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The tight junction: a multifunctional complex

Eveline E. Schneeberger[1] and Robert D. Lynch[2]

[1]*Molecular Pathology Unit, Massachusetts General Hospital, Charlestown 02129; and* [2]*Department
of Biological Sciences, University of Massachusetts at Lowell, Lowell, Massachusetts 01854*

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10.1152/ajpcell.00558.2003.—Multicellular organisms are separated from the ex-
ternal environment by a layer of epithelial cells whose integrity is maintained by
intercellular junctional complexes composed of tight junctions, adherens junctions,
and desmosomes, whereas gap junctions provide for intercellular communication.
The aim of this review is to present an updated overview of recent developments
in the area of tight junction biology. In a relatively short time, our knowledge of the
tight junction has evolved from a relatively simple view of it being a permeability
barrier in the paracellular space and a fence in the plane of the plasma membrane
to one of it acting as a multicomponent, multifunctional complex that is involved
in regulating numerous and diverse cell functions. A group of integral membrane
proteins—occludin, claudins, and junction adhesion molecules—interact with an
increasingly complex array of tight junction plaque proteins not only to regulate
paracellular solute and water flux but also to integrate such diverse processes as
gene transcription, tumor suppression, cell proliferation, and cell polarity.

tight junction structure; function; regulation

THE INTEGRITY of the epithelial cell layer(s) that protects mul-
ticellular organisms from the external environment is main-
tained by intercellular junctional complexes composed of tight
junctions (TJ), adherens junctions, and desmosomes, whereas
gap junctions provide for intercellular communication. The
transmembrane proteins constituting these junctions are linked
to components of the cytoskeleton, thereby establishing con-
nections to other cell-cell and cell-substratum adhesion sites. In
addition, a growing number of cytoplasmic scaffolding mole-
cules associated with these junctions are involved in regulating
such diverse processes as transcription, cell proliferation, cell
polarity, and the formation of a regulated diffusion barrier.
Because there have been several recent reviews summarizing
data regarding the composition and function of TJs (95, 101,
135, 143), the scope of the current review is focused on
relatively newer observations supporting the concept that the
TJ forms a dynamic, multimolecular, multifunctional complex.

STRUCTURE OF THE TIGHT JUNCTION

As the apical member of the junctional complex (43), the TJ
forms a continuous, circumferential, beltlike structure at the
luminal end of the intercellular space, where it serves as a
gatekeeper of the paracellular pathway (Fig. 1*A*). On the
cytoplasmic side of the TJ, the TJ plaque is the site of a
growing number of TJ-associated protein complexes. Within
the confines of the TJ, the cell membranes of adjacent epithe-
lial cells are brought into intimate focal contact sites in which
the intercellular space is obliterated. Freeze-fracture electron
microscopy reveals these to be a network of strands within the

plane of the plasma membranes of neighboring cells (Fig. 1*B*)
(132). When viewed in lightly fixed epithelia, the TJ strands
appear as rows of 10-nm particles with an ∼18-nm center-to-
center spacing (4). Depending on the level of fixation, the type
of cell in which they are located (127, 128), and their molecular
composition (52), the TJ particles may partition during freeze
fracture predominantly onto either the protoplasmic or exoplas-
mic lipid leaflet.

Findings that the number of parallel strands and the com-
plexity of the TJ network varies greatly among different
epithelia led to the suggestion that there is a direct relationship
between these and the measured transepithelial electrical resis-
tance (TER) (30). However, further analysis of the data led
Claude (29) to conclude that the relationship between the
number of strands and TER is not a linear but a logarithmic
one. Furthermore, she predicted that the strands contain pores
that fluctuate between an open and closed conformation, sug-
gesting that the TJ strands appear to be remarkably dynamic
(Fig. 1*C*) (29), a prediction that recently has been supported by
real-time observations in live cells (Fig. 1*D*) (126). The lack of
correlation between the number of strands and TER was shown
clearly in studies utilizing two strains of Madin-Darby canine
kidney (MDCK) cells that differed in the magnitude of their
TER: MDCK I cells (TER $>1,000$ $\Omega\cdot cm^2$) and MDCK II cells
(TER ~100 $\Omega\cdot cm^2$). Neither strain revealed a discernible
difference in the number and complexity of TJ strands or in
their content of zonula occludens (ZO)-1 (134), indicating that
other factors must govern the barrier properties of the TJ
strands.

For purposes of discussion, the growing number of compo-
nents constituting the multimolecular TJ complex may be
divided into three groups: *1*) integral TJ proteins that bridge the
apical intercellular space and form a regulated permeability
barrier; *2*) TJ plaque proteins, many of which express PDZ

domains (PSd/SAP90, discs large, ZO-1) that serve as links between the integral TJ proteins and the actin cytoskeleton and as adapters for the recruitment of cytosolic molecules implicated in cell signaling; *3*) a miscellaneous group of cytosolic and nuclear proteins, including regulatory proteins, tumor suppressors, and transcriptional and posttranscriptional factors (Table 1) that interact either directly or indirectly with TJ plaque proteins to coordinate such diverse functions as the regulation of paracellular solute permeability, cell proliferation, cell polarity, and tumor suppression.

Fig. 1. *A*: electron micrograph of a tight junction (TJ) between two type I pneumocytes in a newborn lamb lung. The intercellular space is obliterated at discrete sites (arrowheads) in the area of the TJ. An adherens junction (AJ) is present below the TJ. *B*: freeze-fracture replica of a TJ similar to that depicted in *A*. The TJ consists of an anastomosing network of strands that form irregular interstrand compartments. In a few segments, where less platinum is deposited, the strands consist of rows of 10-nm-diameter particles (arrowheads). Scale bar, 120 nm. *C*: model of the TJ network in which claudins in the TJ strands determine the ion selectivity of the TJ and the anastomosing TJ strands provide for electrically separate compartments in the TJ. Assuming that the TJ "pores" open and close randomly, TJ strands organized in this way could account for a logarithmic relationship between the number of strands and transepithelial resistance (TER). TER is an instantaneous measure of ion flux (red arrow), whereas measurement of noncharged solute flux (blue dots) is a much slower process, measured over a period of minutes to hours (elapsed time, horizontal arrow) and may traverse a different junctional pathway, as suggested in *D*. *D*: dynamic behavior of TJ strands is shown in time-lapse images of L-fibroblasts transfected with green fluorescent protein-tagged claudin-1 cDNA. Red coloring was used to mark domains that merge over time. The claudin-1 strands reorganized continuously while retaining the integrity of the network as a whole. Numbers in *right upper corners* denote elapsed time in minutes. [Panel *D* is reprinted from Ref. 126. Copyright 2003 National Academy of Sciences, U.S.A.]

INTEGRAL MEMBRANE PROTEINS OF THE TIGHT JUNCTION

Identification of the integral proteins constituting the TJ strands was particularly challenging, given that all attempts to raise antibodies that would recognize mammalian TJ proteins in this evolutionarily highly conserved structure had failed (5). It was not until membrane preparations from an avian source (chicken liver) were used as immunogens that the Tsukita group, in a series of groundbreaking studies, first identified occludin (50) and, subsequently, the family of claudins (47). During this period the junction adhesion molecule (JAM), a member of the immunoglobulin superfamily, was also found to be expressed at the TJs of both endothelial and epithelial cells (93).

Occludin. Occludin is an ~60-kDa tetraspan membrane protein that forms two extracellular loops separated by a short cytosolic loop, and both amino- and carboxy-terminal domains are cytosolic (Fig. 2). The carboxy-terminal domain is rich in serine, threonine, and tyrosine residues, which are targets for a number of protein and tyrosine kinases. The two extracellular loops have an unusual amino acid composition, consisting in the first loop of a high content (~61%) of tyrosine and glycine residues, whereas the second loop is rich (~18%) in tyrosine residues. Very few charged amino acids are present in either loop, and at neutral pH they are predicted to have no net charge (50). Alignment of the amino acid sequence of occludin from five mammalian species indicates a ~90% identity between human, dog, and mouse but a ~50% divergence from that of rat-kangaroo and chicken occludin (5). An alternatively spliced isoform of occludin (occludin 1B) has a tissue distribution that appears to be identical to that of occludin (106).

