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Bile duct ligation in the rat causes upregulation of ZO-2 and decreased colocalization of claudins with ZO-1 and occludin

I. Piotr Maly · Lukas Landmann

Abstract As the only barrier between blood and bile compartments hepatocellular tight junctions play a crucial role in cholestasis-induced increase of biliary permeability. The molecular basis of this reversible defect is not known. We, therefore, examined expression, phosphorylation, distribution and colocalization of the junctional proteins occludin, claudin-1-3, ZO-1 and ZO-2 in rats after bile duct ligation and release of ligation. In control rats, claudin-1 and ZO-2 displayed a lobular gradient with highest expression levels in periportal cells, whereas claudin-2 showed a reciprocal distribution. Other proteins were evenly expressed in the liver lobule. Ligation resulted in upregulation of ZO-2 (2.7-fold), ZO-1 (1.4-fold) and occludin (1.2-fold) but not of claudins. Only ZO-2 showed increased phosphorylation. Distribution patterns were unchanged except for a strong accumulation of ZO-2 in perivenous hepatocytes. Colocalization analysis demonstrated that perivenous ZO-2 was the only protein examined revealing strongly increased overlap with occludin and ZO-1, whereas claudins and other proteins displayed a decrease. All changes were partially reversed by release of ligation. We conclude that differential expression of claudin-1-2 and ZO-2 has functional implications for bile formation. The moderately increased ZO-1 and occludin levels account for the known elongation of tight junction strands. The highly increased expression and changed distribution of ZO-2 suggests that ZO-1 is partly substituted by ZO-2, an alteration possibly causing impaired barrier function.

Keywords Bile secretion · Cholestasis · Tight junctions · Hepatocytes · Transepithelial permeability

Abbreviations

TJ	Tight junctions
Cldn	Claudin
BDL	Bile duct ligation
BDL-R	Release of ligation
AP	Alkaline phosphatase

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Introduction

Tight junctions (TJ) are the only barrier between bile and blood compartments preventing regurgitation of secreted bile constituents by the paracellular pathway. Since bile salts, the main constituents of bile, are concentrated more than 1,000-fold in canaliculi as compared to portal blood, TJ maintain and control steep concentration gradients. In addition, they participate in electrochemical and osmotic equilibration of newly secreted bile by allowing selective movement of water and small solutes (e.g., cations) from blood to bile (Landmann and Stieger 2001). TJ appear as a series of fusion points between the outer leaflets of the membranes of two adjacent cells in ultrathin sections and as a network of anastomosing strands composed of membrane particles in freeze-fracture replicas (Landmann and Stieger 2001). They contain at least four types of transmembrane proteins and more than 30 cytoplasmic scaffolding, signaling and cytoskeletal proteins (Schneeberger and Lynch 2004). Two types of transmembrane proteins, occludin and

claudins (Cldn), which are members of a multigene family with at least 24 members in mammals, have been identified as major constituents of TJ strands (Furuse et al. 1993, 1998). Both molecules contain four transmembrane domains with two extracellular loops but do not show any sequence similarity. There is strong evidence supporting the view that claudins control the barrier and its selective properties (Van Itallie and Anderson 2006). In contrast, occludin does not play a significant role in barrier function as demonstrated by occludin-deficient mice (Schulzke et al. 2005). Hepatocytes are known to express the transmembrane proteins occludin and Cldn-1-3 (Morita et al. 1999; Rahner et al. 2001). Transmembrane proteins interact with a cytosolic plaque of peripheral membrane proteins that, in turn, is closely associated with the cytoskeleton. The major plaque proteins are three closely related MAGUK proteins, ZO-1, -2, -3, containing three PDZ-domains, an SH3-GUK region, and an actin binding site (Fanning and Anderson 1999). These proteins share binding sites for claudins, occludin and ZOs. Direct experimental evidence has shown that ZO-1 and/or ZO-2 are necessary for polymerization of claudins and strand formation in the apical area of the lateral plasma membrane (Fanning et al. 2007; Umeda et al. 2006).

A point mutation in the first, claudin-binding PDZ domain of ZO-2 has been demonstrated to be associated with familial hypercholanemia (Carlton et al. 2003). This alteration results in increased serum bile salt levels, decreased claudin binding, and increased TJ depth, changes that were suggested to cause increased junctional permeability for bile salts. Similar changes are well known characteristics of cholestasis, a pathologic condition which is accompanied by impaired structural and functional integrity of hepatocellular TJ (Landmann and Stieger 2001). Bile duct ligation (BDL), a severe form of cholestasis in rats, results in TJ with decreased numbers of strands, increased junctional depth, and with dramatically increased permeability for peroxidase and dextrans of various molecular weights (Rahner et al. 1996). BDL-induced structural defects are completely reversible within 96 h after recanalization but reversal of functional deficiency is detected much earlier (Stieger et al. 1994). In addition, bile duct ligation has been reported to affect expression levels and subcellular distribution of TJ proteins such as ZO-1 and occludin (Fallon et al. 1993, 1995; Kawaguchi et al. 1999).

ZO-2 and its interaction with claudins have not been examined to date in animal models of cholestasis. We, therefore, examined expression and subcellular distribution of ZO-2 as well as ZO-1, occludin, Cldn-1-3 in livers of rats subjected to BDL and its subsequent release (BDL-R). In addition, we applied a novel in situ approach, colocalization analysis, for the assessment of protein interaction. The data show that ZO-2 is the only protein displaying both markedly increased expression and distinctly altered distribution, whereas protein interactions as assessed by colocalization of ZO-1 or occludin with other TJ proteins are little affected. All the changes are reversible. We conclude that ZO-2 plays a key role in the regulation of paracellular permeability.

Materials and methods

Antibodies

Rabbit polyclonal antibodies recognizing Cldn-1-3, occludin, and ZO-1-2 were purchased from Invitrogen-Zymed (LuBioScience GmbH, Lucerne, Switzerland). For Cldn-1 the mono-specific antibody that does not crossreact with Cldn-3 was used. Mouse monoclonals against occludin and ZO-1 from the same supplier were used for 2-channel detection. Secondary goat antibodies conjugated to Cy2 or TxR were of multilabeling grade and purchased from Amersham Pharmacia Biotech (GE Healthcare GmbH, Otelfingen, Switzerland) or Jackson ImmunoResearch (La Roche, Switzerland). For Western blotting alkaline phosphatase (AP)-conjugated AffiniPure goat anti-mouse or -rabbit secondary antibodies were purchased from Jackson.

Animals and induction of cholestasis

All experiments were approved by the local supervisory board and performed according to strict federal law guidelines regulating animal experimentation. Male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) weighing 190–250 g were housed in light, temperature and humidity-controlled animal quarters. Animals were fasted the night before experimentation.

