). Also, in its amino-terminal half, ZO-1 is highly homologous to the tumor suppressor *discs-large-1* (344). Notably, *discs-large-1* mutations in *Drosophila* alter septate junction morphology and cause neoplastic overgrowth of epithelial cells in the imaginal discs (for a review, see Ref. 350).

Other junctional MAGUK comprise members of the MAGI (membrane-associated guanylate kinase with inverted orientation) family. MAGI possess multiple PDZ domains and colocalize with ZO-1 in MDCK cells (143). Interestingly, MAGI-2 and -3 associate with the tumor suppressor PTEN (**C1**) and enhance PTEN-dependent inactivation of AKT (356), thus possibly favoring apoptosis. However, in spite of this circumstantial evidence, the mechanisms whereby ZO-1 and other junctional MAGUK might contribute to the control of cell proliferation and survival remain unclear. An attractive hypothesis is that transmembrane TJ proteins and associated MAGUK might sequester regulators of growth at the plasma mem-

brane, thereby preventing their translocation to the nucleus and their effect on cell proliferation.

TJ components can also interact functionally with members of the Ras family and with Ras effectors (e.g., Raf and MAPK), which are involved in the regulation of cell growth. For instance, expression of H-Ras (but not K-Ras) in MDCK cells disrupts TJ (56). Conversely, overexpression of occludin reverts the Raf-induced transformation of an epithelial cell line (189). In addition, the MEK1 inhibitor PD98059 induces the assembly of functional TJ and AJ (56). Also, as mentioned above, AF-6 is both a TJ component and a Ras effector (359; **C5**). Finally, it is also worth mentioning that ZO-1 (114) and symplekin (165) have been detected in the nucleus and that, conversely, the transcription factor ASH-1 localizes to the TJ (225). To date, however, the biological consequences of these observations remain elusive.

D. TJ Assembly

1. General principles of TJ assembly

Assembly of TJ molecules into nascent strands parallels the development of the barrier function. Consequently, the increase of electrical resistance in cell monolayers is commonly measured *in vitro* to study TJ assembly. We briefly discuss the experimental systems in sections **III D 2** and **III E 2**. Here, we summarize some general principles and open questions that emerge from these studies. First, it is conceivable that several molecular interactions described in section **III C** are instrumental in assembling TJ. However, the precise sequence of molecular events is far from being fully understood.

Second, research efforts that aimed at determining the primary site of TJ assembly did not reach definitive conclusions. One can envision two alternative sites of assembly, i.e., either from within the cell or at the plasma membrane. According to the former model, cytoplasmic molecules bind transmembrane proteins inside the cell before targeting them to developing TJ strands. Conversely, in the latter model, clusters of transmembrane proteins act at the cell surface as nucleation sites for cytoplasmic partners. Although the argument has not been settled, more evidence lends support to the former model. For instance, ZO protein complexes are detectable in the cytoplasm of cells that have not yet established TJ (22). In addition, in *occludin*-transfected fibroblasts, the presence of endogenous ZO-1 is a prerequisite for localizing occludin to TJ-like structures (326). In contrast, neither occludin displacement from TJ (354) nor null mutation of the *occludin* gene (277) abolishes the junctional localization of ZO-1.

Third, under several circumstances, AJ likely play a crucial role in the assembly of TJ. In epithelial cells, blocking antibodies that disrupt E-cadherin-based AJ also

cause dissociation of TJ (35). In addition, as mentioned above, ZO-1 associates with the AJ molecule β -catenin during early steps of TJ assembly (263). To date, however, the precise role of AJ in TJ biogenesis remains unexplained. Cadherins might merely hold adjoining cells together to allow transmembrane TJ molecules to bind each other. Alternatively, cadherins and associated molecules might trigger a series of intracellular events (e.g., vinculin and α -actinin binding) that lead to F-actin reorganization and eventually to TJ assembly. Along this line, it is worth mentioning that the normal development of TJ in *E-cadherin*-null blastocysts challenges the idea that cadherins are invariably essential for TJ assembly (246). However, in addition to cadherins, recent evidence suggests a role for the four members of the nectin family (which are Ig-like cell adhesion molecules that localize to the AJ) in the AJ-regulated assembly of TJ. In particular, during the formation of junctional complexes, adhesive interactions mediated by nectin ectodomains cause recruitment of ZO-1 (363) and JAM-A (101). The interactions of nectin with AF-6, AF-6 with ZO-1, and ZO-1 with JAM-A may represent a plausible molecular basis for the involvement of nectin in TJ assembly.