In epithelial cells with high TER, Western blot analysis of occludin reveals multiple higher molecular weight bands ranging from 62 to 82 kDa in size; of these, only the low-molecular-weight occludin is extractable with NP-40 (125). A combination of phosphatase digestion and phosphoamino acid analysis indicates that occludin is serine/threonine phosphorylated (125). Phosphorylated occludin appears to be preferentially expressed in TJs, whereas nonphosphorylated occludin is detected by immunofluorescent staining in a punctate pattern
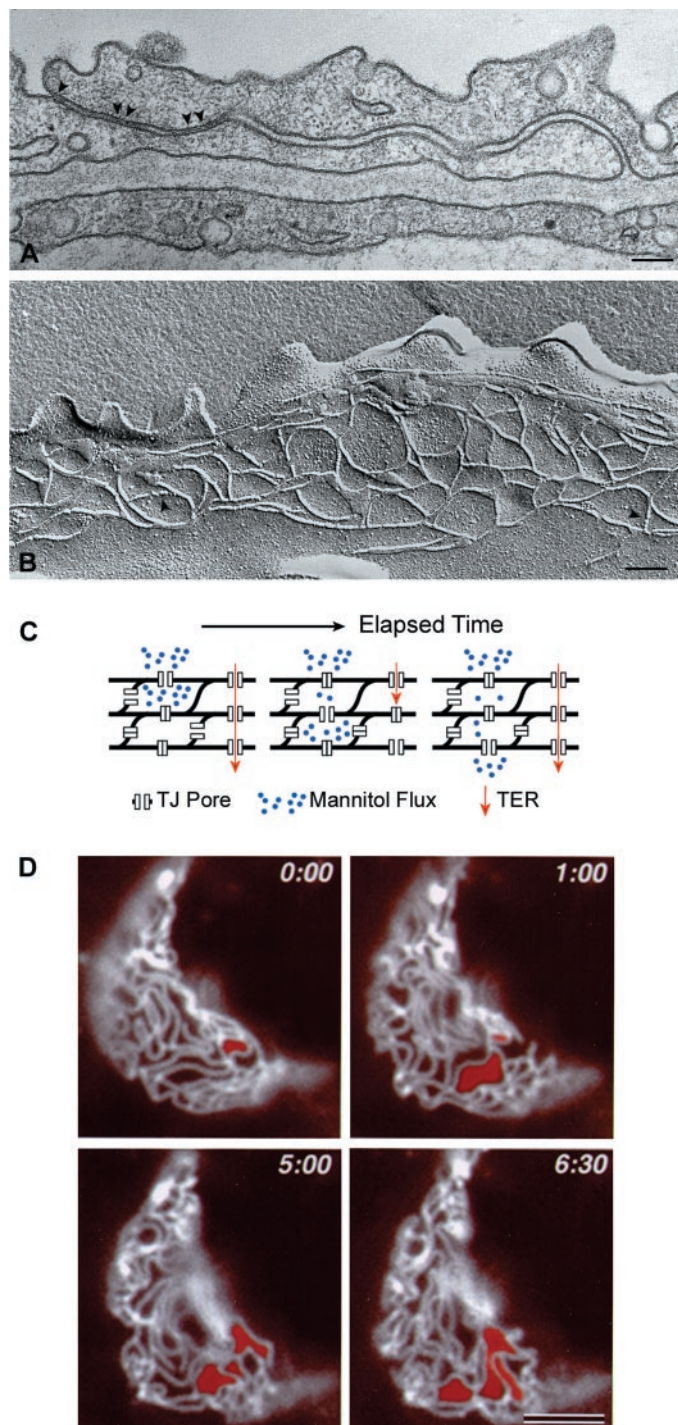
Table 1. *Non-PDZ-expressing proteins recruited to TJ plaque*

Protein	Interacting Partners	Function	Ref.
Cingulin	JAM-1, ZO-1, ZO-2, ZO-3	Cross-linking TJ proteins to the actin cytoskeleton, bundling of F-actin filaments	18, 36
Symplekin	Colocalizes with ZO-1 at TJ; also present in nucleus	Processing of pre-mRNA and its polyadenylation	69, 83
ZONAB	ZO-1, CDK4	Y-box transcription factor; by binding CDK4, ZONAB may regulate cell proliferation, may also regulate ErbB2 expression	11, 14
HuASH1		Transcription factor	108
GEF-H1		TJ-associated guanine nucleotide exchange factor; regulation of paracellular permeability but not TER	20
aPKC	PAR-3, PAR-6	Establishment of cell polarity	40
PP2A		TJ-associated protein phosphatase that regulates phosphorylation of aPKC, ZO-1, occludin, and claudin-1	110
Heterotrimeric G proteins	ZO-1, ZO-2	Signaling molecules that participate in the regulation of TJ barrier function	100
Rab3b, Rab13		Recruited to junctional complexes following cell-cell contact, may be involved in vesicle targeting to cell-cell adhesion sites	94, 152, 166
Sec6/Sec8		Vesicle targeting to sites of cell-cell adhesion	58
PTEN	MAGI-2, MAGI-3	Tumor suppressor; dephosphorylates phosphatidyl phosphates	160, 161
7H6	Not known	Not known	167

TJ, tight junction. See text for definitions of protein abbreviations.

along the basolateral membrane (125). Stimuli that induce occludin phosphorylation, the kinases involved, and the effect of occludin phosphorylation on TJ barrier function have been partially defined. Evidence that protein kinase C (PKC) is involved is supported by observations that addition of the PKC agonist 1,2-dioctanoylglycerol, together with phorbol 12-myristate 13-acetate, to epithelial cells incubated in low-calcium medium results in partial assembly of TJs and occludin phosphorylation. Moreover, this cellular response is inhibited by PKC-specific inhibitors (6). These results suggest that a classical (cPKC) or a novel PKC (nPKC) is involved, because both of these classes of PKC are activated by diacylglycerol and phorbol esters (99). However, phorbol ester-insensitive atypical PKC (aPKC) also participates in occludin and ZO-1 phosphorylation during de novo TJ formation (110) (see below). After recovery from ATP depletion (142) or during calcium repletion (26), TJ assembly and the concomitant rise in TER have been linked to tyrosine phosphorylation of occludin.
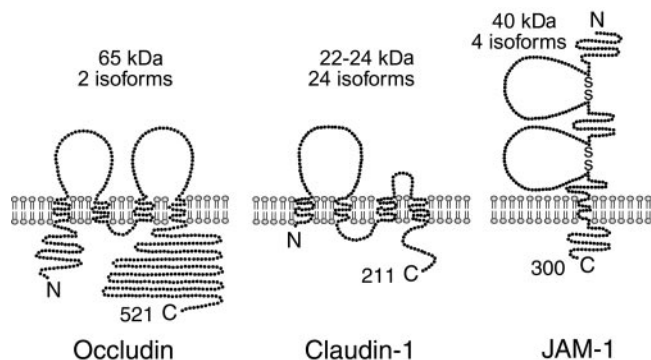

Fig. 2. Integral membrane TJ proteins. Both occludin and claudin-1 are tetraspan proteins that share no sequence homology. The first extracellular loop of occludin is rich in tyrosine and glycine residues, and neither loop has a significant number of charged amino acid residues. By contrast, the amino acid composition of the 2 extracellular loops of claudin varies significantly among different claudins, resulting in a wide range of isoelectric points. Junction adhesion molecule-1 (JAM-1) spans the plasma membrane once and has 2 extracellular Ig type domains, of which the first NH₂-terminal loop interacts with its homologous partner on the adjacent cell. The number of amino acids in each molecule is indicated.

Furthermore, the nonreceptor tyrosine kinase c-Yes coimmunoprecipitates with occludin and has been implicated in regulating TJ barrier function (26). Further studies are required to define the temporal sequence and conditions under which the various kinases and phosphatases are active in regulating TJ protein phosphorylation.

Occludin clearly localizes to TJs (67, 121), and its overexpression increases TER in mammalian epithelial cells (15, 97). However, transfection of insect cells with occludin cDNA produced only focal adhesion sites in cytoplasmic multilamellar structures, but a typical TJ network was not observed (46). Moreover, disruption of both occludin alleles by homologous recombination resulted in embryonic stem cells that not only differentiated into polarized epithelial cells but also formed an effective barrier to the diffusion of a low-molecular-weight tracer. Freeze-fracture replicas of these cells displayed well-developed TJ networks (122), indicating that occludin is not required for the formation of TJ strands. The lack of occludin is not without consequence, however. Occludin null mice express a complex phenotype, but one without obvious structural or functional TJ abnormalities (123) (Table 2). These observations prompted the Tsukita group to reexamine the junctional fraction prepared from the chicken liver, which led to the discovery of two 22-kDa proteins: claudin-1 and claudin-2 (47). The function(s) of occludin, however, remains to be defined.