Surgical procedures for experiments with and without release of ligation have been detailed previously (Rahner et al. 1996). Briefly, rats were laparotomized under pentobarbital anesthesia (50 mg/kg body wt, i.p.). The common bile duct was wrapped in several layers of sterile cellophane and doubly ligated (BDL). In controls, the bile duct was similarly exposed and manipulated but not tied (Ctrl). BDL lasted for 48 h. Release of ligation was performed 2 days after BDL by removing threads and cellophane in a second laparotomy followed by a 48 h equilibration interval (BDL-R). All three protocols were applied to at least six animals per group.

Hepatobiliary serum parameters were determined by routine analysis (Chemisches Labor, Universitätsspital Basel). BDL induced elevated concentrations of all markers and caused a slight loss of body weight. BDL-R resulted in partial reversal of altered parameters and minimal gain of body weight. All changes were highly significant.

Preparation of tissue

Animals were anesthetized and the livers flushed free of blood. Individual livers were examined by correlative biochemical and morphological analysis (Landmann et al. 1998). After tying the hila, the right lateral and caudal lobes were excised and homogenized immediately for biochemical examination (see below). The remainder of the liver was fixed by perfusion with freshly prepared 2% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 5 min at room temperature. Liver tissue was cut into small blocks and immersed for an additional hour in fixative before preparation of cryostat sections.

Homogenates

Liver lobes were homogenized in 63 volumes of ice-cold 50 mM Tris–HCL buffer, pH 8.4, containing 5% SDS, 1% DTT, 1 tablet of Complete Mini Protease Inhibitor Cocktail (Roche, Mannheim, Germany) and 100 µl of Halt™ Phosphatase Inhibitor Cocktail (Pierce, Rockford, IL, USA) in 10 ml solution. After homogenization, the samples were heated to 96°C for 10 min and cooled down. To 0.9 ml of the sample 0.1 ml of 2 M acrylamide was added and the proteins alkylated for 30 min at room temperature (Galvani et al. 2001). The homogenates (final dilution 1:70) were stored at −80°C. Preliminary experiments showed that total protein mass per weight was not different in the three groups (data not shown).

For AP treatment (Chan and Stinson 1986; Sakakibara et al. 1997), the livers were homogenized in 63 volumes of Tris–HCl buffer containing 100 mM NaCl, 0.5% Triton X-100, Protease Inhibitor Cocktail EDTA-free and 10 mM $MgCl_2$. After homogenization, 100 µl of AP (alkaline phosphatase from calf intestine, 1 U/µl, Roche, Mannheim, Germany) were added to 900 µl homogenate and the samples were incubated for 1 h at 37°C. The reaction was stopped by addition of 0.05 g SDS, 0.01 g DTT and heating to 96°C for 10 min.

Polyacrylamide gel electrophoresis and immunoblotting

Electrophoresis on 0.35-mm thick SDS-polyacrylamide slab gels (4%) bound to glass plates was performed as described (Maly and Nitsch 2007). Samples were transferred for 16 h from ultra thin-layer SDS polyacrylamide gels to nitrocellulose membranes (0.2 µm pore size, Bio-Rad, Reinach, Switzerland) using the diffusion blotting technique. Membranes were blocked for 1 h with TBS-T containing 3% BSA, washed and immunoreacted for 1 h with the appropriate antibodies (1:2,000 for rabbit polyclonals recognizing occludin, Cldn-1, -2, -3, ZO-2 and 1:2,000 for mouse monoclonal anti-ZO-1) according to standard protocols. In negative controls, the primary antibody was substituted by nonimmune mouse or rabbit IgG (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). AP-conjugated, species-specific secondary antibodies were used at the dilutions recommended by the manufacturer. Immunoreactive bands were detected by the BCIP/NBT phosphatase substrate system and analyzed with a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

Image recording and processing

Ten micrometer cryostat sections were immunostained by sequential incubation in primary and secondary antibodies diluted 1:200 for monoclonal, 1:100 for polyclonal, and 1:100 for conjugated antibodies in PBS containing 1% BSA for 1 h at room temperature. Capricious antibodies such as Cldn-2 were used over night at 4°C. After washing in PBS, specimens were mounted in Mowiol containing 1% propylgallate. Wide field micrographs were taken on a ZEISS Axiophot equipped with an epifluorescence module.

For colocalization analysis specimens were examined by confocal microscopy (TCS 4D, Leica Microsystems, Mannheim, Germany). Photomultiplier settings were adjusted to the brightest signal and maintained during data acquisition of all three experimental groups. Images were recorded with an apochromatic 100×/nA 1.4 objective lens. Image stacks were sampled according to the Nyquist theorem (Webb and Dorey 1995).

In order to avoid false-positive colocalization results, deconvolved data sets were analyzed as described earlier (Landmann and Marbet 2004). Deconvolution, an image restoration procedure eliminating straylight and suppressing background (Van der Voort and Strasters 1995), was performed with the Huygens Professional software package (Scientific Volume Imaging A.V., Hilversum, The Netherlands) operating in the maximum likelihood estimation mode and using measured point spread functions. Subsequently, every singular plane of an image stack was analyzed with the colocalization module of the Imaris software package (Bitplane AG, Zurich, Switzerland). In order to avoid subjective bias, thresholds for colocalization, a crucial step (Landmann and Marbet 2004), were determined automatically by a thresholding device excluding intensity pairs that exhibit no correlation (Pearson's coefficient < 0, Costes et al. 2004). Colocalizing volumes and Pearson's correlation coefficients were quantified in a region of interest that was defined as occludin or ZO-1 signal. Correlation coefficients, which describe how well two channels are related by a linear equation, were chosen to compensate for variable signal intensity. Quantitative data are means ± SD based on at least three animals, each of which provided 3–6 data sets. Using Student's t test $p < 0.05$ was considered statistically significant.

Results

Distribution of TJ proteins

The subcellular localization of TJ proteins was examined by indirect immunofluorescence. As expected, the transmembrane proteins occludin, Cldn-1, -2, and -3 as well as the plaque proteins ZO-1 and -2 were expressed in hepatocytes and outlined bile canaliculi on two sides as uninterrupted contours (Fig. 1). ZO-1, occludin (Fig. 2a) and Cldn-3 were evenly expressed by all cells in the liver lobule. In contrast, Cldn-2 showed a lobular gradient as reported earlier (Rahner et al. 2001) with expression levels that were markedly increased in perivenous as compared to periportal hepatocytes. The monospecific antibody used here demonstrated that Cldn-1, too, was unevenly distributed but, in contrast to Cldn-2, with an expression pattern that increased from perivenous to periportal cells (Fig. 2c). ZO-2 showed a similar distribution with stronger expression in TJ of periportal hepatocytes. Moderate amount of this protein were found in nuclei (Fig. 2b).

