Mini-Review

Pores in the Wall: Claudins Constitute Tight Junction Strands Containing Aqueous Pores

Shoichiro Tsukita and Mikio Furuse

Department of Cell Biology, Kyoto University Faculty of Medicine, Yoshida-Konoe, Sakyo-ku, Kyoto 606-8501 Japan

In vertebrates, tight junctions (TJs)[1] play a central role in sealing the intercellular space in epithelial and endothelial cellular sheets (Schneeberger and Lynch, 1992; Gumbiner, 1993; Anderson and van Itallie, 1995). Without TJs, these cellular sheets cannot function as barriers to establish compositionally distinct fluid compartments. To maintain homeostasis, various materials must be selectively transported across these cellular sheets. Two distinct pathways are known for this transport: the transcellular and paracellular pathways in which materials move across plasma membranes and TJs, respectively (Spring, 1998; Fig. 1). The molecular machinery involved in transport through the transcellular pathway (i.e., channels, pumps, transporters, etc.) has been identified and well characterized in molecular terms. In sharp contrast, our knowledge regarding paracellular transport is limited mainly because of the lack of information on the molecular architecture of TJs. However, recent identification of TJ-specific integral membrane proteins prompted us to consider the molecular mechanism behind the barrier of TJs as well as transport across TJs.

Fig. 1 shows the structural aspects of TJs. The key structure in TJs is the TJ strand (or fibril) within plasma membranes, which was visualized by freeze-fracture replica electron microscopy (Staehelin, 1973). Each TJ strand laterally and tightly associates with that in the apposing membrane of adjacent cells to form a paired strand, where the intercellular space is obliterated (so-called kissing points of plasma membranes in ultrathin sectional images; Farquhar and Palade, 1963). This paired strand has been suggested to be responsible for the intercellular sealing in epithelial/endothelial cellular sheets. Furthermore, detailed electrophysiological analyses suggested the existence of aqueous pores within the paired TJ strands (Fig. 1; Diamond, 1977; Claude, 1978; Reuss, 1992; Gumbiner, 1993). This mini-review will present an overview of the recent progress in our understanding of the structure and functions of TJ strands in molecular terms.

Claudins Constitute Paired TJ Strands

TJ strands have been suggested by some investigators to be predominantly lipid in nature (i.e., inverted cylindrical lipid micelles; Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982; Verkleij, 1984), but many cell biologists have long searched for proteinaceous components constituting TJ strands. Thus, when occludin, a novel ~65-kD integral membrane protein with four transmembrane domains, was identified as the first component of TJ strands (Furuse et al., 1993; Ando-Akatsuka et al., 1996), this identification was regarded as the Holy Grail in this field (Gumbiner, 1993), and successive studies emphasized the importance of occludin in the structure and functions of TJs (Balda et al., 1996; McCarthy et al., 1996; Chen et al., 1997; Wong and Gumbiner, 1997). However, it has gradually become clear that the molecular architecture of TJ strands is more complex than expected (Balda et al., 1996; Hirase et al., 1997; Moroi et al., 1998). Especially, the fact that the occludin-deficient visceral endoderm cells still bear well developed network of TJ strands indicated that, as yet, unidentified membrane proteins or lipid molecules constitute TJ strands (Saitou et al., 1998).

In 1998, two novel integral membrane proteins (~23 kD) with four transmembrane domains were identified from the junction-enriched fraction as components that copartitioned with occludin (Fig. 2; Furuse et al., 1998a). These proteins, named claudin-1 and -2, were structurally related (~30% identical at the amino acid sequence level), but showed no sequence similarity to occludin. The most characteristic feature of these molecules was that they reconstituted TJs in fibroblasts when singly transfected (Furuse et al., 1998b). The reconstituted TJs were morphologically indistinguishable from those in situ, although they were not zonula but puncta occludens; ultrathin and freeze-fracture replica electron microscopy identified the formation of kissing points and paired strands, respectively, between adjacent transfectants (Furuse et al., 1998b; Kubota et al., 1999).