Claudins. Database searches and genomic cloning have identified up to 24 members of the claudin family (103, 143), and their genes are predicted to encode proteins of 20–27 kDa, none of which show any sequence homology to occludin (47). Claudins, like occludin, are tetraspan proteins with relatively short cytoplasmic amino and carboxy termini flanking a first extracellular loop of ~53 amino acids and a second shorter loop of ~24 amino acids in length (Fig. 2). In contrast to occludin, the isoelectric point (pK_i) of the two extracellular loops ranges in the first loop from a pK_i of 4.17 of claudin-16 to 10.49 of claudin-14 and in the second loop from a pK_i of 4.05 of claudin-2, -7, -10, and -14 to 10.5 of claudin-13 (103). The COOH terminus of all claudins, except claudin-12, ends in YV, a site that binds to the PDZ domains of proteins including

Table 2. *Phenotypic alterations in TJ protein-deficient mice and transgenic mice*

Mouse	TJ Protein	Phenotype	Ref.
Null	Occludin	Growth retardation, male sterility, failure to suckle pups, chronic inflammation, and hyperplasia of gastric epithelium, brain calcification, thinning of compact bone	123
Null	Claudin-1	Death at birth due to cutaneous dehydration and absence of claudin-1 in TJs of stratum granulosum; occludin and claudin-4 remaining in TJs failed to form a barrier to a 600-kDa tracer	49
Null	Claudin-5	Selective increase in permeability of the blood-brain barrier to <800-kDa tracers	109
Null	Claudin-11	Male sterility, slowed central nervous system conductance, hind limb weakness; loss of TJ strands in myelin sheaths and between Sertoli cells	57
Transgenic	Claudin-6	Death within 2 days of birth; aberrant expression of late epidermal differentiation markers and modified epidermal claudin profile	144

ZO-1, -2, and -3 (73). When the claudin-1 YV binding site was blocked by addition of an epitope tag and the epitope tagged construct was overexpressed in epithelial cells, aberrant tight junction strands formed along the lateral membrane, without associated ZO-1. Aberrant TJ strands did not form when nontagged claudin-1 was overexpressed (96). Whereas these results suggest that interaction with ZO-1 is required for the proper incorporation of claudin-1 into the TJ, other mechanisms may also be involved. The number and diversity of claudins and the wide range of pK_i values of the claudin extracellular domains are to be expected for proteins that determine the range of ion selectivity in different epithelia. Precisely how the claudins and occludin are organized in the TJ strands and in the 10-nm particles observed in freeze-fracture replicas is unclear, and how they interact is only partially understood, as indicated below.

Mouse L-fibroblasts transfected with either claudin-1 or claudin-2 cDNA formed intercellular homophilic, Ca^{2+}-independent adhesion sites (86) along their cell borders that appeared as patches of TJ strands in freeze-fracture replicas (51). By contrast, L-fibroblasts transfected with occludin cDNA expressed punctate, homophilic adhesion sites that formed small, discrete arrays of TJ strands (51) and displayed negligible cell-cell adhesion activity (86). However, when occludin and claudin-1 were cotransfected, occludin was readily incorporated into the TJ strands formed by claudin-1 (51). Interactions between different claudins within TJ strands was further examined in L-fibroblasts cotransfected with different paired combinations of claudin-1, -2, or -3. All paired combinations were detected in fracture-labeled TJ strands, indicating that lateral associations among these three claudins are readily formed. However, when L-transfectants expressing one of the three claudins were cocultured with those expressing a different claudin, paired association was noted between claudin-1 and -3 and claudin-2 and -3, but not between claudin-1 and -2, indicating that there appear to be steric constraints preventing the interaction of the extracellular domains of certain claudins on adjacent cells (52).

A number of oligomerized membrane proteins, including connexins, occludin, claudins, synaptophysin, and tetraspanins, share a tetraspan topology but have very different functions. For example, the gap junction serves as a regulated conduit between the cytoplasm of neighboring cells, whereas the TJ forms a regulated barrier in the paracellular space. The connexon subunit of the gap junction forms a 10-nm intramembrane particle composed of six connexin molecules. To determine whether claudin hexamers could account for the 10-nm particles observed in the TJ strands,

claudin-4 was expressed in Sf9 insect cells, a strategy that provided an experimental model free of other confounding TJ proteins (102). Of a large number of detergents tested, only perfluoro-octanoic acid partially maintained claudin-4 oligomeric structure during sucrose velocity centrifugation. SDS-PAGE analysis of the collected fractions detected monomers and polymers ranging in size from dimers to hexamers. Because the lipid composition of Sf9 insect cell membranes allowed tetraspan connexin to oligomerize (133) but only perfluoro-octanoic acid partially preserved claudin-4 hexamers, it is suggested that claudin oligomers by themselves form quite unstable configurations. This prediction is supported, in part, by recent observations indicating that green fluorescent protein-tagged claudin-1, expressed in L-fibroblasts and examined in real time, formed paired claudin strands that displayed a remarkably dynamic behavior (Fig. 1D) (126). The TJ strands continually broke and annealed in an end-to-end and side-to-side manner. However, fluorescence recovery after photobleaching data indicated that individual claudin molecules were not themselves mobile (126). These remarkable observations await confirmation in native TJs.

JAM-1. JAM-1, a 43-kDa glycosylated protein that belongs to the IgG superfamily, is characterized by two extracellular V-type Ig domains, a single transmembrane domain, and a short intracellular COOH terminus that has a PDZ binding motif (Fig. 2) (93) that binds to the PDZ3 domain of ZO-1 (77) and the PDZ9 domain of multi-PDZ domain protein 1 (MUPP-1) (61) (Fig. 3). JAM-1 forms homophilic contacts between the first V-type Ig domain of apposing JAM-1 molecules (85). It is expressed at both endothelial and epithelial TJs where it facilitates the migration of monocytes through the paracellular pathway. Three additional family members, JAM-2 (8), JAM-3 (113), and JAM-4 (65), have been identified. JAM-2 is expressed on endothelial cells, whereas JAM-3 on T cells functions as a counter receptor (7), thereby facilitating lymphocyte migration between endothelial cells (37). JAM-4 is localized to the renal glomerulus and intestinal epithelial cells. In vitro binding studies indicate that JAM-4 binds to PDZ1 and PDZ4 of membrane-associated guanyl kinase inverted (MAGI-1) proteins (Fig. 3), and transfection of L-fibroblasts with JAM-4 confers Ca^{2+}-independent adhesion (65).

When exogenous JAM-1 is expressed in fibroblasts, Ca^{2+}-independent cell-cell adhesion sites form. These appear in freeze-fracture, immunolabeled preparations as immunogold-labeled patches of membranes that are devoid of intramembrane particles (77), suggesting that JAM-1 forms laterally associated planar aggregates. Freeze-fracture immunolabeling of epithe-
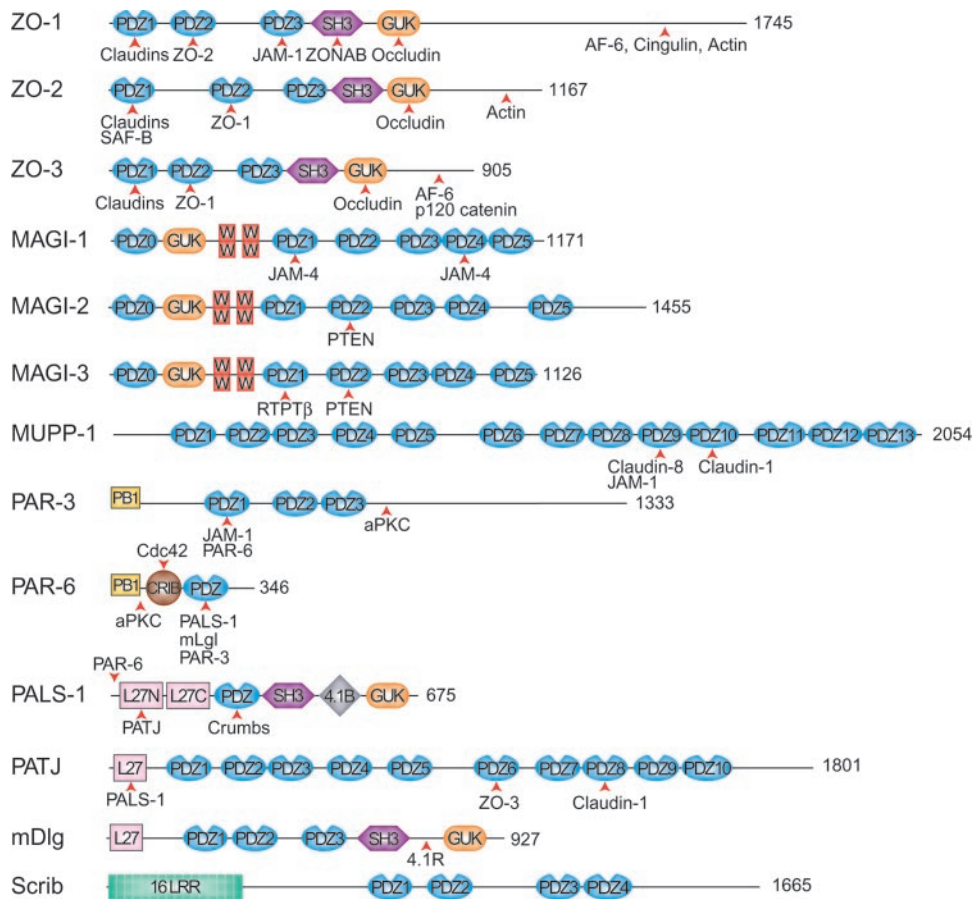
Fig. 3. Diagrammatic representation of cytosolic, TJ-associated, PDZ-expressing proteins. Their binding partners, where known, are indicated. The number of amino acids is indicated at *right*. ZO, zonula occludens; ZONAB, ZO-1-associated nucleic acid binding protein; GUK, guanylate kinase; SH3, Src homology region 3; AF-6, ALL-1 fusion partner from chromosome 6; SAF-B, scaffold attachment factor-B; MAGI, membrane-associated guanyl kinase inverted proteins; PTEN, phosphatase and tensin homologue; RTPTβ, receptor protein tyrosine phosphatase-β; MUPP-1, multi-PDZ domain protein 1; PAR, partitioning-defective protein; PB1, Phox and *Bam*1 domain; aPKC, atypical protein kinase C; Cdc42, cell division cycle 42; CRIB, CDC42/Rac interactive binding domain; PALS, protein associated with Lin-7; mLgl; mammalian lethal giant larvae; PATJ, PALS-1-associated tight junction; L27, Lin-2, -7 domain; mDlg, mammalian discs large; Scrib, Scribble (Vartul); LRR, leucine-rich repeat.

