

# Physiology and Function of the Tight Junction

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Understanding of tight junctions has evolved from their historical perception as inert solute barriers to recognition of their physiological and biochemical complexity. Many proteins are specifically localized to tight junctions, including cytoplasmic actin-binding proteins and adhesive transmembrane proteins. Among the latter are claudins, which are critical barrier proteins. Current information suggests that the paracellular barrier is most usefully modeled as having two physiologic components: a system of charge-selective small pores, 4 Å in radius, and a second pathway created by larger discontinuities in the barrier, lacking charge or size discrimination. The first pathway is influenced by claudin expression patterns and the second is likely controlled by different proteins and signals. Recent information on claudin function and disease-causing mutations have led to a more complete understanding of their role in barrier formation, but progress is impeded by lack of high resolution structural information.

**T**ight junctions form the continuous intercellular barrier between epithelial cells, which is required to separate tissue spaces and regulate selective movement of solutes across the epithelium. Although there are now >40 proteins (Schneeberger and Lynch 2004; Yamazaki et al. 2008) identified within the tight junction, the claudin family of transmembrane proteins, named from the Latin *claudere* to close, has emerged as the most critical for defining tight junction selectivity. Here, we review evidence that claudins regulate permselectivity (including size, electrical resistance, and ionic charge preference) derived from studies in cultured epithelial cell models and the phenotypes of

knockout mice and human mutants. We highlight the physiologic relevance of selectivity but only briefly discuss how it might be physiologically regulated and altered in pathologic situations. We develop the perspective that the barrier is usefully described as having two pathways: first a system of charge-selective claudin-based pores that are 4 Å in radius and a second pathway created by larger discontinuities in the barrier and that lacks charge and size discrimination. The two pathways may be controlled by different proteins and signals. This article focuses on claudins and physiology and is meant to be read as a companion to the article in this collection contributed by

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M. Furuse, which focuses on the molecular structure, proteins, and cell biology of the tight junction (Furuse 2009). The reader is also referred to comprehensive reviews on physiology (Diamond 1978; Powell 1981; Van Itallie and Anderson 2006), pathophysiology (Turner 2006; Schmitz et al. 1999; Nusrat et al. 2001), regulation (Tsukita et al. 2008; Gonzalez-Mariscal et al. 2008), and molecular components of the junction (Schneeberger and Lynch 2004; Krause et al. 2008; Gonzalez-Mariscal et al. 2003).

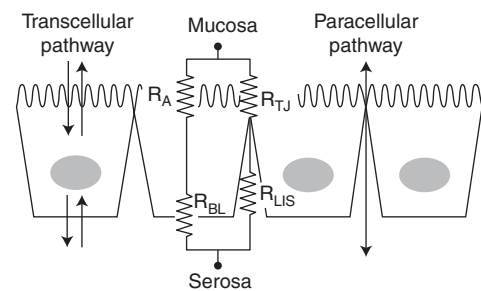
### EVOLUTION OF IDEAS ABOUT PARACELLULAR TRANSPORT AND THE RELEVANCE OF TIGHT JUNCTION PERMSELECTIVITY

Explicit description of a sealing contact between epithelial cells can first be found in the biologic literature in the latter part of the 19th century (Cereijido and Anderson 2001). Staining of epithelial tissues, such as the intestine, with vital dyes revealed a distinct region at the apical end of the lateral cell interspaces referred to as the “terminal bar.” This was thought to be an absolute barrier preventing anything from passing between cells. By the early 20th century, studies began to acknowledge that some material, for example macrophages and water, could cross epithelia through the paracellular space.

Our current understanding of how material selectively crosses epithelia originates with the pioneering work of Hans Ussing and associates, beginning in the 1940s. Ussing addressed the question of how sodium ions are moved in a directional fashion across the epithelium of frog skin, resulting in a steady-state electrical potential across the epithelium (Koefeld-Johnsen and Ussing 1958; Ussing and Zerahn 1951). Their first conceptual breakthrough, the so-called “two membrane model,” stated that the apical and basolateral surfaces had different conductance properties, namely, that sodium enters the cell across the apical membrane down its concentration gradient (later shown to be through Na channels) but was transported in an energy-dependent

fashion out the basal lateral surface (later shown to be by the NaK-ATPase). Ironically, they chose to study an epithelium with one of the electrically tightest junctions in nature, leading them initially to discount the possibility of ion movements between cells. Subsequent inconsistencies between their model and the data led to the realization that  $\text{Cl}^-$  ions must be following  $\text{Na}^+$  in a passive fashion through the tight junction (based on electrical circuit modeling, they called this the shunt pathway) coupled to the electrical gradient generated by active  $\text{Na}^+$  transport (Ussing and Windhager 1964). This was the beginning of understanding about how transcellular and paracellular transport are physiologically coupled (Fig. 1).

