Doctoral Thesis

Dynamics and modulation of tight junctions

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Dynamics and Modulation of Tight Junctions

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH
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DOCTOR OF NATURAL SCIENCES

presented by
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AAS atomic absorption spectroscopy
AJ adherens junction
BHK-21 bovine hamster kidney cell line
Caco-2 human colon carcinoma cell line
CIAP calf intestine alkaline phosphatase
CLSM confocal laser scanning microscopy
DS desmosome
DAPI 4’, 6-diamidino-2-phenylindole
EBSS Earle’s balanced salt solution
FCS fetal calf serum
GFP green fluorescent protein
JAM junctional adhesion molecule
LCM low calcium medium
mAb monoclonal antibody
MAGUK membrane-associated guanylate kinase
MDCK Madin Darby canine kidney cell line
MEM Eagle’s minimum essential medium
Occ-CGFP occludin fused to the C-terminus of GFP
pAb polyclonal antibody
PB pefabloc SC
PBS phosphate buffered saline
SB sodium butyrate
TEER transepithelial electrical resistance
TJ tight junction
ZO-1 zonula occludens 1 (TJ-associated protein)
ZO1-CGFP ZO-1 fused to the C-terminus of GFP
ZO1-NGFP ZO-1 fused to the N-terminus of GFP
ZO1(1279-1745)-CGFP 1.4 kb C-terminal ZO-1 fragment fused to the C-terminus of GFP
SUMMARY

To examine the dynamics of tight junctions (TJs) in living cells, chimera between the TJ-associated protein ZO-1 and green fluorescent protein (GFP) were constructed. If ZO-1 fused to the C-terminus of GFP (ZO1-CGFP) was stably expressed in MDCK cells, it was fully incorporated into TJs and colocalized with endogenous ZO-1. The GFP tag did not influence cell growth, transepithelial electrical resistance, and paracellular mannitol transport. The morphology of the transfected cells was unchanged. The ZO1-CGFP MDCK cell line thus represents an excellent tool to study TJ dynamics in living cells. The influence of the external calcium ion concentration on the formation and dynamics of TJs in living cells was thus explored. Upon opening of the TJs under short-term treatment with EGTA (up to 20 min), the localization of ZO1-CGFP at the membrane persisted. The rim-like pattern around the individual cells appeared fuzzier than in non-treated cells. Long-term calcium depletion resulted in the localization of ZO1-CGFP in the cytoplasm and the nucleus. After restoration of normal Ca\(^{2+}\) concentrations, cell-cell contacts were restored and the localization of ZO1-CGFP was indistinguishable from the one in control cells kept at normal Ca\(^{2+}\) concentrations.

The dynamics of TJs and adherens junctions (AJs) under EGTA treatment was also investigated in fixed MDCK cells. For this purpose the “calcium chelation” method was applied, which comprises treatment with EGTA and permits the reversible opening of TJs. The distribution and colocalization of junctional proteins were studied with confocal laser scanning microscopy using a deconvolution algorithm for high-resolution images. Colocalization was analyzed for pairs of the following proteins: ZO-1, occludin, claudin-1, E-cadherin and F-actin. Significant differences were found for the analyzed pairs in control cells as compared to EGTA-treated cells with respect to the position of the colocalization maxima within the cell monolayers as well as to the amount of colocalized voxels. Under EGTA treatment, colocalization for most TJ and AJ protein pairs, namely ZO-1/occludin, ZO-1/claudin-1, claudin-1/occludin, E-cadherin/occludin and E-cadherin/claudin-1 dropped
below 35% of the control value. Only for the ZO-1/E-cadherin pair, the amount of colocalized voxels increased and a shift to a more basal position was observed. Briefly, during the opening of TJs and AJs, ZO-1 colocalized with E-cadherin in the lateral membrane region, whereas in established cell-cell contacts, ZO-1 colocalized with occludin and claudin-1 in the junctional complex. The combination of deconvolution with colocalization analysis of confocal data sets offers a powerful tool to investigate the spatial relationship of TJ and AJ proteins during assembly and disassembly of cell-cell contacts.

To study the role of the transmembrane proteins occludin and claudin in the formation of the TJ seal, synthetic peptides corresponding to their two extracellular loops were assayed for their ability to alter TJs in MDCK cells. The occludin loop II and claudin loop I peptide increased the mannitol flux in a dose-dependent manner and slightly decreased the transepithelial electrical resistance (TEER) indicating that the TJ permeability barrier was disrupted. The effect of the peptides was not caused by general cell toxicity and the cell morphology was not altered. Observations with smaller fragments of the occludin loop II and claudin loop I peptide suggest that the whole loop is involved in the occludin interaction, whereas the claudin interaction could be tracked to a smaller area of the loop. These results suggest that occludin as well as claudin-1 are implicated in the formation of the TJ permeability barrier. Possibly the extracellular loop of occludin and claudin-1 are involved in binding proteins of adjacent cells, either through homophilic or heterophilic interactions. This indicates that peptides corresponding to the second extracellular loop of occludin and the first extracellular loop of claudin can influence TJs because they compete specifically at the extracellular loops of the occludin and claudin and therefore could be used as specific reagents to manipulate the permeability of TJs.
ZUSAMMENFASSUNG


1. INTRODUCTION

In an epithelial sheet, tight junction (TJ) strands surround each cell. They form a gasket-like belt between the cells near the apical surface and constitute the major barrier in the paracellular pathway of the epithelial tissues. TJs are dynamic structures. In response to various stimuli, they can open and close to allow or prevent the passage of solutes and fluid across an epithelium. Knowledge about TJ regulation and permeability can have important pharmaceutical applications. It may provide useful strategies for drug delivery by enhancing the paracellular transport while preventing damage to the epithelial barrier.

1.1. EPITHELIA AND ENDOTHELIA AS PERMEATION BARRIERS

The main physiological permeation barriers to be crossed by drug compounds are epithelia and endothelia. Multicellular organisms contain various compositionally distinct fluid compartments. These compartments are surrounded by epithelial or endothelial sheets, which function as barriers to maintain the distinct internal environment of each compartment. For example, the renal tubules and the intestine are lined with epithelial sheets and blood vessels with endothelial sheets. Within these sheets, cells are mechanically linked with each other to maintain the structural integrity. The intercellular space between adjacent cells is sealed to prevent the diffusion of solutes (Tsukita et al., 2001). A set of specialized intercellular junctions forms the junctional complex, which is responsible for the sealing (Farquhar & Palade, 1963). The junctional complex of epithelial cells is located at the most apical part of the lateral membrane and consists of three components: tight junctions (TJs), adherens junctions (AJs) and desmosomes (DS). AJs and DS mechanically link adjacent cells, whereas TJs are responsible for intercellular sealing. Molecules can cross the intact epithelial and endothelial cell sheets by two pathways: the transcellular transport through the cells and the paracellular transport through the TJs. The majority of drugs crosses the cellular barriers by the transcellular pathway. This pathway is reserved to
lipophilic compounds. The paracellular pathway between adjacent cells in the sheet is tightly sealed to prevent the diffusion of solutes. Only small and hydrophilic molecules (radii < 11 Å) can enter the paracellular pathway due to restriction of TJs. Over the past few years, there has been an explosion in research aimed at creating new drug-delivery systems for new, more complex drugs such as peptides and proteins. Several studies have shown that one of the possible mechanisms of permeation enhancers is to loosen the TJs of epithelial membranes, thereby increasing the paracellular transport of poorly absorbed drugs. Indeed, attempts to increase paracellular transport by loosening TJs have been hampered by unacceptable side effects (Hochman & Artursson, 1994; Fasano, 1998). Most currently used permeation enhancers (TJ regulators), such as EGTA (Wang et al., 2000) and Zonula occludens toxin (Fasano et al., 1997), activate multiple signal transduction pathways in target cells. This results in a complex signal pattern, which is difficult to control and limits the applicability of TJ modulators as absorption enhancers. Therefore understanding of the regulatory role and TJ permeability may provide useful strategies for drug delivery.

1.2. TIGHT JUNCTIONS

1.2.1. General aspects

In epithelial cells, TJs are located at the most apical region of lateral membranes. TJs were first identified by electron microscopy of ultra-thin
sections. They appear as a set of discrete sites of focal contacts between the plasma membranes of adjacent cells. At these “kissing points” the intercellular space completely disappears, whereas in AJs and desmosomes, the apposing membranes are 15-20 nm apart (Fig. 2a). When observed by freeze fracture electron microscopy, TJs look like a continuous anastomosing network of intramembranous fibrils or TJ strands (Fig. 2b). Fibrils on one cell presumably interact with fibrils on an adjacent cell to close the paracellular space and define the paracellular permeability characteristics (Farquhar & Palade, 1963; Staehelin, 1973).

![Fig. 2: Junctional complex and tight junctions in epithelial cells. a) Electron micrograph of the junctional complex with the tight junction (TJ, circled), adherens junction (AJ) and desmosomes (DS). (Mv, microvilli). Scale bar, 200 nm. b) Freeze-fracture electron microscopic image. TJ appear as a set of continuous, anastomosing intramembranous strands of fibrils (arrowheads) on the P face with complementary grooves on the E face (arrows). (Ap, apical membrane; Bl, basolateral membrane). Scale bar, 200 nm. c) Schematic, three-dimensional structure of TJs. Each TJ strand within a plasma membrane associates laterally with another TJ strand in the apposed membrane of an adjacent cell to form a paired TJ strand, obliterating the intercellular space (“kissing points”). Adapted from Tsukita et al. (2001).]

For much of the last century, investigators were aware that the sealing properties of the TJs were variable and regulated, yet they lacked a molecular explanation. Two models have been proposed to explain the structure and function of TJ strands. They have been suggested by some investigators to be predominantly lipid in nature (Kachar & Reese, 1982; Verkleij et al., 1984). In the “lipid model”, lipids organized in inverted cylindrical lipid micelles are proposed to constitute TJ strands. In the “protein
model”, TJ strands represent units of integral membrane proteins that are polymerized linearly within lipid bilayers. Cell biologists have long searched for protein components constituting TJ strands. Recent identification of TJ-specific integral membrane proteins strongly supports the “protein” model (Furuse et al., 1993; Furuse et al., 1998a), although it can not be excluded that specific lipids might also be important for the formation of TJ strands (Nusrat et al., 2000).

1.2.2. Function and regulation of TJs

Tight junctions form a seal between the apical and the basolateral membrane domain and thus create a barrier to the diffusion of solutes through the paracellular pathway (reviewed in Madara, 1998; Spring, 1998). They also restrict diffusion of integral proteins and lipids within the lipid bilayer and therefore maintain polarity in epithelial cells (reviewed in Cereijido et al., 1998). In addition to these classical functions of TJs, a series of observations suggests that they are also involved in cellular signaling events and participate in the regulation of cell growth and differentiation (Balda & Matter, 1998; Balda & Matter, 2000). An other function of TJs was suggested to be targeting of transport vesicles from the Golgi network vesicle for the development of cellular polarization (Hsu et al., 1999; Lapierre et al., 1999).

TJs are not simply impermeable barriers: they show ion as well as size selectivity, and vary in tightness in a tissue-dependent manner probably due to different expression pattern of TJ proteins (Schneeberger & Lynch, 1992; Gumbiner, 1993). Furthermore TJs are highly dynamic structures regarding their physiological behavior. The function of TJs is modulated by classic second messenger and signaling pathways. Secondary messengers, such as calcium, calmodulin, cAMP, phospholipase C, kinases, nitric oxide, and G-proteins have been implicated in regulating the assembly and permeability of TJs (reviewed by Heiskala et al. 2001; Anderson & Van Itallie, 1995). Signal transduction pathways are triggered by endogenous factors, such as cytokines and growth factors and exogenous factors, such as bacterial toxins.
and dietary constituents. As TJs respond to a series of physiological, pathological and pharmacological challenges, they represent a favorite point of attack for permeation enhancers.

1.2.3. Molecular structure of TJs

The TJ fibrils are now known to be formed by at least two types of transmembrane proteins. Occludin was the first transmembrane protein identified (Furuse et al., 1993). In 1998, the transmembrane proteins claudin-1 and-2 were found (Furuse et al., 1998a). Until now, more than 20 members of the claudin family were identified (Heiskala et al., 2001; Morita et al., 1999a). JAM, the junctional adhesion molecule, a novel member of the immunoglobulin superfamily, is believed to mediate homotypic cell adhesion and to regulate monocyte transmigration. JAM was reported to be localized at the TJ region (Martin-Padura et al., 1998), but it remains unclear whether it is directly incorporated into TJ strands (Tsukita et al., 1999b). Assembly, scaffolding and regulation of the paracellular seal are presumably accomplished by the plaque of proteins on the cytoplasmic surface of TJs. These plaque proteins include the scaffolding MAGUK (membrane associated guanylate kinase) proteins ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991) and ZO-3 (Haskins et al., 1998). A more heterogeneous group of TJ-associated proteins are the myosin-related cingulin (Citi et al., 1988), 7H6 antigen (Zhong et al., 1993) and symplekin (Keon et al., 1996). These proteins remain without known function. Furthermore proteins related to signal transduction and vesicle targeting have been localized to TJs: the Ras target AF-6 (Yamamoto et al., 1997), ASIP, an atypical protein kinase C interacting protein (Izumi et al., 1998), the transcription regulator ZONAB, a ZO-1-associated nucleic acid-binding protein (Balda & Matter, 2000), rab3B (Weber et al., 1994), the vesicular-transport-related Sec6/8 complex (Grindstaff et al., 1998) and the vesicle-associated membrane protein (VAMP)-associated protein VAP-33 (Lapierre et al., 1999).
1.2.4. ZO-1, a TJ-associated protein

The best-studied protein within the cytoplasmic domain of TJs is the 210-225 kD phosphoprotein zonula occludens 1 (ZO-1) that is likely to constitute the major backbone of the TJ plaque. As a result of alternative splicing two isoforms (ZO-1α−, ZO-1α+) are known, which a differentially expressed in tissues showing a variable degree of TJ “plasticity”. ZO-1α− was found in endothelial cells, podocytes and Sertoli cells, whereas ZO-1α+ was present in all other epithelia (Balda & Anderson, 1993). Under steady state conditions ZO-1 is phosphorylated on serine residues (Howarth et al., 1994) and becomes tyrosine phosphorylated in response to certain extracellular stimuli (Staddon et al., 1995). ZO-1 is specifically enriched at the points of TJs in polarized epithelial and endothelial cells. Interestingly, ZO-1 is also found at the cytoplasmic undercoat of AJs in non-epithelial cells (Howarth et al., 1992; Itoh et al., 1993). Using a “calcium switch” assay (see 1.2.6) to control TJ formation in epithelial cells, Rajasekaran et al. (1996) showed that ZO-1 associates initially with AJ components prior to final localization at TJs. It has also been reported that a fraction of ZO-1 accumulates in the nucleus in growing epithelial cells (Gottardi et al., 1996). These results suggest that ZO-1 may serve multiple purposes within the cell such as cell differentiation and regulation of transcription. ZO-1 belongs to the MAGUK superfamily, whose members are associated with the plasma
membrane, typically at sites of specialized cell-cell contacts. The MAGUK family is characterized by one or more postsynaptic density protein-95/disc large tumor suppressor gene/ZO-1 (PSD-95/DLG/ZO-1; PDZ) domains, a src homology (SH-3) domain, and an enzymatically inactive guanylate kinase-like (GUK) domain (Anderson, 1996). The presence of multiple PDZ domains within one protein provides a scaffold for the recruitment of several proteins. Thus, ZO-1 directly binds ZO-2 as well as ZO-3 (Haskins et al., 1998; Wittchen et al., 1999), two additional TJ-associated members of the MAGUK family. They share strong sequence homology with ZO-1, especially within the MAGUK portions of the molecules. Furthermore, claudins bind with their C-termini to the first PDZ domain of ZO-1 (Itoh et al., 1999), whereas the C-terminus of occludin interacts with ZO-1 at the GUK domain (Furuse et al., 1994). ZONAB binds to the SH3 domain of ZO-1 (Balda & Matter, 2000) and the proline-rich C-terminal half of ZO-1 was shown to be directly associated with actin filaments (Fanning et al., 1998; Itoh et al., 1999). Through the recruitment of various types of protein to the TJs using this PDZ-containing protein, a huge macromolecular complex is formed at the cytoplasmic surface of TJ strands. The physiological function of this complex is still unclear. It probably cross-links the actin cytoskeleton to the sealing proteins and might therefore have a role in the regulation of TJ functions. Similar accumulations of PDZ-containing proteins are involved in synaptic signal transduction and its regulation (Hata et al., 1998). Since such macromolecular complexes are not well developed at cadherin-based AJs, it is tempting to speculate that

**Fig. 4: ZO-1, a member of the MAGUK family and its different domains.** ZO-1 binds with its different domains to ZO-2, claudins, ZONAB, occludin and actin. Domains are represented by closed boxes and the regions responsible for intermolecular association are indicated by arrowheads. PDZ, protein-protein interaction domain first described in the proteins PSD-95, DLG and ZO-1; GUK, guanylate-kinase like domain; SH3, src homology region 3. Adapted from Tsukita et al. (2001).
the macromolecular complex formed at TJs is central in the intercellular adhesion signaling of epithelial and endothelial cells. And it could be involved in the regulation of their proliferation, differentiation and polarization (Tsukita et al., 2001).

1.2.5. Occludin and claudin: Transmembrane proteins of TJs

Barrier sealing properties are quite variable among cell types in terms of solute and water flux, electrical resistance and charge selectivity. A molecular explanation for this variability appears closer following identification of the transmembrane proteins occludin and members of the claudin family.

Occludin, a 65 kD transmembrane phosphoprotein, was the first component of TJ strands identified (Furuse et al., 1993). No occludin-related genes have been identified yet, but two isoforms of occludin are generated by alternative splicing (Muresan et al., 2000). The primary amino acid sequence of occludin predicts four membrane-spanning regions, two extracellular loops, a short N-terminal and a long C-terminal domain. Among these domains, the first extracellular loop is characterized by an unusually high content (60%) of tyrosine and glycine residues. Both extracellular loops of occludin consist of uncharged residues with the exception of a few charged residues adjacent to the transmembrane regions. Sequence conservation across different species is found in the N-terminus, the extracellular loop, and the transmembrane segments, while sequence diversity lies in the cytoplasmic loop and C-terminus. High conservation of the loops suggests an important structural function.