Cholestasis caused dilated canaliculi with surprisingly little change in TJ protein distribution (Fig. 3). All proteins examined were expressed exclusively in the TJ and did not show redistribution (Fig. 3, top). The notable exception was ZO-2 which was decreased in nuclei and accumulated strongly in TJ of perivenous hepatocytes thus showing an almost homogenous lobular distribution (Fig. 3, bottom).
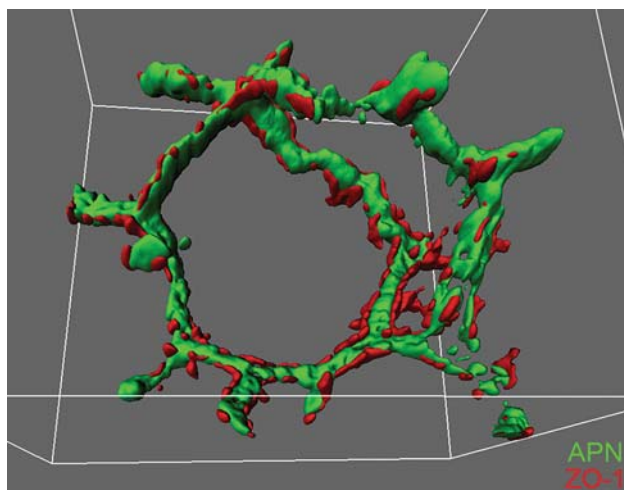

Fig. 1 Surface rendering of a 25 × 25 × 25 µm data set demonstrating the hexagonal arrangement of canaliculi around hepatocytes and their sealing by two sets of TJ. The canalicular plasma membrane was probed with an antibody recognizing the domain-specific membrane protein aminopeptidase N (*green*, Stieger et al. 1994). *Red signal* shows the distribution of the TJ plaque protein ZO-1. The discontinuous appearance of the TJ lines is an artifact caused by thresholding of signals
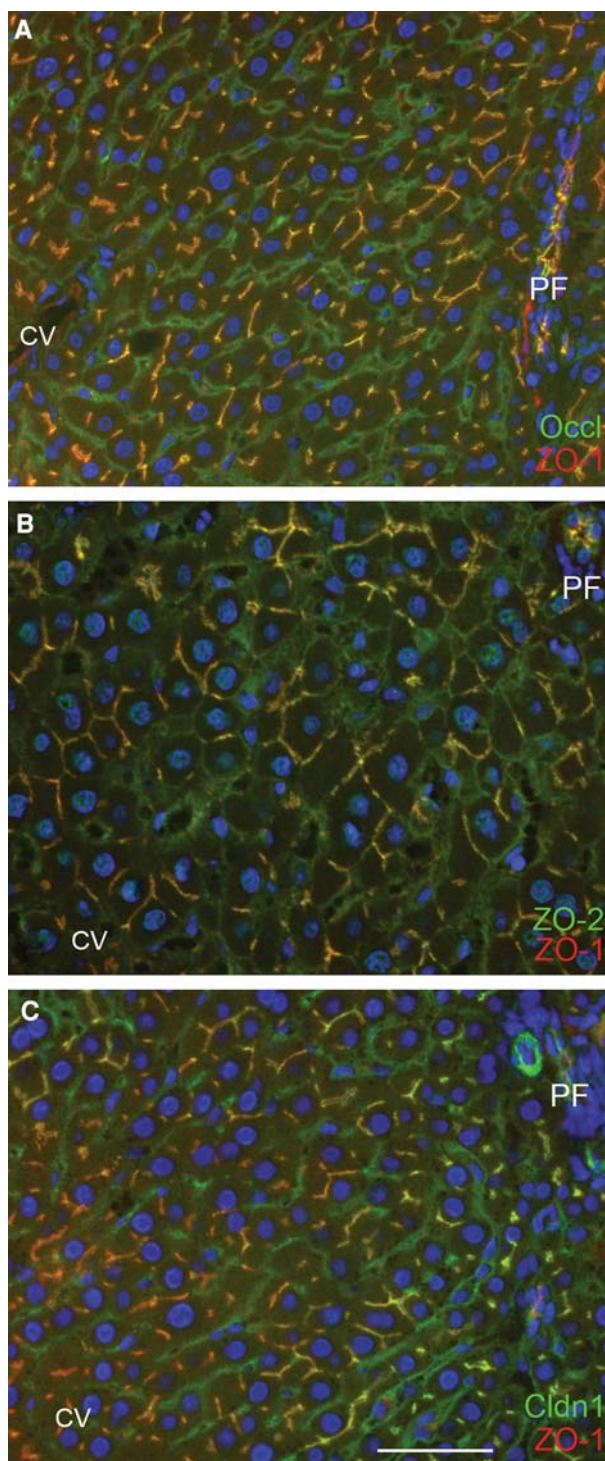

Fig. 2 Distribution of TJ proteins in normal rat liver. (**a**) The transmembrane protein occludin (*green*) and the plaque protein *ZO-1* (*red*) are evenly expressed along the porto-central axis which results in yellow overlap of equal intensity. In contrast, *ZO-2* (**b**) as well as *Cldn-1* (**c**) are more strongly expressed in periportal hepatocytes. Yellow overlap with ZO-1 (*red*) turns gradually into orange in the central zone reflecting the relative decrease of the protein labeled with green fluorescence. Nuclei are stained with DAPI. The *weak green signal* in endothelial cells is unspecific and caused by crossreacting secondary antibodies. *CV* central vein, *PF* portal field. *Bar* = 50 µm

