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# Tight Junctions of the Blood–Brain Barrier

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**SUMMARY**

1. The blood–brain barrier is essential for the maintenance and regulation of the neural microenvironment. The blood–brain barrier endothelial cells comprise an extremely low rate of transcytotic vesicles and a restrictive paracellular diffusion barrier. The latter is realized by the tight junctions between the endothelial cells of the brain microvasculature, which are subject of this review. Morphologically, blood–brain barrier-tight junctions are more similar to epithelial tight junctions than to endothelial tight junctions in peripheral blood vessels.

2. Although blood–brain barrier-tight junctions share many characteristics with epithelial tight junctions, there are also essential differences. However, in contrast to tight junctions in epithelial systems, structural and functional characteristics of tight junctions in endothelial cells are highly sensitive to ambient factors.

3. Many ubiquitous molecular constituents of tight junctions have been identified and characterized including claudins, occludin, ZO-1, ZO-2, ZO-3, cingulin, and 7H6. Signaling pathways involved in tight junction regulation comprise, among others, G-proteins, serine, threonine, and tyrosine kinases, extra- and intracellular calcium levels, cAMP levels, proteases, and TNF$\alpha$. Common to most of these pathways is the modulation of cytoskeletal elements which may define blood–brain barrier characteristics. Additionally, cross-talk between components of the tight junction– and the cadherin–catenin system suggests a close functional interdependence of the two cell–cell contact systems.

4. Recent studies were able to elucidate crucial aspects of the molecular basis of tight junction regulation. An integration of new results into previous morphological work is the central intention of this review.

**KEY WORDS:** tight junction; blood–brain barrier; morphology; freeze–fracture; cadherins; catenins; occludin; cytoskeleton.

## INTRODUCTION

Homeostasis of the microenvironment in the neuronal parenchyma is essential for normal function of the brain. The structure responsible for this homeostasis is called the blood–brain barrier which protects the neuropil against neurotoxic compounds, the considerable variations in the composition of the blood, and the breakdown of concentration gradients between blood and brain. The barrier includes the endothe-

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lial blood–brain barrier on one side and the blood–cerebrospinal fluid barrier on the other. A common feature of all subtypes of the plasma–brain interstitial fluid barrier is the elaborate network of complex tight junctions (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Nabeshima *et al.,* 1975; van Deurs and Koehler, 1979; Møllgård and Saunders, 1986; Rascher and Wolburg, 1997). Tight junctions have been investigated morphologically by freeze–fracturing and ultrathin sectioning and physiologically by measurements of paracellular permeability and transepithelial/transendothelial electrical resistance.

It has been suggested that there is a logarithmic relationship between the number of tight junction strands and the transepithelial electrical resistance (Claude, 1978; Claude and Goodenough, 1973). It was concluded that the complexity of the network of strands could be used for prediction of the physiological parameters permeability and transepithelial electrical resistance (Marcial *et al.,* 1984). Most of the studies on tight junctions were performed in epithelial cells. However, Nagy *et al.* (1984) convincingly showed that the endothelial cells of brain capillaries, as the site of the blood–brain barrier, possess the most complex tight junctions in the vascular system, which follows the prediction of the model of Claude (1978).

There is unequivocal evidence for both the lipidic (Hein *et al.,* 1992; Grebenkämper and Galla, 1994) and the proteinaceous (van Meer *et al.,* 1996; van Meer and Simons, 1986) nature of tight junctions, so it is quite surprising that an integrative model for tight junctions is still lacking. Transmembrane components of the tight junction, occludin and claudin-1 and -2, were finally identified and characterized (Furuse *et al.,* 1993, 1998; Ando-Akatsuka *et al.,* 1996). Although the molecular structure of tight junctions generally appears to be similar in all barrier systems, there are some differences between epithelial and endothelial tight junctions, on one hand, and between tight junctions of peripheral and blood–brain barrier endothelia, on the other hand. For example, blood–brain barrier endothelial cells differ from epithelial cells by the intercalation of components of adhesion and tight junctions and reveal cadherins along the entire intercellular cleft (Schulze and Firth, 1993). Despite the presence of many similar junctional components in epithelial and endothelial cells, the tight junctions differ in terms of morphology, regulation, and determination of their barrier characteristics.

## STRUCTURAL BASIS OF TIGHT JUNCTIONS

### Morphology of Non-Blood–Brain Barrier Tight Junctions

In conventional ultrathin sections, the tight junctions are considered to form pentalaminar layers that result from the fusion of the external leaflets of the partner cell membranes. Depending on the orientation of the section, the tight junctions mostly appear as a chain of fusion (''kissing'') points or as a domain of an occluded intercellular cleft of variable length (Farquhar and Palade, 1963; Brightman and Reese, 1969). In contrast to ultrathin sections, the freeze–fracture technique allows the investigation of the microarchitecture in the plane of the membrane. Tight junctions are quite variable considering their fracture properties in terms of their

association with the one or the other membrane leaflet. After conventional fixation using glutaraldehyde, epithelial tight junctions are associated predominantly with the protoplasmic fracture face (P-face) (Fig. 1b), forming a network of strands and leaving grooves at the external fracture face (E-face) which are occupied by very few particles (see, e.g., Bentzel *et al.,* 1980; Martinez-Palomo *et al.,* 1980; Griepp *et al.,* 1983; Madara and Dharmsathaphorn, 1985; Noske and Hirsch, 1986; Kniesel and Wolburg, 1993).