2. Methods for the study of TJ assembly

Methodological approaches are mostly (but not exclusively) performed in epithelial cells. The “calcium switch” is a frequently used experimental system for studying TJ assembly (207). In this *in vitro* assay, extracellular calcium is first removed from monolayers of confluent cells to disassemble intercellular junctions. Then, calcium is added back to allow junctions to form again and establish the barrier function. During the two steps, the molecular associations of TJ proteins are dynamically regulated. For instance, in the presence of low calcium, ZO-1 becomes more soluble in nonionic detergents, which likely reflects detachment from F-actin, and associates with intracellular vesicles. Then, upon switching back to normal calcium, ZO-1 returns to the lateral membrane. Eventually, ZO-1 reaches the TJ strands and acquires again its characteristic insolubility, which is suggestive of tight linkage to the cytoskeleton.

Another system, the “ATP depletion” is based on the observation that TJ integrity is lost during cell ischemia (318). To mimic this condition *in vitro*, epithelial cells are treated with inhibitors of glycolysis and oxidative phosphorylation, such as 2-deoxy-D-glucose and sodium azide, respectively. Depletion of ATP correlates with a rapid fall of TER. At odds with the calcium switch, ZO-1 remains localized at the lateral membrane, and its solubility in nonionic detergents is even reduced. The latter event is probably mediated by the interaction of ZO-1 with the actin-associated protein fodrin. Then, upon ATP repletion, TJ becomes progressively resealed and ZO-1 be-

comes more soluble, perhaps because it loses its association with fodrin. The different behavior of ZO-1 during the calcium switch and the ATP depletion assays might unveil alternative mechanisms of TJ assembly that predictably operate under different contexts (81).

Finally, aggregation assays are deployed to study the concomitant establishment of TJ and polarity (210). If monolayers of thyroid cells are detached and kept in suspension, they form aggregates that resemble polarized follicles, in which TJ define apical and basolateral domains. In addition, in the presence of soluble matrix proteins or hormones, cells may change their polarity, switching from “closed” to “inverted” follicles (with apical domains facing either the internal lumen or the outer environment, respectively). Remarkably, such a profound reversal in cell polarity is accompanied by displacement (but not disruption) of TJ (236).

E. TJ Function (I): Paracellular Permeability

1. General principles: TJ as barriers or channels?

Every day, large amounts of fluid traverse epithelial barriers (e.g., in small intestine and kidney tubules) to diffuse from lumen into interstitial spaces. Subsequently, fluids cross endothelial barriers twice to enter and eventually to leave the bloodstream. As epithelial and endothelial membranes are impermeable to hydrophilic molecules, fluid adsorption cannot depend on diffusion through membranes, but has to follow alternative routes. One of these routes is the “transcellular” pathway (i.e., through the cytoplasm of individual cells), which is mediated by channels and vesicles (for a review, see Ref. 214). The other route is the “paracellular” pathway (i.e., through LIS of adjacent cells), which is mediated by the TJ.

In the past, the idea of paracellular diffusion across TJ sounded counterintuitive, as TJ had been regarded to as a sealing barrier for the LIS. Clearly, this view correctly applies to TJ of nonadsorptive epithelia (e.g., in urinary bladder), which undoubtedly represent a waterproof barrier. Here, the tightness of TJ and the polarized (i.e., apical-to-basal) extrusion of Na^+ establish high TER and cause basolateral accumulation of Na^+ . In contrast, TJ of adsorptive epithelia (e.g., in kidney tubules and small intestine) and TJ of endothelial cells only restrict (but do not block) the passage of fluids, and establish lower TER. In these cells, the finding that electrical currents flow in correspondence of the LIS (41) provided convincing proof that TJ were indeed channels for paracellular fluxes (295). Hence, the older notion of TJ as impermeable barriers has been gradually integrating the more recent concept of “paracellular channels” (353).