In the 1960s, investigators began to study transport across other epithelia, such as the gallbladder, where the paracellular resistance was much lower than in frog skin (reviewed in Diamond 1977). Because the transepithelial electrical resistance (TER) was so much lower than frog skin, there was initial concern that these tissues were damaged during experimental preparation and this explained their low resistance. Gradually, it was accepted that intact normal epithelia display a wide range of electrical resistances (Table 1). Because the cell membranes are generally of very high resistance



**Figure 1.** Electrical circuit model of the series and paracellular resistances across trans- and paracellular pathways of an epithelial cell monolayer. Transcellular transport is controlled by transporters in the apical and basolateral surfaces. The resistance of these series elements is typically much higher than that of the parallel elements of the paracellular pathway. Thus, the overall resistance of an epithelium is defined by  $R_{TJ}$ , which is defined by the composition of claudins in the tight junction (TJ).

**Table 1.** Electrical characteristics of some epithelia and endothelia

Tissues <sup>a</sup>	Species	R <sub>cell</sub> Ω × cm <sup>2</sup>	R <sub>paracellular</sub> Ω × cm <sup>2</sup>	P <sub>Na</sub> /P <sub>Cl</sub> <sup>b</sup>
Proximal tubule	dog	–	6–7	1.4
Gallbladder	rabbit	229	21	3.3
Duodenum	rat	–	98	–
Jejunum	rat	67	51	10.0
Ileum	rabbit	115	100	2.5
Distal colon	rabbit	730	385	0.6
	mouse surface	132	3,200	–
	crypt	429	–	–
Brain endothelium		1,300		
Gastric fundus	Necturus	2,826	10,573	–
Urinary bladder	rabbit	160,000	300,000	–
<b>Cell lines<sup>c</sup></b>				
Caco-2	human colon	125–250	–	3.0
LLC-PK <sub>1</sub>	pig prox. tubule	100	–	0.6
MDCK	dog	60–4000	–	10.0

<sup>a</sup>All values can be found in Powell (Powell 1981) or Crone (Crone and Christensen 1981).

<sup>b</sup>Permeability ratio of Na<sup>+</sup> versus Cl<sup>−</sup>. P<sub>Na</sub>/P<sub>Cl</sub> in free solute is 0.66. Paracellular pathways with ratios above this value are more permeable for Na<sup>+</sup> than Cl<sup>−</sup>, i.e., cation-selective.

<sup>c</sup>Values for cell lines are the personal observations of Dr. C. Van Itallie. Modified, with permission, from Van Itallie and Anderson (2004) (© American Thoracic Society).

(R<sub>apical</sub> and R<sub>basolateral</sub>), it is the TJ (R<sub>TJ</sub>) that determines whether the transepithelial resistance is high or low (Fig. 1). Theoretically, the lateral intercellular space (R<sub>lis</sub>) could contribute a resistance in series with the tight junction, but there is little evidence that this is physiologically significant.

TER differs by several orders of magnitude between so-called “tight” and “leaky” epithelia (Table 1). For example, the epithelium of the mammalian proximal tubule is only about 6 Ω·cm<sup>2</sup> compared with the toad urinary bladder at 300,000 Ω·cm<sup>2</sup> (Powell 1981; Van Itallie and Anderson 2006). The relevance of TER is that epithelia with “tight” tight junctions can maintain the high electrochemical gradients produced by active transcellular transport. This configuration is used to produce either highly concentrated or diluted secretions, as in the distal nephron, which can produce urine with osmolarity several fold higher or lower than plasma. In contrast, epithelia with “leaky” tight junctions move large amounts of isosmotic fluids. A good example is provided by the human gastrointestinal tract, which secretes and then reabsorbs about 10 L of fluid each day (Boron and Boulpaep 2005). Most of

the gastrointestinal track is of low TER, except in the distal colon where steeper electrochemical gradients are required to reabsorb NaCl and water and form solid stool.

Study of leaky epithelia revealed another interesting and variable property, namely that tight junctions (TJs) have ionic charge selectivity. Charge selectivity is most relevant in leaky tight junctions where higher amounts of ions flow. Almost all leaky TJs show a preference for Na<sup>+</sup> over Cl<sup>−</sup>, and the permeability ratio (expressed as P<sub>Na</sub>/P<sub>Cl</sub>) ranges from about 10 to 0.1 among different epithelia and experimental cultured cell models (Table 1). This represents only a modest ability to discriminate compared with membrane ion channels. For comparison, some amiloride-sensitive Na<sup>+</sup> channels (ENaC) show a 1000-fold preference for Na<sup>+</sup> over K<sup>+</sup>, an identically charged ion of similar size (Hille 2001). However, even a 10-fold cation to anion discrimination has significant physiologic implications. For example, epithelia that secrete salt and water initiated by an apical Cl<sup>−</sup> channel (such as CFTR in the airway, lacrimal glands, etc.) are found to have a threefold to 10-fold preference for Na<sup>+</sup> over Cl<sup>−</sup>. This allows paracellular passage of Na<sup>+</sup>

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to follow secreted  $\text{Cl}^-$  while relatively discriminating against back diffusion of the  $\text{Cl}^-$ , which would defeat the ability to secrete NaCl and water.