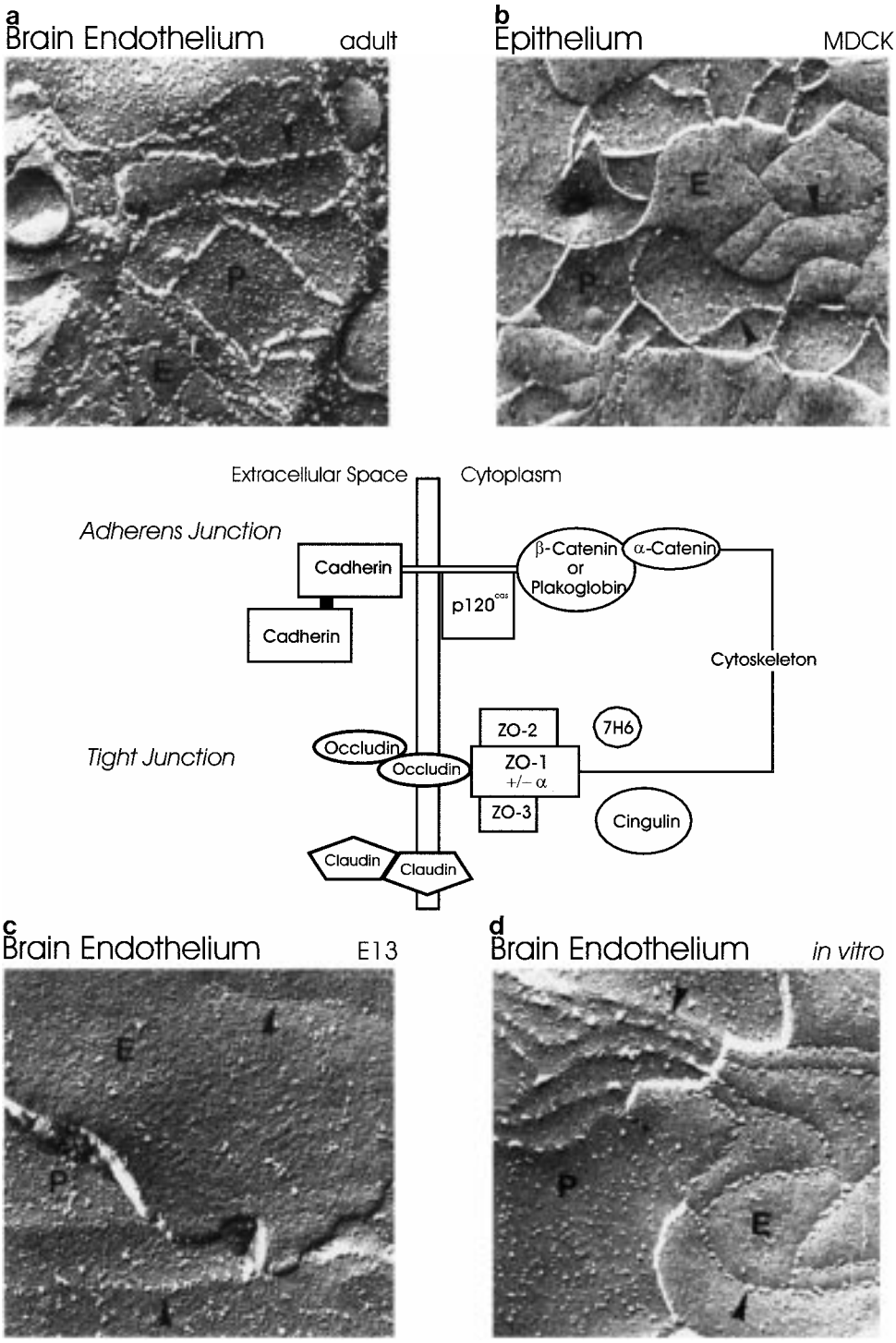
Conspicuously, most published freeze–fracture data on tight junctions show that when particles occur at the E-face, they are arranged in chains; when occurring at the P-face, they frequently, at least in epithelia, are formed as smooth continuous cylindrical profiles. This difference may be explained by the hypothesis that the discontinuous and irregular appearance of tight junction particles on E-faces are due to multiple linkage sites of protein complexes to the cytoskeleton (Suzuki and Nagano, 1991; Lane *et al.,* 1992), and it seems reasonable that the continuous P-face-associated strands rather may represent a lipidic metastructure. Generally, it is believed that the cylindrical profiles of tight junctions consist of double strands which are arranged in an offset manner and that the fracture plane runs in between the partner strands (Hirokawa, 1982; Lane *et al.,* 1992).

In cultured epithelial cells, high electrical resistance and low permeability are in concert with a stable tight junction morphology, which is identical to the situation *in vivo* (Griepp *et al.,* 1983; Madara and Dharmsathaphorn, 1985; Gonzales-Mariscal *et al.,* 1985; Gumbiner and Simons, 1986). Although Stevenson *et al.,* (1988) stressed that tight junctions of low- and high-resistance Madin Darby canine kidney (MDCK) cells are morphologically identical, the depicted tight junction strands of low-resistance cells were discontinuous at the P-face revealing particles on the E-face, whereas the tight junctions of high resistance cells were highly P-face-associated with almost no particles on the E-face. Accordingly, Mandel *et al.,* (1993) and Bacallao *et al.* (1994) described MDCK cells after ATP depletion to suffer from deterioration of both paracellular barrier function and P-face association of the tight junctions, which is accompanied by a reorganization of the actin cytoskeleton.