Identification of claudin-1 and -2 indicated the existence of a novel gene family (Furuse et al., 1998a). To date, 24 members of this gene family have been identified through database searches (Tsukita & Furuse, 1999a; Mitic et al., 2000). Claudins are small 20-27 kD transmembrane proteins. By immuno-electro microscopy, they could be localized within the continuous linear fibrils of the TJs. Claudins share a similar membrane topology with occludin even though they are smaller and their second extracellular loops
and intracellular N- and C-terminals are significantly shorter. They do not show any sequence similarity to occludin. Among the claudins, the cytoplasmic C-terminal tails displays the greatest variability in the primary structure within the claudin superfamily proteins. The amino acid sequences of the first and fourth transmembrane segments and the first and second extracellular loops are highly conserved. The first extracellular loop is larger and more hydrophobic than the second extracellular loop and is believed to bridge the intercellular space (Furuse et al., 1998a; Morita et al., 1999a).

Occludin was originally thought to be the main sealing protein of the TJ (Furuse et al., 1993). It clearly exhibits some barrier functions, as the electrical resistance and the number of TJ strands increased when occludin was overexpressed in cultured epithelial cells (Balda et al., 1996; McCarthy et al., 1996). Furthermore, disruption of occludin interactions by the addition of peptides corresponding to sequences of the extracellular loops resulted in increased TJ permeability (Wong & Gumbiner, 1997; Lacaz-Vieira et al., 1999). Additionally, occludin-transfected fibroblasts exhibited some cell-adhesion activity (Van Itallie & Anderson, 1997). However, several observations could not be explained by the properties of occludin alone. First, until now only one occludin protein has been identified, which is expressed in essentially all TJs. Therefore occludin is unlikely to account for the tissue differences in barrier properties. Second, endothelial cells in non-neuronal tissue and Sertoli cells bear TJs but express only trace amounts of occludin (Hirase et al., 1997; Moroi et al., 1998). Third, and most convincing, is the finding that stem cells lacking occludin are still capable of forming intercellular barriers (Saitou et al., 1998). Therefore, the discovery of the claudin family has offered new possibilities for the understanding of the sealing between cells. Claudins possess several functional characteristics consistent with a role in barrier formation. They show an intrinsic ability to polymerize into linear fibrils. The transfection of a single type of claudin into TJ-null fibroblasts resulted in the formation of extensive networks as shown in freeze fracture images (Furuse et al., 1998b). This is in contrast to occludin, which forms only short fragments of strands. The claudins exhibit stronger calcium-independent adhesion than
occludin when transfected into TJ-null fibroblasts (Kubota et al., 1999), indicating that they can form the transcellular contacts presumably required to seal the intercellular space. Claudins can have restricted expression patterns and display variable tissue distribution (Morita et al., 1999b; Morita et al., 1999c), which is consistent with the idea that differential expression might explain the variable permeabilities observed among different tissues and cell types.

![Diagram of Occludin and Claudin-1](image)

**Fig. 5: Predicted structure of occludin and claudin-1.** Human occludin and claudin-1 contain both four transmembrane domains with N- and C-termini in the cytoplasm. When compared with occludin, the N-terminal cytoplasmic domain, the second extracellular loop and the C-terminal cytoplasmic domain of claudin are fairly short. Acidic (●), basic (▲) and uncharged (○) residues at neutral pH are indicated. Adapted from Furuse et al. (1998a), Mitic & Anderson (1998) and Tsukita et al. (1999b).

Detailed electrophysiological analyses suggested the existence of aqueous pores of different size within the paired TJ strands (Gumbiner, 1993). The dimension of these pores has been estimated to be in the range between 8 and 20 Å (Mitic & Anderson, 1998). As permeability properties of individual paired TJ strands appear to be fairly variable in different epithelia, it is likely that most claudin species can constitute not only the wall, but they could also be involved in the formation of pores. According to a study of a limited number of claudins (Furuse et al., 1999), they are all
capable of homophilic adhesion between cells, and some but not all pairs are capable of heterophilic adhesion. Since claudins are not able to laterally associate with any claudin family proteins in adjacent cells, mismatches in the juxtaposed heteropolymers of claudins between paired TJ strands may lead to the formation of aqueous pores. A spectacular insight into this function of claudins was provided by the discovery that mutations in the gene for paracellin-1 (claudin-16) are the cause of a rare human renal magnesium-wasting syndrome (Simon et al., 1999). Paracellin-1 is found exclusively in the thick ascending loop of Henle, where calcium and magnesium are resorbed from the tubule through a paracellular route. When paracellin-1 is absent, magnesium is lost in the urine, which leads to hypomagnesaemia and seizures. The most obvious interpretation of these results is that paracellin-1 functions as a magnesium-selective channel through the TJ barrier. Coincidentally, the extracellular amino acid chains of claudin-16 are among the most acidic of all claudins. It is therefore tempting to speculate that the extracellular loops of the claudins position their variable residues to influence passage of ions through the aqueous space.

1.2.6. Models to study TJ dynamics

Classically, studies on biogenesis as well as on the opening and resealing of TJs have been performed with monolayers of epithelial cells. They were based on the so called “calcium switch” and the “calcium chelation” method, respectively. The “calcium switch” method implies prolonged calcium starvation after the seeding of cells and subsequent restoration of normal calcium concentrations in the medium. It is used to study de novo assembly of TJs and the development of cell polarity (Cereijido et al., 1978; Dekkers et al., 1998). The “calcium chelation” method comprises a short-term treatment of confluent cells with EGTA, which induces the opening of TJs. After restoration of normal Ca$^{2+}$ levels, TJs are reformed (Martinez-Palomo et al., 1980). This method is used to investigate both disruption and
resealing and thus permits to study modulation of the TJs under a variety of experimental conditions (Balda and Matter, 1998).

1.3. PROTEIN CHIMERA WITH GREEN FLUORESCENT PROTEIN FOR STUDIES IN LIVING CELLS

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is rapidly becoming an important reporter molecule for monitoring gene expression and protein localization in vivo, in situ and in real time. The utility of GFP in research applications derives from its intrinsic ability to generate fluorescence in living tissues, in the absence on any cofactors. Wild-type GFP is a stable, protease resistant protein containing 238 amino acids. It has an 11-stranded β-barrel threaded by an α-helix running up the axis of the cylinder. The chromophore is attached to the α-helix and is buried almost perfectly in the center of the cylinder, which has been called a β-can. The chromophore is a p-hydroxy-benzylideneimidazolinone formed by the cyclization and oxidation of Ser-65, Tyr-66 and Gly-67 in the native protein (Tsien, 1998). The original wild-type GFP protein was greatly improved through alteration of mammalian codon usage and introduction of point mutations to enhance brightness and photostability. Cell biological applications of GFP may be divided into the uses as a tag or as an indicator. In tagging applications, GFP is directly linked to a protein to be tracked and the resulting protein chimera can be observed inside living cells in the fluorescence microscope (Chalfie et al., 1994).

Fig. 6: 3D-structure of GFP, showing 11 β-strands forming a hollow cylinder through which is threaded a helix bearing the chromophore, shown in ball-and-stick representation. Adapted from Ormö et al. (1996).
Fortunately, GFP can be tagged to many proteins to either the N- or the C-terminus without affecting their function. Perhaps because of the tightly folded barrel structure of GFP, it is so self-contained that it does not flip around sticking to bits of the protein to which it is linked. In recent years, the use of GFP fusion proteins has emerged as a powerful technique. Different topics such as cadherin-mediated cell-cell adhesion (Adams et al., 1998), organization and dynamics of the cytoskeleton (Choidas et al., 1998) as well as the dynamics of gap junctions (Holm et al., 1999; Jordan et al., 1999) have been explored.

1.4. AIM OF THE STUDY

1.4.1. A ZO-1 fusion protein to study the dynamics of TJs in living cells

Until now direct visualization of TJ dynamics in living cells was not possible. TJs could only be studied in fixed cells after immunofluorescent labeling of TJ proteins. We report for the first time on the use of a GFP-TJ protein to visualize dynamic processes in living cells. A fusion protein between ZO1 and GFP was expressed in the MDCK epithelial cell line, which forms a homogeneous TJ network (Rothen-Rutishauser et al., 1998a). To test whether the relatively large GFP-tag would influence the functionality of the ZO-1 chimeric protein, the stably transfected cells were characterized with regard to overall cell morphology, cytoarchitecture, protein expression, binding properties of ZO-1 with other TJ-associated proteins, paracellular transport, and transepithelial electrical resistance (TEER). As we could show, the ZO1-CGFP fusion protein was behaving like the endogenous ZO-1 protein. The new ZO1-CGFP MDCK cell line was thus used to investigate the influence of the external calcium ion concentration on the formation and dynamics of TJs in living cells (Riesen et al., 2002).
1.4.2. Studies on the TJ and AJ dynamics in fixed cells by colocalization analysis in the confocal microscope

In epithelial cells, TJs and AJs are related spatially as well as functionally. AJs are located basally to the TJs (Farquhar & Palade, 1963; Boller et al., 1985). They are involved in the mechanical linkage of adjacent cells, as well as in the regulation of the development of cell surface polarity (Nelson, 1994). AJs consist of clusters of transmembrane proteins, which belong to the cadherin family. They function as calcium-dependent adhesion molecules and are intracellularly linked to catenins, which in turn promote anchoring to the actin cytoskeleton. In epithelia, the subclass E-cadherin also called uvomorulin is expressed in AJs (for reviews see Takeichi, 1991; Tsukita et al., 1992). It has been suggested that cadherin is the initial organizer of TJ strands. On one hand, evidence comes from the fact that extracellular antibodies against cadherin inhibit formation of TJs (Vestweber & Kemler, 1985; Gumbiner et al., 1988). On the other hand, the TJs and AJs may be physically linked, particularly during formation of TJs, via ZO-1, which binds to the cadherin-associated proteins α-catenin (Itoh et al., 1997) and β-catenin (Rajasekaran et al., 1996). During epithelial cellular polarization, E-cadherin and ZO-1 are simultaneously recruited to the primordial form of spot-like junctions at the tips of cellular processes, which show no concentration of occludin. As cellular polarization proceeds, occludin gradually accumulates at the ZO-1-positive spot-like junctions and E-cadherin is sorted out (Yonemura et al., 1995; Ando-Akatsuka et al., 1999). It has been shown with fibroblasts transfected with either occludin or claudins that the cell-adhesive activity of TJs itself is not particularly strong, which may be the reason why cadherin cell-adhesion activity is required for TJ formation and maintenance (Kubota et al., 1999).

Detailed information about the opening and resealing of TJs and AJs is still scarce. In the present study we applied the “calcium chelation” method (see 1.4.1). The chelation of extracellular Ca\(^{2+}\) induces disassembly of TJs (Martinez-Palomo et al., 1980) and AJs (Kartenbeck et al., 1982). The behavior of different TJ and AJ proteins as well as F-actin was investigated during the opening and resealing of TJs and AJs by confocal laser scanning...
microscopy (CLSM). A colocalization analysis was performed for pairs of ZO-1, occludin, claudin-1, E-cadherin and F-actin at different stages of the opening and resealing in MDCK cells, a well characterized cell line with respect to cytoskeleton, TJ and AJ proteins (González-Mariscal et al., 1985; Rothen-Rutishauser et al., 1998a). Data acquisition from fluorescently labeled probes was performed at high resolution. A deconvolution algorithm was applied to reduce noise and blur on one hand and to increase resolution on the other. Colocalization analyses of the protein pairs revealed significant differences in the behavior of the investigated proteins (Rothen-Rutishauser & Riesen et al., 2002).

1.4.3. Modulation of TJs with peptides homologous to the two extracellular loops of occludin and claudin-1

The transmembrane proteins of the claudin family as well as occludin are the major constituents in TJ strands. Claudins and occludin are all capable of homophilic, and some claudins of heterophilic adhesion between cells (Furuse et al., 1999). Claudins and occludin consist of four transmembrane segments, three cytoplasmic domains and two extracellular loops (Furuse et al., 1993; Furuse et al., 1998a). Homophilic and heterophilic interaction occurs most probably at these loops. Thus one would predict that peptides corresponding to the extracellular loops of occludin and claudin, respectively, would compete for binding the loops interfering with the hydrophobic protein-protein interactions. Therefore TJs would be perturbed in a very specific manner, provided that the peptides assume the proper configuration in solution and the affinity is high enough to allow displacement of the original protein-protein-interactions. It has been shown that interference with extracellular loops of occludin, by treatment with synthetic peptides corresponding to the two extracellular domains, disrupted the TJ permeability barrier in Xenopus kidney epithelial cell lines (Wong & Gumbiner, 1997; Lacaz-Vieira et al., 1999). Similar methods have been used to inhibit the function of other cell adhesion molecules; for example, small peptides containing the extracellular loop sequences for connexins
Introduction

delay gap junction formation (Dahl et al., 1994; Warner et al., 1995), and a cadherin extracellular peptide inhibits embryo compaction (Blaschuk et al., 1990).

Recent findings suggest that claudins rather than occludin appear to be the leading players in the formation of TJ strands and probably form the major “backbone” of the TJ strands (see 1.2.5). We therefore studied particularly the role of claudin beside the one of occludin in the formation of the TJ seal.

To test whether the sealing of the paracellular pathway by TJs in epithelial monolayers depends on a homologous binding between claudin and occludin, synthetic peptides homologous to the first and second extracellular loops of human occludin and claudin-1, respectively, were assayed for their ability to alter TJs in MDCK cells. These cells were chosen because they have a well established TJ network and they express in addition to claudin-1 at least claudin-2 and -4 (Furuse et al., 2001). Due to the high conservation in the extracellular loops of claudins (Morita et al., 1999a), we concentrated on claudin-1. Paracellular transport, TEER and the morphology of MDCK cells incubated with those peptides were studied to check their ability to modulate TJs.
2. MATERIALS AND METHODS

2.1. REPORTER GENE CONSTRUCTS

2.1.1. ZO-1 constructs

To obtain either C- or N-terminal fusion proteins of ZO-1 to GFP, the human cDNA of ZO-1 was subcloned into an enhanced GFP vector. The complete coding region of ZO-1 (Willott et al., 1993, GenBank accession no. L14837) was PCR amplified (see 2.1.3) from a Bluescript plasmid containing ZO-1 cDNA (kindly provided by Dr. J.M. Anderson, Dept. of Internal Medicine and Cell Biology, Yale School of Medicine, New Haven, CT, USA).

For the ZO-1 fusion to the C-terminus of GFP (ZO1-CGFP) the primers were:

5’-GGTACCCCCGGGAAAGTGGTCAATAAGGACAG-3’ to create a XmaI site and 5’-GTCGACGAGCTCTGGAGAGAGACAAGATGTC-3’ to create a SacI site at the 5’ and 3’ ends of ZO-1, respectively.

For the ZO-1 fusion to the N-terminus of GFP (ZO1-NGFP) the primers were:

5’-GGTACCCCCGGGAAAGTGGTCAATAAGGACAG-3’ to create a XmaI site and 5’-GTCGACGAGCTCCTGGAGAGAGACAAGATGTC-3’ to create a SacI site at the 5’ and 3’ ends of ZO-1, respectively.

The PCR products were subcloned into the pEGFPC1 and pEGFPN2 vectors (Clontech, BD Biosciences, Basel, Switzerland) after digestion with XmaI and SacI (see 2.1.4).

To construct a C-terminal ZO-1 fragment fused to the C-terminus of GFP (ZO1(1279-1745)-CGFP), the ZO-1 cDNA digested with Bgl II was cloned, with no further modifications, into the Bgl II site of pEGFPC1 (see 2.1.4).

All ZO-1 constructs with GFP were in the same reading frame as GFP with no intervening stop codons. They were restriction mapped to confirm the integrity of the plasmids.
2.1.2. Occludin construct

To obtain a C-terminal fusion protein of occludin to GFP, the dog cDNA of occludin was subcloned into an enhanced GFP vector. The complete coding region of occludin (Ando-Akatsuka et al., 1996; GenBank accession no. U49221) was PCR amplified (see 2.1.3) from a Bluescript plasmid containing occludin cDNA (kindly provided by Dr. M. Furuse, Dept. of Cell Biology, Kyoto University Faculty of Medicine, Kyoto, Japan). For the occludin fusions to the C-terminus of GFP (Occ-CGFP) the primers were:

5’-GTCGACGAGCTCCCATGTCATCGAGGCCTT-3’ to create a SacI site and 5’-GGTACCCCGGGATGTTTTCTGTCTATCATAGTC-3’ to create a XmaI site at the 5’ and 3’ ends of occludin, respectively.

The PCR product was subcloned into the pEGFPC1 vector (Clontech) after restriction enzyme digestion with SacI and XmaI (see 2.1.4). The occludin construct with GFP was in the same reading frame as GFP with no intervening stop codons. It was restriction mapped to confirm the integrity of the plasmids.

2.1.3. Amplification of ZO-1 and occludin constructs

Polymerase chain reactions (PCR) were carried out in 50 µl with the following components: 5-20 ng template DNA (see 2.1.1, 2.1.2), 1x PCR buffer 1 (containing MgCl₂, Roche Diagnostics, Rotkreuz, Switzerland), 0.3 µM of each primer (Intron, Kaltbrunn, Switzerland), 0.2-0.5 mM PCR Nucleotide Mix (Promega, Catalys AG, Wallisellen, Switzerland), 2.5-7 U high fidelity proofreading polymerase (Expand™ Long Template PCR System, Roche Diagnostics). Reactions were run on a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk CT, USA) using the following conditions: denaturation at 94°C for 1.5 min followed by 25 cycles of denaturation at 94°C for 20 s, annealing at 52°C for 40 s, extension at 68°C for 5 min and a final elongation step at 68°C for 7 min. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Basel,
Switzerland), ligated in the GFP vector as described in 2.1.4 and the plasmid was propagated in *E. coli* (see 2.1.4).

### 2.1.4. Plasmid manipulations

**Plasmid digestion.** For preparative digestions and to confirm the integrity of the plasmids, 10-100 µg plasmid DNA were digested with 2-10 U of the appropriate enzyme in the corresponding 1x restriction buffer for 2 h. For digestion with *XmaI*, mixes were supplemented with 1x bovine serum albumin (BSA, Promega). *SacI* and *Bgl II* were from Pharmacia Biotech (Duebendorf, Switzerland) and *XmaI* from Promega.