The TJ was first visualized at the ultrastructural level in 1963 by Farquhar and Palade (Farquhar and Palade 1963). They were able for the first time to resolve the apical junction complex (terminal bar) into three morphologically discrete contacts from the apical-most occluding or TJ (zonula occludens) followed by the cadherin-based adherens junction and the more basally positioned desmosomes. Gap junction plaques are also commonly found within the TJ contacts. The TJ zone is formed by a variable number of close cell–cell contacts or “kisses,” which range from a single continuous contact (e.g., some endothelial cells) to a half dozen (jejunum) or dozens of contacts in the most extreme mammalian example (Sertoli cells). Subsequent visualization of the tight junction in freeze fracture EM images revealed that membrane kisses correspond to continuous rows of 10-nm transmembrane particles, which are presumed to contain an oligomerized array of claudins. The physical barrier to paracellular diffusion occurs where rows of particles adhere and seal across the intercellular space. The existence of multiple strands is speculated to provide redundancy to the barrier. Although there is no experimental proof for this idea, it seems very reasonable to assume that multiple barriers in series would provide a fail-safe barrier during cell dynamics, such as when cells move relative to each other (Matsuda et al. 2004) or single cells leave the epithelia sheet during apoptosis (Madara et al. 1980). In the early 1970s, Philippe Claude proposed that TER has a logarithmic dependence on the number of strands in series, the so-called Claude hypothesis (Claude 1978). By a quirk of etymology, the Claude hypothesis is no longer accepted but the TER is now thought to depend on the profile of different claudins expressed.

To summarize, the field of TJs before the discovery of claudins was well described at a physiologic level. Comparing epithelia: Cell-specific TJ barriers allow the passage of

varying levels of electrical current and non-charged solutes. Based on how paracellular ion selectivity varied with extracellular pH, several insightful investigators in the 1970s had already proposed that the junction was created by pore-forming proteins with variable side-chain chemistries (Wright and Diamond 1968). A major transformation (in fact a resurrection) of the field occurred with discovery of the barrier and pore-forming proteins.

### TRANSMEMBRANE TJ PROTEINS AND THE CLAUDIN FAMILY

The TJ has a surprisingly complex protein composition compared with other cell–cell junctions and is composed of at least 40 different proteins (Schneeberger and Lynch 2004; Gonzalez-Mariscal et al. 2003; Yamazaki et al. 2008). This complexity is a consequence of its many interrelated roles in cell polarity (Cereijido et al. 1998), signaling (Gonzalez-Mariscal et al. 2008; Van Itallie and Anderson 2006), transcriptional regulation, and cell cycle (Balda and Matter 2003; Tsukita et al. 2008) and vesicle trafficking (Yeaman et al. 2004), in addition to creating the paracellular barrier. Excellent reviews of the cytoplasmic plaque proteins and nonclaudin transmembrane proteins can be found in Gonzalez-Mariscal et al. (2003) and Schneeberger and Lynch (2004). A recent proteomics study suggests that there are many more transmembrane proteins yet to be characterized (Yamazaki et al. 2008). The role of these other transmembrane proteins remains an active area of investigation; however, all current evidence supports a central role for claudins in defining electrical resistance and permselectivity.

### Nonclaudin Transmembrane Proteins

In addition to claudin family members, there are currently three other transmembrane proteins localized within the adhesive barrier strands and which in theory might directly influence the barrier. Occludin (Furuse et al. 1993) and tricellulin (Ikenouchi et al. 2005) are related tetraspanning proteins of currently unknown

function. Occludin knockdown (KD) cell lines (Yu et al. 2005) and even knockout (KO) mice have no definable barrier defects (Saitou et al. 2000). KD cell lines reform TJs more slowly after a Ca-switch and have elevated levels of active GTP-bound RhoA (Yu et al. 2005), leading to speculation of a role for occludin in cytoskeletal dynamics. KO mice have a collection of defects, but there are no obvious barrier changes when assessed by TER, steady-state potential difference, electrical impedance, or solute tracer flux (Schulzke et al. 2005). An alternative role for occludin in coordinating transmembrane signaling is suggested by the findings that it binds the TGF- $\beta$  type III receptor (Barrios-Rodiles et al. 2005) and manipulation of occludin in cultured cells affects Raf-1 signaling (Wang et al. 2005). Human tricellulin mutations result in a non-syndromic form of deafness by an unknown mechanism (Riazuddin et al. 2006). There is a third protein in the human database homologous to occludin and tricellulin (MARVELD3, Genbank ID: 91862, personal observation). Perhaps functional redundancy among these proteins has obscured the role of the individual proteins. Finally, there are several IgG superfamily members within the stand contacts (CAR [Coyne and Bergelson 2005], CLP24 [Kearsey et al. 2004]); the best studied being JAM-A, which appears to stabilize the barrier (Bazzoni 2003). In support of this idea, KD of JAM-A in cultured epithelia monolayers is reported to induce leakiness for large paracellular tracer molecules (Liu et al. 2000). The intestinal epithelium of JAM-A KO mice is extremely sensitive to disruption in inflammatory models (Laukoetter et al. 2007; Vetrano et al. 2008).