A different type of tight junction is represented by the endothelial cells of the leaky peripheral vasculature. The complexity of the tight junction network is low; there are many open ends and few anastomosing strands (Simionescu *et al.,* 1976; Hüttner and Peters, 1978). In addition, the particles of the tight junctions are associated predominantly with the E-face (Fig. 1b); this property is maintained also in culture (Fallier-Becker *et al.,* 1991). On the P-face, the tight junctions can be recognized only by ridges poor in particles (Simionescu *et al.,* 1976). We conclude that the anchorage of the tight junction particles to the cytoskeleton is too weak—even after chemical fixation—to avoid the disruption of cytoskeletal elements from the tight junction particles. In conclusion, it is generally not advisable to correlate structure and function if comparing tight junctions of different origin.

## Morphology of Blood–Brain Barrier Tight Junctions

In freeze–fracture replicas, the blood–brain barrier tight junctions of mammalian species are characterized first of all by the highest complexity found in the


a
Brain Endothelium
adult
b
Epithelium
MDCK
E
P
E
P
Extracellular Space
Cytoplasm
Adherens Junction
Cadherin
Cadherin
β-Catenin
or
Plakoglobin
α-Catenin
p120cas
Cytoskeleton
Tight Junction
Occludin
Occludin
ZO-2
7H6
ZO-1
+/– α
ZO-3
Cingulin
Claudin
Claudin
c
Brain Endothelium
E13
d
Brain Endothelium
in vitro
E
P
P
E

vasculature of the body (Nagy *et al.,* 1984). In addition, the P-face association of blood–brain barrier tight junctions is high (approx. 55%) compared to that observed in endothelial cells of peripheral blood vessels (approx. 10%; Figs. 1a and d). The altered particle distribution in brain microvessel tight junctions may be indicative of a strong tight junction–cytoskeleton interaction.

Interestingly, the blood–brain barrier endothelial tight junctions in submammalian species are associated almost completely with the P-face and are therefore reminiscent of the epithelial type of tight junctions (Shivers, 1979; Nico *et al.,* 1992; Gerhardt *et al.,* 1996). Morphologically, in these species there is no difference between endothelial and glial (epithelial) types of the blood–brain or blood–cerebrospinal fluid barrier. The phylogenetically ancient elasmobranchs show a glial blood–brain barrier (Bundgaard and Cserr, 1981) and, also, teleosts still have, in addition to an endothelial blood–brain barrier, tight junctions in astroglial and ependymal cells (Sandri *et al.,* 1978; Wolburg *et al.,* 1983). The tanycytes of the circumventricular organs including the choroid plexus epithelium can be regarded as a late ''remnant'' of this old glial barrier type which also in mammals have retained to epithelial type of tight junctions (van Deurs and Koehler, 1979; Mack *et al.,* 1987). Thus, there is a segregation of glial barrier from endothelial barrier tight junctions. The first ones conserve the ancient, glial, or epithelial type of blood–brain barrier tight junctions; the second ones represent a novel type of blood–brain barrier tight junctions, which is marked by an altered equilibrium between the adhesion of tight junction molecules in the intercellular cleft and the anchorage of these molecules in the cytoplasm.

## Modulation of Blood–Brain Barrier Tight Junctions During Development

One of the most important landmarks of developing microvasculature in the rodent brain are the disappearance of fenestrations and the appearance of tight junctions in the endothelium between E11–13 (Bauer *et al.,* 1993; Stewart and Hayakawa, 1994). Early embryonic brain capillaries are still permeable to substances excluded from the neuronal milieu in the adult (Fabian and Hulsebosch, 1989; Johanson, 1980; but compare with Saunders, 1992; Saunders *et al.,* 2000). The external blood–brain barrier of pial vessels in rat embryos up to E20 shows low transendothelial electrical resistance (Butt *et al.,* 1990) and therefore can be regarded

**Fig. 1.** (Opposite) Tight junctions as visualized by the freeze–fracture technique. E, E-face; P, P-face. Arrowheads indicate tight junction structures. The illustration of the molecular composition of the tight and adherens junction region is simplified. The bar is 0.01 $\mu$m for all micrographs. (a) Mature endothelial tight junctions from brain capillaries *in situ.* P-face association of tight junction particles is predominant, which suggests a strong interaction with the cytoskeleton. $a^-$ and $a^+$ isoforms of ZO-1 are expressed. (b) Tight junctions of the high-resistance strain of MDCK cells cultured for 11 days. P-face association of tight junction particles is highly predominant, which suggests a strong interaction with the cytoskeleton. $a^-$ and $a^+$ isoforms of ZO-1 are expressed. (c) (lower left) Premature (E13) endothelial tight junctions from brain capillaries *in situ.* Almost no tight junction particles were found on either the E- or the P-face; it is not known which isoforms of ZO-1 are expressed in the earliest stages of tight junction formation in blood–brain barrier endothelial cells. (d) Tight junctions from brain endothelial cells, after 10 days in culture. E-face association of tight junction particles is predominant, which suggests a weak attachment of tight junction particles to the cytoskeleton. The $a^-$ isoform of ZO-1 is exclusively expressed.

as immature. Also, in the chick cerebellum and spinal cord, a stepwise progression of the endothelial barrier to HRP from the superficial to the medullary region has been described between E12 and E15 (Wakai and Hirokawa, 1978). In the developing mouse, Stewart and Hayakawa (1987) demonstrated a gradual decline of both the permeability index (defined as the ratio of brain/plasma HRP activity divided by the blood vessel density) and the interendothelial cleft index (defined as the proportion of the junctional profile that is composed of junctional clefts). Schulze and Firth (1992) more closely characterized the maturation of the blood–brain barrier in the rat as an increase in the ratio of ''narrow zones'' to ''wide zones'' in the interendothelial clefts. Similarly, tight junctional membrane domains of pial microvessels narrowed over developmental periods. However, it is worth mentioning that in one group of pial vessels endothelial junctions remain separated by a small (2.8 nm) cleft, and in another group junctional membranes fuse (Cassella *et al.,* 1997).