Fig. 3 Distribution of TJ proteins was surprisingly little altered by bile duct ligation (*BDL*) or release of ligation (*BDL-R*). This is demonstrated in the *top two rows* by periportal (*PF*) and central (*CV*) hepatocytes stained for *Cldn-3* (*green*) and occludin (*red*). The only TJ component displaying alterations was *ZO-2* (*green*, *bottom two rows*) that was upregulated by bile duct ligation in cells surrounding the central vein and decreased after release of ligation (*bottom row*, *center* and *right*). This is reflected by the differential contribution of *red signal* (*ZO-1*) to the overall appearance of the TJ. Nuclei are stained by DAPI (*blue*). *BD* bile ducts in portal fields, *CV* central veins. *Bar* = 20 μm
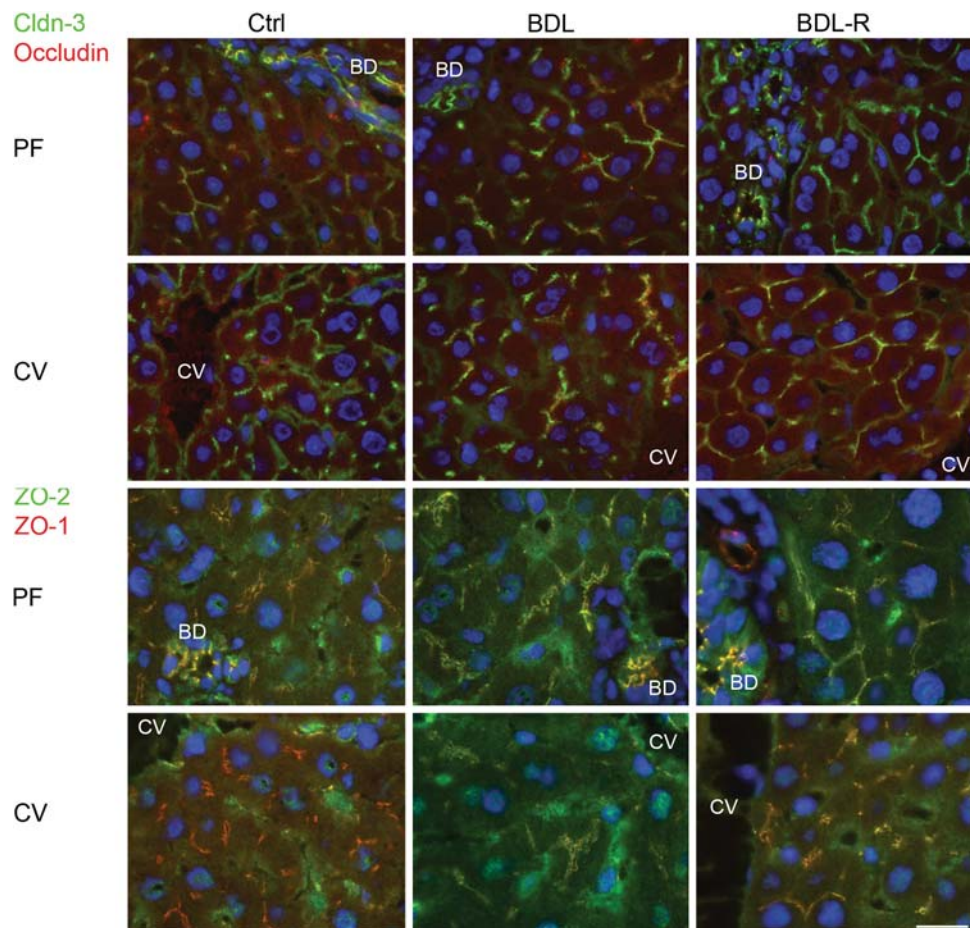
Western blot analysis

We next examined expression levels of TJ proteins in Ctrl, BDL and BDL-R livers by immunoblotting (Fig. 4). Blots of total liver homogenates were probed with the appropriate antibodies which identified bands at approximately 25 kDa (claudins; Furuse et al. 1998), 65 kDa (occludin; Furuse et al. 1993), 160 kDa (ZO-2; Gumbiner et al. 1991) and 220 kDa (ZO-1; Stevenson et al. 1986). Multiple banding reflects different splicing forms and/or phosphorylation states. Densitometric analysis of Western blots (Fig. 4, bottom) showed that ZO-2 displayed the strongest response to BDL and was increased more than 2.5-fold. This increase was slightly decreased after BDL-R. Additional BDL-induced increases were 1.4- and 1.2-fold in ZO-1 and occludin, respectively. These moderate alterations, however, were not reversed by BDL-R. Claudins showed minor changes that were not significant.

Phosphorylation

Liver homogenates were treated with AP in order to demonstrate BDL-induced differences in protein phosphorylation (Fig. 5). AP treatment decreased the molecular mass of ZO-1 in all three groups but did not change its banding pattern brought about by two different splicing forms (Willott et al. 1992). This indicates moderate phosphorylation which was not changed by any treatment.

In contrast, the sharp, dense band of ZO-2 was accompanied by an AP-sensitive slower wide and diffuse band after BDL and even more so after BDL-R which demonstrates increased phosphorylation.

Occludin bands were quite broad and included a sharp, dense, lower band and a wide, diffuse band above it. This pattern was very similar in control, BDL and BDL-R animals. After AP treatment, the slow, broad band was significantly reduced whereas the molecular mass of the dense, lower band was not altered. This shows that occludin was phosphorylated on multiple sites in control, BDL and BDL-R animals and that its phosphorylation pattern did not differ between groups. Similar, but less conspicuous results were obtained for Cldn-3 and Cldn-2, whereas AP had no effect on Cldn-1. Thus, the only evidence for altered phosphorylation patterns of TJ proteins was found in ZO-2.

Colocalization analysis

Integral and peripheral TJ proteins interact both with themselves and with each other in order to establish a functional
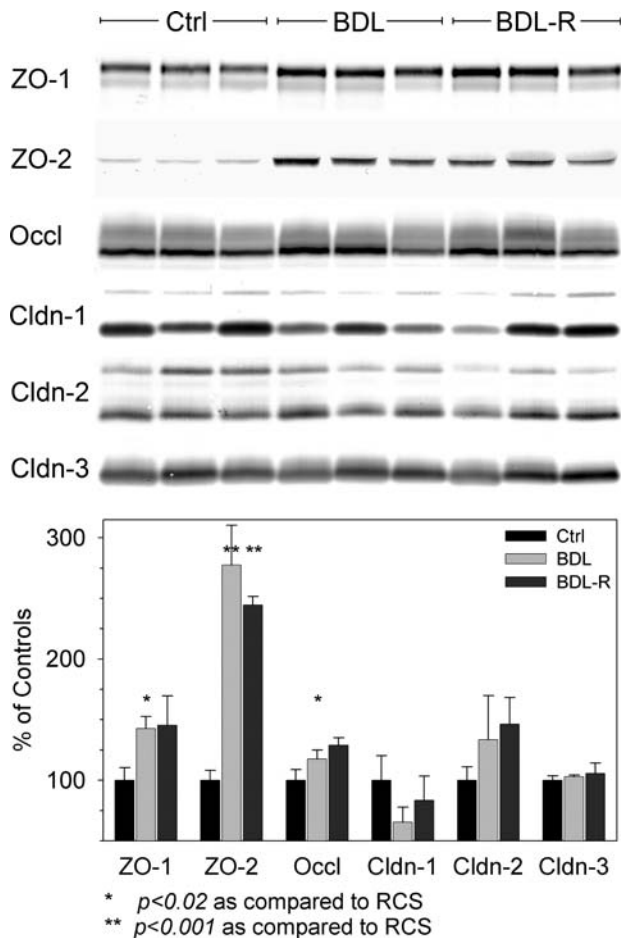
Fig. 4 Effect of bile duct ligation (*BDL*) and its subsequent release (*BDL-R*) on the expression of several tight junction proteins in the rat liver. **a** Whole liver homogenates were analyzed for *ZO-1*, *ZO-2*, *occludin*, *claudin-1*, *claudin-2* and *claudin-3* by SDS electrophoresis and blotting. Immunoreactive bands were detected by the BCIP/NBT phosphatase substrate system. **b** Semiquantitative data were generated by scanning of bands with a Personal Laser Densitometer and shows, that the expression of ZO-2 increased after BDL up to 2.7-fold as compared to control animals. ZO-1 and occludin increased only moderately (1.4-fold and 1.2-fold, respectively) but there are no significant changes in the level of claudins expressed after BDL and BDL-R
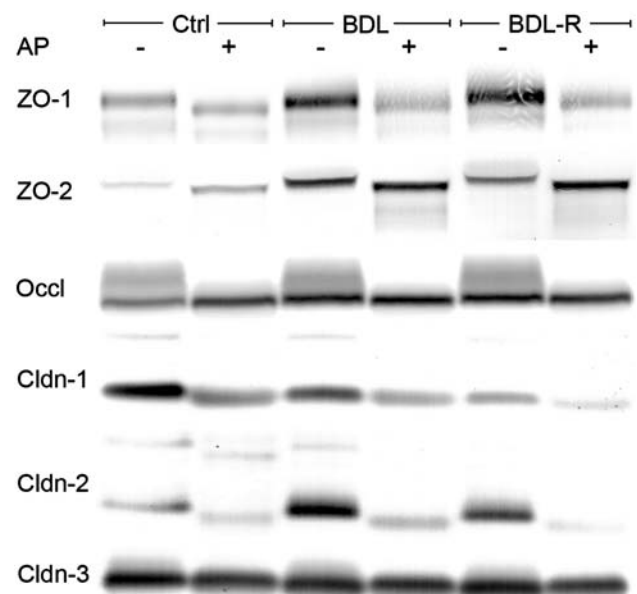