### The Claudin Family

The first claudins were discovered in 1998 by Furuse and Tsukita through traditional biochemical fractionation of membrane fractions from liver (Furuse et al. 1998). When expressed in claudin-null fibroblasts, claudins formed the characteristic linear strands of 10-nm particles observed in TJs and conferred cell-to-cell

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adhesion (Kubota et al. 1999). Mammalian claudins range from 20 to 27 kDa and have four transmembrane helices: a short internal amino-terminal sequence (2–6 residues), two extracellular domains (loop 1 is 49–52 residues and loop 2 is 16–33 residues), and a longer and more variable cytoplasmic tail (21–63 residues) (Fig. 2B). Claudins are recognized by the signature residues W-GLW-C-C in the first extracellular loop, although the function of these highly conserved residues remains unknown (Fig. 2A). The carboxy-termini of claudins contain a PDZ-binding motif and several have been shown to bind PDZ domains within the cytoplasmic scaffolding proteins ZO-1,-2, and -3 (Itoh et al. 1999), MUPP1 (Hamazaki et al. 2002; Jeansonne et al. 2003) and PATJ (Roh et al. 2002). ZO-1 has three PDZ domains, MUPP1 13, and PATJ 10, suggesting that there exists a dense Velcro-like trap of PDZ interactions under the claudin strands. However, there is actually no clear evidence that PDZ binding is required for targeting of claudins to the TJ in epithelial cells or for claudins to confer physiologic effects when expressed in cultured cell models. In a single case, when claudin-1 was expressed in cultured MDCK cells without a functional PDZ motif, this resulted in ectopic strands on the lateral cell surface (McCarthy et al. 2000).

Claudins are members of the much larger pfam00822 or PMP-22/EMP/MP20/Claudin family. These claudin relatives share the tetra-spanning topology and W-GLW-C-C signature motif in the first extracellular loop. Beyond these structural similarities, their functions appear highly divergent and only some are believed to create intercellular barriers. The most homologous to claudins are MP20 (eye lens specific membrane protein) (Steele et al. 1998); epithelial membrane proteins (EMP-1, -2, -3) (Jetten and Suter 2000), and peripheral myelin protein 22 (PMP22) (Notterpek et al. 2001). PMP-22 is highly expressed in Schwann cells and required for myelin formation (Bronstein 2000). Several forms of human peripheral polyneuropathies arise from PMP22 mutations, deletions, or gene duplications (Brancolini et al. 2000) (Table 2). Surprisingly,





**Table 2.** Genetic diseases of tight junction proteins

Gene	Disease	Pathology/Mechanism	Ref.
Cldn-1	Ichthyosis, sclerosing cholangitis	Affects skin and bile ducts	(Hadj-Rabia et al. 2004)
Cldn-14	Nonsyndromic deafness, <i>DFNB29</i>	Cochlear hair cell degeneration	(Ben Yosef et al. 2003)
Cldn-16			
Human	HHNC <sup>a</sup>	Defective renal Mg <sup>++</sup> reabsorption	(Simon et al. 1999)
Bovine	interstitial nephritis		(Hirano et al. 2000)
Cldn-19	Renal Mg <sup>++</sup> loss and vision loss	Similar to Cldn-16	(Konrad et al. 2006)
PMP22	Peripheral polyneuropathies	Demyelination	(Gabreels-Festen and Wetering 1999)
	HNPP <sup>b</sup>	Gene deletion	
	Charcot-Marie-Tooth Type 1A	Gene duplication	
	Dejerine-Sottas syndrome	Point mutations	
ZO-2	Familial hypercholanemia	Defective PDZ-claudin binding	(Carlton et al. 2003)
Tricellulin	Nonsyndromic deafness	Loss of ZO-1 binding	(Riazuddin et al. 2006)

<sup>a</sup>Hypomagnesemia hypercalciuria with nephrocalcinosis

<sup>b</sup>Hereditary neuropathy with liability to pressure palsies. Modified, with permission, from Van Itallie and Anderson 2006 (© Annual Reviews [www.annualreviews.org](http://www.annualreviews.org)).

Five claudin-like sequences have been identified in *Caenorhabditis elegans* (Asano et al. 2003) and RNAi-mediated ablation of claudin-like protein 1 disrupted the barrier between epithelial cells of the hypodermis.