At E13, tight junction particle density as evaluated by freeze–fracturing of rat brain endothelial cells was found to be extremely low on both membrane leaflets (Fig. 1c). We have proposed that the basic structure of a tight junction network consisting of membrane grooves and ridges on the E- and P-face of low complexity represent preformations of tight junctions (Kniesel *et al.,* 1996). This hypothesis is also supported by our finding of continuous grooves on the E-face in combination with corresponding discontinuities of the P-face strands in developing or cultured retinal pigment epithelium (unpublished data). Thus, membrane grooves appear not only to be caused by particles leaving the E-face during freeze–fracturing, as it is known from E-face pits of gap junctions.

During rat brain development, association of tight junction particles is also altered in the cerebral capillaries from a predominant E-face association in stages E15 and E18 to a predominant P-face association in P1 and adult. A high degree of E-face association is documented also for human embryos (Møllgård and Saunders, 1986) but was not discussed by the authors. The predominant P-face association commencing between E18 and P1 is in good agreement with the rapid increase in the transendothelial electrical resistance found in pial vessels of the rat at E21 (Butt *et al.,* 1990).

## Modulation of Blood–Brain Barrier Tight Junctions in Cell Culture

For an investigation on the induction and regulatory mechanisms of the blood–brain barrier, *in vitro* models of the blood–brain barrier have been established (see, e.g., Méresse *et al.,* 1989; Rubin *et al.,* 1991; Tontsch and Bauer, 1991; Abbott *et al.,* 1992; Wolburg *et al.,* 1994). Some studies have shown that astrocytes or related neuroepithelial cells participate in the induction of barrier properties in endothelial cells (for review, see Wolburg and Risau, 1995). Arthur *et al.* (1987) and Wolburg *et al.* (1994) provided evidence for the release of humoral factors by astrocytes which were suggested to contribute to tight junction formation. In contrast, Tao-Cheng *et al.* (1987) found a direct contact between astrocytes and endothelial cells required.

It is evident that the paracellular barrier is less elaborate in cultured cells.

Whereas the tight junction complexity of capillary fragments was maintained or reinduced in cultured endothelial cells by certain treatments the P-face association could never be restored to *in vivo* levels and the modulation of P-face association correlated well with the physiological measurements (Wolburg *et al.,* 1994), suggesting an important role for the P-face association at least as an indicator for a functional blood–brain barrier. The low P-face association of tight junctions in untreated cultured blood–brain barrier endothelial cells is identical to that in endothelial cells in extracerebral blood vessels *in vivo* and *in vitro.* As discussed before, the degree of P-face association may reflect the state of interaction between cytoskeletal and tight junction components, although the molecular mechanisms of P-face association determination are still enigmatic.

## Molecular Organization of Blood–Brain Barrier Tight Junctions

At first glance, the molecular constitution of blood–brain barrier tight junctions as characterized to date is quite similar to that found in epithelial tight junctions (for review, see Anderson and van Itallie, 1995; Balda and Matter, 1998; Mitic and Anderson, 1998). This might be surprising, since the dynamics of tight junction synthesis, the regulatory sensitivity to extrinsic factors and the morphological appearance of epithelial and endothelial blood–brain barrier-tight junctions differs in major aspects.

Several tight junction-associated protein components have been identified. The tight junction components occludin (Furuse *et al.,* 1993; Ando-Akatsuka *et al.,* 1996), claudin-1 and -2 (Furuse *et al.,* 1998), ZO-1 (Stevenson *et al.,* 1986), ZO-2 (Jesaitis and Goodenough, 1994), ZO-3/p130 (Balda *et al.,* 1993; Haskins *et al.,* 1998), 7H6 (Zhong *et al.,* 1994), and cingulin (Citi *et al.,* 1989) were detected in epithelial as well as in endothelial blood–brain barrier tight junctions, whereas symplekin was exclusively found at the junctional zone in epithelial cells (Keon *et al.,* 1996).

## The Transmembranous Tight Junction-Constituent Occludin

Occludin was initially isolated from junction-enriched membrane fractions of the chick liver as a transmembranous tight junction protein of approx. 65 kDa (Furuse *et al.,* 1993), which exists in several isoforms. Four membrane-spanning $\alpha$-helices were deduced from hydrophobicity plots. In consequence, there are putatively two extracellular loops, whereas the amino and carboxy termini are both located intracellularly. Occludin was expected be a constituent of the tight junction strands or particles, which indeed was confirmed at the electron microscopic level by immunogold localization of occludin at tight junction kisses in ultrathin sections (Furuse *et al.,* 1993) as well as on freeze–fracture replicas (Fujimoto, 1995; Hirase *et al.,* 1997; Saitou *et al.,* 1997).

Occludin shows high interspecies variability between chicken and mammals (Ando-Akatsuka *et al.,* 1996), sharing less than 50% identity in amino acid sequence. In contrast, human, murine and canine occludins are more closely related, showing approximately 90% identity. Beside the high content of tyrosine and glycine in the first extracellular loop (approx. 60%), the most conserved region of occludin

comprises the carboxy terminal ZO-1 binding domain, an $\alpha$-helical coiled coil structure, putatively linking occludin to the cytoskeleton.