lial cells indicates that at steady state, native JAM-1 is localized at, but is not an integral member of, the TJ strands (77). Although in vitro binding studies have revealed that ZO-1 acts as a link between JAM-1 and claudins via its PDZ3 and PDZ1 domains, respectively, recent binding studies indicate that MUPP-1 also links JAM-1 to claudin-1 via its respective PDZ9 and PDZ10 domains (61) (Fig. 3). Interestingly, a regulator of cell polarity, partitioning defective protein (PAR-3), binds by one of its PDZ domains to the COOH terminus of JAM-1 (38, 77). Thus it appears that JAM-1 is tethered to claudins via ZO-1 and that JAM-1 in turn recruits PAR-3, with its associated aPKC and PAR-6 complex, to the TJ. However, as discussed below, the recruitment of the PAR-3/aPKC/PAR-6 complex to the TJ is a sequential multistep process (105).

TJ assembly is initiated by homotypic interactions between E-cadherin and nectin on the surface of adjacent cells. Like JAM-1, nectin is a Ca^{2+}-independent adhesion molecule that is a member of the Ig superfamily. The cytosolic domains of nectin and E-cadherin do not associate with each other directly. Instead, ALL-1 fused gene on chromosome-6 (AF-6, afadin), which binds to the carboxy terminus of nectin, is linked to β-catenin, which in turn binds to the carboxy terminus of E-cadherin. During this early phase of TJ assembly, JAM-1 is recruited to nectin adhesion sites through the interaction of ZO-1 and AF-6, their respective cytoplasmic tail-binding proteins (45), suggesting that nectin is involved in the recruitment of JAM-1 to sites of TJ assembly. Once TJs are fully assembled, JAM-1 and nectin segregate to their respective junctional complexes. Other cytoplasmic proteins bound to JAM-1 include calcium/calmodulin-dependent serine protein kinase (CASK) (92) and cingulin (18), which may serve a signaling and tethering function, respectively.

Coxsackie virus and adenovirus receptor. Coxsackie virus and adenovirus receptor (CAR), like JAM-1 and nectin, is another member of the Ig superfamily (21) that forms homotypic adhesion sites close to the TJ. Although its precise relationship to the TJ is somewhat controversial (31, 147), CAR coimmunoprecipitates with ZO-1. CAR recruits ZO-1 to the cell membrane and, when overexpressed in epithelial cells, augments TER (31). It is noteworthy that CAR and a number of other TJ proteins are targets of viral, bacterial, and parasitic pathogens.

THE TIGHT JUNCTION AS A REGULATED PERMEABILITY BARRIER

The ability of epithelia to create a diffusion barrier between cellular compartments of very different fluid and solute composition is controlled by essentially two pathways: *1*) the transcellular pathway, which is governed by energy-dependent transporters and channels that are asymmetrically distributed on the apical and basolateral cell membranes; and *2*) the paracellular pathway, in which integral TJ proteins span the apical intercellular space and regulate the passive diffusion of ions and small noncharged solutes via the paracellular space (116). Although the mechanisms involved in transcellular transport are well studied, it is only relatively recently that those controlling the paracellular route have begun to be

elucidated. In addition to serving as a regulated gate/barrier in the paracellular pathway, the TJ also functions as a fence in the plane of the plasma membrane, where it contributes to the maintenance of asymmetrically distributed integral membrane proteins and lipids.

Claudins participate in the formation of ion selective pores. Recent studies in which cell membrane-impermeant polyethylene glycol oligomers were used indicated that restrictive pores, 0.43–0.45 nm in radius, are present within TJs of intestinal cell monolayers (151). Whereas these data suggest that the TJ barrier is permeable to small, noncharged solutes, pores in the TJ are capable of discriminating between ions of similar charge, and their permeability is dependent on both ion concentration and ambient pH (139, 165). With few exceptions, TJs in most epithelia are cation selective (116). However, both the TER and solute permeability of TJs vary widely among different tissues and even among cells in the same tissue (39, 84). The existence of only two isoforms of occludin and the small number of charged residues in their two extracellular domains suggest that occludin is unlikely to contribute directly to the formation of ion-selective pores in the TJ. By contrast, considering the large number of different claudins whose extracellular loops have a wide range of pK_i, it is reasonable to postulate that the charged amino acid side chains in the extracellular domains of one or more claudins could form ion-selective pores in a given TJ.

In an insightful study, Furuse et al. (48) showed that MDCK I cells (TER $>10,000 \, \Omega \cdot cm^2$) express claudin-1 and -4 but not claudin-2, whereas MDCK II cells (TER $<200 \, \Omega \cdot cm^2$) express claudin-1, -2, and -4. When MDCK I cells were transfected with claudin-2 cDNA, there was a 20-fold decline in TER. Expression of the introduced claudin-2, however, did not change the number of TJ strands, the cell density, or the TJ permeability to a relatively large, 4-kDa, noncharged solute. Interestingly, the 10-nm particles, observed in the freeze-fractured TJs, preferentially partitioned onto the E-face in both control MDCK II cells and the claudin-2-transfected MDCK I cells but not in MDCK I cells transfected with the empty vector. This finding suggests that the interaction of claudin-2, with either TJ plaque proteins or other integral TJ proteins, differs from that of other claudins.

A variety of strategies have been applied to further examine the molecular basis for the ion selectivity of selected claudins. The induced overexpression of claudin-4 in MDCK II cells increased TJ strand complexity and decreased paracellular conductance, a change that was attributed specifically to a decrease in Na^+ but not Cl^- permeability. The expression levels of claudin-1, -2, and -3, occludin, and ZO-1 remained unchanged in these cells (146). Site-specific mutagenesis was used to more specifically examine the role of charged amino acid residues in the first extracellular domain of claudins. When the positive charge on position 65 of claudin-4 was replaced with a negative charge and the construct expressed in MDCK II cells, Na^+ permeability increased. Conversely, when three negative charges on the first loop of claudin-15 were replaced either singly or in combination by positive charges, ion selectivity changed from a preference for Na^+ to one for Cl^- (32). These informative observations must be viewed with some caution because site-directed mutagenesis may lead not only to a change in net charge but also to an altered molecular conformation. Furthermore, overexpression of an exogenous TJ protein may alter the organization of native proteins within TJ strands.

The first of these concerns was partially overcome by constructing chimeras of claudin-2 and -4 (33). Expression in MDCK II cells of claudin-2 chimeras, in which one or both extracellular domains were replaced with those of claudin-4, increased both the number of TJ strands and TER and decreased the permeability of Na^+ relative to Cl^-. By contrast, expression of claudin-4 chimeras, in which one or both extracellular domains of claudin-2 replaced those of claudin-4, increased the number of TJ strands, but alterations in TER and charge selectivity were only modest. These observations support a model in which the extracellular domains of the claudins are sufficient to form ion-selective pores in the paracellular pathway (33). However, the electrophysiological effect of expressing extracellular domains of claudin-4 on claudin-2 was greater than that of overexpressed native claudin-4, suggesting the interesting possibility that other domains, including the COOH terminus, may play a role in modulating ion selectivity.

The perturbing effect of overexpressing modified TJ proteins (32, 33, 96, 146) was minimized by using retroviral infection (165), a strategy that leads to highly efficient insertion of a relatively low copy number of the transgene per cell. With the use of this approach, MDCK II cells that lack endogenous claudin-8 were transfected with claudin-8 cDNA epitope tagged at its NH_2 terminus. This led to the selective downregulation of endogenous claudin-2 expression without affecting that of other endogenous claudins. Further studies are required to determine whether altered rates of synthesis and/or degradation of these two claudins might account for these observations. It is noteworthy that expression of claudin-8 reduced TJ permeability to mono- and divalent cations but had no effect on anion or neutral solute permeability, suggesting that claudin-8 acts primarily as a cation barrier (165). It also confirms indirectly the observations that claudin-2 forms cation-selective channels that are capable of changing a "tight" TJ to a leaky one (2, 48).