Experimental evidence notwithstanding, the idea of permeable TJ has been difficult to reconcile with the

general theory of fluid transport across epithelia. According to the theory, the movement of fluids is vectorial, i.e., it is directed from the apical to the basolateral side of the epithelium. In addition, the flux of solvent depends on the movement of solute, and the adsorbate at the basal side is isosmotic to the solution applied on the apical side (169). Consequently, a major assumption of the theory is that TJ be absolutely impermeable, to prevent back-flow of solutes to the apical compartment during the vectorial transport. Forty years ago, a model postulated that a compartment of high osmolality (conceivably the LIS) is the precise site, where transport of solutes is coupled to movement of fluids. In the model, active transport of Na^+ (which is pumped out of the cell into the LIS by the basolateral $\text{Na}^+\text{-K}^+\text{-ATPase}$) creates an osmotic force that drives water from the cell cytosol into the LIS. Then, accumulation of isosmotic fluid builds up a hydrostatic pressure that eventually pushes the adsorbate into the interstitial tissue (74). In this context, an impermeable TJ is essential to impose anatomical asymmetry to the LIS. Otherwise, a leaky TJ would diminish the osmotic gradient and preclude fluid adsorption.

The realization that the assumption of impermeable TJ was only partially correct led to the formulation of alternative (but so far unproven) model systems. Hence, although to date the local osmotic model remains the widely accepted explanation for fluid movements, novel methodology ought to be developed to investigate the physical and chemical properties of TJ and thus incorporate the leakiness of TJ in the model (295). Another unsettled issue is the relative contribution of the transcellular and paracellular routes. If TJ are also channels, then trans- and paracellular movements should be viewed as coordinated (rather than separate) steps in fluid adsorption. In this respect, the polarized cell can be modeled as an electric circuit containing two resistive arms in parallel, i.e., the transcellular arm (which comprises apical and basolateral resistances in series) and the paracellular arm (which consists of TJ and LIS resistances in series, Ref. 199).

In addition to refined physiological measurements, it is essential to define the molecular anatomy of paracellular channels within TJ strands. In this respect, it has long been known that there is a quantitative correlation between number of strands and TER (63). More precisely, TER increases in a logarithmic manner with the number of strands. The simplest model assumes that there is a single channel in each strand and that channels fluctuate between open and closed states. If each channel within n strands has the same probability of being either open (P_{open}) or closed (P_{close}), then permeability along the paracellular route depends on $(P_{\text{open}})^n$, thus accounting for the logarithmic correlation of TER with the strand number (62).

However, in addition to the number of strands, qualitative features (e.g., expression, combination, and mixing ratios of different claudins) may also be critical. As mentioned in section III B1 B, differences in TER between MDCK I and II (which have a similar number of TJ strands, Ref. 300) correlate with differences in claudin-2 expression (102). More in general, functional properties of claudins predictably render them capable of acting as building blocks for both strands and channels. First, claudins form polymers both within (in *cis*) and between (in *trans*) strands, thus providing the backbone of the strand and the adhesive forces for intercellular cohesion, respectively. Second, although some claudins (e.g., claudin-1 and -2) can be assembled in *cis* into heteropolymeric strands, the same claudins within these strands cannot bind heterophilically in *trans* (107). It has been proposed that such points of defective contact may correspond to putative channels (319).