Low quantities of occludin were detected by immunofluorescence also during blood–brain barrier development in guinea pig at postnatal day 8 (P8), whereas occludin was found to be strongly expressed at P70 (Hirase *et al.,* 1997). This finding was quite unexpected, since well elaborated, although not mature, tight junctions were demonstrated by freeze–fracturing as early as embryonic day 15 (E15) in rat blood–brain barrier endothelial cells (Kniesel *et al.,* 1996). Moreover, the transendothelial electrical resistance of pial vessels in the rat indicates a functional blood–brain barrier from E20 (Butt *et al.,* 1990), when tight junctions are morphologically mature. Actually, occludin could be demonstrated at premature tight junctions of the rat blood–brain barrier as early as E15 on the electron microscopic level (Kniesel *et al.,* unpublished data). It is not clear if this discrepancy is due to an altered molecular composition of tight junctions, interspecies variability, or methodological approaches.

Epitope-blocking experiments using transepithelial electrical resistance and permeability as functional indicators clearly show the second extracellular loop of occludin to be crucial for the maintenance of the paracellular barrier (Wong and Gumbiner, 1997). A newly discovered feature of occludin is a calcium-independent adhesiveness (Van Itallie and Anderson, 1997), which is mediated by the first extracellular loop of occludin and depends on the presence of the submembranous tight junction component zonula occludens protein 1 (ZO-1). Under low calcium conditions, ZO-1 and occludin were shown to colocalize in cytoplasmic vesicles and also partly at intercellular clefts in MDCK cells. The remaining membrane-bound complexes may be sufficient for the retained adhesive function of occludin, while barrier properties are lost (McCarthy *et al.,* 1996; Van Itallie and Anderson, 1997).

In transfection experiments by Furuse *et al.,* (1996) using insect-derived Sf9 cell lines, occludin was not transferred to the cell membrane, instead occludin-positive multilamellar bodies were induced. Since ZO-1, which is normally directly linked to the carboxy terminus of occludin (Furuse *et al.,* 1994), is absent in Sf9 cells, it was attributed as a prerequisite for occludin to be targeted to cellular membrane and directed to its final destination in the intercellular cleft. This hypothesis is supported indirectly (a) by the immunolocalization of ZO-1 in the developing tight junctions of rat brain capillaries at a stage, when tight junction particles are still lacking in freeze–fracture replicas (Kniesel, unpublished data), and (b) more directly by occludin transfection experiments. In these experiments, fibroblast cell lines were used which either do or do not express ZO-1-containing adherens-like junctions but, in any case, express no endogenous occludin (Van Itallie and Anderson, 1997). Only in the presence of ZO-1 and adherens junction components, occludin was targeted to cellular membranes (Van Itallie and Anderson, 1997).

A study using a chimeric protein comprising only the cytoplasmic domain of occludin could demonstrate that this part of occludin contains a basolateral targeting signal and is able to mediate endocytosis (Matter and Balda, 1998). Additionally, it could be shown that occludin was initially inserted in the basolateral membrane region during tight junction genesis.

On the other hand, endogenous occludin in MDCK cells, transfected with

carboxy-truncated forms of chicken occludin, which lack the ZO-1 binding region, surprisingly was transferred to the tight junctions (Balda *et al.,* 1996). The first evidence to solve this virtual contradiction came from experiments with carboxy-truncated mutants of occludin in early *Xenopus* embryos. It could be shown that exogenous occludin oligomerizes with endogenous occludin *in vivo* during tight junction assembly (Chen *et al.,* 1997). Furthermore, the carboxy terminus of occludin proved to be essential for barrier function in *Xenopus,* since tight junctions containing mutant occludin were leaky (Chen *et al.,* 1997).

Molecular mechanisms of tight junction modulation are mostly enigmatic until now. Occludin itself is an excellent candidate as a regulatory target. In MDCK cell lines, occludin exists in multiple phosphorylation states. It could be shown that mainly serine phosphorylation efficiently stabilizes occludin in its membrane-bound location (Sakakibara *et al.,* 1997). In consequence, serine phosphorylation at least determines specificity and stability of membrane-associated occludin.

Hitherto, occludin has been assumed to be essential for tight junction integrity. But unexpectedly, occludin-deficient embryonic stem cells formed intact tight junctions which completely resemble wild-type tight junctions in freeze–fracture replicas (Saitou *et al.,* 1998). Accordingly, the authors concluded that occludin is not the main structural component of tight junctions. Other molecules such as the recently found claudins (Furuse *et al.,* 1998) may be responsible for the integrity of the tight junctions. Occludin seems to act more in a yet undefined regulatory context than as a major structural tight junction protein.

## Submembranous Tight Junction-Associated Proteins

ZO-1, a 220-kDa phosphoprotein, was the first tight junction-associated protein identified and characterized (Stevenson *et al.,* 1986). Although its localization to the tight junction is not exclusive (Howarth *et al.,* 1992; Itoh *et al.,* 1993; Aaku-Saraste *et al.,* 1996), there is no tight junction without ZO-1. In cellular systems with less elaborate or no tight junctions at all, ZO-1 is found enriched in regions of the adherens junctions (Itoh *et al.,* 1993), where it may interact with components of the cadherin–catenin system (Rajasekaran *et al.,* 1996, Itoh *et al.,* 1997). Its expression level and localization does not correlate with the physiological efficiency of the paracellular barrier function (Stevenson *et al.,* 1988).