Another integral membrane protein with a single membrane-spanning domain, named JAM, was shown to be lo-

Address correspondence to Shoichiro Tsukita, M.D., Ph.D., Department of Cell Biology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan. Tel.: 81-75-753-4372. Fax: 81-75-753-4660. E-mail:htsukita@mfour.med.kyoto-u.ac.jp

[1]*Abbreviations used in this paper:* CPE, *Clostridium perfringens* enterotoxin; TJ, tight junction.

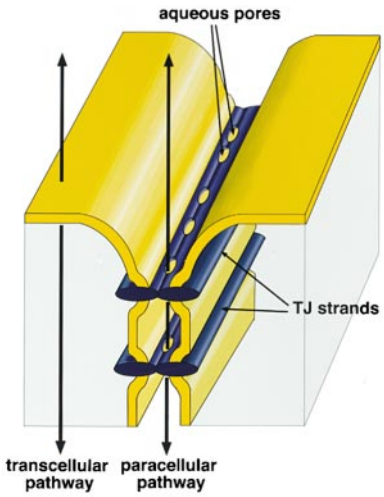

Figure 1. Schematic drawing of TJs. At the most apical region of lateral membranes, TJs occur between two adjacent cells. TJs are composed of paired TJ strands, in which each TJ strand laterally and tightly associates with that in the apposing membrane of adjacent cells. Paired TJ strands are thought to contain aqueous pores. In transcellular and paracellular pathways, materials move across plasma membranes and TJs, respectively.

calized at TJs (Martin-Padura et al., 1998). Although the nature of this molecule has remained elusive, preliminary freeze-fracture replica analyses indicated that this molecule itself has no ability to reconstitute TJ strands in fibroblasts (Itoh, M., unpublished data).

The Claudin Family, A Newly Emerging Gene Family

Identification of claudin-1 and -2 indicated the existence of a novel gene family. To date, 18 members of this claudin family have been identified, and the list of claudins is still increasing (Morita et al., 1999a; Simon et al., 1999; Tsukita and Furuse, 1999). Some of these molecules were previously identified as RVP1 (claudin-3; Briehl and Miesfeld, 1991), CPE-R (claudin-4; Katahira et al., 1997), TMVCF (claudin-5; Sirotkin et al., 1997), and OSP (claudin-11; Bronstein et al., 1996), although their physiological functions have remained unclear. All of these claudin family members have not yet been examined in detail, but the data obtained to date by immunolabeling and/or transfection experiments favored the notion that all members of the claudin family are directly involved in the formation of TJ strands in situ.

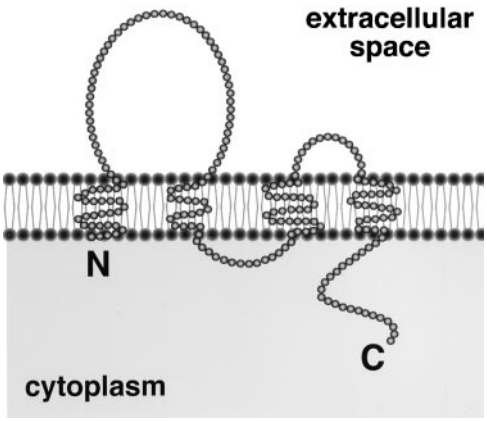

Figure 2. Membrane folding model of claudin-1. Both NH_2 and COOH termini are located in the cytoplasm. Each circle corresponds to each amino acid residue.

Northern blotting showed that tissue distribution patterns of each claudin member are distinct (Furuse et al., 1998a; Morita et al., 1999a). For example, claudin-1 and -2 are expressed at high levels in the liver and kidney, whereas claudin-3 mRNA is detected mainly in the lung and liver. Claudin-4, -7, and -8 are primarily expressed in the lung and kidney. These findings together with those of immunofluorescence microscopy suggested that, in various tissues, >2 species of claudins are coexpressed in single cells. On the other hand, it also became clear that some types of cells express their own specific claudin species. One example is claudin-5/TMVCF, which consists of TJ strands specifically in endothelial cells of blood vessels (Morita et al., 1999c). To date, claudin-5/TMVCF has not been found in nonendothelial cells. Another example is claudin-11/OSP, which is primarily expressed in the brain and testis. This claudin species was shown to constitute TJ strands between lamellae of myelin sheaths of oligodendrocytes in the brain and those between adjacent Sertoli cells in the testis (Morita et al., 1999b). Recently, claudin-11/OSP–deficient mice were successfully generated (Gow et al., 1999). In these mice, TJ strands were absent in myelin sheaths of oligodendrocytes and Sertoli cells, conclusively indicating that, in these types of cells, TJ strands are mainly composed of a single specific claudin, claudin-11/OSP. These findings naturally raised questions regarding the physiological relevance of the existence of so many claudin species. This point will be discussed later.