To avoid transfection entirely and to establish whether ion channels mediate the ion conducting properties of TJs, permeability studies were conducted on three cell lines that differed in the magnitude of their measured TER. The cells examined included MDCK II cells ($<100 \, \Omega \cdot cm^2$), T84 cells ($\sim1,000 \, \Omega \cdot cm^2$), and MDCK I cells ($>5,000 \, \Omega \cdot cm^2$) (139). The studies were conducted on confluent monolayers that were at steady state, under conditions that minimized perturbations associated with changes of medium, pH, and temperature. Under these controlled conditions, TJs were found to share the biophysical properties of ion channels, including size and charge selectivity, dependence on ion concentration and pH, as well as the presence of ionic competition (139). These observations in the aggregate indicate that specific claudins appear to determine the ion selectivity of pores in TJs.

Role of occludin in the TJ. The role of occludin in TJs and its contribution, if any, to the function and/or regulation of the ion pores in TJs is unclear. Overexpression of occludin in MDCK cells increased TER (reduced ion permeability) but also, paradoxically, increased the transepithelial flux of mannitol (15, 97). Similar unexpected functional changes were observed after activation of RhoA GTPase in MDCK cells expressing a G protein-coupled prostaglandin receptor (63), whereas overexpression of either Rac1 or RhoA GTPase re-

sulted in disruption of TJ structure and function (82). It is noteworthy that overexpression of a recently identified TJ-associated Rho GTPase-specific guanine nucleotide exchange factor (GEF/H1) increased the paracellular permeability to hydrophilic solutes without affecting TER (ion permeability) (20). The effect of GEF/H1 activity on the state of occludin phosphorylation and function in the TJ, however, was not examined.

The possibility that p160^{ROCK}, one of the key effectors of RhoA, might regulate occludin phosphorylation and TJ permeability was examined in endothelial cells. Occludin phosphorylation was detected under both p160^{ROCK}-dependent (application of lysophosphatidic acid) and -independent (histamine treatment) conditions, both of which were associated with stress fiber formation, myosin light chain phosphorylation, and increased transmonolayer flux of a relatively large protein tracer. However, whereas inhibition of this RhoA-activated kinase blocked the cytoskeletal changes in both treatment groups and prevented lysophosphatidic acid-induced myosin light chain and occludin phosphorylation as well as the increased tracer flux, it had no effect on either the histamine-induced occludin phosphorylation or the histamine-induced augmented tracer flux. These observations suggest that occludin may be a target for receptor-initiated signaling and that occludin phosphorylation may regulate TJ permeability independently of cytoskeletal activity (66).

Expression of occludin in L-fibroblasts that lack cadherin-based adhesion sites and ZO-1 conferred little adhesiveness (86, 145). However, when occludin was expressed in normal rat kidney cells and Rat-1 fibroblasts, which have well-developed ZO-1-containing adherens junctions, increased Ca^{2+}-independent adhesion was observed, indicating that when interacting with ZO-1, occludin has an adhesive function (145). Further evidence for an adhesive function of occludin was observed in experiments in which the addition of peptides, whose amino acid sequence corresponded to that of the second (159) extracellular loop of occludin, was associated with disruption of the TJ barrier. Occludin also appears to act as a fence in the plane of the membrane. Expression in MDCK cells of truncated occludin lacking its cytosolic COOH terminus resulted in the diffusion of apically applied BODIPY-labeled sphingomyelin along the lateral membrane, whereas the labeled lipid remained on the apical surface of cells expressing intact occludin (15). Because the truncated occludin was overexpressed, the possibility that the organization of endogenous TJ components was perturbed cannot be excluded.

The amino terminus of occludin is the target of the E3 ubiquitin-protein ligase Itch, which causes the ubiquitination of occludin (140). Furthermore, inhibitors of proteosome activity reduce occludin degradation and prolong its half-life. By contrast, the turnover of claudin-1 is unaffected by inhibition of proteosome activity, suggesting that the turnover of these two integral TJ proteins is regulated by different mechanisms. Within the 150-amino acid ZO-1 binding domain of the carboxy terminus of occludin, there is a 27-amino acid coiled coil domain that binds ZO-1, aPKC-ζ, the nonreceptor tyrosine kinase c-Yes, and the regulatory subunit of phosphatidylinositol 3-kinase, as determined by in vitro binding assays (111). Such interactions require confirmation by in vivo coimmunoprecipitation experiments. Further clues about the function of occludin were

obtained from studies in which expression of the Ras effector Raf-1 (89) or transformation of epithelial cells by Ras (27) was associated with both the loss of TJs and cell contact growth control. Introduction and expression of the occludin gene in Raf-1-activated cells (89) or treatment of the Ras-transformed cells with the MEK1 (MAPK and ERK kinase 1) inhibitor PD-98059 (27) reestablished both functional TJs and phenotypic differentiation. Together, these observations suggest that occludin may act to coordinate the activity of the cytoskeleton with a variety of signaling pathways, essential for the maintenance of the epithelial cell phenotype.

TRANSCRIPTIONAL REGULATION OF INTEGRAL TJ PROTEIN GENE EXPRESSION AND PHENOTYPIC CONSEQUENCES OF CLAUDIN GENE DELETION OR MUTATION

Transcriptional regulation of the occludin and claudin promoters. Insight into the regulation of occludin and claudin promoter activity is beginning to emerge. As alluded to above, expression of occludin may be regulated at the transcriptional level by a number of different factors, including those in the Ras pathway (27, 89, 91) as well as by the inflammatory cytokines, tumor necrosis factor-α, and interferon-γ (91). In addition, the transcription factors β-catenin/Tcf complex (104) and Cdx homeodomain protein/hepatocyte nuclear factor-1α (124) have been shown to bind directly to the claudin-1 and claudin-2 promoters. These factors appear, therefore, to provide for regulation of TJ activity under a wide variety of physiological and pathological conditions.

A different set of conditions is required, however, during embryological development and neoplastic transformation. In the embryo when mesoderm and the neural crest develop, epithelial cells undergo an epithelial to mesenchymal transition (EMT). Similarly, in neoplastic transformation of epithelial cells, there is decreased intercellular adhesion, increased cell motility, and a loss in cell polarity. This requires that the expression of TJ and adherens junction proteins be downregulated. The zinc finger transcription repressor Snail has recently been implicated in EMT. Snail binds to the E-box motif, 5′-CA(G/C)(G/C)TG, which is found in the E-cadherin promoter (17, 25), with the result that E-cadherin expression is suppressed and adherens junctions are disrupted. Interestingly, the occludin and claudin-3, -4, and -7 promoters similarly contain one, six, eight, and eight E-boxes, respectively. When Snail was overexpressed in epithelial cells, there was complete repression of occludin, claudin-3, -4, and -7, and E-cadherin expression at both mRNA and protein levels, and this was accompanied by the disruption of both TJs and adherens junctions with subsequent induction of EMT (72). E12/47 and SIP1 are examples of other repressors causing a decrease in E-cadherin expression by binding to its promoter E-boxes, suggesting that upregulation of non-Snail repressors may also alter TJ protein expression in epithelial tumors (24, 34, 114). Although our understanding of the transcriptional regulation of TJ proteins during embryogenesis and carcinogenesis is still rudimentary, the mechanisms involved are an area of current active investigation.

Phenotypic alterations in occludin- and claudin-deficient and transgenic mice. To define the role of occludin and individual claudins in the TJs of different tissues, gene deletion (49, 57, 109, 121, 144) and transgene strategies (144) have been applied. As shown in Table 2, occludin-deficient mice display a complex phenotype, indicating that this member of the TJ has a more complex and yet to be clearly defined function (123). Similarly, mice deficient in claudin-1 (49), claudin-5 (109), or claudin-11 (57) revealed novel roles in the TJs of specific tissues, whereas overexpression of claudin-6 resulted in aberrant expression of both epidermal differentiation markers and of other claudins.

Human claudin gene mutations. Elegant genetic analyses of families with consanguineous marriages have uncovered the specific function of two additional claudins. Positional cloning on a cohort of individuals suffering from an autosomal recessive, renal Mg^{2+} wasting disease identified mutations in a human gene *PCLN-1* (paracellin-1). The paracellin-1 protein (claudin-16) is expressed exclusively in the epithelial cells of the thick ascending limb of Henle of the kidney (130). Although a wide variety of mutations and premature terminations were detected in the *PCLN-1* gene of these individuals, all suffered from Mg^{2+} wasting, indicating impaired tubular reabsorption of this divalent cation via ion pores in the claudin-16-expressing renal epithelial TJs.

Similarly, the analysis of two large families with multiple consanguineous marriages and a high incidence of inherited deafness revealed two different claudin-14 mutations that co-segregated with recessive deafness. This led to the recognition that claudin-14 in the sensory epithelium of the cochlea in the inner ear is critical for maintaining the ionic composition of the endolymph (153).

TJ PLAQUE PROTEINS AND THE COORDINATION OF SIGNALING AT THE TJ

In addition to their prime function as a regulated permeability barrier in the paracellular pathway and a fence in the plane of the membrane, TJs play a pivotal role in organizing such diverse processes as morphogenesis, cell polarity, cell proliferation, and differentiation, which requires the coordination of signals impinging on and emanating from the plasma membrane. Mounting evidence suggests that the cytosolic TJ plaque is one of the sites in which such signaling is coordinated (13, 95).