### Claudin Cell–Cell Adhesion and Assembly

There remains almost no information about how individual claudins assemble into higher order structures to create 10-nm membrane particles, polymerize into the strands observed in freeze-fracture EM, or adhere across the cell–cell interspace. Currently, two papers suggest claudins, like connexins in the gap junction, form hexamers. This is based on a study of human claudin-4 expressed in insect cells (Mitic et al. 2003) and on purified native MP20, the distant claudin relative found in the lens (Jarvis and Louis 1995). There is consistent evidence that claudins can form homotypic adhesive plaques, and two studies provide evidence that this homotypic cell–cell adhesion can occur through residues in the center of the second extracellular loop (Daugherty et al. 2007; Piontek et al. 2007). Molecular modeling of claudin-5 complemented by mutagenesis suggests that adhesion requires an interaction of complementing hydrophobic residues along a helix in the center of the second loop

(Piontek et al. 2007). A model of the barrier in Figure 2B is based on evidence that claudins are cell-to-cell adhesion molecules and form small pores through the barrier, but there is currently insufficient evidence to speculate on how they oligomerize to form the particles seen in freeze fracture EM images.

Most cell types express several different claudins, which assemble into the same adhesive strands, yet the generality of heterotypic binding across the intercellular space remains an unresolved question. There is limited evidence that a subset of claudins can interact heterotypically across cell contacts; for example, claudin-1 can bind claudin-3 but not claudin-2 (Furuse et al. 1999), but there is currently no information that allows generalizations about which claudins can interact or if adhesion involves additional proteins (Fig. 2A,B). Some claudins are observed to coimmunoprecipitate with a list of proteins (e.g., claudin-7 with EpCAM [Le Naour and Zoller 2008]; claudin-11 with  $\beta$ 1 integrin and tetraspanins [Tiwari-Woodruff et al. 2001]), although the biologic implications remain unknown. Further understanding of how various functions (selectivity, adhesion, and assembly) are organized within the protein is severely limited by a current lack of structural information.

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### PERMSELECTIVITY AND THE TWO PATHWAY MODEL: SMALL SELECTIVE PORES AND NONSELECTIVE BREAKS

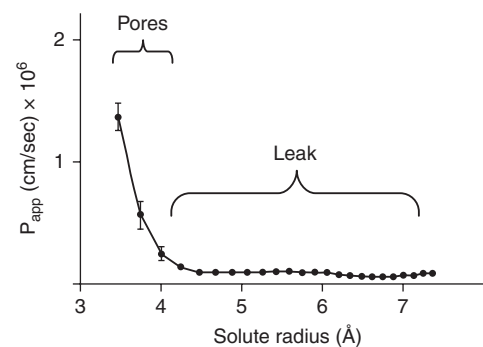
The term “permselectivity” is used to describe variations among TJs in electrical resistance, ionic charge and size discrimination, and the magnitude of solute permeability (Table 1) (Powell 1981). TER and solute flux are in part a function of cell geometry. For example, a monolayer with smaller cells has more cell–cell contact length per unit area through which electrical current and solutes can pass. Assuming the same claudins are expressed, a monolayer with smaller cells will show lower TER and higher solute flux. Geometry of the individual cell–cell contacts can also affect TJ length per unit area. Some cells touch with straight junction contacts, whereas others make elaborate interdigitations. For example, those between cells in mammalian thin ascending limb of the loop of Henle are extremely redundant, which increases TJ length per unit area of the epithelium at least 10-fold over strength contacts. Presumably, this arrangement enhances the space for paracellular transport without requiring larger epithelium. However, at the protein level, it is now recognized that a critical determinant of permselectivity is the profile and levels of different claudins expressed in a given tight junction.

#### Size

In general, description of paracellular size selectivity in the cell biology and physiology literature has been rather imprecise, in contrast to studies in the pharmaceutical literature, which have specifically focused on how transepithelial drug absorption is influenced by solute size and chemistry. Research in the former fields is often concerned only with TJ assembly or disassembly, rather than with subtleties of the permselectivity. In most studies, a single tracer size is used to report paracellular flux, which limits the ability to determine experimental changes in the size-dependence of permeability. Tracer charge is usually ignored as is a possible transcellular contribution from transcytosis. These subtleties are important because information

about alterations in size and charge dependence may have important pathologic implications, for example in determining if specific cytokines or bacterial toxins might have increased access across the paracellular space.

Now that the actual barrier-forming proteins have been discovered, it is instructive to characterize permeability as a continuous function of solute size (Watson et al. 2001; Watson et al. 2005; Van Itallie et al. 2008). This is performed by characterizing the permeability for solutes of progressively larger molecular radii. Apparent Permeability [ $P_{app} = (dQ/dt) / (\text{Concentration Gradient} \times \text{Area})$ ] takes into account the chemical driving force for each solute and surface area, and, unlike simple flux ( $dQ/dt$ ), can be compared among tissues and laboratories. When size profiling is performed using a noncharged solute like graded polyethylene polymers (PEGs), it becomes obvious that permeability has two components (Fig. 3). There is a higher capacity pathway with a steep size dependence for solutes less than about 4 Å in radius (Knipp et al. 1997; Watson et al. 2001; Van Itallie et al. 2008). This pore-pathway shows ionic charge discrimination and its magnitude varies among epithelia. This is the pathway that carries most of the