**Isolation of DNA from agarose gels.** Digests were run on preparative 0.7-1.2 % agarose gels in 0.5x TBE buffer (see 2.1.5) according to Sambrook et al. (1989). A small part of the DNA band, was visualized by ethidium bromide staining and exposing to a long-range ultraviolet light source. The DNA band of interest band (unstained) was excised using a scalpel and purified using the Qiaquick PCR Gel Extraction Kit (Qiagen) according to the manufacturers specifications. The yield of isolated DNA was estimated by running 0.8-1.2 % agarose gels in 1x TAE buffer (see 2.1.5) and comparing the band intensity to DNA of known quantity.

**Dephosphorylation of 5’ ends.** To inhibit self-ligation, the 5’ends of linearized vector were dephosphorylated with calf intestine alkaline phosphatase (CIAP, Pharmacia Biotech). Usually, dephosphorylation was carried out directly after digestion with the restriction enzyme since the digest buffers were also suitable for the dephosphorylation reaction. To 50 µl end volume, 0.1 U CIAP were added to 1-20 µg linearized vector DNA and incubated at 37°C for 30 min. CIAP was inactivated by incubation at 85°C for 15 min.

**DNA Ligation.** DNA fragments were quantified on agarose gels as described above and the amount of insert DNA needed for a given amount of vector DNA was calculated using the following formula:
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ng insert = \((\text{ng vector} \cdot \text{insert size in kb})/\text{vector size in kb}) \cdot (\text{insert/vector ratio})\).

Generally, ligations were performed in 10 µl volumes using 50-100 ng of vector and insert/vector ratios of 3:1, 1:1 and 1:3. Vector DNA, insert DNA (dephosphorylated at 5’ ends as described above), 1x ligation buffer and 1-2 U T4 DNA ligase (Promega) were mixed and incubated overnight at 16 to 22°C. As a control vector without insert was ligated.

Transformation of plasmid DNA. For the propagation of plasmid DNA, two E. coli strains, namely XL-1 Blue and TG1 were used. Competent E. coli (see below) were thawed on ice and divided into 60 µl aliquots. 3 µl of ligation mix or 1-10 ng of supercoiled plasmid was added and gently mixed. The transformation mix was incubated on ice for 20 min, heat-shocked at 42°C for 2 min and incubated on ice for 1-2 min. 200 µl LB medium (see 2.1.5) containing 2 µl 2M MgSO\(_4\) and 2 µl 1M KCl were added and the cells were incubated at 37°C with shaking for 1 h. 100-200 µl of the transformation mix were spread on plates with 50 µg/ml kanamycin (Fluka, Buchs, Switzerland) and incubated overnight at 37°C.

Plasmid DNA isolation (small amounts). For the analysis of recombinant clones, a standard miniprep protocol was used (Sambrook et al., 1989). It was modified to analyze simultaneously small amounts of plasmids in lots of clones. Single colonies were picked with sterile toothpicks, spread on plates with 50 µg/ml kanamycin with a single stroke and incubated overnight at 37°C. On the following day, half of the colony was placed in 18 µl distilled water, mixed and lysed with 3 µl phenol-TE (Sigma). The lysate was briefly mixed by vortexing for 10 s and centrifuged at 13000 g for 3 min. 10 µl of the supernatant were analyzed by agarose gel electrophoresis.

Plasmid DNA isolation (large amounts). For the preparation of large amounts of plasmid DNA suitable for transfection, the Maxiprep Kit (Qiagen) was used. The preparations were done according to the protocol supplied by the manufacturer. Plasmid DNA was dissolved in TE buffer pH 7.4 at an approximate concentration of 1 µg/µl. Quantification was done
by agarose gel electrophoresis and UV measurement. For UV measurements, the plasmid DNA was diluted 1:40 in 5 mM Tris-HCl buffer pH 8.5. Absorption at 260 nm and 280 nm was measured. Plasmid DNA was considered to be clean if the ratio 260:280 was more than 1.8. The amount of plasmid DNA was calculated using 1 OD$_{260}$ unit = 50 µg/ml DNA. Plasmid DNA for transfections was stored in aliquots at -20°C.

**Competent cells.** XL-1 Blue and TG 1 cells were streaked out on LB plates from a glycerol stock and incubated overnight at 37°C. Single colonies were transferred in 3 ml LB medium (see 2.1.5) containing 30 µl 2M MgSO$_4$ and 30 µl 1M KCl and incubated overnight at 37°C with gentle shaking. On the following day, 1 ml of one overnight culture was used to inoculate 100 ml LB medium containing 1 ml 2M MgSO$_4$ and 1 ml 1M KCl. Cells were grown for about 1-2 h at 37°C to an OD$_{600}$ of 0.4-0.5. Cells were incubated on ice for 10 min, pelleted at 2000 g for 10 min at 4°C and resuspended in 20 ml cold TfBI medium (see 2.1.5), followed by 10 min on ice. Cells were pelleted again and resuspended in 4 ml ice-cold TfBII medium (see 2.1.5) by gently stirring. 200 µl aliquots were stored at -80°C.

### 2.1.5. Buffers and solutions for plasmid manipulations

Solutions that are not listed here were made according to standard procedures (Sambrook et al., 1989).

**TAE buffer.** A stock solution (50x) was prepared with 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA. Distilled water was added to the final volume of 1 L and pH was adjusted to 8.5. The working solution (1x) contained 0.04 M Tris-acetate and 0.01 M EDTA.

**TBE buffer.** For the stock solution (10x) 108 g Tris base, 55 g boric acid and 40 ml 0.5 M EDTA (pH 8.0) was added to ~ 800 ml of distilled water. The pH was adjusted to 8.3 with boric acid and the final volume was brought to
1 L with distilled water. The working solution (0.5x) contained 0.045 M Tris-borate and 0.01 M EDTA.

**TE buffer.** 10 ml 1 M Tris (pH 7.4, 7.6 or 8.0), 2 ml 0.5M EDTA (pH 8.0) and destilled water were mixed to a final volume of 1 L. The working solution (1x) contained 10 mM Tris and 1 mM EDTA.

**LB media/agar.** For LB medium, 10 g Bacto-Tryptone (Difco, Chemie Brunschwig AG, Basel, Switzerland), 5 g yeast extract (Difco) and 8 g NaCl were dissolved in distilled water, the pH was adjusted to 7.0 with 5 N NaOH (~ 0.2 ml) and brought to a final volume of 1 L. Medium was sterilized by autoclaving for 20 min at 121°C on liquid cycle. For LB plates, 15 g/L Bacto-Agar (Difco) was added to the LB medium and sterilized by autoclaving for 20 min at 121°C on liquid cycle. When the medium was removed from the autoclave, it was gently swirled to distribute the melted agar evenly throughout the solution. The medium was cooled down to 50°C before adding 50 μg/ml thermolabile kanamycin.

**TfB I.** Medium was prepared by mixing the following volumes of sterile solutions: 3 ml 1 M CH₃COOK, 10 ml 0.5 M MnCl₂, 10 ml 1 M RbCl, 1 ml 1 M CaCl₂, 15 ml glycerol and 61 ml distilled water. The working solution (1x) contained 30 mM CH₃COOK, 50 mM MnCl₂, 100 mM RbCl, 10 M CaCl₂ and 15 % glycerol.

**TfB II.** Medium was prepared by mixing the following volumes of sterile solutions: 200 µl MOPS, 200 µl RbCl, 1.5 ml 1 M CaCl₂, 3 ml glycerol and 15.1 ml distilled water. The working solution contained 10 mM MOPS, 75 mM RbCl, 10 M CaCl₂ and 15 % glycerol.

### 2.2. CELL CULTURE TECHNIQUES

#### 2.2.1. Cell cultures and their cultivation methods

MDCK cells, strain II (passage # 220-240) were grown on plastic TPP®-flasks (Techno Plastic Products AG, Winiger AG, Wohlen,
Switzerland) in Eagle’s minimum essential medium (MEM, ICN, EGT Chemie AG, Tägerig, Switzerland) with Earl’s salts supplemented with 10 % fetal calf serum (FCS, GibcoBRL, Life Technologies, Basel, Switzerland), 1.7 mM L-glutamine (GibcoBRL), 0.2% NaHCO₃ as well as 100 units/ml penicillin and 100 µg/ml streptomycin (GibcoBRL).

Caco-2 cells (ATCC HTB-37, passage # 21-24) were cultivated in minimum essential medium with Earle’s Salts (EMEM, GibcoBRL), 0.1 mM non essential amino acids and 2 mM L-glutamine, supplemented with 0.15 % NaHCO₃, 20 % FCS, 1.0 mM sodium pyruvate (Fluka), 100 units/ml penicillin and 100 µg/ml streptomycin.

BHK-21 fibroblasts (ATCC CCL-10, passage # 68-72) were grown in Dulbecco’s minimum essential medium (DMEM, GibcoBRL) containing 10 % FCS, 1.7 mM L-glutamine, 0.15% NaHCO₃ as well as 100/ml units penicillin and 100 µg/ml streptomycin. Cells were incubated in a 5 % CO₂ atmosphere at 37°C and subcultivated twice weekly.

Experimental cultures for immunostaining and TEER measurements were grown on Falcon® cell culture inserts with a Cyclopore® membrane (0.4 µm, 4.2 cm² or 0.9 cm² as indicated, Becton Dickinson Labware, Le Pont De Claix, France). For imaging of living cells, they were grown on Lab Tek™ chambered coverglass systems (Nunc, Life Technologies, Basel, Switzerland). For the studies of TJs and AJs under EGTA treatment, experimental cultures were seeded at a density of 10⁵ cells/cm² and propagated on Falcon® cell culture inserts (4.2 cm²) for 11 days, which corresponds to stage III cells (Rothen-Rutishauser et al., 1998a).

### 2.2.2. Growth curves

To establish growth curves, cells were seeded at a density of 5x10⁴ cells/cm² in TPP® 6- well plates (Techno Plastic Products AG), unless otherwise stated. At the times indicated, cells were trypsinized in triplicates and cell numbers were counted in a Neubauer counting chamber.
2.2.3. TEER measurements

The transepithelial electrical resistance (TEER) was measured at 37°C with a Millicell-ERS system (MERS 000 01, Millipore Cooperation, Bedford MA, USA) with a chopstick electrode (MERSSTX01, Millipore) or an Endohm-24S electrode (World Precision Instruments Inc., Sarasota FL, USA). At the times indicated, the resistance was measured (at least 2 h after changing the medium) and the TEER value of the support membrane without cells was subtracted. The resistance values of the cell layers [Ω cm²] were obtained by multiplication with the surface area of the inserts.

2.2.4. Phase contrast microscopy

Growing cells were regularly observed in a phase contrast microscope (Leitz Fluovert FU, Leica, Germany). Pictures were taken with a Leica camera system (Wild MPS52).

2.2.5. Viability test

Viability of the cells was tested with a trypan blue test according to Spector et al. (1998). Normal healthy cells are able to exclude the dye, but trypan blue diffuses into cells in which the membrane integrity has been lost. Briefly, cells were trypsinized and diluted in EBSS, pH 7.4 (Earle’s balanced salt solution, GibcoBRL) to 1.5-2.0 x 10⁵ cells/ml. Thereafter, the cell suspension was mixed with the same volume of trypan blue dye (37°C, 0.4 % w/v in EBSS, Sigma) and incubated at 37°C for 5 min. The total cell number as well as the non-viable (blue) cells were counted in a Neubauer counting chamber and the viability was calculated as follows:

\[
\text{cell viability (\%)} = \frac{\text{total cells} - \text{non-viable (blue) cells}}{\text{total cells (stained and unstained)}} \cdot 100 \quad \text{Eq. 1}
\]
2.3. TRANSFECTION OF DIFFERENT MAMMALIAN CELLS AND SELECTION OF STABLE CELL LINES

Cell lines (MDCK, Caco-2, BHK-21 see 2.2.1) were transfected with Lipofectamine (GibcoBRL). Between 1-2 µg of DNA (see 2.1) was used with 6 µl of Lipofectamine. As recommended by the manufacturer, transfections were performed in OptiMem I medium (GibcoBRL). Cells were incubated at 37°C and 5% CO₂ in the presence of the DNA-liposome complexes. After 5 h, medium was changed to standard growth medium. After 24 h of incubation, the cells were rinsed with fresh medium and incubated for another 24 to 48 h. Transfectants were examined by CLSM to detect expression of GFP fusion proteins.

For the isolation of cells stably expressing GFP constructs MDCK cells were used. Positive transformants were obtained by selection of transiently transfected cells in growth medium containing 600 µg/ml G418 (Promega). Clonal isolates were obtained through dilution of stable transfectants and picking of clones by cloning rings. About 10 positive clones from each plasmid were analyzed more closely regarding their fluorescence patterns in the microscope and the Western blot profiles. The best clone was chosen and amplified. Cells were maintained in medium containing 600 µg/ml G418 for a few more cell cycles. To maintain them as a permanent cell line, 100 µg/ml G418 (Promega) were added to the medium. To enhance the expression of fusion protein in stably transfected ZO1-CGFP MDCK cells, sodium butyrate (SB) was added to the medium 16-24 h prior to experiments at the concentrations indicated (up to 5 mM).

2.4. ANTIBODIES AND FLUORESCENT REAGENTS

The mouse anti occludin mAb (# 33-1500), the rabbit anti ZO-1 pAb (# 61-7300) and the rabbit anti claudin-1 pAb (# 71-7800) were purchased from Zymed Laboratories (Gebr. Maechler, Basel, Switzerland). The rat anti E-cadherin (uvomorulin) mAb (#U3254) was from Sigma. F-actin was labeled with phalloidin-coumarin (#C-606) or TRITC-phalloidin (# R-415) from Molecular Probes (Leiden, Netherlands). The following secondary
antibodies were from Chemicon (Juro, Lucerne, Switzerland): the rat anti ZO-1 mAb (# MAB1520), goat anti rat IgG cy5, goat anti rat IgG cy3, goat anti rabbit IgG cy5, goat anti mouse IgG cy5. Goat anti mouse IgG cy3 was purchased from Sigma and goat anti rabbit IgG cy3 from Amersham (Little Chalfont, UK). Cell nuclei were visualized with 4,6-Diamidino-2-Phenylindole (DAPI, Hoechst) in fixed cells, or with Hoechst 33342 (Molecular Probes) in living cells.

2.5. IMMUNOFLUORESCENT LABELING

Cells were labeled and prepared for CLSM according to Rothen-Rutishauser et al. (1998a). Briefly, cell layers were fixed for 15 min at room temperature in 3 % paraformaldehyde in PBS (phosphate buffered saline pH 7.4: 130 mM NaCl, 10 mM Na₂HPO₄/KH₂PO₄). Fixed cells were treated with 0.1 M glycine in PBS for 5 min. They were permeabilized with 0.2 % Triton X-100 in PBS for 15 min, before they were incubated at 37°C with the primary antibody for 60 min and with the secondary antibody for 90 min. Antibodies were diluted in PBS containing 3 % BSA as listed in Tab. 1. Preparations were mounted in 0.1 M Tris-HCl (pH 9.5): glycerol (3:7) containing 50 mg n-propyl-gallate per ml (Sigma). As a control, the specificity of the antibodies and the labeling procedure were tested with the secondary antibodies only.

For claudin-1 stainings, the protocol was slightly modified in order to prevent non-specific staining: after fixation, cells were permeabilized with 0.05 % Triton X-100 in PBS for 5 min and then washed 2 times in PBS followed by one washing step with PBS containing 5 % non-fat dry milk. Samples were incubated at 37°C with the primary antibody for 60 min and with the secondary antibody for 30 min. After washing 3 times with PBS containing 5 % non-fat dry milk, cells were mounted as described above. Antibodies were diluted in PBS containing 5 % non-fat dry milk as described in Tab. 1.
Tab. 1: Combinations of primary and secondary antibodies, fluorescent reagent and their dilutions used for colocalization studies

<table>
<thead>
<tr>
<th>primary antibodies</th>
<th>secondary antibodies and fluorescent reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZO-1/E-cadherin</td>
<td>goat anti rabbit IgG cy5 (1:50)</td>
</tr>
<tr>
<td></td>
<td>goat anti rat IgG cy3 (1:50)</td>
</tr>
<tr>
<td>ZO-1/F-actin</td>
<td>goat anti rat IgG cy5 (1:50)</td>
</tr>
<tr>
<td></td>
<td>TRITC-phalloidin (1:10)</td>
</tr>
<tr>
<td>ZO-1/occludin</td>
<td>goat anti rabbit IgG cy5 (1:50)</td>
</tr>
<tr>
<td></td>
<td>goat anti mouse IgG cy3 (1:50)</td>
</tr>
<tr>
<td>ZO-1/claudin-1</td>
<td>goat anti rat IgG cy5 (1:50)</td>
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<td></td>
<td>goat anti rabbit IgG cy3 (1:100)</td>
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<tr>
<td>claudin-1/occludin</td>
<td>goat anti rabbit IgG cy5 (1:100)</td>
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<td></td>
<td>goat anti mouse IgG cy5 (1:50)</td>
</tr>
<tr>
<td>E-cadherin/occludin</td>
<td>goat anti mouse IgG cy5 (1:50)</td>
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<td></td>
<td>goat anti rat IgG cy3 (1:50)</td>
</tr>
<tr>
<td>E-cadherin/claudin-1</td>
<td>goat anti rabbit IgG cy5 (1:50)</td>
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<td></td>
<td>goat anti rat IgG cy3 (1:50)</td>
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<tr>
<td>F-actin/occludin</td>
<td>goat anti mouse IgG cy5 (1:50)</td>
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<td></td>
<td>TRITC-phalloidin (1:10)</td>
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<tr>
<td>F-actin/claudin-1</td>
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<td>TRITC-phalloidin (1:10)</td>
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<td>F-actin/E-cadherin</td>
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<tr>
<td></td>
<td>TRITC-phalloidin (1:10)</td>
</tr>
</tbody>
</table>

a Antibodies were diluted in PBS or non-fat dry milk as described above.

For the two triple stainings, the combinations of the following primary and secondary antibodies were used: Samples were either stained with a combination of the primary antibodies rat anti ZO-1/rabbit anti claudin-1/mouse anti occludin and the secondary antibodies goat anti rat IgG cy5/goat anti rabbit IgG cy3/goat anti mouse IgG cy2. Or the staining was done with a combination of rat anti E-cadherin/rabbit anti ZO-1 and was detected by the combination of goat anti rat IgG cy5/goat anti rabbit IgG cy2/TRITC-phalloidin.