Among the growing number of protein complexes identified in the TJ plaque is an array of adapter proteins that have in common the expression of one or more PDZ domains. PDZ domains are a sequence of 80–90 amino acids that fold to form a hydrophobic groove to which the COOH-terminal T/SXV residues of the target protein bind (41). However, PDZ domains may also heterodimerize with other PDZ domains. The PDZ-expressing proteins identified in or near the TJ plaque are depicted in Fig. 3 and include *1*) the zonula occludens proteins ZO-1, ZO-2, and ZO-3, which belong to a family of membrane-associated guanylate kinase (MAGUK) homologues; *2*) the membrane-associated guanylyl kinase inverted proteins MAGI-1, MAGI-2, and MAGI-3; *3*) the multi-PDZ domain protein 1 MUPP-1; *4*) the partitioning defective proteins PAR-3 and PAR-6, members of the PAR3/aPKC/PAR6 polarity complex; *5*) the MAGUK protein associated with Lin-7 (PALS1) and the PALS1-associated tight junction (PATJ), a multi-PDZ domain protein, both of which are members of the Crumbs/PALS1/PATJ polarity complex (119); *6*) mammalian lethal giant larvae (mLgl) and Scribble (Scrib), members of the Scrib-(Vartul)/mammalian discs large/mLgl (Scrib/mDgl/mLgl) polarity complex (Fig. 4); and *7*) a miscellaneous array of proteins lacking PDZ domains that are also localized to the TJ plaque (see Table 1). It is noteworthy that a number of these TJ plaque proteins, including ZO-1 (56), ZO-2 (141), ZONAB (11), symplekin (69, 138), and a human homologue of the *Drosophila ash1* gene (108), are detected, under certain conditions, both at the TJ and in the nucleus, suggesting that they may transduce signals from the plasma membrane to the nucleus, where they appear to regulate gene expression.

ZO proteins. The MAGUK family of proteins plays an important role in signal transduction by clustering critical membrane proteins including those of synapses, ion channels. and TJs (41). The three TJ-associated ZO proteins (ZO-1, -2, and -3) contain unique motifs not shared by other MAGUK family members, including nuclear localization and export signals as well as a leucine zipper-like sequence (141). ZO-1, a 225-kDa MAGUK protein, was the first TJ-associated protein to be identified (136) and was subsequently found to be localized also in adherens junctions of cells that lack TJs (70, 76). There are two ZO-1 isoforms; one is ZO-1α^+ and the other is ZO-1α^-, which lacks an internal 80-amino acid residue domain contained in ZO-1α^+ (155). Whereas both isoforms are expressed in most epithelial cells, the exclusive expression of ZO-1α^- is associated with TJs that are more labile, including those of endothelial and Sertoli cells (9), as well as with the specialized junction of glomerular podocytes (87). The NH_2-terminal half of ZO-1 consists of three PDZ domains: one Src oncogene homology region 3 (SH3) domain, and one catalytically inactive guanylate kinase (GUK) homologue (Fig. 3). The COOH-terminal half of ZO-1 has an acidic region, a differentially spliced α-domain, and a terminal proline-rich region (154).

ZO-1 has a number of binding partners. Its PDZ1 domain binds to claudins (73), whereas its PDZ2 domain interacts with both ZO-2 and ZO-3 as well as with the gap junction protein connexin43 (53). The fact that ZO-1 coimmunoprecipitates with ZO-2 or ZO-3 indicates that these proteins form independent complexes in vivo (59, 158). ZO-1-associated kinase (ZAK), a serine protein kinase (10), and ZO-1 nucleic acid binding protein (ZONAB), a Y-box transcription factor (11, 14), are localized to the TJ plaque by binding to the SH3 domain of ZO-1, whereas the GUK domain of ZO-1 is the binding site for the COOH terminus of occludin (42). ZO-1 serves as a link between occludin and the actin cytoskeleton via its proline-rich COOH terminus (42), although occludin also binds actin directly (158). ZO-1 interacts with a number of other TJ-associated proteins, including the Ras target AF-6 (162), cingulin (36), and JAM-1 in epithelial cells (18) (Fig. 3), and with α-catenin in nonepithelial cells (75). These diverse interactions indicate that ZO-1 has a scaffolding function. Its NH_2-terminal portion clusters both transmembrane junctional proteins and other PDZ-expressing proteins, whereas its COOH-terminal domain serves as an anchor to the actin cytoskeleton. In MDCK I cells transfected with truncated ZO-1 expressing the three PDZ domains but lacking the GUK occludin binding domain, the modified ZO-1 failed to localize at the plasma membrane. Moreover, these transfectants under-
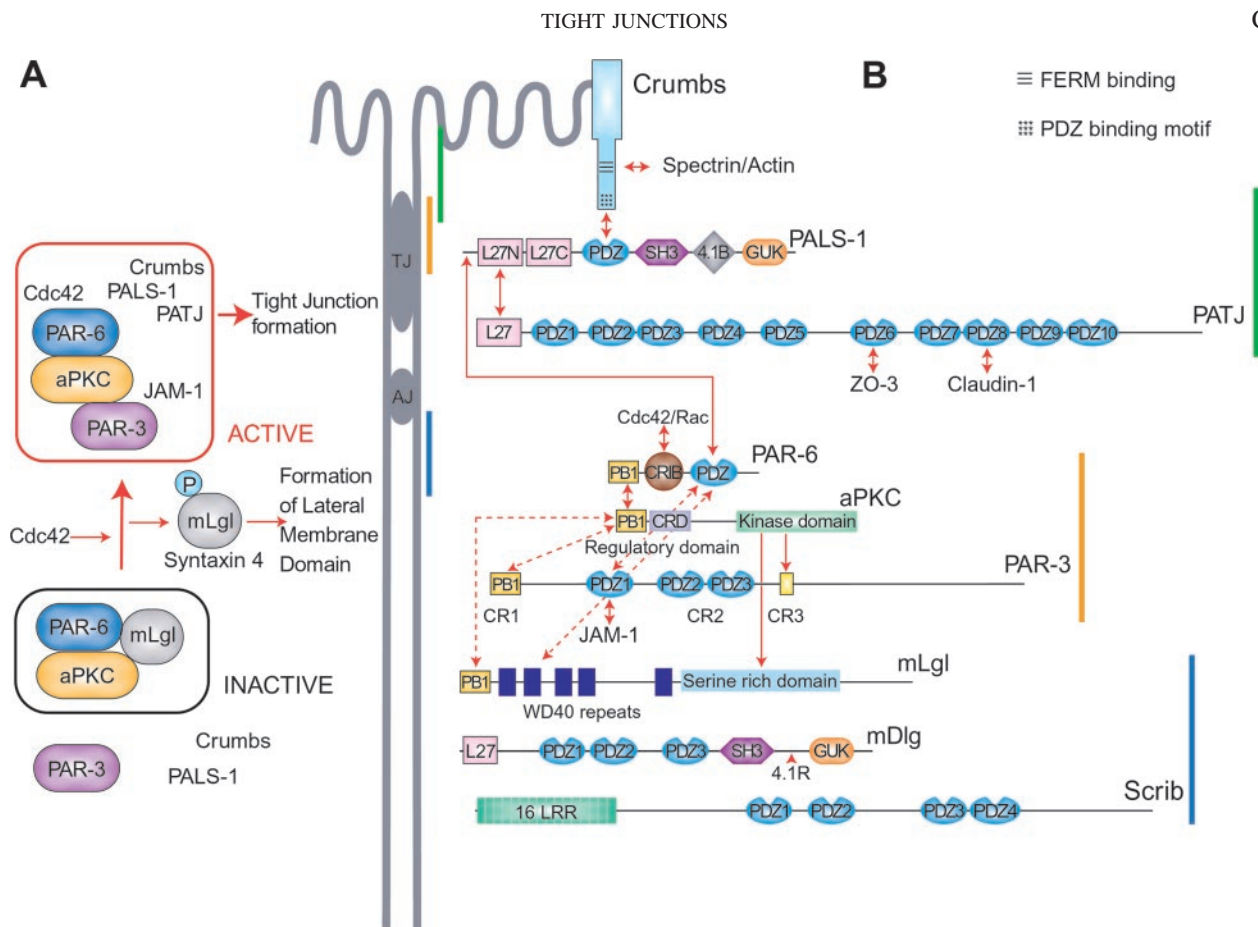
Fig. 4. Three multimolecular complexes contribute dynamically, either directly or indirectly, to TJ assembly and the establishment of cell polarity. In *B*, single arrows denote aPKC-mediated phosphorylation of the indicated substrate molecule. Solid double arrows indicate an adhesive interaction, whereas dashed double arrows specify transient adhesive interactions. In *A*, the diagram depicts the competitive interaction of PAR-3 and mLgl with the PAR-6/aPKC complex and its effect on TJ assembly. After it has detached from the PAR-6/aPKC complex, phosphorylated mLgl localizes to the lateral cell membrane, where it binds syntaxin 4. The colored vertical lines indicate the location of the three polarity complexes relative to the TJ in cell monolayers at steady state. FERM, band 4.1/ezrin/radixin/moesin; CR, conserved region; CRD, cysteine-rich domain. [Panel *A* is adapted from Ref. 163. Copyright 2003, with permission from Elsevier.]

went an epithelial-to-mesenchymal transformation, which was associated with activation of the β-catenin signaling pathway (117). The interaction of ZO-1 and ZONAB is interesting not only because these two proteins appear to be involved in regulating the ErbB2 proto-oncogene promoter (14) but also because their interaction controls both epithelial cell proliferation and cell density through ZONAB's interactions with the cell division kinase CDK4. A reduction in nuclear ZONAB, induced by ZO-1 overexpression, lowers the level of nuclear CDK4 (11). Although the precise mechanisms involved remain to be defined, the following model has been proposed. ZO-1 may be viewed as a gauge of cell density and, possibly, cell shape. When a certain cell density threshold is achieved, cell proliferation is stopped by the ZO-1-mediated cytoplasmic sequestration of ZONAB and CDK4 at the TJ (11).