Fig. 5 In order to demonstrate possible ligation- (*BDL−*) and release- (*BDL-R−*) associated differences in the state of protein phosphorylation, homogenates were treated with *AP*. AP treatment caused apparent size shifts of *ZO-1*, *ZO-2*, *occludin*, and in less degree of *claudin-2* and *claudin-3*. However, the observed size shifts were similar in all tree groups

barrier. We, therefore, applied a novel approach using high-resolution confocal microscopy in combination with quantitative colocalization analysis in order to detect changes in the molecular composition of TJ.

Absolute values differed widely for the various protein pairings in control livers (Table 1) with colocalizing volumes ranging from 2 to 33%. As expected, the values were higher between integral (occludin vs. Cldn-1, -3) or peripheral membrane proteins (ZO-1 vs. ZO-2) than between peripheral and transmembrane proteins (ZO-1 vs. Cldn-1, -3). Correlation coefficients which reached rather high values of close to 0.7 showed a similar pattern. Both parameters displayed the lowest values in low expression sites of

Table 1 Absolute volumes of colocalization and correlation coefficients between TJ proteins and ZO-1 or occludin in control livers

	% of ZO-1	% of occludin	CC with ZO-1	CC with occludin
Cldn-1 periportal	19.4 ± 2.5	21.4 ± 4.0	0.42 ± 0.03	0.50 ± 0.05
Cldn-1 pericentral	12.2 ± 4.8	8.0 ± 5.6	0.26 ± 0.05	0.23 ± 0.19
Cldn-2 pericentral	17.5 ± 5.5	33.0 ± 4.1	0.39 ± 0.04	0.63 ± 0.03
Cldn-2 periportal	6.9 ± 3.4	16.1 ± 2.6	0.23 ± 0.08	0.42 ± 0.02
Cldn-3	13.2 ± 3.8	22..3 ± 3.4	0.32 ± 0.04	0.40 ± 0.03
Occl	31.7 ± 8.5		0.61 ± 0.03	
ZO-1		24.2 ± 9.3		0.68 ± 0.03
ZO-2 periportal	29.2 ± 3.3	19.1 ± 6.6	0.61 ± 0.02	0.48 ± 0.08
ZO-2 pericentral	1.9 ± 1.4	9.1 ± 7.9	0.19 ± 0.04	0.33 ± 0.19

The relationship of trans- and peripheral MPs was monitored in a region of interest defined by ZO-1 signal; assembly of integral membrane proteins in a volume corresponding to occludin signal. Data are from three animals, each represented by six image stacks

proteins with a lobular gradient while maximum values indicated a strong affinity of ZO-1 for ZO-2 and occludin.

Absolute numbers depend on various factors including the quality of antibodies and the relative amount of protein expressed in the region of interest. Therefore, values were normalized to controls. The data demonstrate (Figs. 6, 7) that the volumes of colocalization between transmembrane as well as between integral and peripheral membrane proteins were decreased by BDL by 15–60% with most pairings displaying significant differences. BDL-R resulted in partial reversal. Though the general trend was identical in occludin and ZO-1, the two proteins differed in the degree of alterations. Whereas ZO-1 showed larger BDL-induced decreases for Cldn-1 and Cldn-2, occludin displayed a more pronounced decrease for Cldn-3 (Fig. 7). The only notable exception to this pattern was ZO-2 which showed transitorily increased overlap with both ZO-1 and occludin in pericentral but not periportal hepatocytes (Figs. 6, 7).

Discussion

Differential distribution of TJ proteins

All TJ proteins examined are expressed by hepatocytes and localize to both sides of the canaliculi (Fig. 1). Occludin, Cldn-3 and ZO-1 are expressed evenly by all cells of the liver lobule. In contrast, Cldn-1 and ZO-2 display a lobular gradient with expression levels increasing from perivenous to periportal hepatocytes (Fig. 2), whereas Cldn-2 is expressed in a reciprocal manner, i.e., more strongly in perivenous than in periportal cells. Expression patterns of occludin, ZO-1, Cldn-2 and -3 agree with earlier observations (Rahner et al. 2001). The availability of monospecific antibodies recognizing Cldn-1 and ZO-2 demonstrates for the first time a lobular gradient of these proteins. Thus, hepatocellular TJ contain a homogeneously expressed backbone consisting of occludin, Cldn-3 and ZO-1. This scaffold is superimposed with the periportal Cldn-1 that is gradually substituted by the perivenous Cldn-2. Interestingly, Cldn-1 and -2 form intercellular contact with themselves and with Cldn-3 but not with each other in transfected fibroblasts (Furuse et al. 1999). It is, therefore, conceivable that their reciprocal expression safeguards the formation of contact points along the entire lobule. In the cytoplasmic plaque the ubiquitous ZO-1 is overlaid by ZO-2 in the periportal but not perivenous zone.