For colocalization studies, pairs of proteins were labeled as listed in Tab. 1. For imaging of the ZO1-CGFP MDCK cells, the protocol was slightly modified to reduce bleaching of GFP fluorescence. In brief, cells grown on cell culture inserts or Lab Tek™ chambered coverglass systems were fixed.
for 5 min and permeabilized in 0.2 % TritonX-100 in PBS for 3 min. Samples were incubated at 37°C with the primary antibody for 30 min and with the secondary antibody for 45 min. After additional three washes, the cells were mounted with glycerol-based Slow Fade Light™ Antifade mounting medium (Molecular Probes).

2.6. CONFOCAL LASER SCANNING MICROSCOPY

A Zeiss LSM 410 inverted microscope was used (lasers: HeNe 633nm, HeNe 543nm, Ar 488/514nm and Ar UV 364nm; Zeiss, Oberkochen, Germany). Optical sections at intervals of 0.15 or 0.3 µm were taken with a 63x/1.4 Plan Apochromat water objective. Image processing was done on a Silicon Graphics O2 workstation using IMARIS, a 3D multi-channel image processing software for CLSM data sets (Bitplane AG, Zuerich, Switzerland).

For studies with living cells and long-term observations, the transfected cells grown on Lab Tek™ chambered coverglasses systems, were placed in a temperature-controlled chamber at 37°C. Medium was replaced at least every 30 min.

The contrast and brightness settings of the microscope were kept constant during the course of image acquisition throughout a related series.

2.7. DECONVOLUTION AND COLOCALIZATION ANALYSIS

Deconvolution and colocalization analyses were done as described in Rothen-Rutishauser et al. (1998b). Briefly, CLSM data were recorded simultaneously for pairs of fluorescence signals at the Nyquist frequency of the microscope, which corresponds in our microscope to a lateral sampling distance of 50 nm and an axial sampling distance of 150 nm. In order to remove blur, to improve resolution and to reduce noise the Huygens 2 software was applied using a theoretical point spread function (Scientific Volume Imaging B. V., Netherlands). Colocalization analysis was carried
out with the software "Colocalization" (Bitplane AG). For the statistical analysis, confocal images were recorded as described above. For each pair of proteins, the number of colocalized voxels for each layer was calculated for 5 different membrane areas (area: approx. 10 µm x 4 µm, thickness of optical section: 0.15 µm).

### 2.8. SDS PAGE AND IMMUNOBLOTTING

For immunoblot analysis, cell extracts were prepared by direct addition of Laemmli sample buffer without β-mercaptoethanol (Laemmli, 1970). For sample preparation, cells were washed 2 times with PBS (see 2.5) and harvested by scraping (approx. 50-100 µl buffer/cm² cells). Samples were stored at –80°C. The total protein concentration in the samples were determined with the Bio-Rad DC protein assay (Bio-Rad, Glattbrugg, Switzerland). Before SDS PAGE analysis, β-mercaptoethanol was added to the samples, which were heated for 5 min at 95°C. Approximately 20 µg protein per slot were loaded onto mini-gels (Mini ProteanII, Bio-Rad). After separation, proteins were transferred onto nitrocellulose membranes (Hybond-C extra, Amersham). The blot was blocked for 60 min at room temperature with TBBS buffer (20 mM Tris, 500 mM NaCl, 0.1 % Tween, pH 7.4) containing 5 % non-fat dry milk. Blotted membranes were probed with a rabbit anti ZO-1 pAb (Zymed # 61-7300), a rabbit anti occludin pAb (Zymed # 71-1500), a rat anti E-cadherin mAb (Sigma # U3254), rabbit anti claudin-1 pAb (Zymed # 71-7800), a rabbit anti all actin pAb (Sigma # A2066) and a mouse anti GFP mAb (Clontech # 8371-1). The primary antibodies were diluted 1:1000 in TBBS buffer containing 1 % non-fat dry milk and the blot was incubated for 90 min at room temperature. After 5 washing steps at room temperature, 5 min each with TBBS buffer, the blot was incubated for 45 min with the secondary goat anti rabbit, rat or mouse antibodies, conjugated to alkaline phosphatase (Pierce, Socochim SA, Lausanne, Switzerland), diluted 1:30’000. After incubation with the secondary antibody at room temperature and another 5 washing steps at room temperature, bands were detected by chemiluminescence using the
Immun-Star™ substrate (Bio-Rad). The blotted membrane was exposed to X-ray films (Super RX, Fuji, Dielsdorf, Switzerland). For quantification of bands detected with the ZO-1 Ab by densitometry, a computer-assisted imaging device MCID M5 software (Imaging Research Inc., Canada) was used.

2.9. IMMUNOPRECIPITATION

For immunoprecipitation, MDCK and ZO1-CGFP MDCK were grown for 3 d and transfected cells were treated with 1 mM SB for 20 h before cell lysis. For radio-labeling, both cell lines were first incubated with Met/Cys-free medium (Sigma) for 1 h, and then labeled with Promix™ [35S] Met/Cys (Promega) for 24 h. After labeling, cells were lysed in Triton X-100 lysis buffer (0.25 % TritonX-100, 10 mM Tris-HCl, 120 mM NaCl, 25 mM KCl, 2 mM EDTA, 2 mM EGTA), and proteins in the lysate were immunoprecipitated with a rabbit anti human ZO-1 pAb (Zymed # 6-7300). Precipitated proteins were resolved by 7.5 % SDS PAGE, transferred onto nitrocellulose membranes and the signals were detected by autoradiography. The same blots were also immunostained with a rat anti mouse ZO-1 mAb (Chemicon # MAB1520) and a rabbit anti canine ZO-2 pAb (Zymed # 71-1400), respectively.

2.10. “CALCIUM SWITCH” METHOD

The “calcium switch” was performed as described previously (Nigam et al., 1992). Briefly, ZO1-CGFP MDCK cells, at a density of 100’000 cells/cm² for “low density” and 200’000 cells/cm² for “high density”, were allowed to attach in MEM on Lab Tek™ chambered coverglass systems for 1 h at 37°C. The coverglasses were then carefully washed with MEM modified for spinner cultures (SMEM, Sigma), a Ca²⁺-free medium, and kept in low calcium medium (LCM) for 24 h at 37°C. The switch experiments were initiated by replacing LCM with normal MEM.
LCM was prepared according to Gumbiner & Simmons (1986). Briefly, SMEM with 10 % FCS was dialyzed in a Slide-A-Lyzer® 10K Dialysis Cassette (Pierce) at 4°C against 0.15 M NaCl, then 0.15 M NaCl with 0.2 mM EDTA, followed by two changes with 0.15 M NaCl. Each change was done against a 100-fold volume for 10-12 h. The dialyzed medium was diluted with SMEM containing 1.7 mM L-glutamine, 0.2 % NaHCO₃ as well as 100 units/ml penicillin and 100 µg/ml streptomycin. The final LCM contained 1 % FCS.

The calcium concentration in LCM was approximately 2 µM as measured by atomic absorption spectroscopy (AAS) on a Perkin Elmer 2100 AAS (λ = 422.7 nm). For ASS measurements, 0.1 % lanthanum chloride (Fluka # 61490) was added to the samples to avoid interference with phosphate. Standard curves were established with CaCO₃ between 0.5 and 50 µM calcium. For all solutions, deionized water was used.

2.11. “CALCIUM CHELATION” METHOD

For the “calcium chelation” method, confluent ZO1-CGFP MDCK cells (3 d after seeding, 20 h with 1 mM SB) grown on Lab Tek™ chambered coverglass systems or MDCK cells, grown for 11 d on cell culture inserts, were treated with EGTA at 37°C as described by Cereijido et al. (1978). In brief, the normal, Ca²⁺-containing (1.8 mM) MEM was replaced with MEM without FCS supplemented with 2.0 mM EGTA (EGTA medium). After 20 min, the EGTA medium was replaced with normal, Ca²⁺-containing MEM and reconstitution of TJs was observed.

For studies with living ZO1-CGFP MDCK cells, Hoechst 33342 (Molecular Probes) was added to the cells to visualize cell nuclei 15 min before the addition of EGTA. Additionally, the effect of EGTA on the TJs was visualized with rhodamin-dextran, a marker for the paracellular transport. ZO1-CGFP MDCK cells were overlaid with 1.0 mg/ml TRITC-Dextran (MW 4400, Sigma) before the addition of EGTA and z-scans were performed.
2.12. PARACELLULAR TRANSPORT STUDIES

To test the tightness of cell layers, transport studies with $^{14}$C-mannitol were performed in either a Costar® two-chamber vertical diffusion system (see 2.12.1) or, across Falcon® cell culture inserts in 12-well plates (see 2.12.2).

2.12.1. Mannitol flux assay in the Costar® two-chamber vertical diffusion system

Cells were grown on polycarbonate Snapwell™ insert membranes (Costar # 3407, Integra Biosciences AG, Wallisellen, Switzerland) as indicated in 2.2.1. Insert membranes with the cells were fixed in the Costar system, and the donor and receiver compartments each filled with 7.0 ml of MEM containing 1 % FCS. The temperature was kept at 37°C. The contents of each compartment were mixed by a flow of carbogen ($O_2/CO_2 = 95/5$) adjusted to 15 ml/min. Radiolabeled mannitol (1.5 µM, 1.9 GBq/mmol, NEN, Life Sciences, Zaventem, Belgium) was added to the donor compartment, i.e. on the apical side of the cells. Samples of 100 µl were collected from the receiver compartment at the times indicated (up to 10 samples per chamber). Sample volumes were not replaced. At the end of the experiment (120 min), the final mannitol concentration in the donor compartment was determined. Samples were mixed with 3 ml scintillation cocktail and analyzed in a multi-purpose scintillation counter (Beckman CS 6500, Fullerton CA, USA).

2.12.2. Paracellular mannitol flux assay across Falcon® inserts in six well plates

Transport studies were performed in 12-well plates (Falcon # 3503) at 37°C on a rocker (Gasser Apparatebau & Laborzubehör, Teufen, Switzerland) with gentle agitation at maximal angle of 10° with a frequency of 20 min$^{-1}$. MDCK cells were grown on Falcon cell culture PET inserts (0.9 cm$^2$, 0.4 mm, # 3180) for 5 days. The apical chamber contained 0.4 ml and the
basolateral chamber 1.5 ml of MEM. The radiolabeled mannitol was added to the donor compartment, i.e. the apical side of the cells. The initial concentration of $^{14}$C-D-mannitol in the donor compartment was approximately 2 µM (1.9 GBq/mmol, NEN). Samples of 50 µl were collected from the receiver compartment, i.e. the basolateral side of the cells at the times indicated. Sample volumes were not replaced. At the end of the experiment (60-80 min), the final mannitol concentration in the donor compartment was determined. The inserts with the cells were washed twice with transport medium and also analyzed by LSC. Samples were mixed with 3 ml scintillation cocktail and analyzed by a multi-purpose scintillation counter (Beckman CS 6500). No significant absorption of mannitol was found to the cell layers (< 0.05 %).

### 2.12.3. Calculation of the apparent permeability, $P_{app}$ value

$P_{app}$ values were calculated from the linear increase of $Q_r(t)$ (the “cumulative compound amount in the receiver chamber-time” curve according to Martin (1993):

$$P_{app} = \frac{dQ_r}{dt} \cdot \frac{1}{c_o} \cdot \frac{1}{A} [\text{cm/s}]$$  

Eq. 2

where $P_{app}$ [cm/s] is the apparent permeability coefficient, $c_o$ [mol/ml] the initial drug concentration in the donor compartment and $A$ [cm$^2$] the area of the cell layer, i.e the insert membrane. The slope $dQ_r/dt$ was determined by linear regression from the linear range of the $Q_r(t_n)$ curve. $Q_r(t_n)$ was calculated according to Eq. 3.

$$Q_r(t_n) = c_r(t_n) \cdot V_r(t_n) + \sum_{i=1}^{n-1} [c_r(t_i) \cdot V_s(t_i)] [\text{mol}]$$  

Eq. 3

where $V_s(t_i)$ is the sample volume at the time point $t_i$, which was kept constant at 0.05 ml.
2.13. PEPTIDE SYNTHESIS

Different peptides were synthesized corresponding to the entire first and second putative extracellular domains of occludin and claudin-1, respectively. Additionally, peptide fragments from the occludin loop II and claudin-1 loop I peptides with overlapping sequences were synthesized. The control peptide aureocin had a similar low content of charged amino acids as the other peptides. For peptide sequences see Tab. 2.

Tab. 2: Peptide sequences with its number of amino acids and molecular weight

<table>
<thead>
<tr>
<th>peptide</th>
<th>sequence</th>
<th>aa</th>
<th>mw</th>
</tr>
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<tbody>
<tr>
<td>claudin-1 31-81 (loop I)</td>
<td>RIYSYAGDNIVTAQAMYEGLWMSCVSQSTGQVQCKVFDSLNLSTLQATR-NH₂</td>
<td>51</td>
<td>5636</td>
</tr>
<tr>
<td>claudin-1 146-160 (loop II)</td>
<td>QEFYDPMTPVNARYE-NH₂</td>
<td>15</td>
<td>1859</td>
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<tr>
<td>claudin-1 31-49</td>
<td>RIYSYAGDNIVTAQAMYEGY-NH₂</td>
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<tr>
<td>claudin-1 47-65</td>
<td>EGYEGLWMSCVSQSTGQVQCK-NH₂</td>
<td>21</td>
<td>2319</td>
</tr>
<tr>
<td>claudin-1 63-81</td>
<td>QCKYFDSLNLSTLQATR-NH₂</td>
<td>19</td>
<td>2124</td>
</tr>
<tr>
<td>claudin-1 90-135 (loop I)</td>
<td>DRGYGTSLLGSGVPSGGSFGSGYGSYGYSYGYYGYGHGGYTDPR-NH₂</td>
<td>46</td>
<td>4687</td>
</tr>
<tr>
<td>claudin-1 196-243 (loop II)</td>
<td>GVNPTAQSSGLYGSQIYALCNQFYTPAATGLYVDQYLYHYCVVDQPE-NH₂</td>
<td>48</td>
<td>5296</td>
</tr>
<tr>
<td>claudin-1 196-214</td>
<td>GVNPTAQSSGLYGSQIYA-OH</td>
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<td>1899</td>
</tr>
<tr>
<td>claudin-1 211-229</td>
<td>QIYALCNQFYTPAATGLYV-OH</td>
<td>19</td>
<td>2136</td>
</tr>
<tr>
<td>claudin-1 225-243</td>
<td>TGLYVDQYLYHYCVVDQPE-OH</td>
<td>19</td>
<td>2305</td>
</tr>
<tr>
<td>aureocin</td>
<td>MGALIKTGAKIIGSGAAGGLGTYIHKLGK-OH</td>
<td>31</td>
<td>2955</td>
</tr>
</tbody>
</table>

Peptides were synthesized by the group of Prof. A. Beck-Sickinger, Institute of Biochemistry, University of Leipzig (Germany). Peptides were produced according to Langer et al. (2001). Briefly, peptides were synthesized by automated, multiple solid-phase peptide synthesis with a robot system (Syro, MultiSynTech, Bochum, Germany) on a polystyrene-1% divinylbenzene polymer matrix using a 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (rink amide-) or a 4-benzyl-oxybenzyl alcohol (Wang) resin (both from Novabiochem, Läufelfingen, Switzerland).
Analyses of the products were performed by analytical reversed-phase HPLC (Merck-Hitachi, Darmstadt, Germany) and the correct mass was determined by ion-spray mass spectrometry (SSQ 710, Finnigan MAT, Bremen, Germany). The purity of the peptides was approx. 10 % for longer (46-51 aa) and 80 % for smaller peptides (15-21 aa).

### 2.14. TREATMENT OF THE CELLS WITH PEPTIDES

Peptides were prepared as 50 mg/ml stock solutions in DMSO. MDCK cells were seeded at a density of 5 x 10^5 cells/cm² on Falcon® cell culture inserts (0.9 cm², # 3503) and cultured for 3 d, unless otherwise stated. Peptides were added to both sides of the cell culture inserts with a final concentration of 1 mM peptide (unless stated otherwise), 1 % DMSO and 50 µM pefabloc SC (PB, Fluka), a serine protease inhibitor in MEM. After incubation for 48 h, transport studies were performed with ^14^C-mannitol (see 2.12.2). A DMSO control, which contained 1 % DMSO and 50 µM PB only was included in each set of experiments. P\_{app} values were calculated (see 2.12.3) and the P\_{app} values were normalized to the corresponding DMSO control which was set to 100 %.

### 2.15. STATISTICS

Comparisons between values (TEER values, number of colocalized voxels and P\_{app} values) were carried out using a two-sample test. The significance of the difference was investigated on the condition the data obey a Gaussian standard normal distribution with \( n_1 \) and \( n_2 \) independent samples, where \( x_1 \) and \( x_2 \) are the averages of \( n_1 \) and \( n_2 \) samples with a standard deviation of \( \sigma_1 \) and \( \sigma_2 \).

\[
-z_{\alpha/2} \cdot \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}} < x_1 - x_2 < +z_{\alpha/2} \cdot \sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}} \quad \text{Eq. 4}
\]

The two-sample test was conducted at a level of significance of \( \alpha = 0.05 \).
3. RESULTS

3.1. A ZO1-GFP FUSION PROTEIN TO STUDY THE DYNAMICS OF TIGHT JUNCTIONS IN LIVING CELLS

3.1.1. Establishment of a stably transfected MDCK cell line

3.1.1.1 Transient expression of ZO1-GFP in different mammalian cells

Constructs composed of full-length human ZO-1 fused to the C (ZO1-CGFP) - or N-terminus (ZO1-NGFP) of GFP, respectively, were prepared and expressed in MDCK and Caco-2 cells. Additionally, cells were transfected with a C-terminal ZO-1 fragment that was fused to the C-terminus of GFP (ZO1(1279-1745)-CGFP). As a control, we transfected TJ-deficient BHK-21 fibroblasts. Living cells were examined 2 d after transfection by CLSM to investigate the localization of the GFP constructs.