Studies on claudins are now better defining the possible contribution of these molecules to the “barrier” function of the TJ. First, overexpression of some claudins is often associated with the expected reduction of permeability. For instance, claudin-1 overexpression in MDCK cells increases TER (213). In addition, null mutation of *claudin-1* gene increases permeability to water in the epidermis of newborn mice (103). Similarly, claudin-4 overexpression in MDCK cells decreases conductance, which is accompanied by a selective decrease in permeability to sodium and an increase in the complexity of TJ strands (325). Notably, the effects of claudin-4 on permeability are recapitulated by a chimera consisting of the first extracellular domain of claudin-4 on claudin-2, thus indicating that this domain is sufficient to determine the paracellular properties of claudin-4 (67). Second, mutational analysis suggests that some claudins might act as an electrostatic barrier to cation. In this respect, a mutation that replaces a positive to a negative charge in the first extracellular loop of claudin-4 increases permeability to sodium (66). At variance with claudin-4, claudin-15 likely acts as a selective barrier for anions, as mutations that replace three negative to positive charges in the first extracellular loop of claudin-15 increase the permeability to chlorine ions (66). Third, the anatomical localization of some claudins further suggests a role as barriers. For instance, claudin-8 and claudin-3 are preferentially expressed in the distal segments of the nephron, which are characterized by low paracellular permeability (167).

However, complementing this view, other data indicate that claudins may also act (perhaps surprisingly at first sight) as “channels,” rather than barriers, to water and solutes. First, overexpression of claudin-2 in MDCK I cells in vitro enhances TER and converts strands from a tight to a leaky type (106). Second, overexpression of claudin-6 in transgenic mice enhances permeability to water (321). Along the same line, mutations in *claudin-16*

decrease permeability to cations, thus impairing resorption of magnesium and causing hypomagnesiemia (293). Third, anatomical localization of some claudins suggests a role as channels. For instance, claudin-2 is selectively expressed in the proximal nephron segment, which is characterized by high permeability to solutes (90). As a general consideration, it should be taken into account that overexpression experiments may alter the equilibrium between claudins within the TJ strands. For instance, claudin-8 overexpression in MDCK II cells reduces permeability to cations. However, the effect might be due either to a direct action of claudin-8 as “cation barrier” or to the reduced expression of the “cation channel” claudin-2 that follows claudin-8 induction (365).

Clearly, unlike conventional ion channels, paracellular channels within TJ strands are parallel (and not perpendicular) to the plane of the plasma membrane (309). However, like ion channels, TJ channels have putative pores of similar size, are selective for cations over anions, and are blocked by high-molecular-weight cations. It is expected that future work will aim to define how individual claudins (and other TJ molecules as well) may assemble within the TJ strands to provide junctions with either stringent (barrier) or permissive (channels) structures. In this respect, real time microscopy techniques in living cells should provide interesting information. For instance, a recent report analyzed claudin-1-based TJ strands in transfected L fibroblasts (using claudin-1 fused with green fluorescent protein) and highlighted a dynamic behavior and a functional reorganization of the TJ strands (280).

2. Methods for the measurement of paracellular permeability

Techniques used to analyze paracellular permeability comprise the measurement of ionic currents and nonelectrolytic fluxes. For a discussion of the biophysical basis and the experimental protocols, the reader is referred to more detailed reviews (52, 199, 295).

The ability of ions to carry electrical currents in aqueous solutions is exploited to measure the resistance of epithelial or endothelial monolayers. The experimental system consists of passing a pulse of direct current across a monolayer and of measuring the resulting change of voltage. If the amplitude of the current is known, then Ohm's law allows calculating the resistance of the monolayer. Similarly, when using alternating currents, it is possible to measure the impedance. The circuit model outlined in section III E 1 (with the transcellular and paracellular resistive arms in parallel) represents the conceptual basis for the assay. The contribution of the transcellular arm (which comprises apical and basal membrane resistors in series) is usually ignored, as long as TER values are lower than the resistance of biological membranes ($\sim 1,000 \Omega \cdot \text{cm}^2$). In addition, when estimating the

paracellular arm (which comprises TJ and LIS resistors in series), the contribution of the LIS resistor is ignored, unless TER values are very low (i.e., $< 25 \Omega \cdot \text{cm}^2$) or the LIS is exceptionally narrow, long, and tortuous. In all these extreme conditions (i.e., very high or very low TER), neglecting the contribution of either membrane or LIS might overestimate the TJ resistor (126). However, in the most commonly used tissues (e.g., small intestine and gallbladder) and cell lines (e.g., MDCK and Caco-2), TER averages few hundreds of ohms times square centimeter. Thus, to a good approximation, measurements in this range of values give a fair assessment of paracellular conductance through the TJ.