Claudins Are Functionally Involved in the TJ Barrier

Now that the paired TJ strands can be reconstituted from a single gene product, claudin, the relationship between the TJ strand formation and the TJ barrier function could be experimentally evaluated. However, as noted above, since the reconstituted TJs did not surround individual fibroblast transfectants continuously, the barrier function of these TJs could not be measured using these cellular sheets. Gene knockout is a promising approach to analyze the functions of TJs, as recently shown in claudin-11/OSP–deficient mice (Gow et al., 1999), but it is technically difficult to directly examine the barrier functions of TJs in mice in detail.

Recently, to solve these technical difficulties, the bacterial peptide toxin, *Clostridium perfringens* enterotoxin (CPE), was used (Sonoda et al., 1999). As described above, claudin-4 was initially identified as a CPE receptor (CPE-R), and it was reported that the COOH-terminal half of this peptide (C-CPE) specifically bound to claudin-4/CPE-R (Katahira et al., 1997). When this C-CPE was applied to cultured epithelial cells (i.e., MDCK cells that express mainly claudin-1 and -4), claudin-4 was specifically

removed from TJs with a concomitant decrease in transepithelial electrical resistance as well as in the number of TJ strands. These findings provided concrete evidence of the direct involvement of claudins in the barrier functions of TJs.

We have now concluded that claudins constitute the wall between adjacent epithelial/endothelial cells, which is responsible for sealing in the paracellular pathway. However, as noted above in situ, this wall contains pores, leading to the question of the chemical natures of these pores.

Aqueous Pores within TJ Strands

Analysis of hereditary hypomagnesemia yielded insight that may allow us to answer this question. The renal resorption of Mg^{2+} is known to occur predominantly through the paracellular pathway in the thick ascending limb of Henle but, in these patients, this paracellular flux is blocked, resulting in severe hypomagnesemia. Positional cloning has identified a member of the claudin family (claudin-16/paracellin-1) as a gene responsible for this disease (Simon et al., 1999), and this claudin species was shown to be exclusively expressed in the thick ascending limb of Henle. This finding led to a very intriguing conclusion: claudin-11/paracellin-1 is directly involved in a selective paracellular conductance for Mg^{2+} ions of claudin-based TJ strands.

It is possible that only claudin-16/paracellin-1, but not other claudins, can form pores through its homotypic interaction on the claudin-based wall. However, as the permeability properties of individual paired TJ strands appear to be fairly variable in different epithelia (Diamond, 1977; Gumbiner, 1993), it is more likely that most claudin species can constitute not only the wall, but also the pores in the wall. Recent detailed analyses of the manner of interaction of heterogeneous claudin species within and between TJ strands provided important information to clarify this point (Furuse et al., 1999). (1) When two of claudin-1, -2, and -3 were coexpressed in L fibroblasts, claudins were copolymerized into individual TJ strands (heteropolymers) in any combination (Fig. 3 a). (2) When two of the L transfectants singly expressing claudin-1, -2, or -3 were cocultured, claudin-3–based strands (homopolymers) laterally associated with claudin-1– or claudin-2–based strands (homopolymers) in a heterotypic manner, but such heterotypic paired strands were not formed between claudin-1– and claudin-2–based strands (Fig. 3 b). (3) When L transfectants singly expressing claudin-1 were cocultured with those coexpressing claudin-1 and -2, claudin-1 homopolymers laterally associated with claudin-1/2 heteropolymers to form paired strands (Furuse, M., and S. Tsukita unpublished data; Fig. 3 c). These observations suggested a possible explanation as to how claudins constitute the wall and the pore simultaneously. In Fig. 4, two types of paired strands are supposed, claudin-1/3–based and claudin-1/2–based paired strands, in which individual strands, i.e., heteropolymers consisting of claudin-1 and -3 or claudin-1 and -2, respectively, are laterally associated. In the former type of paired strands, claudin-1 and -3 could adhere both in homotypic and heterotypic manners (Fig. 3 b). However, in the latter type, claudin-1 could not adhere with claudin-2 in a heterotypic manner, resulting in the formation of pores within paired strands. This is purely speculative at this stage, but as in