Fig. 7: Localization of different GFP constructs by CLSM in unfixed, transiently transfected MDCK, Caco-2 and BHK-21 cells, respectively. Cells were grown on Lab Tek™ chambered coverglass systems and mounted on the microscope at 37°C as described in Methods. (a) ZO1-CGFP in MDCK cells; (b) ZO1-NGFP in MDCK cells; (c) ZO1(1279-1745)-CGFP in MDCK cells; (d) GFP only in MDCK cells; (e) ZO1-CGFP in Caco-2 cells; (f) ZO1-CGFP in BHK-21 cells. Optical sections (x,y) are shown with x,z- and y,z-projections, respectively.
In transiently transfected MDCK cells, both ZO1-CGFP (Fig. 7a) and ZO1-NGFP (Fig. 7b) were preferentially transported to the plasma membrane, where they colocalized with endogenous ZO-1 (data not shown). In contrast to ZO1-CGFP, ZO1-NGFP showed some cytoplasmic fluorescence. The C-terminal ZO-1 fragment (ZO1(1279-1745)-CGFP) was found at the lateral membrane, but was also present throughout the cytoplasm (Fig. 7c). MDCK cells expressing a construct encoding GFP alone displayed diffuse fluorescence throughout the cytoplasm and the nucleus (Fig. 7d). In Caco-2 cells, ZO1-CGFP (Fig. 7e) showed the same expression pattern as in MDCK cells. Expression of ZO1-CGFP in BHK-21 cells (Fig. 7f) was found throughout the cytoplasm, but no fusion protein was seen in the nucleus. In the following, the ZO1-CGFP construct was chosen for stable transfection.

3.1.1.2  **Transient expression of Occludin-GFP in MDCK cells**

A construct composed of full-length dog occludin fused to the C-terminus of GFP (Occ-CGFP), was prepared and expressed in MDCK cells. Living cells were examined 2 d after transfection by CLSM. In MDCK cells transiently transfected with an Occ-CGFP construct, occludin-CGFP was transported to the plasma membrane, but was also present throughout the cytoplasm (Fig. 8).

**Fig. 8: Localization of a occludin-CGFP by CLSM in unfixed, transiently transfected MDCK cells.** Cells were grown on Lab Tek™ chambered coverglass systems and mounted on the microscope at 37°C as described in methods. An optical sections (x,y) is shown a with x,z- and y,z-projection, respectively.
Occ-CGFP transfected MDCK cells were analyzed 4 d after plating with SDS PAGE and immunoblots (Fig. 9). In MDCK cells (lane 1) as well as in Occ-CGFP MDCK cells (lane 2) a band around 65 kD was detected with a rabbit anti occludin pAb. In Occ-CGFP MDCK cells an additional band at 85 kD was observed. The 85 kD correspond to the predicted molecular mass of the Occ-CGFP fusion protein. A strong overexpression of Occ-CGFP in the stably transfected cell line was not only seen by Western blot analysis but also in the CLSM (not shown).

![Fig. 9: Expression of the Occludin-GFP fusion protein by Western blots. Lysates of non-transfected MDCK cells (lane 1) and Occ-CGFP transfected MDCK cells (lane 2) were probed in an immunoblot with a polyclonal antibody against occludin. For experimental details see Methods.](image)

### 3.1.1.3 Stably transfected ZO1-CGFP MDCK cells

**Characterization by microscopy.** Stably transfected ZO1-CGFP MDCK cells, treated for 18 h with 1mM SB to enhance the expression, and non-transfected MDCK cells were analyzed both in the phase contrast microscope and in the CLSM. With phase contrast microscopy no difference in the overall cell morphology was found between the two cell lines (Fig. 10a and b). For CLSM non-transfected and stably transfected MDCK cells were fixed with 3 % paraformaldehyde for 5 min at 25°C on day 3 after plating. Preparations were triple labeled with phalloidin-coumarin for F-actin, a rabbit anti ZO-1 pAb and a rabbit anti occludin pAb, respectively. No difference in actin filament organization was found as judged by F-actin staining between stably transfected MDCK cells expressing ZO1-CGFP (Fig. 10c) and non-transfected MDCK cells (Fig. 10d). Complete colocalization of ZO1-CGFP with antibody labeled endogenous ZO-1 (Fig. 10e), and antibody labeled occludin (Fig. 10f), respectively, could be shown in ZO1-CGFP MDCK cells.
Fig. 10: Comparison of non-transfected and ZO1-CGFP MDCK cells by microscopy. Cells were cultivated for 3-4 d and transfected cells were treated with 1 mM SB for 18 h before analysis (for details see Methods). Phase contrast microscopy of living (a) ZO1-CGFP and (b) non-transfected MDCK cells. For CLSM, cells were fixed and stained as indicated. CLSM of (c) ZO1-CGFP and (d) non-transfected MDCK cells stained for F-actin. Colocalization (yellow) of ZO1-CGFP (green) with endogenous antibody-labeled TJ proteins (e) ZO-1 (red) or (f) occludin (red) in ZO1-CGFP transfected MDCK cells. Micrographs c, d, e and f represent single optical sections (x,y) with xz- and yz-projections, respectively.
Western blot analysis. The stably transfected ZO1-CGFP MDCK cells and non-transfected MDCK cells were characterized with SDS PAGE and immunoblots after 4 d in culture (Fig. 11). In ZO1-CGFP MDCK cells, a band at 240 kD was detected with a GFP antibody (lane 3), whereas a ZO-1 antibody revealed a major protein band at 210 kD and a minor band at 240 kD (lane 7). The intensive band at ~210 kD detected with the ZO-1 antibody represents the endogenous ZO-1 protein. It is present in transfected and non-transfected MDCK cells. The band at ~240 kD is only present in transfected cells. The 240 kD correspond to the predicted molecular mass of the ZO1-CGFP fusion protein. Lysates of non-transfected MDCK cells were GFP negative (lane 1), whereas in MDCK cells transfected with cDNA encoding for GFP alone, a band at 30 kD was resolved (lane 2). As a control, lysates of TJ-deficient BHK-21 fibroblasts were used which express only small amounts of ZO-1 (lane 8) as compared to non-transfected MDCK cells (lane 5). At 70 kD, a non-specific band was seen with the GFP antibody in non-transfected as well as in transfected MDCK cells (lanes 1-3).

To study the level of ZO1-CGFP expression in stably transfected MDCK cells, total cell lysates of non-transfected and ZO1-CGFP MDCK cells were
probed in a Western blot analysis using a ZO-1 antibody. Transfected ZO1-CGFP MDCK cells, treated for 18 h with different concentrations of SB (up to 5 mM), which is known to for non-specifically enhancing protein expression, and non-transfected MDCK cells were analyzed 4 d after plating. The ZO1-CGFP contributed between 10 and 50 % (estimate from densitometry) to the total amount of ZO-1 protein. Expression of ZO1-CGFP protein increased with increasing SB concentration (Fig. 12) with a concomitant reduction of the expression of endogenous ZO-1. No overexpression of total ZO-1 was found in transfected cells as compared to non-transfected MDCK cells. For further experiments, 1 mM SB, which produces adequate fluorescence intensity for CLSM and preserves the viability, was chosen as standard concentration. SB concentrations ≥ 2 mM resulted in a decreased viability of the cells (data not shown).

Additionally, the time-dependent expression of ZO1-CGFP was investigated by Western blots and CLSM. ZO1-CGFP MDCK cells, treated for 18 h with 1 mM SB, were cultured for 1, 3, 8 and 10 days, respectively and compared with non-transfected MDCK cells (Fig. 13). A time-related fusion protein expression occurred in ZO1-CGFP MDCK cells. ZO1-CGFP protein expression decreased from day 3 after seeding. At day 10, no fusion protein could be detected any more. The age-related decrease for ZO1-
CGFP expression was also observed by CLSM. By day 8 in culture, no ZO1-CGFP was expressed in ZO1-CGP MDCK cells anymore (not shown).

**Fig. 13: Time-dependent ZO1-CGFP expression in stably transfected MDCK cells.** Lysates of MDCK and ZO1-CGFP cells (1 mM SB) were probed with a polyclonal ZO-1 antibody. Cells were cultured for 1, 3, 8 and 10 days (representative picture out of 2 independent experiments).

**Immunoprecipitation.** For further characterization, non-transfected and ZO1-CGFP MDCK cells labeled with [35S]Met/Cys were immunoprecipitated with a polyclonal ZO-1 antibody (Fig. 14). Immunoprecipitates were analyzed by autoradiography (lanes 1,2). The identities of the bands were verified by Western blotting with the respective antibodies (lanes 3,4). In non-transfected MDCK cells (lanes 1,3) and ZO1-CGFP MDCK cells (lanes 2,4), analysis of ZO-1 immunoprecipitated protein complexes showed the presence of a band at 160 kD and 210 kD, corresponding to the molecular weights of ZO-1 and ZO-2, respectively. Transfected cells exhibited an additional band at 240 kD. The ratio between ZO-1 and ZO-2 in MDCK cells is similar to the ratio of the sum of ZO1-CGFP and ZO-1 to ZO-2 in transfected MDCK cells.

**Fig. 14: Co-immunoprecipitation of ZO-1 and ZO-2 in non-transfected and ZO1-CGFP MDCK cells.** Non-transfected MDCK cells (lanes 1, 3) and ZO1-CGFP MDCK cells (lanes 2, 4) were labeled with [35S]Met/Cys, lysed and precipitated with a polyclonal ZO-1 antibody. Precipitates were submitted to SDS PAGE and bands were detected by autoradiography (lane 1,2) or by Western blots with both ZO-1 and ZO-2 antibodies (lane 3,4).
Results

Growth curves, TEER and mannitol transport. Non-transfected MDCK cells and ZO1-CGFP MDCK cells were further characterized with respect to their growth curves, TEER values and paracellular transport characteristics. Cells were seeded at a density of $5 \times 10^4$ cells/cm$^2$. Transfected cells were treated with 1 mM SB for 18 h prior to use and compared to non-transfected cells. Both cell types grew confluent within two days and reached a plateau of $5 \times 6 \times 10^5$ cells/cm$^2$ after 10 days in culture (Fig. 15).

![Fig. 15: Growth characteristics of non-transfected and ZO1-CGFP MDCK cells.](image)

Cells were cultivated under standard conditions and transfected cells were treated with 1 mM SB for 18 h before measurements (for details see Methods). Representative growth curves of MDCK (○) and ZO1-CGFP MDCK treated with 1 mM SB (▲). Each data point represents the mean ± standard deviation of 3 independent experiments.

The TEER measurements in both cell lines revealed a plateau of about $180 \, \Omega \, \text{cm}^2$ 3 days after plating (not shown). Both MDCK cells and ZO1-CGFP MDCK cells, treated at day 3 with 1 mM SB for 24 h, showed an increase in TEER from 180 to $300 \, \Omega \, \text{cm}^2$ as compared to non-treated cells (Fig. 16). As we could show with immunoblots this increase in TEER did not correspond to an increased expression of occludin, claudin-1, or actin (data not shown). Transport studies were performed with $^{14}$C-mannitol to get an indication about the tightness of the cell layer in transfected and non-transfected MDCK cells, respectively. Similar Papp values ($\sim 2 \, \text{cm/s} \times 10^{-6}$) were obtained for both cell lines.
3.1.2. In vivo dynamics of ZO-1 after prolonged calcium depletion in low calcium medium

We examined the effect of long-term Ca\(^{2+}\) depletion on ZO1-CGFP MDCK cells by using the “calcium switch” (see Methods). Cells were trypsinized, seeded at either “low density” (100’000 cells/cm\(^2\)) or “high density” (200’000 cells/cm\(^2\)), and grown in LCM for 24 h. The localization of ZO1-CGFP was studied by CLSM in living cells over a period of 6 h. At the time of switching from LCM to normal calcium conditions (0 h), cells seeded at “low density” were spread to some extent and exhibited no cell-cell contacts. The ZO1-CGFP was diffusely organized in the cytoplasm (Fig. 17) and was not found at the plasma membrane in the region of the junctional complex as seen in confluent monolayers under normal calcium conditions (see Fig. 10). In cells seeded at “high density”, some cells exhibited cell-cell contacts. At these contacts, ZO1-CGFP was found concentrated at the plasma membrane even before restoration of normal Ca\(^{2+}\) concentrations. After switching from LCM to normal MEM, time-lapse series revealed the formation of new contacts between neighboring cells between 3 and 6 h after the Ca\(^{2+}\) switch. The ZO1-CGFP appeared at the plasma membrane exclusively at the sites of cell-cell contact. Some of the fluorescence remained in the cytoplasm even if TJs had been established after 6 h. After 12 h ZO1-CGFP fluorescence was predominantly found at
**Fig. 17**: Time-lapse series of ZO1-CGFP MDCK cells treated with the “calcium switch” method. The kinetics of ZO1-CGFP distribution in ZO1-CGFP MDCK cells, plated in LCM for 24 h and subsequently switched to normal medium, was studied in the CLSM. Cells were seeded at “low density” (100’000 cells/cm²) or “high density” (200’000 cells/cm²) and the pictures were taken at the times indicated. Optical sections (x,y) with xz- and yz-projections through the whole cell layer are shown. Cells plated at “high density” already made some contacts in LCM medium (*). After switching from LCM to normal MEM, time-lapse series revealed the formation of new cell-cell contacts (arrows). In cells without cell-cell contacts, ZO1-CGFP was not found at the plasma membrane (arrowheads).
the membrane (not shown). Note that in cells after Ca\(^{2+}\) depletion and during subsequent formation of new cell-cell contacts, ZO1-CGFP was also found in the nuclei, whereas it was not localized in the nuclei in confluent cells (not shown).

3.1.3. **In vivo dynamics of ZO-1 under short time EGTA treatment**

The influence of EGTA treatment on ZO1-CGFP MDCK cells was visualized by CLSM. “Ca\(^{2+}\) chelation” experiments were performed as described (see Methods). The opening of TJs, which occurred as a consequence of Ca\(^{2+}\) chelation, and the reformation after reconstitution of the normal Ca\(^{2+}\) concentration, was followed by time-lapse imaging of the fluorescence of ZO1-CGFP in living cells (Fig. 18). Before EGTA treatment, ZO1-CGFP was exclusively localized near the apical side at the cell-cell contacts. After 15 to 20 min EGTA treatment, the majority of cells had rounded up and detached from their neighbors. Intercellular junctions were disrupted as can be clearly seen in the xz- and yz-projections. In all cells, ZO1-CGFP remained associated with the plasma membrane in a rim-like staining pattern. Junctional areas bordering the contacts between three or four cells appeared to be most susceptible to the effects of EGTA, since they were first to display changes in ZO1-CGFP localization. During EGTA treatment, the ZO1-CGFP label at the membrane appeared to be fuzzier than in untreated control cells and after restoration in normal medium, respectively (Fig. 18, insets). After replacement of the EGTA medium with normal MEM, cells flattened and made again contact with their neighbors. Within 90 min, the ZO-1 network was almost restored, except for some spots at the multicellular contacts.
Results

Fig. 18: Effect of EGTA treatment on confluent ZO1-CGFP MDCK cells (“calcium chelation” method). Stably transfected ZO1-CGFP MDCK cells cultured for 3 days were treated with EGTA and living cells were studied in the CLSM with the time-lapse mode. Pictures were taken at the times indicated and fluorescence was followed: ZO1-CGFP (green) and nuclei (blue). Single optical sections (x,y) with xz- and yz-projections through the whole cell layer are shown. For each time point a 3D reconstruction (“shadow projection”) is shown.

The effect of EGTA was additionally visualized with rhodamin-dextran. The “Ca\(^{2+}\) chelation” experiments were performed as described (see above and Methods). Before the addition of EGTA, rhodamin-dextran was added. The opening of the TJ was followed by time-lapse imaging of the ZO1-CGFP and dextran fluorescence in living cells (Fig. 19). Before EGTA treatment, dextran was not able to pass the MDCK monolayer and ZO1-CGFP was seen as a single contact point in the middle of two neighboring cells. After 4 min, ZO1-CGFP was seen as two points between two
neighboring nuclei. As a consequence of the disruption of the TJs, cells rounded up and dextran entered the gaps between the cells.

Fig. 19: Opening of tight junctions followed with rhodamin-dextran in ZO1-CGFP MDCK cells. Stably transfected ZO1-CGFP MDCK cells cultured for 3 d were treated with the “calcium chelation” method and living cells were studied in the CLSM. Pictures represent cross-sections (z-sections) of the cell layer (for details see Methods). Pictures were taken at the times indicated and fluorescence was followed: ZO1-CGFP (green), dextran (red) and nuclei (blue).


### 3.2. Dynamics of Tight and Adherens Junctions under EGTA Treatment as Studied in Fixed Cells by Co-localization Analysis in the Confocal Microscope

#### 3.2.1. Dynamics of TJs upon Ca^{2+} chelation

MDCK cells were grown for 11 days and the "Ca^{2+} chelation" experiment was performed as described in Methods. The addition of EGTA medium resulted in a decrease in TEER (Fig. 20). TEER dropped to 30% of the initial value (~200 $\Omega$ cm$^2$) after 20 min EGTA treatment. Replacement of the EGTA medium with MEM, i.e. restoration of the normal Ca$^{2+}$ concentration, lead to the recovery of TEER to 60 - 70% of the initial value within 60 min. After 3 h it was back to the control value. According to this TEER profile, four time points were defined to follow the opening and resealing of TJs and AJs: (A) control at 0 min, before addition of EGTA medium, (B) after 20 min exposure to EGTA medium, (C) after 1 h recovery in normal, Ca$^{2+}$-containing medium, and (D) after 6 h recovery in normal medium.

![TEER Values over Time](image.png)

**Fig. 20: Effect of calcium chelation on TEER.** MDCK cells were grown for 11 d and treated with the "Ca^{2+} chelation" method. TEER values were determined at the times indicated. Four time points were defined for further studies: (A) control at 0 min before addition of EGTA medium; (B) after 20 min in EGTA medium; (C) 1 h after replacing EGTA medium with normal, Ca$^{2+}$-containing medium; (D) 6 h after replacing EGTA medium with normal, Ca$^{2+}$-containing medium. Each data point represents the mean ± standard deviation of 5 individual cell culture inserts of one representative experiment.

To start with, the influence of EGTA treatment on MDCK cells was visualized by CLSM. The dynamics of TJs was studied in fixed cells with an antibody against the TJ protein ZO-1 (see Methods). Fig. 21 shows
image restorations in the SFP mode of the four time points defined above. Images represent cumulative projections of 40 optical sections of 0.15 µm each. In normal medium (time point A), TJs appeared as a continuous network (Fig. 21a, a’). Under Ca²⁺ chelation, cells began to detach from each other and to round up (time point B, Fig. 21b).