In addition, the use of tracers of known molecular weight allows determining the flow of fluids across the paracellular route and estimating the size of the TJ “pores.” The most commonly used tracers are either radioactive (e.g., D- ^{14}C)mannitol and [^3H]inulin) or fluorescent (e.g., fluorescein isothiocyanate-labeled dextrans of different molecular weights). These nonelectrolyte tracers usually do not cross the transcellular pathway. In these experiments, the flux of solutes of graded size and molecular weight is measured as a function of molecular size and radius. Clearly, TER and fluxes are inversely related, even if there are differences in the kinetics of either TER increase or flux decrease during TJ sealing. For instance, expression of an occludin mutant in MDCK II cells increased both TER and paracellular permeability, thus suggesting that paracellular fluxes may occur even in the presence of electrically sealed TJ (24).

3. Regulation of permeability by extracellular agents

The presence of a multiprotein complex at the TJ, as well as the association of TJ with the perijunctional actin-myosin ring, are the most likely candidate targets for permeability regulating agents (see for a review, see Ref. 243). One of the most physiologically relevant examples of TJ regulation is represented by the effect of nutrients, specifically glucose, which activates the Na^+ -glucose cotransport system. Addition of glucose to the apical surface of a segment of small intestine results in enhanced paracellular permeability and reduced TER (254). These functional responses are accompanied by morphological events, such as internal dilatation of TJ and dissociation of ZO-1 from the TJ (15). Although the precise biochemical events at the TJ are still unknown, the effect of the Na^+ -glucose cotransport likely involves activation of the Na^+/H^+ exchanger at the apical membrane, phosphorylation of myosin light chain, and ultimately contraction of the perijunctional actin-myosin ring leading to TJ opening (322).

Among extracellular stimuli acting on TJ, there are several inflammatory cytokines. First, interferon- γ enhances permeability of the T84 epithelial cell line (200),

reduces ZO-1 expression, causes redistribution of occludin and ZO-2, and disrupts apical actin (364). Second, the combination of interferon- γ and tumor necrosis factor- α enhances permeability of microvascular endothelial cells (40) and reduces the junctional distribution of JAM-A in umbilical vein endothelial cells (252). In contrast, other noninflammatory cytokines, such as epidermal growth factor, exert a positive effect on TJ in epithelial cells (327).

Interestingly, it has been recently reported that protein CagA from *Helicobacter pylori*, the causative agent of peptic ulcer disease, associates with ZO-1 and JAM-A. In this way, CagA induces TJ disassembly, which in turn facilitates bacterial attachment and eventually disruption of the epithelial barrier (2). Finally, another recent report highlights a possible link between TJ formation and vascular angiogenesis, which is potentially relevant to the function of the blood-brain barrier. Specifically, conditioned medium from astrocytes overexpressing the PKC substrate *src*-suppressed C-kinase substrate blocks angiogenesis and, in parallel, increases TJ proteins and decreases permeability in endothelial cells (186).

F. TJ Function (II): Cell Polarity

1. General principles of cell polarity: TJ as cause or effect of polarity?

The term *polarity* refers in general to the asymmetric distribution of molecules within the cell, as it is found in diverse cell types (e.g., neurons) and unicellular organisms (e.g., budding yeasts). Here, we refer to polarity as to the subdivision of the plasma membrane into apical and basolateral domains in epithelial and endothelial cells. The functional relationship between TJ and polarity is supported by a series of observations. First, TJ are precisely localized at the boundary between apical and basolateral domains. Second, TJ are composed of integral membrane proteins, which conceivably function as a "fence" to limit the diffusion of proteins and lipids within the plane of the membrane. Third, in the context of the fluid transport theory (see sect. III E1), TJ play a primary role, not only by preventing back-flow of the adsorbate, but also by maintaining the polarized distribution of the apical Na⁺ channels and the basolateral Na⁺-K⁺-ATPase, thus ensuring vectorial transport of isosmotic fluids. However, the issue is still controversial. In particular, the fence function of TJ is likely restricted to some membrane lipids. In contrast, several proteins can achieve and maintain a polarized distribution even in the absence of TJ.