ZO-2, a 160-kDa polypeptide, coimmunoprecipitates with ZO-1 and, like ZO-1 (59), has three PDZ domains: an SH3, a GUK domain, and a proline-rich COOH terminus (19, 80). Its peptide map and turnover rate, however, are distinct from ZO-1 (59). Although there is a 51% sequence identity with ZO-1, ZO-2 contains a 36-residue, alternatively spliced region at the COOH terminus, and the COOH-terminal proline-rich region has only 25% amino acid identity with ZO-1 (19). Like ZO-1, specific regions in the NH$_2$ terminus of ZO-2 interact with claudins and occludin, and with α-catenin in nonepithelial cells (74), whereas the COOH terminus binds to actin (74, 158). Recruitment of ZO-2 to TJs involves the interaction of its PDZ1 domain with the COOH terminus of claudins (74). ZO-2 is also targeted to the nucleus in subconfluent cultures, where its PDZ1 domain interacts with the DNA binding protein scaffold attachment factor-B (SAF-B), which is involved in transcriptional regulation (Fig. 3). The gene expression that is altered by this interaction, however, requires further study. Interestingly, ZO-1 does not associate with SAF-B, suggesting that these two MAGUK proteins have nonredundant functions (141).

ZO-3, a 130-kDa protein, was identified as a coimmunoprecipitant of ZO-1 (12). There is a 47% amino acid identity among ZO-1, ZO-2, and ZO-3. The amino acids located between the PDZ2 and PDZ3 domains of ZO-3 are uniquely enriched in proline residues (64). Like ZO-1 and ZO-2, ZO-3 localizes to the TJ; however, complexes form in vitro between ZO-1 and ZO-2 and between ZO-1 and ZO-3, but not between ZO-2 and ZO-3 (158). Interaction with occludin is not required

for recruitment of ZO-1, ZO-2, or ZO-3 to the TJ; in occludin-deficient embryonic stem cells, these three MAGUK proteins are distributed normally at the TJ (122). ZO-3 has a number of binding partners, including the Ras target protein AF-6 (afadin) and p120 catenin, both of which bind to the COOH-terminal half of ZO-3 (157). Overexpression of the NH$_2$-terminal half of ZO-3 (NZO-3) in MDCK cells delays both the recruitment and assembly of TJ and adherens junction proteins at the plasma membrane (156). It has been suggested that ZO-3 may alternate between an "open" or "closed" conformation in which intramolecular interactions either expose or block access to the COOH terminus, thereby regulating p120 catenin binding to ZO-3 (157) When NZO-3 is overexpressed, it binds to and blocks access to the COOH terminus of endogenous ZO-3, with the consequence that p120 catenin is available to inhibit RhoA signaling, either directly or indirectly, which in turn results in increased cell motility (157).

MAGI-1, -2, and -3 proteins. The MAGI-1, -2, and -3 proteins represent another family of PDZ-expressing proteins that are localized to the TJ plaque (Fig. 3). The inverted arrangement refers to the fact that, in contrast to the ZO proteins, in MAGI the GUK domain is in the NH$_2$ terminus, followed by two W-W domains and five PDZ domains at the COOH terminus (28). MAGI proteins lack an SH3 domain. MAGI-1, ZO-2, and MUPP-1 bind and may counteract viral oncoproteins (54, 55, 88), and MAGI-2 and MAGI-3 interact with the lipid phosphatase tumor suppressor PTEN (phosphatase and tensin homologue) (160, 161). These observations suggest that the TJ plaque is an important site for the recruitment of growth suppressors. More recently, the receptor tyrosine phosphatase-β (RPTPβ) has been shown to bind to the PDZ2 domain of MAGI-3. Although MAGI-3 itself is not tyrosine phosphorylated, it associates with tyrosine-phosphorylated proteins, suggesting that MAGI-3 in its role as a scaffolding molecule may be involved in linking this receptor tyrosine phosphatase to its substrates at the plasma membrane (1).

MUPP-1. MUPP-1 is a TJ-associated cytosolic protein with 13 tandem PDZ domains. MUPP-1 is exclusively localized to the TJ of polarized epithelial cells, where it binds to claudin-1 via PDZ10 (61) and to claudin-8 via PDZ9 (79). Like ZO-1, MUPP-1 also is linked to JAM-1 via its PDZ9 domain, suggesting that it is a multivalent scaffolding protein that may recruit a number of other, yet to be determined proteins to the TJ (61), where they may participate in the regulation of epithelial cell growth and differentiation.

ROLE OF POLARITY COMPLEXES IN TJ ASSEMBLY

Studies with the *Caenorhabditis elegans* worm and the *Drosophila melanogaster* fruit fly have uncovered a rich trove of protein complexes that play a central role in asymmetric cell division, anterior-posterior polarity in embryogenesis, apical-basolateral polarity of epithelial cells, and the regulation of junction assembly (120). Among these are the PAR-3/aPKC/PAR-6, Crumbs3/PALS1/PATJ, and Scrib/mDlg/mLgl complexes, which are highly conserved throughout evolution. Their mammalian homologues participate in the assembly of TJs and the establishment of apical-basal epithelial cell polarity.

PAR-3/aPKC/PAR-6. PAR-3, also known as aPKC isotype-specific interacting protein (ASIP), and PAR-6 are PDZ do-main proteins (Fig. 3) that regulate cell polarity and TJ assembly (78), whereas aPKC (ζ, λ) belongs to a family of Ca^{2+}- and diacylglycerol-independent PKCs that do not respond to phorbol esters. Initiation of TJ assembly requires Ca^{2+}-dependent, E-cadherin-mediated cell-cell contact (60, 129) and does not involve the PAR-3/aPKC/PAR-6 complex. Instead, this complex appears to be involved in regulating a later stage of TJ assembly (137), because overexpression of PAR-3 appears to accelerate this process (68). Conversely, overexpression of a dominant negative aPKC has no effect on E-cadherin interactions but blocks the assembly of TJs and disrupts the polarized distribution of Na$^+$-K$^+$-ATPase (137). Immunolocalization studies of epithelial cell monolayers at steady state indicate that the PAR-3/aPKC/PAR-6 complex is localized on the apical side of the TJ (Fig. 4*B*) (68). Recruitment of the PAR-3/aPKC/PAR-6 complex to the TJ (164) is mediated by the binding of PAR-3 to JAM-1 (38, 77) and phosphorylation of PAR-3 by aPKC (68). Recent data indicate that the NH$_2$-terminal conserved region of PAR-3 (amino acids 1–86) promotes its self-association, which in turn facilitates recruitment of PAR-3/aPKC/PAR-6 complexes to the assembling TJ (105). The kinase activity of aPKC is enhanced (164) when Cdc42, a Rho family GTPase involved in regulating epithelial cell polarity, binds to the Cdc42/Rac interactive binding (CRIB) domain of PAR-6 (Fig. 4*B*) (81, 90). In the later stages of TJ assembly, aPKC also phosphorylates ZO-1, occludin, and claudin-1 (110). Protein phosphatase 2A (PP2A), the first serine/threonine phosphatase to be localized at the TJ, negatively controls TJ activity by dephosphorylating aPKC, ZO-1, occludin, and claudin-1 (110). Thus PP2A may play a pivotal role in controlling the activity of the PAR-3/aPKC/PAR-6 signaling complex, thereby contributing to the regulation of TJ assembly and establishment of cell polarity.