**Figure 3.** The two pathway model. Idealized data show the permeability of noncharged polyethylene glycol molecules of different sizes across an epithelium. The pathway for molecules below 4 Å is formed by claudin pores that are size and charge selective. The pathway for molecule above 4 Å in diameter shows no charge selectivity and appears to be defined by the status of cell-to-cell adhesion and the cytoskeleton.



electrical current during physiologic transport and during experimentally imposed electrical field (reflected in the TER), thus its magnitude and charge selectivity are key determinants of physiologic transport. As described below, claudins confer the charge selectivity, but there is less information on what determines the magnitude of flux through the pores. However, different claudins may control the level of porosity because it has been observed that expression of claudin-2 in cultured epithelial cells selectively increases the permeability for solutes smaller than 4 Å, whereas claudins 4 and 18 do not (Van Itallie et al. 2008). In vivo, deletion of claudin-5 from mice results in a size-selective increase in permeability of TJs in blood vessels (Nitta et al. 2003). It is important to recognize that the permeability for noncharged and charged solutes are not necessarily directly correlated. Because pores in different epithelia can have different charge discrimination, one can observe an apparent paradox in which the solute permeability is very high but the ion permeability is very low (high TER). Taken together, the present data suggest that claudins create the system of small discriminating pores, and by an unknown mechanism regulate the magnitude of flux (Fig. 3).

Solutes that are larger than 4 Å can also get through intact TJs. The magnitude of this component varies among epithelia and shows no charge discrimination (Knipp et al. 1997; Artursson et al. 1993). The distinction between the pore and nonpore pathways has been overlooked in most of the literature because permeability is typically measured only by tracers, which are larger than the pores, namely mannitol (4.2 Å in radius), inulin (15 Å), and a graded series of fluorescently labeled dextrans (4 kDa, 10 kDa, 40 kDa, etc). This larger pathway is speculated to represent small temporary breaks in the otherwise continuous TJ contacts. Interestingly, flux through this pathway can be enhanced by proinflammatory cytokines without altering the pore pathway (Watson et al. 2005). The nonpore pathway is sensitive to cytoskeletal disruption (Bruewer et al. 2004; Ivanov et al. 2005) and any form of cellular injury (Nusrat

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et al. 2000). There is significant evidence that the “intactness” of the TJ is controlled by cytoskeletal dynamics (Hartsock and Nelson 2008), myosin light chain activity (Ma et al. 2005), and any factor affecting cell homeostasis. As investigators dissect the role of individual proteins and signals in regulating the barrier, it will be useful to characterize barriers in terms of the pore and nonpore pathways.

### Claudin Charge Selectivity and TER

All of the available data supports the idea that the first extracellular loop of claudins creates an “electrostatic selectivity filter,” controlling overall resistance and charge selectivity of the small pores (Fig. 2). In contrast, admittedly limited data suggest that the second loop is involved in cell–cell adhesion (Blasig et al. 2006; Piontek et al. 2007). Charged amino acid side chains on the claudins limit similarly charged ions in solution without opposing permeability of oppositely charged ions. Most of these studies have been performed by expressing a single foreign claudin in the background of all the other claudins expressed in a cultured epithelial cell monolayer, most often MDCK and LLC-PK<sub>1</sub> cells or by siRNA knockdown of selected claudins (Hou et al. 2006). A change from the baseline TER and dilution potential (a measure of cation/anion selectivity) is interpreted to reveal selectivity of the transfected or silenced claudin compared with the background. These studies are somewhat qualitative. Lacking the electrophysiologic tools used to characterize membrane channels, such as patch clamping, it has been impossible to assign a specific conductance to individual claudins.

A total of 15 claudins have now been tested with good consensus. Claudins 2 (Furuse et al. 2001; Colegio et al. 2002; Amasheh et al. 2002) and 10 (Van Itallie et al. 2006) tend to make tight monolayers leakier. Claudins 1 (Inai et al. 1999; McCarthy et al. 2000), 4 (Van Itallie et al. 2001), 5 (Wen et al. 2004; Amasheh et al. 2005), 7 (Alexandre et al. 2005), 8 (Angelow et al. 2006; Yu et al. 2003; Jeansonne et al. 2003), 11 (Van Itallie et al. 2003), 14 (Ben Yosef et al. 2003), 15 (Colegio

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et al. 2003; Van Itallie et al. 2003), 16 (Hou et al. 2005; Ikari et al. 2008), 18 (Jovov et al. 2007), and 19 (Hou et al. 2008) tend to make leaky monolayers tighter. Claudins 2, 15 (Amasheh et al. 2002; Colegio et al. 2003), 16, and 19 (Hou et al. 2008) have a preference for cations over anions. Claudin-10a (Van Itallie et al. 2006) prefers anions. Where tested, the effect on TER is dose-dependent and each claudin reaches a saturating influence on TER (Van Itallie et al. 2001), although the reason for this is not clear. Most reports simply express the change in barrier permselectivity that results from the maximal induction level. Some studies suggest that the transfected claudin adds to those already present, whereas a single study convincingly shows that claudin-8 can replace endogenous claudin-2 (Yu et al. 2003). Potencies vary, with claudins 14 (Ben Yosef et al. 2003) and 18 (Jovov et al. 2007) producing the electrically tightest barriers. A single study has simultaneously expressed two different claudins, showing that claudins 16 and 19 exert properties in a cooperative and not just additive fashion, consistent with the idea that claudins might form heterotypic pores (Hou et al. 2008).