ZO-1 exists in several splice variants. Two of these variants, $a^+$ or $a^-$ were thought to be characteristic for the ''epithelial'' or the ''endothelial'' type of tight junction, respectively (Willot *et al.,* 1992; Balda and Anderson, 1993). It could be demonstrated that the banding pattern of ZO-1 differed in brain and aorta endothelial cells *in vitro,* whereas the ZO-1-pattern of epithelial LLC-PK1 cells seemed to be similar to that found in brain endothelial cells (Hirase *et al.,* 1997). Thus, functional relevance for the specific distribution of the ZO-1 $a^+$ and $a^-$ isoforms is likely. Possibly, there is a correlation between the morphological tight junction–parameter P-face association and the expression of $a^+$ isoforms of ZO-1. It is tempting to speculate that $a^+$ isoforms stabilize the interaction between cytoskeletal and junctional components in a way that intracellular binding forces exceed those in the intercellular space. In consequence, tight junction particles tend to associate pre-

dominantly with P-faces. The strong intracellular anchoring obviously correlates with physiological barrier properties (Wolburg *et al.,* 1994; Kniesel *et al.,* 1996; Hirase *et al.,* 1997).

ZO-2, a 160-kDa protein, turned out as an ubiquitous component of epithelial and endothelial tight junctions (Jesaitis and Goodenough, 1994). Unlike ZO-1, ZO-2 is restricted exclusively to the tight junction region and is, for example, not found in the fascia adherens of cardiac myocytes as was shown for ZO-1 before (Itoh *et al.,* 1993). The functional significance of ZO-2 is not clear, but like ZO-1 it gets phosphorylated on tyrosine residues after EGF induction or v-src transfection (Van Itallie *et al.,* 1995; Takeda and Tsukita, 1995).

Recently, a protein p130 known to coprecipitate with the ZO-1/ZO-2 complex (Balda *et al.,* 1993) was characterized in detail and named ZO-3 in order to emphasize its close homology to ZO-1 and ZO-2. ZO-3 was demonstrated to bind occludin and ZO-1 directly, but not ZO-2 (Haskins *et al.,* 1998).

ZO-1, ZO-2, and ZO-3 are members of the family of membrane-associated guanylate kinase homologues (MAGUKs) (Willott *et al.,* 1993; Jesaitis and Goodenough, 1994; Haskins *et al.,* 1998), whose elements may play an important role in signal transduction (Woods and Bryant, 1991; Anderson, 1997). MAGUK proteins share at least three defining core domains: a SH3 (src-homology 3) domain, a guanylate kinase, and a PDZ domain. The latter is named after the postsynaptic density-95 protein (PSD), the *Drosophila* lethal Dlg (disk large) tumor suppressor gene product, and ZO-1.

Commonly, SH3 domains bind signaling proteins and/or cytoskeletal elements, guanylate kinases catalize the ATP-dependent transformation of GMP to GDP, but the homologous domains in some MAGUKs are enzymatic inactive since binding sites for either ATP and/or GMP are lacking. PDZ domains are known to mediate specific binding to carboxy-terminal cytoplasmic ends of transmembrane proteins. Recently, ZO-1 could be demonstrated to interact with the gap junction protein connexin 43 via its second PDZ domain (Giepmans and Moolenaar, 1998). Binding of MAGUKs to the cytoskeleton could be demonstrated for p55 and hDlg via the band 4.1-protein (Lue *et al.,* 1994) and for ZO-1 via fodrin (Itoh *et al.,* 1993).

## Peripherally Tight Junction-Associated Proteins

Cingulin was identified as a more peripherally located, ubiquitous tight junction component. Cingulin exists as coiled–coil dimers in two isoforms of 108 and 140 kDa and is phosphorylated on serine residues (Citi *et al.,* 1989). The phosphorylation state of cingulin in MDCK cells could not be influenced by treatments with the phorbol ester PMA, the protein kinase C inhibitor H7, or the calcium chelator EGTA, indicating that cingulin phosphorylation is not directly targeted by cAMP, protein kinase C, or calcium-mediated pathways (Citi and Denisenko, 1995). On the other hand, protein kinase C inhibition by H7 prevents localization of cingulin at cell–cell contacts and generally reduces the amount of cingulin after tight junction reassembly *in vitro* (Denisenko *et al.,* 1994; Citi and Denisenko, 1995; Denker and Nigam, 1998).

Another tight junction-associated protein is the 7H6 antigen, a protein with a

molecular mass of 155–175 kDa. 7H6 is expressed at epithelial and endothelial tight junctions (Zhong *et al.,* 1994; Satoh *et al.,* 1996) and its localization as well as its phosphorylation state was found to correlate with barrier function (Zhong *et al.,* 1994; Satoh *et al.,* 1996).

## MODULATION OF TIGHT JUNCTION CHARACTERISTICS AND SECOND-MESSENGER PATHWAYS

### Regulation of Tight Junction *De Novo* Formation

Low concentrations ($<4\ \mu M$) of extracellular calcium causes the loss of tight junctions, paracellular barrier function, and cell polarity, as well as the dislocation of tight junction-associated proteins in cultured epithelial and endothelial cells (D'Angelo Siliciano and Goodenough, 1988). Barrier properties are reinduced within hours by the addition of calcium as indicated by the reestablishment of a complex tight junction network, accompanied by an increasing transcellular electrical resistance (Gonzales-Mariscal *et al.,* 1990; Contreras *et al.,* 1992).