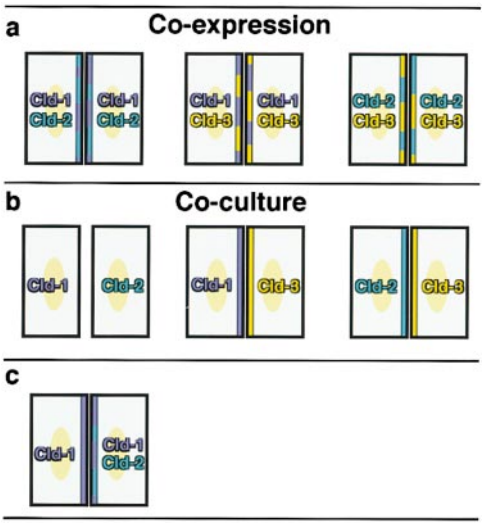

Figure 3. Manner of interaction of heterogeneous claudin species within and between TJ strands. (a) L transfectants coexpressing two of claudin-1, -2, and -3 were cultured. (b) Two of the L transfectants singly expressing claudin-1, -2, or -3 were cocultured. (c) L transfectants expressing claudin-1 were cocultured with those coexpressing claudin-1 and -2.

situ TJ strands are composed of various combinations of at least 18 claudin species, it is tempting to hypothesize that the tightness of paired TJ strands is determined by the number/type of species of claudins and their mixing ratio in strands.

Perspective

Individual TJ strands (paired TJ strands) differ in permeability depending on cell type. For example, as a model system, the tightness of MDCK cells has been compared between type I and II clones; MDCK I cells have a fairly tighter TJ barrier than MDCK II cells, but no significant difference was detected in the number of TJ strands between these two clones (Stevenson et al., 1988). In this mini-review, we proposed a model that may explain the variation of the tightness of individual paired TJ strands based on the combination of claudin species. This model may be oversimplified. In most of the TJ strands in situ, occludin is incorporated in the claudin-based strands (Fujimoto, 1995; Furuse et al., 1998b). It is not clear how claudins and occludin are arranged to form individual TJ strands. Furthermore, the information is not available re-

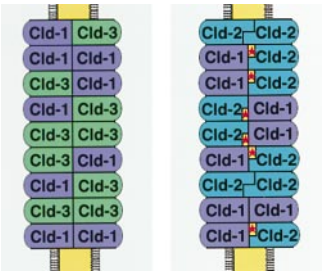

Figure 4. Two types of paired strands: one composed of claudin-1 and -3 (left), and the other composed of claudin-1 and -2 (right). Asterisks, putative pores. See text for details.

garding the stability of individual TJ strands. If TJ strands are repeatedly broken and annealed dynamically, their stability would also be an important determinant of the tightness of individual strands. In this connection, the interaction between claudins/occludin and the underlying cytoskeleton should be analyzed in detail (Anderson and van Itallie, 1995; Itoh et al., 1999; Tsukita et al., 1999).

The tightness of TJs is determined not only at the level of individual strands, but also at that of strand networks (Claude and Goodenough, 1973; Madara and Dharmsathaphorn, 1985). The number of strands as well as the frequency of their ramification have been shown to be important factors to determine the tightness of TJs. However, it remains unclear as to what regulates these factors in molecular terms. As the network pattern of reconstituted claudin-based strands in L fibroblasts differs significantly depending on claudin species, the combination and the mixing ratio of claudin species appear to also be crucial in regulating the tightness of TJs at the level of the network (Furuse et al., 1998b; Morita et al., 1999b,c). In this connection, the regulation of individual claudin species should be examined in detail not only at the transcriptional, but also at the posttranscriptional levels. We are now in a position to begin exploring the molecular mechanism behind the determination and regulation of tightness of TJs (i.e., the transport of materials across TJs).

Submitted: 31 January 2000
Revised: 18 February 2000
Accepted: 23 February 2000

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