Members of the claudin family generate the barrier and control its permeability characteristics (Van Itallie and Anderson 2006; Anderson et al. 2004). Therefore, it is highly probable that differential claudin expression along the porto-venous axis reflects changing physiological properties as demonstrated by the nephron where various claudins are expressed in different segments with different barrier properties (Kiuchi-Saishin et al. 2002). As barrier properties of Cldn-3 have not yet been characterized the functional interpretation of our findings is difficult. However, since Cldn-1 increases transepithelial resistance (McCarthy et al. 2000) while Cldn-2 decreases it and forms cation-selective pores (Amasheh et al. 2002; Colegio et al. 2003), it is reasonable to assume that the Cldn-3-based strands are rendered tighter by the insertion of Cldn-1 in the periportal zone, whereas Cldn-2-containing perivenous TJ are more leaky and permeable for cations. This would imply that secreted bile salts are neutralized electrochemically and osmotically predominantly through TJ in the perivenous zone, i.e., at the very end of the biliary tree, a process that could provide an additional driving force for bile flow.

Upregulation of some but not all TJ proteins is associated with decreased colocalization

BDL has been reported to cause discontinuities in ZO-1 and occludin fluorescence patterns (Fallon et al. 1995; Kawaguchi et al. 1999). Our data (Fig. 3) do not support this observation. Deconvoluted data sets demonstrate, however, that local intensity differences are a general feature of many TJ proteins and can give rise to an interrupted colocalization pattern (Fig. 6). It is likely that low intensity fluorescence was not recognized in unprocessed confocal images due to the high level of background generated by the strongly autofluorescent hepatocytes.

Our data demonstrate that BDL induces upregulation of some but not all transmembrane and plaque proteins (Fig. 4): Whereas claudin expression is not altered significantly by cholestasis, occludin is upregulated by 20%, ZO-1 by 40%, and ZO-2 by 170%. Our ZO-1 data are in line with but less distinct than those reported earlier (Fallon et al. 1993; Kawaguchi et al. 1999). However, our finding that occludin is slightly upregulated is not supported by the 50% decrease reported for this protein (Fallon et al. 1995). The reasons for this discrepancy are not clear.

As demonstrated by stereology, BDL induced increase of TJ length results in junctional strand length increased by 30% (55 vs. 72 km/cm³ liver; Rahner et al. 1996). In contrast, BDL-induced proliferation of cholangiocytes, which express the same TJ proteins, leads only to a twofold increase of TJ length (108 vs. 230 m/cm³; Novak and Landmann unpublished) and, therefore, plays a minor role. In addition, strand quality as assessed by spacing and size of natively frozen membrane particles was not altered by BDL (Rahner et al. 1996). Taken together the data suggest moderately elevated levels of TJ proteins and indicate that newly synthesized occludin and ZO-1, the amount of which is increased parallel to TJ length, is incorporated into the
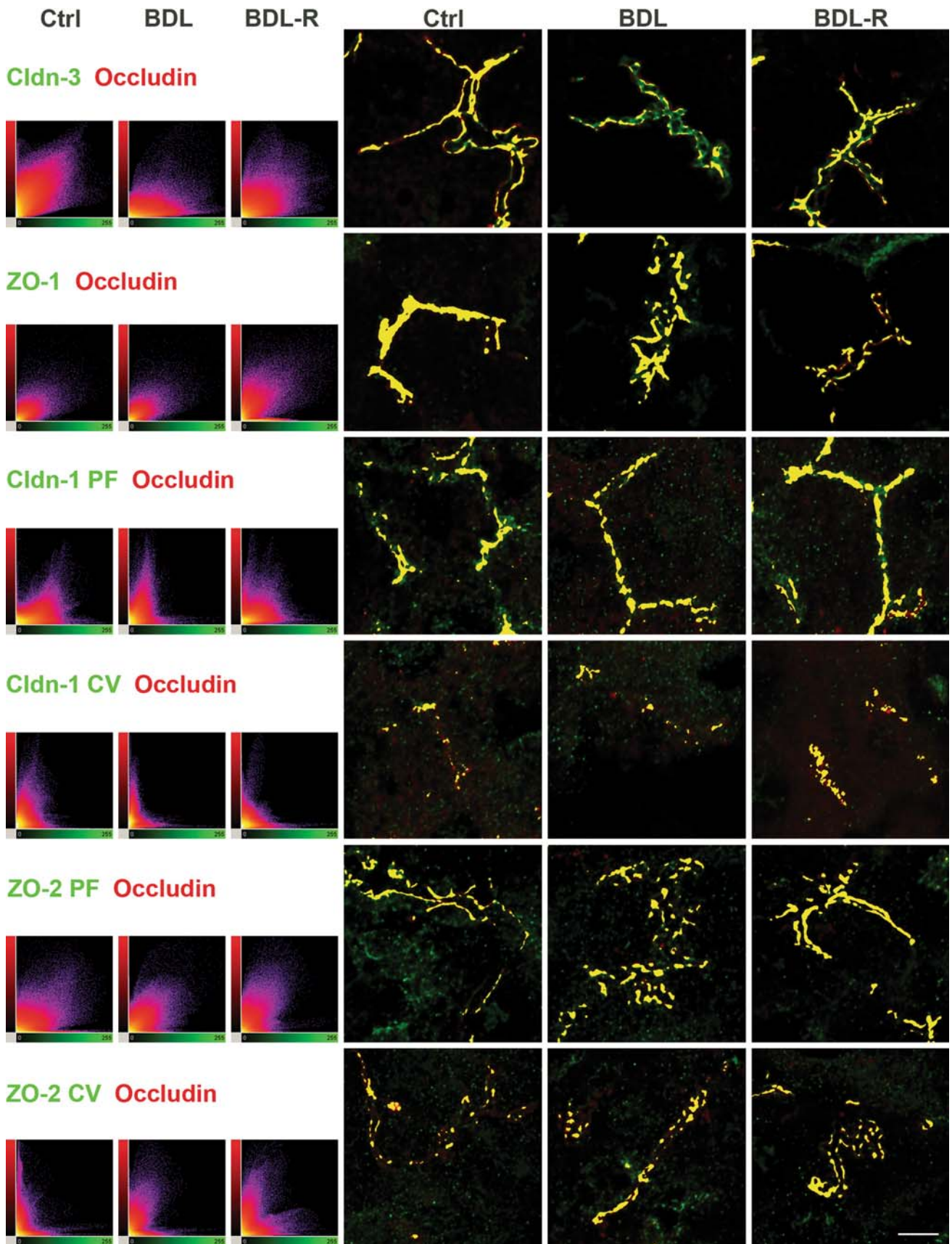
Fig. 6 2D histograms and colocalization patterns of occludin and selected TJ proteins. The data sets are displayed as maximum intensity projections. All proteins (*green*) show fragmented overlap (*yellow*) with occludin (*red*) which is caused by below threshold intensities of one protein of the pairing. Colocalization analysis but not subjective examination reveals differences between the three groups. The histograms demonstrate the general trend of decreased overlap after BDL and to reversal after BDL-R. Only ZO-2 in perivenous cells (*bottom row*) shows more colocalization signal after BDL than in *Ctrl*, while an intermediate pattern is displayed by BDL-R

longer TJ. As claudin expression is not altered significantly, this results in a relatively decreased contribution of claudins and increased amounts of occludin in cholestatic TJ strands, an alteration that may play a role in the increase of TJ permeability.