During calcium-depletion experiments using blood–brain barrier endothelial cells, tight junction complexity is grossly reduced and P-face association of tight junction particles predominantly switch to E-faces (Kniesel, unpublished data) before tight junction particles almost totally disappear, leaving only grooves on E-faces and ridges on P-faces (Kniesel *et al.,* 1996). These steps in tight junction disassembly seem to reverse tight junction-genesis in blood–brain barrier endothelial cells during development in the rat cortex (Kniesel *et al.,* 1996). Although intracellular calcium levels were also modulated during variation of extracellular calcium, which in turn may influence protein kinase C (Stuart *et al.,* 1994), the major calcium effects are likely to be exerted via extracellular calcium binding sites. This notion is supported by the finding that delay of calcium influx by application of $La^{3+}$ has no further impact on the kinetics of the developing transcellular electrical resistance (Contreras *et al.,* 1992).

Nevertheless, the possibility cannot be excluded that variations of extracellular calcium levels may additionally have impact on the transformation of lipid organization from lamellar bilayer phases to hexagonal micelles, and vice versa, thereby modulating a putatively lipidic sealing component of tight junctions (Hein *et al.,* 1992; Grebenkämper and Galla, 1994).

The calcium-sensitive cadherins were suspected of playing a role in calcium-mediated barrier-modulation. Indeed, functional blocking of extracellular epitopes of E-cadherin/uvomorulin in MDCK cells show inhibitory effects on tight junction reformation (Gumbiner *et al.,* 1988; Gumbiner and Simons, 1986). These effects on the tight junction barrier may be either a secondary phenomenon, caused by the loss of intercellular adhesion or a consequence of directly coupled intracellular signaling cascades of the adherens junction and the tight junction system. In fact, ZO-1 could be localized at adherens junction structures in cellular systems with poorly developed or completely absent tight junctions (Itoh *et al.,* 1993; Jesaitis and Goodenough, 1994).

Furthermore, another specific relationship between ZO-1 and the cadherin–catenin system was found in cells devoid of tight junctions expressing ZO-1 mutants *in vitro*. ZO-1 acted as a linker between $\alpha$-catenin and the actin cytoskeleton (Itoh *et al.,* 1997). The functional role with respect to cellular adhesion is unclear, since ZO-1 is exclusively found in the tight junction region in cells possessing well-elaborated tight junctions (Itoh *et al.,* 1993).

A direct interaction between ZO-1 and adherens junction components could be demonstrated in MDCK cells during early stages of tight junction reassembly *in vitro*. But the early complex of ZO-1 with $\alpha$-, $\beta$-, and $\gamma$-catenin is no longer detected after the establishment of the tight junction network (Rajasekaran *et al.,* 1996), suggesting transient ''catenin-storage sites'' for ZO-1 rather than regulatory functions of ZO-1 for cellular adhesion. This notion is supported by the alternative binding of E-cadherin or ZO-1 to $\beta$-catenin complexes, since also $\beta$-catenin–E-cadherin complexes were found devoid of ZO-1.

Tyrosine phosphorylation of ZO-1 (to a lesser extent, also, of ZO-2 and ZO-3/p130) and $\beta$-catenin reversibly accompanies the decline in transepithelial electrical resistance after inhibition of tyrosine phosphatases by phenylarsine oxide in MDCK cells (Staddon *et al.,* 1995; Staddon and Rubin, 1996). In the epithelial cell line A431, EGF-induced ZO-1 phosphorylation on tyrosine residues leads to redistribution and colocalization of ZO-1 and actin to apical sites of the cell. Pretreatment of the cells with cytochalasin D, which destabilizes the cytoskeleton, inhibits both, ZO-1 phosphorylation and its rearrangement (Van Itallie *et al.,* 1995). These findings may be indicative for interdependent regulatory events including mobilization or transfer initiation of tight junction and/or adherens junction proteins as well as for the disintegration or reorganization of complexes as shown for catenin and ZO-1 aggregates (Rajasekaran *et al.,* 1996) and, also, confirm studies on the importance of the cytoskeleton for tight junction integrity (Meza *et al.,* 1980; Madara *et al.,* 1986; Stevenson and Begg, 1994; Kovbasnjuk *et al.,* 1998).

On the other hand, ZO-1 is heavily phosphorylated during tight junction formation at slit diaphragms in glomerular foot processes after protamine sulfate treatment (Kurihara *et al.,* 1992). As well, a more than twofold increase in protein kinase C activity was observed at the membrane during tight junction genesis *in vitro* and the protein kinase C isoform $\times$ colocalizes with ZO-1 in immunofluorescence assays. The development of transcellular electrical resistance as well as ZO-1 phosphorylation during tight junction formation is reduced to a considerable degree by calphostin C, a highly specific inhibitor of protein kinase C (Stuart and Nigam, 1995). Additionally, translocation of ZO-1 to the membrane is delayed. On the other hand, pretreatment of cultures with protein kinase C-inhibitors effectively retards tight junction degradation (Citi, 1992).

This apparent contradiction in the results may be explained by the hypothesis that certain phosphorylation events are responsible for structural dynamics and the translocation of molecular components. However, under steady-state conditions phosphorylation levels are constant. Therefore, no differences in the phosphorylation state of ZO-1, ZO-2, or ZO-3/p130 were detected in high- and low-calcium environments even after activation of protein kinase C by diC8, a diacylglycerol analogue (Balda *et al.,* 1993).

After diC8 stimulation, an increase in transcellular electrical resistance could be demonstrated as well as induction of tight junction formation, including recruitment of ZO-1 to the membrane and F-actin rearrangement even in low-calcium medium. In contrast, the phorbol ester TPA, also a potent activator of protein kinase C, decreases transcellular electrical resistance in epithelial cells (Balda *et al.,* 1991) as well as in blood–brain barrier endothelial cells (Rubin *et al.,* 1991; Wolburg *et al.,* 1994). In blood–brain barrier endothelial cells TPA reduces both P-face association and tight junction complexity (Wolburg *et al.,* 1994). The reason for the opposite effects of diC8 and TPA is not known, but the results suggest that phosphorylation events give rise to alterations of barrier functions, which, nevertheless, go along with reproducable molecular and structural rearrangements.