If functional TJ are present, some lipids (e.g., glycosphingolipids and sphingomyelin) are confined to the apical surface. Hence, a commonly used experimental approach consists of growing cell monolayers on filters, adding a fluorescein- (52) or biotin-labeled (184) lipid to

the apical bathing solution, and following its distribution by confocal microscopy. Combining this assay with the protocol of calcium switch described in section III D2 allows studying the role of TJ in cell polarity. This approach has shown that, within the apical domain, lipid diffusion is only restricted in the outer leaflet of the lipid bilayer. When fluorescent lipid probes are specifically added to the outer leaflet (by means of asymmetric liposomes), they remain localized within the apical domain. In contrast, random insertion of the same lipids in both leaflets (by means of symmetric liposomes) results in their diffusion to the basolateral domain, thus confirming that TJ act as a fence for the diffusion of lipids only within the outer leaflet (328).

Earlier studies had proposed that TJ restrict the diffusion of proteins (260). In addition, other authors reported that basolateral (but, interestingly, not apical) membrane proteins required the presence of TJ, to acquire and maintain asymmetric distribution (332). It should be stressed, however, that one of the major technical problems in these studies is the inability to specifically disassemble TJ, without interfering with TJ-independent mechanisms of protein polarization (see for a review, see Refs. 83, 269). These mechanisms comprise recognition of sorting signals (47), anchorage to glycosylphosphatidylinositol (195), and binding to the cytoskeleton (211, 230). In addition, recent studies have called in question the role of TJ in protein distribution. For instance, TJ disruption following expression of either a deletion mutant of occludin (24) or constitutively active G proteins (160) affects the distribution of membrane lipids and the regulation of paracellular permeability, but does not disturb the polarized distribution of membrane proteins.

Finally, evidence indicates that some TJ components are indeed targeted to the membrane by one of these TJ-independent mechanisms. For instance, addition of the carboxy-terminal cytoplasmic tail of occludin to the Fc receptor results in selective expression of the fusion protein at the basolateral membrane, thus suggesting that occludin contains a basolateral-targeting signal (209). On the basis of these assumptions, it is tempting to speculate that the TJ is an effect (and not a cause) of cell polarity (53).

G. TJ Function (III): In Vivo Studies

1. Genetic deletion of TJ genes in mouse

The function of four TJ molecules, namely occludin, claudin-1, claudin-5, and claudin-11, has been analyzed by genetic deletion in mouse. *Occludin*-deficient mice are born with no gross phenotype. In addition, *occludin*-deficient embryoid bodies differentiate into polarized epithelial cells bearing normal and functional TJ, thus suggest-

ing that occludin is dispensable for TJ formation and function (277). However, further characterization of the occludin-deficient mice has highlighted a much more complex phenotype, which is characterized by postnatal growth retardation. In addition, males are infertile (even when mated with wild-type females), whereas females (albeit fertile) do not suckle the progeny. Interestingly, in spite of normal TJ, pleiotropic abnormalities are found in several tissues, such as hyperplasia of the gastric epithelium, mineral deposition in the brain, and other alterations of testis, salivary glands, and bone (278). This peculiar phenotype suggests that occludin has additional functions that are unrelated to its role at the TJ. Alternatively, signaling events initiated by occludin at the TJ might have more complex consequences than it was previously thought.