Changes in the molecular composition of TJ strands is strongly supported by our colocalization data which demonstrate decreased volumes of overlap and correlation coefficients between claudins and occludin or ZO-1 (Fig. 6, 7). Though colocalization analysis is a useful tool for the assessment of TJ quality on the molecular level, overlap of signals may but does not necessarily indicate protein-protein interaction. As a colocalizing voxel reflects the presence of both proteins in a volume of $50 \times 50 \times 120$ nm, decreased overlap can result from decreased protein concentration(s) or from disturbed protein interactions. It is unlikely that interactions between transmembrane or integral and peripheral membrane proteins are severely impaired because our data do not show significant alterations in TJ protein phosphorylation (Fig. 5). This key

regulator of protein interaction is involved in the proper positioning in TJ of occludin (Sakakibara et al. 1997) and plays a role in the regulation of claudin barrier function (Van Itallie and Anderson 2006). In addition, and more importantly, dissociation of interacting proteins is expected to display severely decreased correlation coefficients which measure the linear relationship of two signals. This was not the case because decreases in coefficients did not exceed the degree of volume alterations (Fig. 7). We conclude, therefore, that the proportion of claudins in TJ strands is reversibly decreased by BDL which might compensate for the increased contribution of occludin.

ZO-2

Due to their multiple binding sites the three MAGUKs, ZO-1, ZO-2, and ZO-3 are regarded as scaffolding proteins with specific, nonredundant functions for each protein though their sequences show a high degree of conservation. Recent evidence demonstrates that polymerization of claudins into TJ strands depends on ZO-1 or ZO-2 (Umeda et al. 2006) and that ZO-1 controls the location of TJ transmembrane proteins by a complex machinery of protein binding and targeting signals (Fanning et al. 2007). Our data show a more than 2.5-fold and reversible upregulation of ZO-2 (Fig. 4) which results in strongly increased colocalizations with occludin and ZO-1 in perivenous but not periportal hepatocytes (Fig. 7). BDL induced incorporation of ZO-2 in TJ of perivenous cells (Fig. 3) suggests that the network of plaque proteins is altered drastically and that perivenous

Fig. 7 Colocalizing volumes (**a**, **c**) and correlation coefficients (**b**, **d**) of TJ proteins and occludin (**a**, **b**) or ZO-1 (**c**, **d**). Quantitative examination demonstrates that colocalization is decreased by bile duct ligation (BDL) and, at least partly, restored after release of ligation (BDL-R). The alterations of correlation coefficients are very similar to those of colocalizing volumes. This supports quantitative changes in protein expression and makes dissociation of interacting proteins unlikely
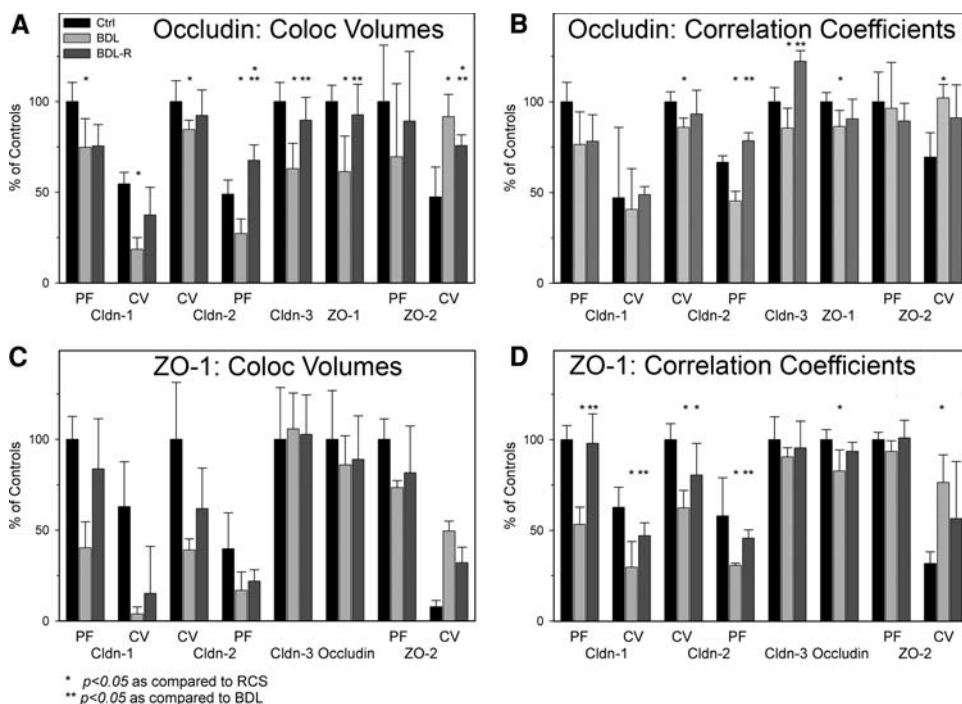
ZO-1 is—at least in part—substituted by ZO-2. As ZO-1 forms homodimers as well as heterodimers with ZO-2 and ZO-3 (Utepbergenov et al. 2006), the exact nature of substitution remains unclear. We can speculate that the observed strong association between ZO-1 and transmembrane proteins might be impaired by ZO-2, a view that could not be proven since no monoclonal antibody against ZO-2 was available. Interestingly, familial hypercholanemia, a disease associated with increased serum bile salt concentration, has been shown recently to be associated with a point mutation in ZO-2 affecting the claudin-binding domain (Carlton et al. 2003). This resulted in decreased affinity of claudins to ZO-2 suggesting that claudin strands are poorly anchored to plaque proteins. In addition, this defect induced the cholestatic features of increased junctional permeability and depth.

Though it remains to be shown if ZO-2 interactions with TJ transmembrane proteins can modulate barrier properties directly or indirectly, we may hypothesize that the known dynamics of TJ strands might be increased by ZO-2. Fibroblasts transfected with GFP-tagged claudin form TJ strands that break and reanneal within minutes as demonstrated by real-time imaging (Sasaki et al. 2003). It has been postulated that breaking and resealing of strands could allow for the movement of solutes across the barrier in a step-wise manner passing a strand at a break and waiting for a gap to open in the next strand while the former gap is resealed (Anderson et al. 2004). It is possible that ZO proteins control the frequency of this process and that ZO-2 could generate breaks more often that ZO-1.