## Regulation of an Established Paracellular Barrier

From transplantation experiments it became evident, that blood–brain barrier characteristics are determined to a great extent by extrinsic factors (for a review, see Wolburg and Risau, 1995). In culture factors released from astrocytes seem to be necessary (Arthur *et al.,* 1987; Tao-Cheng *et al.,* 1987; Tontsch and Bauer, 1991; Dehouck *et al.,* 1994) but are not sufficient (Rubin *et al.,* 1991; Wolburg *et al.,* 1994) to induce and maintain blood–brain barrier characteristics. Until now, the factors which are effective in blood–brain barrier induction remain obscure, since the environment of brain capillaries is complex and there are putatively more influences of distinct origin, which might work synergistically in a defined temporal and spatial pattern.

In freeze–fracture studies, the elevation of cAMP levels resulted in a more complex appearance of the tight junction network, whereas treatment with conditioned medium derived from rat astrocytes rather modulates the adhesion of tight junction particles to the cytoskeleton as indicated by an increase in tight junction particles fracturing to the P-face leaflets (Wolburg *et al.,* 1994). Most effective in inducing paracellular barrier characteristics as defined by transendothelial electrical resistance and/or inulin permeability was the combined treatment with astrocyte-conditioned medium and forskolin (Rubin *et al.,* 1991; Wolburg *et al.,* 1994). Whereas tight junction complexity could be reinduced *in vitro* to the *in vivo* level, neither P-face association of tight junction-particles nor transendothelial electrical resistance did achieve the levels observed *in vivo* (Wolburg *et al.,* 1994; Kniesel *et al.,* 1996) (cf. Figs. 1b and c), suggesting a crucial role for the strong interaction between tight junction particles and cytoskeleton (Wolburg *et al.,* 1994) analogous to the cadherin–catenin system (Kemler, 1993).

Recently, in low-resistance MDCK cells the effect of carboxy-truncated chicken occludin on the transepithelial electrical resistance was investigated (Balda *et al.,* 1996). Paradoxically, the transepithelial electrical resistance was elevated, while the permeability also increased. This finding is most likely due to the existence of paracellular channels, already postulated by Claude (1978), which might be increased in number or opening probabilities by the additional insertion of truncated occludin. The slower diffusion of solutes could occur stepwise from strand to strand by differential opening of ''channels,'' while fast fluctuations in ion concentrations

are excluded over the total depth of the strictly compartimented tight junction network (Claude, 1978; Marcial *et al.,* 1984; Balda *et al.,* 1996; Madara, 1998).

Similar results were obtained by induction of chick occludin expression in low-resistance MDCK cells (McCarthy *et al.,* 1996). Despite the differences in primary sequence between chick and canine occludin, chick occludin was specifically targeted to the tight junction and was capable of elevating the transepithelial electrical resistance as well as the paracellular mannitol flux after an initial decrease in permeability. In freeze–fracture replicas, the increased number of parallel strands and the overall depth of the tight junction indicated the additional insertion of chick occludin (McCarthy *et al.,* 1996).

Recently, the RhoA and Rac1 small GTPases were shown to play a role in the regulation of tight junction structure and function. The organization of tight junction strands in MDCK cells expressing RhoA and Rac1 mutants is grossly altered and the permeability for inulin and anionic or neutral dextran as well as the transcellular electrical resistance is strongly affected (Jou *et al.,* 1998). The inhibition of the rho pathway by *Clostridium botulinum* exotoxin revealed a disorganization of perijunctional actin and ZO-1 in T84 cells and transient expression of rhoC led to actin concentration at intercellular contacts (Nusrat *et al.,* 1995; Madara, 1998).

In blood–brain barrier endothelial cells, the activation of the rho pathway *in vitro* by lysophosphatidic acid (Moolenaar, 1995) disrupted the paracellular barrier (Schulze *et al.,* 1997). The different consequences of inhibition or activation of rho may be due to cell-type specific mechanisms or additional effects of LPA (Moolenaar, 1995).

## CONCLUSION

Although knowledge of the molecular constitution of tight junctions as well as interactions and interdependencies of junctional components accumulates, blood–brain barrier-specific characteristics are still difficult to identify. Several aspects of tight junction genesis and regulation are similar in epithelial and blood–brain barrier endothelial cells, but the differences in structure and sensitivity to extrinsic influences has not yet been explained at the molecular level. Further characterization of tight junction components and/or regulators specifically for blood–brain barrier endothelial cells in comparison to high-resistance epithelial barriers will gain new insights in the fine-tuning of the different systems. The molecular analysis of the blood–brain barrier is of outstanding clinical relevance, since insight into regulatory mechanisms of the paracellular barrier in the brain are of primary significance for the development of new therapeutic strategies. Treatment of brain tumors has to consider both tumor angiogenesis (Millauer *et al.,* 1994; Folkmann, 1995) and the permeability of tumor vessels (Coomber *et al.,* 1987; Heiss *et al.,* 1996; Isenmann *et al.,* 1996). Both are intimately dependent on the brain microenvironment. Applying results of basic research on angiogenesis, permeability-inducing factors and their receptors, adhesion molecules, and the regulation of junctional components in the brain endothelium to the clinical phenomena of tumor

hypervascularity and vascular hyperpermeability should shed some light on the mechanisms underlying these processes.

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