Genetic ablation of *claudin-1* induces neonatal death and appearance of wrinkles in the skin. *Claudin-1*-deficient pups die within 24 h after birth, probably because of massive body dehydration. Surprisingly, however, the morphology of TJ and the distribution of other TJ components in keratinocytes is unaffected in mutant mice. Finally, the organization of the epidermis in layers is normal, the only exception being a more compact and thicker stratum corneum (103).

As mentioned above, claudin-5 is an important adhesive component of vascular TJ, which are particularly well organized in brain vessels. Surprisingly, deletion of the *claudin-5* gene in mice is not accompanied by altered morphology of brain vessels during development. However, there is selective impairment of the blood-brain barrier function against molecules smaller than 800 Da, thus providing an interesting example of size-selective loosening of the barrier (237).

Claudin-11 is expressed in oligodendrocytes and Sertoli cells (219). Upon deletion of the *claudin-11* gene, TJ strands disappear from the myelin sheaths of oligodendrocytes and from Sertoli cells. In addition, mice exhibit both neurological deficits (such as slowed nerve conduction and hindlimb weakness) and male sterility. Thus the barrier function exerted by claudin-11-based strands likely plays an essential role in neurological function and in spermatogenesis (116).

2. Genetic diseases in humans associated with mutations of TJ genes

Mutations in the *claudin-16/paracellin* and *claudin-14* genes have been recently associated with inherited disorders in humans. As mentioned in section III E1, it is still unclear whether specific TJ components may mediate selective conductance to individual ions. Positional cloning has allowed associating a hereditary form of hypomagnesiemia, which is due to renal wasting of Mg^{2+} , with mutations in the *claudin-16/paracellin* gene. The

association is further supported by the specific localization of claudin-16 in the thick ascending limb of Henle, where adsorption of Mg^{2+} occurs (293). Finally, like claudin-16, claudin-14 has a restricted distribution, as it is expressed in the TJ of the sensory epithelium in the organ of Corti. The association of mutations in *claudin-14* with a form of nonsyndromic recessive deafness suggests that TJ play an essential function in the inner ear probably because TJ help compartmentalize endolymph (342). Finally, as mentioned above (see sect. III B2B), mutations in the TJ-associated kinase WNK-4 cause secondary hypertension with pseudohypaldosteronism (346).

H. Conclusions

Here, we have summarized general concepts on the structure and function of TJ, with emphasis on the role of this junctional organelle in the regulation of paracellular permeability and cell polarity. In addition, we have provided an overview of available information on the complex molecular architecture of TJ. As it is clear from the above discussion, complexity not only stems from the ever increasing number of TJ components, but also from the complex manner, in which these components interact with each other and with other molecules in the cell. A further, and intellectually demanding, level of complexity consists in understanding how individual TJ components and their molecular interactions may account for the functional role of TJ, as well as for its regulation and assembly. Integrated knowledge of the molecular basis of TJ function and regulation is still in its infancy, and completion of this challenging task will require the concerted deployment of different experimental approaches. In this respect, refinement of physiological measurements of permeability, morphological studies of molecular localization in real-time and at a single-cell resolution, comprehensive analysis of molecular expression and interaction with genomics and proteomics techniques, and evaluation of in vivo models of permeability are warranted to provide an exhaustive picture of the role of TJ in normal conditions and in disease.

We thank Fabrizio Orsenigo and Cristina Corti for their help in preparing the manuscript.

This work was supported by European Community Grants QLK3-CT-2002-02059, QLG1-CT-1999-01036, QLK3-CT-1999-00020, LSHM-2003-503254, LSHG-CT-2003-502935, and 503573; Italian Association for Cancer Research; Telethon-Italy Grant E.1254; Italian Ministry of Health Grants RF00.73, RF01.185, and RF02 Progetti Speciali; Italian Ministry of University and Research Grants MIUR 2001053777_002, MIUR-FIRB RBNE01F8LT_007, MIUR-FIRB RBNE01T8 C8_004, and MIUR-FIRB RBAU01E5F5; National Research Council Progetto Genomica Funzionale; Agenzia Spaziale Italiana; Parent Project Association, and Association International for Cancer Research Grant 04-095.

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