References

Amasheh S, Meiri N, Gitter AH, Schoneberg T, Mankertz J, Schulzke JD, Fromm M (2002) Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells. J Cell Sci 115:4969–4976

Anderson JM, Van Itallie CM, Fanning AS (2004) Setting up a selective barrier at the apical junction complex. Curr Opin Cell Biol 16:140–145

Carlton VE, Harris BZ, Puffenberger EG, Batta AK, Knisely AS, Robinson DL, Strauss KA, Shneider BL, Lim WA, Salen G, Morton DH, Bull LN (2003) Complex inheritance of familial hypercholanemia with associated mutations in TJP2 and BAAT. Nat Genet 34:91–96

Chan JR, Stinson RA (1986) Dephosphorylation of phosphoproteins of human liver plasma membranes by endogenous and purified liver alkaline phosphatase. J Biol Chem 261:7635–7639

Colegio OR, van Itallie C, Rahner C, Anderson JM (2003) Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture. Am J Physiol 284:C1346–C1354

Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S (2004) Automatic and quantitative measurement of protein–protein colocalization in live cells. Biophys J 86:3993–4003

Fallon MB, Mennone A, Anderson JM (1993) Altered expression and localization of the tight junction protein ZO-1 after common bile duct ligation. Am J Physiol 264:C1439–C1447

Fallon MB, Brecher AR, Balda MS, Matter K, Anderson JM (1995) Altered hepatic localization and expression of occludin after common bile duct ligation. Am J Physiol 38:C1057–C1062

Fanning AS, Anderson JM (1999) PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. J Clin Invest 103:767–772

Fanning AS, Little BP, Rahner C, Utepbergenov D, Walther Z, Anderson JM (2007) The Unique-5 and -6 Motifs of ZO-1 regulate tight junction strand localization and scaffolding properties. Mol Biol Cell 18:721–731

Furuse M, Hirase T, Itoh M, Nagafuchi A, Yonemura S, Tsukita S, Tsukita S (1993) Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 123:1777–1788

Furuse M, Fujita K, Hiiragi T, Fujimoto K, Tsukita S (1998) Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J Cell Biol 141:1539–1550

Furuse M, Sasaki H, Tsukita S (1999) Manner of interaction of heterogenous claudin species within and between tight junction strands. J Cell Biol 147:891–903

Galvani M, Rovatti L, Hamdan M, Herbert B, Righetti PG (2001) Protein alkylation in the presence/absence of thiourea in proteome analysis: a matrix assisted laser desorption/ionization-time of flight-mass spectrometry investigation. Electrophoresis 22:2066–2074

Gumbiner B, Lowenkopf T, Apatira D (1991) Identification of a 160-kDa plypeptide that binds to the tight junction protein ZO-1. Proc Natl Acad Sci USA 88:3460–3464

Kawaguchi T, Sakisaka S, Sata M, Mori M, Tanikawa K (1999) Different lobular distributions of altered hepatocyte tight junctions in rat models of intrahepatic and extrahepatic cholestasis. Hepatology 29:205–216

Kiuchi-Saishin Y, Gotoh S, Furuse M, Takasuga A, Tano Y, Tsukita S (2002) Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. J Am Soc Nephrol 13:875–886

Landmann L, Stieger B (2001) Tight junctions in liver disease. In: Cereijido M, Anderson JM (eds) Tight junctions. CRC Press, Boca Raton, pp 575–597

Landmann L, Marbet P (2004) Colocalization analysis yields superior results after image restoration. Microsc Res Tech 64:103–112

Landmann L, Angermüller S, Rahner C, Stieger B (1998) Expression, distribution and activity of Na$^+$, K$^+$-ATPase in normal and cholestatic rat liver. J Histochem Cytochem 46:405–410

Maly IP, Nitsch C (2007) SDS disc electrophoresis of proteins in homogeneous, low-concentrated polyacrylamide gels. Electrophoresis 28:1508–1513

McCarthy KM, Francis SA, McCormack JM, Lai J, Rogers RA, Skare IB, Lynch RD, Schneeberger EE (2000) Inducible expression of claudin-1-myc but not occludin-VSV-G results in aberrant tight junction strand formation in MDCK cells. J Cell Sci 113:3387–3398

Morita K, Furuse M, Fujimoto K, Tsukita S (1999) Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. Proc Natl Acad Sci USA 96:511–516

Rahner C, Stieger B, Landmann L (1996) Structure–function correlation of tight junctional impairment after intrahepatic and extrahepatic cholestasis in rat liver. Gastroenterology 110:1564–1578

Rahner C, Mitic LL, Anderson JM (2001) Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas and gut. Gastroenterology 120:411–422

Sakakibara A, Furuse M, Saitou M, Ando-Akatsuka Y, Tsukita S (1997) Possible involvement of phosphorylation of occludin in tight junction formation. J Cell Biol 137:1393–1401

Sasaki H, Matsui C, Furuse K, Mimori-Kiyosue Y, Furuse M, Tsukita S (2003) Dynamic behavior of paired claudin strands within apposing plasma membranes. Proc Natl Acad Sci USA 100:3971–3976

Schneeberger EE, Lynch RD (2004) The tight junction: a multifunctional complex. Am J Physiol 286:C1213–C1228

Schulzke JD, Gitter AH, Mankertz J, Spiegel S, Seidler U, Amasheh S, Saitou M, Tsukita S, Fromm M (2005) Epithelial transport and barrier function in occludin-deficient mice. Biochim Biophys Acta 1669:34–42

Stevenson BR, Siliciano JD, Mooseker MS, Goodenough DA (1986) Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 103:755–766

Stieger B, Meier P, Landmann L (1994) Effect of obstructive cholestasis on membrane traffic and domain-specific expression of plasma membrane proteins in rat liver parenchymal cells. Hepatology 20:201–212

Umeda K, Ikenouchi J, Katahira-Tayama S, Furuse K, Sasaki H, Nakayama M, Matsui T, Tsukita S, Furuse M, Tsukita S (2006) ZO-1 and ZO-2 independently determine where claudins are polymerized in tight-junction strand formation. Cell 126:741–754

Utepbergenov DI, Fanning AS, Anderson JM (2006) Dimerization of the scaffolding protein ZO-1 through the second PDZ domain. J Biol Chem 281:24671–24677

Van der Voort HTM, Strasters KC (1995) Restoration of confocal images for quantitative image analysis. J Microsc 178:165–181

Van Itallie CM, Anderson JM (2006) Claudins and epithelial paracellular transport. Annu Rev Physiol 68:403–429

Webb RH, Dorey CK (1995) The pixelated image. In: Pawley JB (ed) Handbook of biological confocal microscopy. 2nd edn. Plenum Press, New York, pp 55–67

Willott E, Balda MS Heintzelmann M, Jameson B, Anderson JM (1992) Localization and differential expression of two isoforms of the tight junction protein ZO-1. Am J Physiol 262:C1119–C1124