**Fig. 21: Effect of calcium chelation on the localization of ZO-1.** Cells were cultured and treated with the "Ca²⁺ chelation" method as described in Methods. Cells were fixed at time points A-D (see Fig. 20) and labeled with an anti ZO-1 antibody (see Methods). Micrographs were taken in the CLSM: a,b,c,d show 3D reconstructions (SFP mode) to illustrate the ZO-1 network at time points A-D; a’, b’, c’, d’ show a selected region (see insets) of a-d at higher magnification. The arrows in b’ point to ZO-1 positive connections between two neighboring cells.

At higher magnification, ZO-1-positive connections could be observed between neighboring cells (Fig. 21b’ arrows). ZO-1 always remained localized at the cell membranes. Fluorescent strands appeared slightly swollen as compared to the control preparation. After replacement of the EGTA medium with normal, Ca²⁺-containing medium contacts between cells were reestablished within 60 min (time point C, Fig. 21c, c’). The slight swelling persisted. A complete network, indistinguishable from the
control kept in normal medium, was restored within 6 h (time point D, Fig. 21d, d’). Inhibition of protein synthesis by addition of cycloheximide to the cell cultures did not influence the outcome of the “Ca^{2+} chelation” experiment (data not shown).

3.2.2. Localization of TJ and AJ proteins during the opening and reformation of cell junctions

The localization of the TJ and AJ proteins ZO-1, occludin, claudin-1, and E-cadherin, as well as F-actin was studied in fixed cells at the time points A-D (see Fig. 20). Preparations were triple stained for ZO-1/occludin/claudin-1 or ZO-1/E-cadherin/F-actin. ZO-1, for which the localization in the four time points has been illustrated above (Fig. 21) was kept as a reference in both types of preparations. Areas of interest were chosen for each time point and optical sections taken at the level of the junctional complex for each of the three labeled proteins (Fig. 22). In control cultures, before EGTA treatment, ZO-1 and occludin were exclusively found at the cell-cell contacts whereas claudin-1 was localized at the cell-cell contacts as well as along the lateral membranes (data not shown). E-cadherin showed a bright signal at the cell-cell contacts and in addition a weak signal in the cytoplasm. A similar distribution was found for F-actin except that the signal in the cytoplasm was clearly stronger and stress fibers were extending through the basal part of polarized cells (not shown). After 20 min incubation in EGTA medium, when the TJs were opened, occludin showed a similar distribution as ZO-1, however, localization at the membrane was more diffuse than seen with ZO-1. In contrast, claudin-1 and E-cadherin were localized throughout the cells, and F-actin was arranged in a typical peripheral belt. One hour after replacement of the EGTA medium with normal, Ca^{2+}-containing MEM, the reformation of the TJ network was in an advanced state.

The complete relocation, indistinguishable from the control, was fast for ZO-1, but took longer for occludin, claudin-1, E-cadherin and F-actin. Only
**Fig. 22: Effect of EGTA treatment on the localization of TJ/AJ proteins and F-actin.** Cells were cultured and treated with the "Ca²⁺ chelation" method. Cells were fixed at the four time points (A, B, C, D) defined in Fig. 20 and triple-stained for ZO-1/occludin/claudin-1 (a) and ZO-1/E-cadherin/F-actin (b), respectively, as described (see Methods). Pictures were taken in the CLSM. Single optical sections (x,y) are shown.
after 6 h recovery (time point D) in normal medium the network was fully restored indistinguishable from the control staining pattern.

### 3.2.3. Colocalization of TJ and AJ proteins during the opening and reformation of cell junctions

Colocalization studies (see Methods) were performed with pairs of proteins in double-labeled samples as indicated in Tab. 1. The colocalization patterns are visualized in 3D views (SFP mode) from the top of the cells (x,y-orientation) comprising the sum of colocalized voxels, and in a projection (x,z-axis) of the same region of interest (Fig. 23). In control cultures (time point A), i.e. before EGTA treatment, two patterns can roughly be distinguished. For ZO-1/E-cadherin, ZO-1/F-actin, ZO-1/occludin, ZO-1/c Claudin-1, Claudin-1/ occludin and F-actin/occludin colocalization was found exclusively at the TJ position, i.e. at cell-cell contacts. The other pairs, namely E-cadherin/occludin, E-cadherin/c Claudin-1, F-actin/c Claudin-1 and F-actin/E-cadherin showed colocalization in the TJ position, but also along the lateral membrane, which is clearly seen in the xz-projections. Additionally, F-actin/c Claudin-1 and F-actin/E-cadherin showed a non-negligible labeling in the cytoplasmic region. With exposure to EGTA medium for 20 min (time point B), the colocalization pattern of all protein pairs changed with the most striking differences in the pairs with ZO-1. For ZO-1/E-cadherin and ZO-1/F-actin, colocalization remained confined to the cell membranes, but was extended beyond the cell-cell contacts along the lateral plasma membrane. In contrast, ZO-1 colocalization with the TJ proteins occludin and Claudin-1 was reduced to a few spots in the region of cell-cell contacts. The same distribution pattern was found for Claudin-1/occludin, E-cadherin/occludin and E-cadherin/c Claudin-1. For F-actin pairs different patterns were seen. In case of F-actin/occludin the coherent network along the cell-cell contacts found in the control (time point A) was replaced by a beaded pattern of colocalization. For F-actin/c Claudin-1 and F-actin/E-cadherin colocalization was reduced in the TJ/AJ area, whereas it remained strong in the cytoplasm. After replacement
Results

ZO-1/E-cadherin

ZO-1/F-actin

ZO-1/occludin

ZO-1/claudin-1

claudin-1/occludin
**Fig. 23: Colocalization of TJ/ AJ proteins and F-actin.** Cells were cultured and treated with the "Ca\(^{2+}\) chelation" method as described in Methods. Cells were fixed at the four time points (A, B, C, D) defined in Fig. 20 and double-stained for pairs of TJ and AJ proteins as well as F-actin (see Methods). Micrographs were taken in the CLSM at fixed settings and colocalization was analyzed for each protein pair. Colocalization is illustrated as a 3D reconstruction (upper micrograph) and with the corresponding x,z-projection (lower micrograph) for each time point and each protein pair. Intensities correspond to the cumulative colocalized voxels.
of EGTA medium with normal, Ca\(^{2+}\)-containing medium, colocalization was gradually restored (time point C). Colocalization patterns indistinguishable from the respective controls were obtained for all pairs tested within 6 h recovery in Ca\(^{2+}\)-containing medium (time point D).

3.2.4. Statistical analysis of colocalization of TJ and AJ proteins during the opening and reformation of cell junctions

To get from the purely descriptive level to a more quantitative statement, the data presented in Fig. 23 were reanalyzed. Five representative membrane regions (area approx. 10 µm x 4 µm, thickness of optical section: 0.15 µm) comprising single cell-cell contacts, i.e. membrane areas, were selected. The number of colocalized voxels of these regions was calculated for each protein pair in each optical section along the z-axis, i.e. through the cell layer (~ 9 µm). Colocalization profiles of all protein pairs are plotted in Fig. 24. The focus is on time point A (control, before addition of EGTA medium) and time point B (after 20 min EGTA medium). The full recovery of TJs, documented in Fig. 23, was confirmed with this analysis, but time point C and D have been omitted for clarity. Despite the relatively large standard deviations very clear patterns emerged. Before Ca\(^{2+}\) chelation (time point A) all tested protein pairs fell roughly into two classes according to their colocalization profile. The four ZO-1 pairs as well as F-actin/occludin exhibited one prominent colocalization peak in the junctional area, i.e. within the layers 1 to 25, close to the apical level. For occludin/claudin-1, E-cadherin/occludin, F-actin/claudin-1 and F-actin/E-cadherin, colocalization was not limited to the junctional area, but extended along the z-axis. A first peak was present in the TJ area in agreement with the other protein pairs. However, a high level of colocalization persisted down to levels 40 to 50. For all protein pairs tested the colocalization pattern changed significantly under Ca\(^{2+}\) depletion. The colocalization peak for ZO-1/E-cadherin and ZO-1/F-actin shifted to a position between layers 30 to 40, while the maximum level of colocalized voxels doubled. For ZO-1/occludin and ZO-1/claudin-1, in contrast, the colocalization peak was
**Fig. 24: Dynamics of TJs and AJs during EGTA treatment.** From the micrographs shown in Fig. 23, five representative membrane regions (area approx. 10 µm x 4µm) were selected and the number of colocalized voxels was calculated for each optical layer. The first layer (#1) corresponds to the optical section at the apical position, the last layer (# 60) to the one at the basal position of the cells. Colocalization plots: time point A (black, control), time point B (gray, 20 min EGTA treatment). Each data point represents the mean ± standard deviation of the number of colocalized voxels of 5 representative membrane regions. Arrows point to regions of cell-cell contacts.
Results

reduced to about 15 % of the control peak with a shift from around layer 15 to around layer 25. With claudin-1/occludin a similar pattern was found as with ZO-1/occludin and ZO-1/claudin-1. The peak at position 15 was shifted to a position around 25, however the reduction was not as prominent as with ZO-1/occludin and ZO-1/claudin-1. The second peak (around position 43) encountered in the control preparation of the claudin-1/occludin pair disappeared under EGTA treatment.

In the case of E-cadherin/occludin and E-cadherin/claudin-1 no significant colocalization remained after EGTA treatment. For F-actin/occludin a shift of the colocalization peak was noted from layer 15 to layer 30 and the level of colocalization was reduced to about 50 %. With F-actin/claudin-1 significant colocalization was found in a broad peak between layer 20 to 50 in the controls. It was reduced to below 20 % of the control level. Finally, with F-actin/E-cadherin the first peak of colocalization (around layer 20) disappeared completely, whereas the second peak remained, though slightly shifted.

For better comparison of the changes in colocalization between the protein pairs the total amount of colocalized voxels (xyz-space; 6 µm^3) was analyzed for selected pairs (Fig. 25). Time point A of each pair was used as internal control and the respective numbers of colocalized voxels at the time point B were normalized to the control values. This analysis shows an increase (between 220 and 280 %) in the number of colocalized voxels for ZO-1/E-cadherin and ZO-1/F-actin. In contrast, for ZO-1/occludin and ZO-1/claudin-1 a significant decrease of colocalized voxels to less than 30% of the control was observed. The same trend was found with claudin-1/occludin, although the difference was not statistically significant in this case. In the case of E-cadherin/occludin and E-cadherin/claudin-1 colocalization disappeared completely under EGTA treatment. For the remaining F-actin/occludin, F-actin/claudin-1 and F-actin/E-cadherin pairs there was a tendency for a reduction of colocalized voxels. In the case of F-actin/occludin it was not statistically different from the control value.
**Fig. 25: Statistical analysis of colocalization during EGTA treatment.** Cells were treated with the "Ca\(^{2+}\) chelation" method and prepared for colocalization analysis in the CLSM as described in Fig. 23. For all pairs, membrane regions (area approx. 10 \(\mu\)m x 4 \(\mu\)m) were analyzed (Fig. 24) and the total number of colocalized voxels calculated (x,y,z-space): time point A (■, control) and time point B (■, 20 min EGTA treatment). For each protein pair the numbers were normalized to the number of colocalized voxels in the control (time point A) and expressed as percentage. Means ± standard deviations of the number of colocalized voxels were calculated for each protein pair from 5 representative membrane regions. Colocalization values of pairs with 95% significance as compared to the respective control, determined by a two-sample test, were labeled with an asterisk.
3.3. MODULATION OF TIGHT JUNCTIONS WITH PEPTIDES HOMO-LOGOUS TO THE TWO EXTRACELLULAR LOOPS OF OCCLUDIN AND CLAUDIN-1

3.3.1. Effect on the paracellular transport

Synthetic peptides corresponding to the entire first and second extracellular loops of occludin and claudin-1 were synthesized and assayed for their ability to impair TJs. First, the effect of the peptides was tested by measurement of the paracellular transport. MDCK cells were cultured and treated with the occludin loop I, occludin loop II, claudin loop I and claudin loop II peptides (1 mM) as described in Methods (2.14). After incubation for 48 h at 37°C, transport studies were performed with 14C-mannitol (for details see 2.12.2).

Treatment of the MDCK monolayers with the occludin loop II and claudin loop I peptides caused a more than two-fold increase of the mannitol flux as compared to the DMSO control. The effect of the two peptides was not significantly different. In contrast, the occludin loop I and claudin loop II peptides, did not alter the flux (Fig. 26).

![Graph showing the effect of peptides on paracellular transport]

Fig. 26: Effect of peptides corresponding to the two extracellular loops of occludin and claudin on the paracellular transport. MDCK cells were cultured and incubated with the respective peptides (1 mM) for 48 h at 37°C. A DMSO control was included in the experiment. Transport studies were performed with 14C-mannitol (see Methods). All data were normalized to the DMSO control. Each data point represents the mean ± standard deviation of at least 3 independent experiments. Statistical significance was calculated with a two-sample test. Values with significant increase in the flux (α = 0.05) as compared to the control, are labeled with an asterisk.
Compared with the other three peptides, the occludin loop I peptide was very water insoluble. Therefore the actual concentration in solution is lower than for the other peptides and it can not be excluded that this peptide would also have an effect on the paracellular transport. Since at day 5 after plating, cells are still in the exponential growth phase, $P_{\text{app}}$ values for the control were between $1.0$ and $2.5 \times 10^{-7}$ cm/s. For better comparison between different experiments, the $P_{\text{app}}$ values were normalized to the DMSO control (included in each set of experiments), which was set to 100%.

To exclude non-specific effects, the control peptide aureocin, containing a comparable low content of charged amino acids as the loop peptides, was tested. At a concentration of 1 mM, it had no effect on the paracellular transport. Additionally, the DMSO control did not show a difference in the flux assay as compared to the MDCK cells without the addition of any adjuvant (Fig. 27).

**Fig. 27: Control experiments to exclude non-specific effects of the peptides.** MDCK cells were cultured and incubated with aureocin (control peptide, 1 mM), with the DMSO control and with no adjuvants at all (control) for 48 h at 37°C as described in Methods. Transport studies were performed with $^{14}$C-mannitol (see Methods). All data were normalized to the DMSO control. Each data point represents the mean ± standard deviation of 3 cell culture inserts.

### 3.3.2. Effect on the transepithelial electrical resistance

Next, we tested if the occludin loop II and claudin loop I peptide would also affect the TEER, another important measure for the tightness of TJs. MDCK cells were cultured on Falcon cell culture inserts (4.2 cm²) and treated with the occludin loop II and claudin loop I peptides (1 mM) as
described in Methods. After incubation for 6 h, 24 h and 48 h at 37°C, the TEER was measured (see 2.2.3). Before the addition of the peptides, the TEER was around 160 Ω cm\(^2\). It decreased after addition of the occludin loop II and claudin loop I peptide to the MDCK monolayers. After 6 h, the TEER was just slightly altered compared to the control, whereas after 24 h, the TEER showed a significant decrease to 120 Ω cm\(^2\) for the peptide-treated monolayers. After incubation for 48 h, the TEER did not significantly decrease as compared to 24 h (Fig. 28). The control peptide aureocin did not alter the TEER (data not shown).

\[\text{Fig. 28: Effect and time course of the occludin loop II and claudin loop I peptide on the TEER.} \]

MDCK cells were cultured and incubated with the claudin loop I peptide (■) and occludin loop II peptide (□) at a final concentration of 1 mM for 48 h at 37°C. A DMSO control (■) was included in the experiment. TEER measurements were performed at the times indicate as described in Methods. Preliminary data from at least two cell culture inserts.

### 3.3.3. Cell morphology and viability

To exclude that the effect of the occludin loop II and claudin loop I peptides was caused by general cytotoxicity, we examined the cell morphology and the viability of the peptide-treated cells and the control. To examine the cell morphology, MDCK cells were cultured in TPP® 24-well plates and treated with the occludin loop II and claudin loop I peptides (1 mM) as described in Methods. After incubation for 48 h at 37°C, cells were observed by phase contrast microscopy.
The overall cell morphology of the DMSO control and the peptide-treated monolayers was similar. They showed a regular cobble-stoned morphology and the monolayers appeared to be intact. Only cell-cell contacts seemed to be darker in the peptide-treated cells as compared to the control (Fig. 29).

To test the viability, MDCK cells were cultured and treated with the respective peptide (1 mM) as described in Methods. After incubation for 48 h at 37°C, the viability of the treated cells was checked with a trypan blue test (see 2.2.5). The viability of the peptide-treated and control cells was between 95.8 and 97.6 % (Tab. 3). There was not seen a significantly increased number of death cells.

Cells excluded the vital dye trypane blue, indicating that they remained intact and alive. As shown by phase contrast microscopy and the viability test, the effect of the occludin loop II and claudin loop I peptides on the modulation of TJs is not caused by general cytotoxicity.
3.3.4. Expression of different TJ and AJ proteins

To examine the potential effects of the occludin loop II and claudin loop I peptides on the TJs at the molecular level, the localization of different TJ and AJ proteins was visualized by CLSM. MDCK were cultured and incubated with the occludin loop II and claudin loop I peptides (1 mM) as described in Methods. After incubation for 48 h at 37°C, monolayers were fixed and triple stained for ZO-1/occludin/claudin-1 and ZO-1/E-cadherin/F-actin, respectively (see 2.5).

Similar ZO-1 and occludin stainings were observed for the occludin loop II- and the claudin loop I-treated cells as well as for the control. ZO-1 and occludin were localized at the membranes. Occludin was additionally found in the cytoplasm. The claudin-1 staining for the peptide-treated cells and the control reassembled the occludin staining. Except for the claudin loop I-treated cells, in few regions the claudin staining seems to be blurred or fuzzier than the DMSO control (Fig. 30a). E-cadherin and F-actin distributions were not changed by occludin loop II- and claudin loop I treatment as compared to the control (Fig. 30b).