There is a general relationship between the chemistry of the first loop and charge selectivity (Fig. 2A). However, the relative influence of specific positions is still under debate. For example, claudin-16 has the highest proportion of negatively charged residues (Fig. 2A, shaped positions) and when expressed in cultured epithelial cell monolayers, results in a strong enhancement in permeability for  $\text{Na}^+$  and  $\text{Mg}^{++}$  but has no effect on  $P_{\text{Cl}}$  (Hou et al. 2005). In the opposite direction, claudin-10a has more basic residues and reduces  $P_{\text{Na}}$ , while simultaneously increasing  $P_{\text{Cl}}$  (Van Itallie et al. 2006). Each claudin has shown a signature pattern of whether it will increase or decrease TER and increase, decrease, or not alter the individual  $P_{\text{Na}}$  and  $P_{\text{Cl}}$ . The ability to increase TER can be based on limiting permeability for just cations (claudin-4 [Van Itallie et al. 2001]) or both cations and anions (claudin-7 [Alexandre et al. 2005]). There is a reassuring correlation between the physiology of particular

tissues, the claudins they express, and the physiology of those claudin as determined experimentally (Holmes et al. 2006). This is best documented along the nephron, where TER and ion selectivity are very well documented. For example, “leaky” claudin-2 is the dominant form in the leaky proximal tubule and is excluded from tighter segments; “tight” claudin-4 and -8 are restricted to the tight collecting ducts (Angelow et al. 2008).

The role of individual positions in influencing charge selectivity has most often been tested by expressing charge-reversing mutations. This approach has led to the conclusion that positions along much of the first loop can influence selectivity and thus this loop is folded to line the pore space through which soluble ions pass. This has been most extensively studied for claudins 15 (Van Itallie et al. 2003) and 16 (Hou et al. 2005). In the case of claudin-15, three widely spaced and negatively charged residues were sequentially reversed and shown to have additive effects (Van Itallie et al. 2003). The “distributed filter” model has been criticized because it is based on introducing abnormal charges rather than simply neutralizing them. Extensive characterization of charge-neutralizing mutations of claudin-2 convincingly showed that the only charged residue that provides the high  $P_{\text{Na}}$  is the aspartic acid at position 65 (Fig. 2A) (Yu et al. 2009). Resolution of the mechanism of selectivity must await high-resolution structural information.

The TJ pore is slightly wider than that of transmembrane pores and consequently shows less ionic discrimination. For comparison, some  $\text{Na}^+$  channels (ENaC) show a permeability ratio for  $\text{Na}^+$  to  $\text{K}^+$  ( $P_{\text{Na}}/P_{\text{K}}$ ) as high as 1000 to 1 (Hille 2001). In contrast, claudin pores show almost no discrimination between  $\text{K}^+$  and  $\text{Na}^+$  and the  $P_{\text{Cl}}/P_{\text{Na}}$  ratio ranges only from about 10 to 0.1 when compared among various in vivo epithelia and cultured cell monolayers. Membrane channels are of a size where the associated water molecules must be stripped off so the ion can fit through the pore (Hille 2001; Yu et al. 2009). The modest ability of TJs in MDCK cells to



discriminate among the alkali metal cations ( $K > Rb > Na > Li > Cs$ , so-called Eisenman selectivity sequence V–VIII) does actually suggest that they are being partially dehydrated as they pass through the pore. The observed Eisenman sequence is slightly different from the rank order of their free mobilities in solution, leading to the conclusion that the pore radius is about 3.6 Å (Yu et al. 2009). This is concordant with the 4 Å radius estimated using noncharged solutes (Watson et al. 2001). It is remarkable that this degree of size and charge selectivity can be maintained while cells in an epithelial sheet continuously move relative to one another.

Several claudins have now been deleted from mice and the resulting phenotypes are consistent with a barrier role for claudins 1 (Ladwein et al. 2005), 5 (Nitta et al. 2003), 11 (Gow et al. 2000), 14 (Ben Yosef et al. 2003), and 15 (Tamura et al. 2008), although the phenotypes are not easily explained as changes in permselectivity. In contrast, deletions of 16 (Himmerkus et al. 2008) and 19 (Hou et al. 2008) phenocopy their human disease mutants and are more easily explained as ion selectivity defects, as described in the following section.