Crumbs3/PALS1/PATJ. Crumbs3/PALS1/PATJ is a second evolutionarily conserved, multiprotein complex that is involved in establishing mammalian epithelial cell polarity. Of the three known human Crumbs homologs, Crumbs3 has been localized to mammalian epithelial cells (120). The link between the Crumbs3/PALS1/PATJ complex and the PAR-3/aPKC/PAR-6 complex is mediated through the PDZ domain of PAR-6 and the amino terminus of PALS1 and, like the PAR-3/aPKC/PAR-6 complex, is regulated by Cdc42 (Fig. 4*B*) (71). Although the transmembrane protein Crumbs3 is expressed on the apical membrane of epithelial cells, it is indirectly linked to the TJ via the MAGUK protein PALS1, which in turn is linked to the multi-PDZ domain protein PATJ (119). The PDZ binding motif at the COOH terminus of Crumbs3 binds to the single PDZ domain of PALS1. The MAGUK protein PALS1 has multiple protein-protein interaction domains (Fig. 4*B*), of which the NH$_2$-terminal L27 domain binds to the L27 domain of PATJ. The COOH termini of ZO-3 and claudin-1 bind to the sixth and eighth PDZ domain of PATJ, respectively, thereby tethering the Crumbs3/PALS1/PATJ complex to the TJ (118). Expression of dominant negative PATJ in MDCK cells causes mislocalization not only of PALS1 but also of members of the PAR-3/aPKC/PAR-6 complex and ZO-1. Conversely, when PAR-6 is overexpressed, PALS1 is prevented from localizing to the TJ (71). Whereas the precise role of the Crumbs/PALS/PATJ complex in TJ regulation remains to be clarified, its linkage to the PAR-3/aPKC/PAR-6 complex suggests that it, too, may be involved in the later stages of TJ assembly.

Scrib/mDlg/mLgl. A third complex, Scrib/mDlg/mLgl, resides on the lateral cell membrane, basal to established TJs (163). However, during the initial phases of cell polarization and TJ assembly, mLgl competes with PAR3 to form an independent complex with PAR-6 and aPKC. The NH$_2$ terminus of mLgl binds to the PDZ domain of PAR-6 and, in addition, interacts with aPKC to transiently colocalize with aPKC/PAR-6 at cell-cell contact sites (Fig. 4A) (163). mLgl has five highly conserved and closely spaced serine residues that are substrates for aPKC-mediated phosphorylation (115). In a calcium switch assay in which epithelial cells are made to form TJs by the addition of Ca^{2+}, mLgl/aPKC/PAR-6 complexes initially localize apically at early cell-cell contact sites. This is accompanied, within 30 min, by a two- to threefold increase in mLgl phosphorylation, which may facilitate the dissociation of mLgl from the aPKC/PAR-6 complex. Approximately 3 h after the addition of Ca^{2+}, mLgl migrates to the lateral membrane, basal to the TJ. Thus it appears that the mLgl/aPKC/PAR-6 complex transiently suppresses TJ assembly. Once dissociated from mLgl, the aPKC/PAR-6 complex is free to form a PAR-3/aPKC/PAR-6 complex that then promotes TJ assembly (Fig. 4A). Thus the selective interaction of aPKC/PAR-6 with and phosphorylation of either mLgl or PAR-3 contributes to the regulation of epithelial cell polarity and TJ assembly. Once mLgl is located basal to the TJ, it can be coimmunoprecipitated with syntaxin 4, an *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) protein involved in vesicle trafficking to the basolateral membrane (107). Although they colocalize in epithelial cells, it is currently not known whether there are direct interactions among mLgl, mDlg, and Scrib.

TIGHT JUNCTION PROTEINS AS TARGETS OF INFECTIOUS AGENTS

Epithelia form one of the primary physical barriers that protect the organism against infectious agents in the environment. Interestingly, a number of TJ proteins are targets and/or receptors for factors expressed and/or released by viruses, bacteria, or parasites. In this section we highlight a few interesting recent observations that serve as examples of the clever strategies developed by these pathogens to bypass the TJ barrier. For the effect of enteric pathogens on TJ barrier function, see a recent review on this topic (22).

Viruses. The attachment and endocytosis of the reovirus is initiated by the interaction of the viral attachment protein σ1 with JAM-1. This interaction stimulates nuclear factor-κB (NF-κB) activation, which results in cellular apoptosis (16). Although it appears counterintuitive that the virus should induce apoptosis in the host cell, reovirus replication requires ∼16 h, whereas the apoptotic cell remains viable for up to 48 h (35). These observations indicate that JAM-1 is capable of transducing signals from the environment to the nucleus and suggest that the TJ may serve as a sensor for environmental pathogens.

A different strategy is used by coxsackie B virus and adenovirus (21), whose receptor, CAR, is situated basal to the TJ. The initial viral infection may occur when there is a break in the epithelium following injury, thereby enabling the virus to gain access to its CAR receptor. A viral capsid protein, known as fiber, binds to CAR, which in turn promotes entry of the virus into epithelial cells where replication occurs. Once the virus is released into the basolateral compartment, however, it must then penetrate the TJ to infect a new host. This process is facilitated by the fact that fiber binds CAR with a higher affinity than that of CAR-CAR interactions on adjacent cells. After the release of replicated viruses into the basolateral space, soluble fiber binds to CAR, which results in disruption of TJs, giving the virus access to the apical extracellular space (147).

Intracellular TJ proteins including PDZ-expressing TJ plaque proteins are the target of oncogenic adenovirus and papillomavirus. The adenoviral EF-ORF1 and papilloma viral E6 oncoproteins both bind via their PDZ binding domains to MUPP-1 (88) and MAGI-1 (54), whereas the adenoviral EF-ORF1 also binds to ZO-2 (55). The oncogenic effect of these interactions is attributed, in part, to the sequestration and degradation of these TJ-associated tumor suppressor proteins.

Bacteria. Bacteria also utilize a variety of ingenious strategies to disrupt the TJ barrier. The enterotoxin produced by *Clostridium perfringens* is a 35-kDa protein responsible for food poisoning in humans (98). The COOH terminus of this peptide binds specifically to both claudin-3 and -4 (44, 131), whereas the NH$_2$ terminus forms pores in the plasma membrane (62). To examine the interaction with TJ proteins specifically and to avoid injury to the plasma membrane, the COOH-terminal half of the enterotoxin was applied to either L-cells transfected with claudin-1, -2, -3, or -4 or to confluent monolayers of MDCK I cells that express claudin-1 and -4. In the L-cell transfectants, the enterotoxin fragment selectively bound to claudin-3 and -4 but not to claudin-1 or -2. Notably, within 4 h of enterotoxin peptide binding to claudin-4 in MDCK I cells, the TJs began to disintegrate, TER fell, and paracellular flux increased (131). It is unclear from these studies, however, whether the interaction of the peptide with claudin-4 caused the depolymerization of proteins in existing TJ strands. Alternatively, because disruption of TJs occurred only when the peptide was added to the basolateral surface, it is conceivable that its interaction with claudin-4 monomers in the membrane prevented their incorporation into TJ strands, thereby leading to a breakdown of TJs.

When *Helicobacter pylori* adhere to the apical-junctional complex of epithelial cells, the CagA protein is translocated from the bacterium into the epithelial cell (112). It is noteworthy that CagA appears to target *H. pylori* to intercellular junctions where both ZO-1 and JAM-1 are recruited to sites of bacterial attachment. Subsequent to this interaction, TJ barrier function is disrupted and cell shape is significantly altered (3). Although the mechanisms that lead to the disruption of the TJ in *H. pylori* infections are not well understood, it is possible that perturbations in the organization of ZO-1 and JAM-1 may lead to abnormal localization and function of important signaling molecules. The clinical consequences of this interaction, if prolonged, are gastric ulcers and/or gastric carcinoma (23).

Parasites. House dust mite allergens are important contributors to the increasing incidence of asthma. In a remarkable set of experiments, cysteine and serine peptidases were identified in the fecal pellets of the house mite *Dermatophagoides pteronyssinus* (*Der p 1*) (148–150). When inhaled, these peptidases specifically cleave occludin and ZO-1, which leads to the breakdown of TJs, the loss of the protective barrier to inhaled allergens, and the initiation of an immune response.

In summary, knowledge of the TJ has evolved from regarding it as a simple permeability barrier bridging the paracellular space to a multifunctional complex comprising a series of protein complexes that are involved in numerous vital and diverse functions of epithelial cells. The claudins, a family of integral TJ proteins, form ion-selective pores within the TJ strands, whereas occludin and JAM-1 may have an adhesive and/or signal transducing function as they interact with various cytosolic complexes. The recent discovery that several polarity complexes, first described in the fruit fly, are conserved in mammalian cells and are closely associated with the TJ clearly indicates that the TJ performs a vital role in establishing epithelial cell polarity. One of the most interesting recent developments is the recognition that a number of TJ-associated cytosolic proteins are also found in the nucleus, where they appear to regulate gene expression and control cell growth and cell density. Several of these proteins may function as tumor suppressors. Localization of a number of kinases, phosphatases, G proteins, and guanine nucleotide exchange factors at the TJ clearly indicates that it plays a vital role in transducing signals impinging on and emanating from the plasma membrane. Much remains to be learned, but these are exciting times in the study of the TJ.

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