To further investigate the mechanism of action of the occludin loop II and claudin loop I peptides on the TJs at the molecular level, Western blot analysis of the TJ proteins ZO-1, occludin and claudin-1 as well as the AJ protein E-cadherin were performed. MDCK were cultured and peptides were added as described above. After 48 h incubation at 37°C, monolayers were lysated and characterized by immunoblots with a ZO-1, an E-cadherin, an occludin, an all actin and a claudin-1 antibody. Actin is shown as a reference that equal protein amounts were loaded (see 2.8).
Fig. 30: Characterization of occludin loop II and claudin loop I peptide-treated and untreated cells by CLSM. MDCK cells grown for 3 days on cell culture inserts, were incubated with the respective peptides (1 mM) and control (DMSO, PF) for 48 h at 37°C. Cells were fixed and triple stained for ZO-1/occludin/claudin-1 (a) and ZO-1/E-cadherin/F-actin (b) as described in Methods. Pictures, taken in the CLSM, show single optical sections (x,y). The contrast and brightness settings of the microscope remained constant during the course of image acquisition throughout a series with equal staining.
There was no alternation of the protein expression in the occludin loop II- and claudin loop I-treated cells as compared to the control. The total cellular levels of ZO-1, E-cadherin, occludin and claudin-1 were comparable in all samples and the phosphorylation pattern of occludin remained unaltered (Fig. 31).

![Fig. 31: Characterization of occludin loop II and claudin loop I peptide-treated cells by Western blots. MDCK cells were cultured, peptides (1 mM) were added and incubated for 48 h at 37°C as described in Methods. For all samples equal protein amounts were loaded. Samples of occludin loop II (1)- and claudin loop I (2)-treated cells as well as the DMSO control (3) were tested. Blots were made with antibodies for ZO-1, E-cadherin, occludin, actin and claudin-1. Representative picture out of 3 independent experiments.](image)

### 3.3.5. Dose-dependent effect of the claudin loop I peptide on the paracellular transport

Furthermore, the dose-dependent response of the claudin loop I peptide on the paracellular transport was assayed. MDCK cells were cultured and incubated with the claudin loop I peptide at a final concentration of 1 mM, 200 µM, 40 µM and 5 µM. After incubation for 48 h at 37°C, transport studies were performed with ^14^C-mannitol (see Methods).

The ability of the claudin loop I peptide to increase the paracellular transport was dose-dependent. A maximal effect on the mannitol flux was found at a final concentration of 200 µM peptide, resulting in a 2.3-fold
increase of the flux as compared to the DMSO control. With a concentration of 40 µM peptide, the flux was only increased 1.2-fold. Therefore, the maximal effective concentration will be between 40-200 µM claudin loop I peptide (Fig. 32). It is important to point out that the claudin loop I peptide is not pure, therefore the maximal effective concentration could be even lower.

**Fig. 32: Dose-dependent effect of the claudin loop I peptide on the paracellular transport.** MDCK monolayers, grown for 3 days, were incubated with a final concentration of 1 mM, 200 µM, 40 µM and 5 µM claudin loop I peptide. A DMSO control was included in the experiment. After incubation for 48 h at 37°C, the paracellular flux was measured with ¹⁴C-mannitol (see Methods). All data were normalized to the control. Each data point represents the mean ± standard deviation of 3 cell culture inserts.

### 3.3.6. Effect of the claudin loop I peptide on the paracellular transport at different growth state of MDCK cells

Additionally, the response of the claudin loop I peptide on the paracellular transport on both newly forming monolayers and confluent cells with an established TJ network was tested. MDCK cells were either seeded at a density of 5 x 10⁴ cells/cm² in normal Ca²⁺-containing medium and after three days, the claudin loop I peptide (200 µM) was added to the confluent MDCK cells. Besides, MDCK cells were seeded at a density of 2 x 10⁵ cells/cm², after 24 h in LCM, normal, Ca²⁺-containing MEM with the claudin loop I peptide (200 µM) was added to the non-confluent MDCK
cells. After incubation for 48 h at 37°C with the peptide, transport studies were performed with 14C-mannitol (see Methods).

Treatment of the confluent or newly forming monolayers with the claudin loop I peptide resulted in a similar increase of the paracellular transport. With both approaches, the claudin loop I peptide caused a more than 2-fold increase in the mannitol flux as compared to the DMSO control. $P_{\text{app}}$ values for the control under normal conditions was $2.5 \times 10^{-5}$ cm/s and for the control after low calcium conditions $1.4 \times 10^{-6}$ cm/s. The difference in the paracellular transport is attributed to differences in the growth state of the cells. Briefly, the effect of the claudin loop I peptide on the paracellular transport was not dependent on the two tested growth state of the cells (Fig. 33). It is important to point out that the cells grown under normal Ca$^{2+}$ conditions, even so they were confluent, were growing exponentially and still develop new cell-cell contacts. Further investigations should therefore be done on confluent MDCK cells in the stationary phase (> day 10 after plating).

**Fig. 33: Effect of the claudin loop I peptide on the paracellular transport on confluent versus non-confluent cells.** Newly confluent MDCK monolayers (■) grown for 3 d and MDCK cells grown for 24 h in LCM (■) were incubated with the claudin loop I peptide (200 µM) for 48 h at 37°C. A DMSO control was included in both approaches. Transport studies were performed with 14C-mannitol (see Methods). All data were normalized to the control. Each data point represents the mean ± standard deviation of 3 cell culture inserts.
3.3.7. Effect of fragments of the occludin loop II and claudin loop I peptides on the paracellular transport

To map the binding site of the occludin loop II and claudin loop I peptides more precisely, smaller partially overlapping peptides were used in the flux assay. Fragment regions were selected such as to prevent intramolecular disulfide bonds. MDCK cells were cultured and treated with the occludin loop II and claudin loop I fragments (1 mM) as described in Methods. After incubation for 48 h at 37°C, transport studies were performed with $^{14}$C-mannitol (see Methods).

First, we compared smaller peptides of the second extracellular loop of occludin with the entire occludin loop II peptide. The whole occludin loop II peptide caused a 2.2-fold increase in the mannitol flux compared to the DMSO control. The occludin fragments 196-214 and 225-243 still showed a significant increase in the mannitol flux compared to the DMSO control, even though the increase was reduced to about 1.5-fold. In contrast, the occludin fragment 211-229 did not significantly alter the flux (Fig. 34). Briefly, the occludin fragment partially lost their effect on the paracellular transport. Therefore the occludin interaction may include the entire loop.

Second, we compared smaller peptides of the first extracellular loop of claudin-1 with the entire claudin loop I peptide. The claudin fragment 47-65 had even a stronger effect on the paracellular transport than the whole claudin loop I peptide. The flux increased almost 3-fold compared to about 2.5-fold for the whole peptide. In contrast, the claudin fragments 31-49 and 63-81 lost their activity (Fig. 34). Therefore we conclude that for the claudin loop I, the interaction seems to be restricted to a smaller region of the extracellular loop.
Fig. 34: Effect of peptide fragments of the occludin loop II and claudin loop I peptides on the paracellular transport. MDCK monolayers grown for 3 d on cell culture inserts were incubated with the respective peptides (1 mM) for 48 h. A DMSO control was included in the experiment. Transport studies were performed with $^{14}$C-mannitol (see Methods). All data were normalized to the DMSO control. Values with mean ± standard deviation are calculated from 3 cell culture inserts of one representative experiment. Statistical significance was calculated with a two-sample test. Values with significant increase in the flux ($\alpha = 0.05$) as compared to the respective control, were labeled with an asterisk. The results for the whole occludin loop II and claudin loop I peptide (■) from Fig. 26 were included.
4. DISCUSSION

4.1. A ZO-1 GFP FUSION PROTEIN TO STUDY THE DYNAMICS OF TIGHT JUNCTIONS IN LIVING CELLS

As we could demonstrate by CLSM, the ZO1-CGFP construct in transiently transfected MDCK shows the same distribution pattern as the endogenous ZO-1 protein. This ZO-1 fusion protein thus provides an optimal tool to analyze TJ dynamics in living cells by fluorescence microscopy. Interestingly enough, only the ZO1-CGFP colocalized completely with the native ZO-1, whereas ZO1-NGFP appeared not only at the cell-cell contacts but also in the cytoplasm. Different reasons could account for this finding such as reduced binding affinities for the binding partners or a slower turnover of the fusion protein as compared to the endogenous species. The N-truncated ZO-1 (ZO1 1279-1745-CGFP) appeared not only at the cell-cell contacts, but all along the lateral membranes and in the cytoplasm. This is consistent with the finding that the C-terminus of ZO-1 binds to actin (Itoh et al., 1999). In contrast to the full-length ZO-1, the N-truncated forms could therefore be localized not only at the cell-cell contacts, but also at other actin-rich structures such as the lateral membranes and the cytoplasm (Fanning et al., 1998).

In transfected BHK-21 cells, a TJ- and AJ-deficient fibroblast cell line, ZO1-CGFP is localized in the cytoplasm only and not in the cytoplasmic membrane. This is in agreement with the finding that ZO-1 requires at least AJ-like structures to be targeted to the membrane (Van Itallie & Anderson, 1997; Itoh et al., 1999).

Since the GFP-tag is relatively large, it cannot be excluded a priori that the protein to be tracked could be affected (Kosemund et al., 2000). For instance, binding and regulative properties of ZO-1 could be altered. Additionally, SB treatment could induce cell differentiation (Fiorino & Zvibel, 1996; Moyer et al., 1999). Still the fused GFP does not appear to interfere with the functions of ZO-1 in the ZO1-CGFP transfected MDCK cells. Also non-transfected MDCK cells and stably transfected ZO1-CGFP MDCK cells treated with 1 mM SB were indistinguishable with regard to growth curves and mannitol transport. Additionally, as could be shown by
Discussion

phase contrast and confocal laser scanning microscopy, cell morphology was unchanged. In particular, no difference in actin filament organization was found as judged by F-actin staining. The ZO-1 localization, expression and binding properties were also not affected.

As reported for other transfected cells, expression of the fusion protein could be stimulated by treatment of ZO1-CGFP MDCK cells with SB. For our system the optimal SB concentration was found to be 1 mM SB. In agreement with published data (Tanaka et al., 1991), concentrations higher than 1 mM resulted in reduced viability of the cells and changes in cell shape. A time-related fusion protein expression occurred in ZO1-CGFP MDCK cells in the presence of 1 mM SB. The ZO1-CGFP fluorescence and protein expression decreased from day 3 after seeding despite of the presence of the CMV promoter. At day 10, no fusion protein expression could be detected any more (data not shown). This could be due to the fact that induction of the ZO1-CGFP fusion protein may be cell cycle-dependent or associated with the degree of cell differentiation (Tanaka et al., 1991; Choidas et al., 1998). In the presence of different SB concentrations (0-5 mM), the ZO1-CGFP fusion protein expressed in stably transfected MDCK cells accounted for 5-50 % of the total amount of intracellular ZO-1. The latter remained unchanged as the endogenous ZO-1 protein was down-regulated with increasing expression of ZO1-CGFP. Similar levels were found in other permanently GFP-transfected cell lines (Choidas et al., 1998). It is interesting to note that treatment of the ZO1-CGFP cell line as well as MDCK cells with 1 mM SB resulted in a 50 % increase of TEER as compared to non-treated cells. As we could demonstrate with Western blots this is not due to overexpression of occludin and claudin-1 (not shown). The increase in TEER was also not reflected in decreased $^{14}$C-mannitol permeability, which is consistent with the notion that TEER measurements are subject to relatively large variations, and thus paracellular transport studies are more reliable and reveal more about the tightness of the TJ network (Ho et al., 2000).

Having shown the suitability of the ZO1-CGFP fusion protein to substitute for endogenous ZO-1, it was exploited to investigate the influence of the external calcium ion concentration. Two experimental set-ups were used:
either a short-term treatment with EGTA (Ca\textsuperscript{2+} chelation) of monolayers exhibiting a complete TJ network, or prolonged Ca\textsuperscript{2+} starvation of freshly seeded cells in LCM.

Studies on the dynamics of TJ opening and reformation by microscopic techniques have previously been focused on fixed cells (Siliciano & Goodenough, 1988; Collares-Buzato et al., 1994). With this approach, it was realized early that complexation of Ca\textsuperscript{2+} with chelators such as EGTA resulted in the opening of TJs (Cereijido et al., 1978; Martinez-Palomo et al., 1980). These findings could now be confirmed with time-lapse imaging of ZO1-CGFP in living cells. Under short-term treatment with EGTA, the localization of ZO1-CGFP at the membrane persisted during 15 to 20 min, however the rim-like ZO1-CGFP pattern appeared fuzzier as compared to control cells. This may be indicative for the loosening of the contacts between TJ-associated partners. After replacement of the EGTA medium with normal MEM, cells flattened again and contacts with neighboring cells were restored within 90 min in a reversible process as judged from the appearance of the TJ network. The fluorescent pattern of ZO1-CGFP during the “Ca\textsuperscript{2+} chelation” experiment is comparable with the results of Siliciano & Goodenough (1988) obtained with fixed cells. Theoretically the Hoechst 33342, a DNA intercalating molecule used for orientation within living cells, could have influenced protein synthesis by interfering with DNA replication. In agreement with Citi (1992), inhibition of protein synthesis by addition of cycloheximide to the cell cultures did not influence the outcome of the Ca\textsuperscript{2+} chelation experiments, both with regard to the morphological changes and TEER (data not shown). This renders an influence of the Hoechst 33342 stain unlikely.

The “calcium switch” method (Siliciano & Goodenough, 1988; Citi, 1992) has previously been applied to gain information on the de novo formation of TJs. It comprises a brief attachment phase of 1 h in normal medium before cells are exposed to LCM. When ZO1-CGFP MDCK cells were exposed to Ca\textsuperscript{2+} starvation for 24 h, cells were spread to some extent and ZO1-CGFP fluorescence was found throughout the cells. In cells seeded at “high density”, cell-cell contacts were still present and ZO1-CGFP was additionally found at the site of cell-cell contacts. This indicates that close
proximity of two neighboring cells is sufficient for ZO-1 to localize at the membrane. This was also verify in fixed cells with ZO-1 antibody staining (not shown). After switching from LCM to normal MEM, time-lapse observations of the ZO1-CGFP cells showed significant changes in the ZO1-CGFP localization. Within 1 to 6 h ZO1-CGFP fluorescence in the cytoplasm decreased and instead appeared at the plasma membrane, where new cell-cell contacts were established between neighboring cells. Within 12 h fully confluent monolayers were formed with a complete network of ZO1-CGFP between cells in agreement with studies by Anderson et al. (1989) and Stuart et al. (1994) with fixed cells. There has been a controversy about the possible appearance of ZO-1 in the nucleus under certain conditions of cell growth (Stevenson et al., 1989; Gottardi et al., 1996). Under LCM conditions, ZO1-CGFP fluorescence in living cells was regularly found in the nuclei. Moreover, it was also found in the nuclei in subconfluent cells and in monolayers which had been mechanically damaged (not shown). The use of ZO1-CGFP permits to localize ZO-1 under conditions that exclude artifacts due to fixation of the cells and crossreactivity of the antibody. Proteins larger then 20-40 kD are normally excluded from the nucleus. However, active transport processes, such as “piggyback” mechanisms and nuclear sorting signals, enable larger proteins to pass through the nuclear pore complex. Sequence analysis of ZO-1 resulted in the identification of two nuclear localization, which could be responsible for nuclear enrichment of ZO-1 sequences (Gottardi et al., 1996; González-Mariscal et al., 1999). The ZO1-CGFP was not localized in the nuclei of transfected BHK-21 cells. Thus it could be that this nuclear sorting signal is not recognized in the fibroblast cell line. Our findings support results by Gottardi et al. (1996) who showed that a nuclear localization of ZO-1 is inversely related to the extent to which cell-cell contacts have been established. The dual localization of ZO-1 either in the nucleus or at the TJs may hint at a role of this protein in the transfer of regulatory signals from the cell surface to the nucleus (Gottardi et al., 1996; González-Mariscal et al., 2000). It remains to be investigated whether differences in the localization of ZO-1 either in the nuclei or at the membranes correlate with
the degree of ZO-1 phosphorylation since posttranslational modifications could regulate the signal transduction capability of ZO-1.

To sum up, stably transfected ZO1-CGFP MDCK cells are useful to study TJ modulation under physiological and pathological conditions in living cells. The most attractive feature of ZO1-CGFP resides in its ability to act as a probe for the TJs in a single preparation over an extended period of time. It is particularly attractive as ZO-1 plays an important role not only as a major scaffold of the TJs but also in signal transduction pathways.
4.2. DYNAMICS OF TIGHT AND ADHERENS JUNCTIONS UNDER EGTA TREATMENT

In the current study the distribution and colocalization of different pairs of TJ and AJ proteins during opening and resealing of cell junctions were investigated in MDCK cells using the “calcium chelation” method (Martinez-Palomo et al., 1980; Collares-Buzato et al., 1994). F-actin was included in this study for its special role in the cytoskeletal organization and anchoring of the TJ complex (Fanning et al., 1998).

The colocalization pattern of all TJ and AJ protein pairs, established by CLSM and subsequent image analysis, changed significantly during the opening of the junctions. Differences were most striking between the four ZO-1 pairs: ZO-1/E-cadherin, ZO-1/F-actin, ZO-1/occludin and ZO-1/claudin-1. In control cell cultures, i.e. before the addition of EGTA, colocalization for all ZO-1 pairs was found exclusively near the apical domain at the cell-cell contacts within the TJ belt. Our data also confirm the colocalization between ZO-1 and F-actin found by Fanning et al. (1998) and Wittchen et al. (1999) using a combination of in vitro and in vivo binding assays. Data are also in agreement with previous colocalization and in vitro binding studies that showed binding of ZO-1 to claudin-1 (Itoh et al., 1999) and occludin (Furuse et al., 1994). It is interesting to note that colocalization of ZO-1/E-cadherin is very similar to that of ZO-1/F-actin although there is no evidence for a direct association of ZO-1 with E-cadherin. During EGTA treatment, i.e. the opening of the junctions, colocalization of ZO-1/E-cadherin and ZO-1/F-actin remained confined to the cell membrane, but extended along the lateral membrane beyond the original areas of cell-cell contacts. These findings could be substantiated by statistical analysis. The amount of colocalized voxels increased significantly (2-3-fold) and a shift of the colocalization maxima to a more basal position was observed. For ZO-1/occludin as well as ZO-1/claudin-1, in contrast, colocalization was reduced to a few spots in the region of the cell-cell contacts (~15 % of the control value). This indicates that upon opening of TJs and AJs the colocalization pattern of ZO-1 with other junctional proteins changes completely. The original colocalization of ZO-1 with occludin and
claudin-1 disappears, an increased colocalization with E-cadherin is established. No information is available from other studies for the possible colocalization of these proteins during the opening of established TJs and AJs. Previous colocalization studies had been concentrating on the establishment of new cell-cell contacts of cultured epithelial cells for ZO-1, occludin and E-cadherin (Yonemura et al., 1995; Rajasekaran et al., 1996; Ando-Akatsuka et al., 1999). These authors could demonstrated that ZO-1 associates initially with AJ components prior to the final localization at TJs and evidence was presented that in ZO-1-positive junctions the amount of E-cadherin decreased while occludin increased as epithelial polarization proceeded. These results are consistent with our findings and can be interpreted that ZO-1 colocalizes with E-cadherin as long as no functional TJs exist. They confirm the importance of ZO-1 in the (re)-formation of TJs during epithelial cellular polarization.