In zebrafish, reduction in claudin-15 through morpholino treatment produces a developmental intestinal defect, which very likely results from loss of ion transport selectivity (Bagnat et al. 2007). In the absence of claudin-15, the gut remains as multiple lumens, which fail to fuse. It is proposed that active ion and water secretion is required for the lumens to enlarge, come into contact, and fuse. In the absence of claudin-15, there may be a back leak of  $Na^+$ , preventing fluid accumulation and expansion. Interestingly, claudin-15 KO mice develop a megaintestine, but this phenotype appears to be a proliferation and not a permeability defect (Tamura et al. 2008).

### Claudin-16 Mutants and Other Inherited Diseases of Tight Junction Proteins

Currently, there are seven human diseases known to be caused by mutations in genes

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encoding tight junction proteins (Table 2). The basis for ZO-2 (Carlton et al. 2003) and tricellulin-based defects (Riazuddin et al. 2006) remains unclear, but the phenotypes of several of the recessive claudin mutants provide additional evidence that claudins provide selectivity for the barrier. We describe the claudin-16 defect in some detail because it provides the best evidence that claudins *in vivo* create ion selective pores.

The first disease-causing claudin mutation was identified through positional cloning of the locus responsible for a rare form of renal magnesium loss, hypomagnesemia hypercalcemia with nephrocalcinosis (HHNC) (Simon et al. 1999; Kaushansky et al. 2007). Patients with this defect show increased urinary loss of  $Mg^{++}$  and  $Ca^{++}$ , and reduced plasma  $Mg^{++}$  levels, leading to weakness and seizures. The gene responsible encodes claudin-16 (originally referred to a Paracellin-1). Claudin-16 is highly restricted to the thick ascending limb of the loop of Henle, the segment of the nephron most involved in reabsorbing filtered  $Mg^{++}$ . Other claudins are also expressed in the same TJs. Paracellular reabsorption is driven by an intraluminal positive electrical potential with respect to the peritubular interstitium. These observations led to an initial hypothesis that claudin-16 is a  $Mg^{++}$  pore and when absent,  $Mg^{++}$  remains in the tubule and is lost in the urine. When expressed in cultured monolayers, wild-type claudin-16 does form a highly cation-selective pore (Hou et al. 2005). Consistent with the electrostatic claudin pore model, the first loop contains many negatively charged residues (Fig. 2), and neutralizing mutations reduce cation permeability (Hou et al. 2005). Many of the human mutations are missense mutations, which fail to traffic properly to the plasma membrane when expressed in cultured cell models (Kausalya et al. 2006) suggesting the defect results from the selective absence of claudin-16, leaving other claudins to define the barrier's ion selectivity. Recently, mutations in the human claudin-19 gene were shown to cause renal  $Mg^{++}$  wasting by a very similar mechanism. In addition, claudin-19 is expressed in the retina and affected individuals

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have defects in retinal development and vision loss (Konrad et al. 2006) (Table 2).

The Mg-pore model is probably too simplistic. The intraluminal positive potential driving  $Mg^{++}$  results from a paracellular back diffusion of  $Na^+$  down its concentration gradient into the lumen. If  $Na^+$  entry is limited in the absence of claudin-16 pores, there would be a reduced electrical gradient to drive  $Mg^{++}$  out. Although this can not be tested in humans, study of isolated perfused nephron segments from claudin-16 KO mice is consistent with a defect in  $Na^+$  permeability (Himmerkus et al. 2008). Whatever the full explanation, claudin-16 is clearly a cation pore and HHNC remains the best example of a disease caused by an ion-selective TJ defect.

It is tempting to also explain hearing loss in the human claudin-14 mutants as an ion selectivity defect, but the evidence is only circumstantial. Claudin-14 is expressed in TJs lining the intrachoclear space, which contains fluid uniquely high in  $K^+$ . This high gradient promotes rapid entry of  $K^+$  into the outer hair cells; this is required for their depolarization during acoustic mechanotransduction. Other forms of deafness are caused by mutations in the transporters that normally secrete  $K^+$  into this space, suggesting that the loss of claudin-14 might cause deafness by allowing the  $K^+$  gradient to dissipate. In fact, when claudin-14 is expressed in cultured MDCK cells, the junctions become very tight by specifically restricting cation permeability (Riazuddin et al. 2006). Finally, claudin-1 mutation results in neonatal ichthyosis-sclerosing cholangitis syndrome (NISCH), but whether this pathophysiology results from a permeability defect is unclear (Hadj-Rabia et al. 2004). Although claudin-1 is very widely expressed among all epithelia, it is difficult to rationalize why patients with these mutations develop predominately a scaling skin disease and obliteration of their bile ducts.

## CONCLUSION

Significant progress has been made over the past decade in understanding the role of claudins in regulation of TER and paracellular ionic

selectivity. In addition, the recognition of the existence of two pathways for paracellular solute flux should allow a more sophisticated analysis of the roles individual TJ proteins play in physiologic and pathologic regulation of permeability of nonionic solutes. However, further insights are hampered by the lack of a three-dimensional model of TJ structure. Studies on the synaptic junction may provide clues and methods for studying TJ organization, but ultimately we will need structural information about the integral membrane proteins of the TJ to ask informative questions about how this complex is organized and physiologically regulated.

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