For the integral membrane proteins occludin and claudin-1 colocalization in control cells was restricted to the cell-cell contacts. These observations confirm the close positional relationship between occludin and claudin-1 as already shown by Furuse et al. (1998a) by means of a colocalization approach with cross-sectional views. During the opening of TJs with EGTA, colocalization disappeared. As indicated above, occludin as well as claudin binds to ZO-1 (Furuse et al., 1994; Itoh et al., 1999). Since the colocalization of occludin and claudin to ZO-1 was reduced, consequently the close proximity of occludin to claudin-1 disappears. The colocalization data for ZO-1, occludin and claudin-1 indicate a complete rearrangement of the TJ-related proteins upon opening of the TJs.

We have also shown that in control cells occludin and claudin-1, respectively, colocalized with E-cadherin near the apical domain at the cell-cell contacts. This is in agreement with previous colocalization studies from Furuse et al. (1998a) who showed in cross-sectional views that E-cadherin was distributed along the lateral membranes and it was distributed more basely than those from occludin and claudin-1. During EGTA treatment however, this colocalization disappeared. No published data are available for these pairs of proteins.
Before EGTA treatment occludin, claudin-1 and E-cadherin, respectively, colocalized with F-actin, similar to observations with F-actin/ZO-1. In contrast to ZO-1/F-actin, there is no indication for a direct binding of occludin, claudin-1 and E-cadherin to the actin filament, whereas it is known that F-actin is associated through ZO-1 with occludin and claudin-1 (Fanning et al., 1998; Itoh et al., 1999) and through α-catenins with E-cadherin (Rimm et al., 1995). This positional close relationship seems to get lost during EGTA treatment since colocalization of the F-actin/occludin, claudin-1/F-actin and F-actin/E-cadherin pairs was reduced. Taken all together we postulate that during EGTA treatment ZO-1 still binds to F-actin whereas the binding of ZO-1 to occludin and claudin-1 is lost and therefore also the spatial relationship of occludin and claudin-1 to actin filaments.

In summary, we could demonstrate that in established cell-cell contacts ZO-1 colocalized with occludin and claudin-1 in the junctional complex, whereas upon disruption of the TJs, enhanced colocalization of ZO-1 with E-cadherin in the lateral membrane region appeared. Furthermore, we showed that colocalization between occludin, claudin-1, E-cadherin and F-actin disappeared during the opening of the junctions.

The origin of the different binding behavior of ZO-1 to occludin, claudin-1 and E-cadherin in established cell-cell contacts and during the opening of the junctions is not clarified yet. The peculiar distribution of ZO-1 can be partly explained by the affinity of its N-terminal half for the C-terminal sequence of occludin (Furuse et al., 1994) and for the C-terminal sequence of α-catenin, which binds directly to E-cadherin (Itoh et al., 1997). However, it still remains unclear why during the opening of the junctions, ZO-1 is recruited to AJ proteins, whereas in polarized epithelial cells ZO-1 is localized at TJs, although α-catenin is still concentrated at the AJs. Further analysis of the molecular basis should clarify this behavior. It is likely that the different binding behavior of ZO-1 to E-cadherin is due to changes in the conformation of the E-cadherin/catenin complex. It has been suggested that Ca\(^{2+}\) does not work as simple bridges between junctions but also affects E-cadherin to undergo Ca\(^{2+}\)-dependent conformational changes (Vestweber & Kemler, 1985; Pokutta et al., 1994; Nagar et al., 1996).
Furthermore Citi (1992) showed that protein kinase inhibitors prevent opening of cell junction. Even though it has been shown that ZO-1 phosphorylation did not change when Ca\(^{2+}\) was removed for a short time (Howarth et al. 1994), there are still many potential points of attack for kinases and phosphatases in the junctional complex.

Colocalization analysis provides information about the spatial arrangement of two proteins. Since the resolution is limited to about 50-100 nm (Rothen-Rutishauser et al., 1998b), we are restricted to show close proximity but not a direct association of two different proteins. Best evidence for a direct association between different junctional proteins would be the demonstration of binding interactions with biochemical techniques, such as immunoprecipitation or co-sedimentation. Few results were obtained with these methods since the junctional transmembrane proteins are polymerized in detergent resistant strands (Itoh et al., 1999) and protein-protein interaction may not be strong enough to be demonstrated by means of immunoprecipitation. Another promising method for quantitative colocalization studies would be FRET (fluorescence resonance energy transfer) analysis using different GFP fusion proteins with TJ and AJ proteins (Pollok & Heim, 1999). It would have to be demonstrated, however, that the different GFP entities would not interfere with the localization and mutual interactions of proteins.

The combination of CLSM and deconvolution with subsequent colocalization analysis and statistical evaluation represents a powerful tool to explore the relative spatial arrangement of protein pairs and provides the means to follow shifts of colocalization maxima along the xz- and yz-axis. Even though we are limited to show close proximity instead of direct association of two different proteins, this method helps, on a comparative basis, to get a better understanding of the dynamics of the junctional complexes.
4.3. MODULATION OF TIGHT JUNCTIONS WITH PEPTIDES HOMOLOGOUS TO THE TWO EXTRACELLULAR LOOPS OF OCCLUDIN AND CLAUDIN-1

As shown in this work, synthetic peptides homologous to the second extracellular loop of occludin and the first extracellular loop of claudin-1, respectively, were able to impair tight junctions when added to epithelial MDCK monolayers as assayed by paracellular transport studies.

To test if the effect of the two peptides could be attributed to specific interactions of the peptides with the extracellular loops of occludin and claudin-1, we first performed paracellular transport studies with mannitol as well as TEER measurements. The incubation of the cells with the occludin loop II and claudin loop I peptides resulted in a more than two-fold increase of the paracellular transport. Peptides corresponding to the first extracellular loop of occludin and the second extracellular loop of claudin-1 and a control peptide with a similar amino acid composition did not alter the paracellular flux. The increase in the paracellular transport corresponded with a slight decrease of the TEER from 160 to 120 $\Omega \text{cm}^2$. Since the decrease of the TEER correlates with an increase in the paracellular transport it rather attributes to an increased paracellular tight junction permeability than an increased transcellular plasma membrane permeability for ions. The results from the flux and TEER studies were first indications that the effect of the two peptides appeared to be due to specific interactions with the loops.

Wong & Gumbiner (1997) have demonstrated that a peptide consisting of the chicken sequence for the second extracellular loop of occludin blocked the TEER when applied to cultured *Xenopus* A6 cells, whereas a peptide corresponding to the first extracellular loop of occludin had no effect. In their assay, only a peptide modified at the two cysteine residues of the extracellular loop to prevent disulfide bonds had an effect, whereas an unmodified form of the peptide was ineffective. They ascribed this difference either to an increased hydrophilicity of the peptide or to the prevention of intermolecular disulfide bonds. These findings are partly consistent with ours. We also showed that the occludin loop I peptide was ineffective. But in contrast to Wong & Gumbiner (1997), we did show an
effect with the occludin loop II peptide even though it was not modified at the cysteine residues. The differences in these results could be interpreted with different experimental conditions between the studies. First, higher DMSO and FCS concentrations were used and therefore better solubility of the peptides and thus a higher effective peptide concentration could be achieved. Second higher peptide concentrations were used and thus a larger fraction of the peptide may remain non-oxidized. Third and important to point out, is that Wong & Gumbiner (1997) used a *Xenopus* cell line combined with the chicken sequence of occludin. In contrast, we combined a dog cell line with a human occludin sequence. Thus the differences could be attributed to species specific sequence requirement. Sequence analysis of the amino acid sequence of mammalian occludins revealed that they were close related to each other (90 % identity), whereas mammalian sequences diverged considerably from those of chicken (50 % identity; Tsukita & Furuse, 1998). It is therefore possible that, since the sequence of the chicken peptide is very diverse from the *Xenopus* protein, it is more important that the peptide is non-oxidized. In our approach the human peptide matches the dog protein much better and therefore binding occurs despite oxidation. Recently, Lacaz-Vieira et al. (1999) assayed synthetic peptides corresponding to small segments of the first loop of occludin which altered the TEER in A6 cell monolayers. Since our occludin loop I peptide was very water insoluble compared to the other peptides tested, we can not exclude that smaller fragments of the occludin loop I peptide with better solubility would have an effect as well.

The peptide corresponding to the claudin loop I was equivalent effective as the occludin loop II peptide. In contrast, the claudin loop II peptide had no effect on the mannitol flux and apparently does not contribute to the formation of TJs. Our findings are consistent with the hypothesis of Furuse et al. (1998a) that since the first extracellular loop of the claudins is larger and more hydrophobic than the second extracellular loop, the first loop is the candidate to bridge the intercellular space. The second loop is suggested to act as a receptor for signaling molecules. Claudin-4 was first identified and cloned as a *Clostridium perfringens* enterotoxin (CPE) receptor (Katahira et al., 1997). Later, it has been shown that claudin-3 and -4 bind
the ligand CPE via their second extracellular loops and confers structural changes in TJs (Sonoda, 1999). Still, the physiological function of this receptor (loop) remains unclear.

To further demonstrate that the effect of the occludin loop II and claudin loop I peptide was caused by specific interactions and not by general cell toxicity, the overall cell morphology and the viability of the cells were tested. The occludin loop II and claudin loop I peptides didn’t neither reduce the viability of the cells, nor was the overall cell morphology affected. These results together with previous findings that not all of the tested peptides affected the paracellular permeability of the TJs, indicate a specific peptide-protein interaction. I therefore assume that the occludin loop II and claudin loop I peptides selectively perturb TJ sealing elements and specifically increase the paracellular transport in MDCK cells, leaving the overall cell morphology intact. According to Wong & Gumbiner (1997), two possible mechanisms of action of the occludin loop II and claudin loop I peptide could account for their perturbation of the TJ barrier. First, the occludin and claudin peptide could bind to the extracellular loops of occludin and claudin. There they would intercalate into the TJs and directly interfere with the normal properties of the functional seal. Second, the peptides, binding to the extracellular loops could cause the release of occludin and claudin from its normal stabilized interactions in the TJ, which subsequently leads to a gradual disassembly of the TJ sealing elements. We therefore investigated the localization of different TJ proteins, actin and E-cadherin by CLSM but data did not provide definite results. The occludin loop II treated cells showed a similar staining pattern for ZO-1, occludin, claudin-1, F-actin and E-cadherin as the control cells and no alteration of the TJ protein ZO-1, occludin, claudin-1 and the AJ protein E-cadherin expression could be shown by Western blot analysis. Our findings support the first proposed mechanism that the peptides intercalate and affect the function of TJs without the depletion of the transmembrane proteins occludin from the TJs. In contrast, Wong & Gumbiner (1997) showed by CLSM in A6 cells that the occludin loop II treated cells had substantially less occludin present at cell boundaries and the depletion of occludin could be confirmed by Western blot analysis. Additionally, Vietor et al. (2001)
showed besides a decreased occludin expression the formation of multilayered, unpolarized cell clusters in infrequently affected cell patches. Next, I tested whether the claudin loop I treated monolayers showed a different expression level compared to the control cells. On the one hand it looks like the claudin loop I peptide would affect the localization of the claudin protein at the TJs. On the other hand there was just sporadically different staining patterns observed. Therefore, it could be that just patches of cells in the monolayers were sensitive to the perturbation of the permeability barrier by peptide treatment. Moreover it can not be excluded that the occludin loop II peptide effected the occludin localization at the TJs like Wong & Gumbiner (1997) and Vietor et al. (2001) showed. It is important to mention that also untreated MDCK monolayers show a non-homogenous staining pattern. Therefore further investigations should be done with several randomly chosen fields within a single preparation. Western blot analysis of the claudin loop I peptide treated cells did not reveal any difference in the expression level compared to control cells. Thus the different staining pattern of the claudin treated cells could be rather attributed to a redistributed from the membrane to the cytoplasm.

The claudin loop I peptide concentration required for a maximal increase of the mannitol flux appears to be high. The apparent $K_i$ of the peptide is in the range of 40-200 µM, so the peptide seems to participate in a relatively low affinity binding interaction. It is conceivable that only a fraction of the peptide is in the conformation permissive for binding, e.g. due to dimerization of the peptides in solution. Alternatively, the entire peptide population may exhibit low affinity due to conformational constraints. Furthermore the maximal effective concentration could be lower since the claudin loop I peptide is not 100 % pure.

First results of the TEER measurements showed that the effect of the claudin loop I peptide was not attained before 24 h. Because of this long time course I suppose that peptides do not compete with established TJs. Therefore we expected that the claudin loop I peptide would have an enhanced effect on the paracellular transport added to non-confluent cells without established TJs. This supposition could not be confirmed. The claudin loop I peptide response on the paracellular transport, with both
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newly forming monolayers and confluent cells with an established TJ network, was equal. Therefore the effect is probably not dependent on the two tested growth states of the cells. It is important to point out that the confluent cells at day 5 were still growing exponentially and developing new cell-cell contacts. Further investigations should therefore be done on steady-state monolayers (> day 10) with fully established TJs.

To map the binding site of the claudin loop I and occludin loop II more precisely, smaller partially overlapping peptides were used in the flux assay. Dahl et al. (1992) stated that if the binding site of a protein comprises only part of the extracellular loop, then one would expect some smaller peptides to exhibit equivalent or even stronger effects than the larger peptides, whereas other small peptides should be ineffective. If the binding site is large or if it is comprised of many small units, then several of the small peptides should inhibit to the same or to a lower extent than the larger peptides. The occludin fragment partly lost their effect on the mannitol flux therefore the peptide-occludin interaction may include the entire loop. In contrast, the claudin fragment 47-65 had an even stronger effect than the whole claudin loop I peptide. The flux increased almost 3 times, whereas for the whole peptide around 2.5 times. Thus the claudin interaction seems to be restricted to a smaller region. These results with smaller fragments of the entire occludin loop II and claudin loop I peptides, also indicate a specific interaction of the peptides to impair TJ integrity.

To sum up, the occludin loop II and claudin loop I peptide increased the mannitol flux indicating that the TJ permeability barrier was disrupted. The paracellular transport was increased in a dose-dependent manner and saturation could be achieved. The effect of the occludin loop II and claudin loop I peptide on the TJ permeability barrier correlated with a slight decrease of the TEER. The effect of the peptides was not caused by general cell toxicity and the overall cell morphology was not affected. Further CLSM should be done to further examine the localization of TJ proteins. The total expression level of occludin, claudin-1, ZO-1 and E-cadherin was not altered. Observations with smaller fragments of the occludin loop II and claudin loop I peptide suggest that the occludin interaction involves the whole loop, whereas the claudin interaction is restricted to a smaller area of
the loop. These results implicate not only occludin as proposed by Wong & Gumbiner (1997), but also claudin-1 in the formation of the TJ permeability barrier. Direct evidence that occludin and claudin confers adhesiveness was obtained by Van Itallie & Anderson (1997) and Furuse et al. (1999) expressing occludin and claudin, respectively, in fibroblasts, which lack TJs. They observed that occludin and as well as claudins are able to form polymers with homophilic adhesion. The ability to form linear polymers in the plasma membrane and interact over an extensive protein surface may be the mechanism by which occludin and claudin create a molecular-level barrier across the paracellular pathways.

Briefly, our results suggest that the extracellular loops of occludin and claudin-1 are involved in binding a protein of the adjacent cell, either through a homophilic or heterophilic interaction with some other binding partners. Thus peptides can influence TJs because they compete specifically at the extracellular loops of the occludin and claudin proteins.

For further studies within this project, emphasis will be put on the effect of claudin peptides on the paracellular TJ barrier permeability. It has been shown by Furuse et al. (1998b) and Kubota et al. (1999) that members of the claudin family contribute more to the TJ barrier than occludin (see 1.2.5). Therefore and since the claudin family contains 20 different species which are supposed to be responsible for the big variety between the tightness of different tissues and cell types, it seems to be more interesting to concentrate on different members of the claudin family than on occludin. It has been seen that the claudin interaction is restricted to part of the loop. Therefore further experiments should be done with fragments of the loop due to easier synthesis and enhanced solubility of smaller peptides. The effect of the claudin loop I peptides should be studied not only on the MDCK II but also on the MDCK I cell line. First, we hope to find stronger effects of the peptides in MDCK I than in MDCK II cells. The MDCK I cell line is known to form tighter TJs than the type II cell line, which is expressed particularly in higher TEER values. For MDCK I cells the TEER values are around 2000 Ωcm² compared to the type II which has TEER values around 200 Ωcm². I therefore expect an increased effect of the
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peptides on the TEER value. Also, it would be interesting to see if the paracellular transport would be increased as well.
Second, it has been shown recently that the differences in the TEER values between the two types might be due to the expression of different claudins. MDCK I express primarily claudin-1 and -4, whereas MDCK II also expresses large amounts of claudin-2 in addition to claudin-1 and -4. When claudin-2 was introduced into MDCK I cells, the TEER value of these MDCK I transfectants felt to the level of MDCK II cells without any changes in the number of TJ strands. Even though the extracellular loops of the claudins are highly conserved, they seem to form TJs with variable properties. Therefore the effect of different peptides corresponding to the first extracellular loops of claudin-1, -2 and -4, respectively, should be tested. Effects of the particular peptides should be tested separately as well as in combinations to test synergism between them.

Heiskala et al. (2001) have suggested using claudins as targets to manipulate the paracellular transport of solutes and drugs in a cell type- and/or tissue-specific manner.
The transient perturbation of the barrier function of the TJs by claudin peptides could be potentially useful in medical therapeutics such as permeation enhancement for poorly absorbed hydrophilic drugs such as peptides and proteins. It may be worthwhile searching for smaller fragments that specifically bind to individual claudin species. Selective removal of specific claudin species from TJ strands with the combination of these peptides would provide a new way to modulate the TJ barrier function in situ and to improve bioavailability of drugs to targeted organs.
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