Connexin45 directly binds to ZO-1 and localizes to the tight junction region in epithelial MDCK cells

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Abstract Zonula occludens protein 1 (ZO-1) is a cytosolic tight junction protein that tethers transmembrane proteins such as occludin, claudin and junctional adhesion molecule to the actin cytoskeleton. The interaction between ZO-1 and claudin or junctional adhesion molecule occurs via the amino-terminal PSD95/Dlg/ZO-1 (PDZ) domains in ZO-1. A yeast two-hybrid screen to search for proteins that interact with the PDZ domains of ZO-1 identified connexin (Cx) 45. Cx45 interacts with the PDZ domains of ZO-1 and ZO-3, but not ZO-2, via a short C-terminal PDZ binding motif (SVWI). In transfected epithelial Madin–Darby canine kidney cells, Cx45 co-localizes with endogenous ZO-1 at or near tight junctions and co-precipitation experiments show that Cx45 and ZO-1 directly interact. Inactivating the C-terminal PDZ-binding motif in Cx45 affects its co-precipitation and co-localization with ZO-1. The growing number of connexins (i.e. Cx43 and Cx45) that can associate with ZO proteins indicate that ZO proteins may play a more general role in organizing gap junctions and/or in recruiting signaling molecules that regulate intercellular communication. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Gap junction; PDZ domain; Protein–protein interaction

1. Introduction

Intercellular communication is paramount in allowing cells to successfully accomplish their task in a coordinated manner in multicellular organisms and tissues. One mechanism of intercellular communication relies on the formation of gap junctions, protein channels that span the lipid bilayers of adjacent cells and thus allow the direct exchange of ions, second messengers and metabolites between neighboring cells. Gap junction permeability plays a critical role in cellular differentiation, development and metabolic and electrophysiological processes (reviewed in [1–4]).

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Abbreviations: Cx, connexin; ZO protein, zonula occludens protein; PDZ domain, PSD95/Dlg/ZO-1 domain; MDCK cell, Madin–Darby canine kidney cell

Gap junctions are composed of subunit proteins encoded by the connexin multigene family, comprising over a dozen distinct connexin genes. Connexins span the lipid bilayer four times, with their N- and C-termini facing the cytoplasm (reviewed in [5,6]). Six connexin molecules assemble into a hemichannel or connexon, forming a pore in the lipid bilayer. Interaction of two connexons on adjacent cells establishes an intercellular aqueous pore that allows the exchange of small molecules between cells. A few to more than 10^5 connexons aggregate into tightly packed plaques to form gap junctions. Connexons may contain a single (homomeric connexon) or multiple (heteromeric connexon) connexin types. In addition, adjacent cells may contribute identically or differently composed connexons to form homotypic or heterotypic intercellular channels. This unique feature to generate diversity in terms of subunit composition, stoichiometry and docking is thought to specify the physiological properties of individual gap junctions.

Oligomerization of connexins and assembly into connexons occurs early in the secretory pathway [7]. How connexons are then transported to the plasma membrane is less well understood. There is ample evidence, however, that specific types of connexons can localize to specialized plasma membrane domains in different cell types [1]. During oocyte maturation and follicle development, granulosa cells extend processes to the oocyte, leading to the formation of intermediate junctions between the two cells. Connexin (Cx) 37 is restricted to gap junctions between granulosa cells and the oocyte, whereas Cx43 is found in channels linking adjacent granulosa cells [8]. In Schwann cells, Cx32 localizes to the incisures of Schmitt–Lanterman and the paranodal regions bordering the nodes of Ranvier [9,10]. In cardiac myocytes, Cx43 is present in intercalated discs [11], which connect myocytes in an end-to-end orientation. Intercalated discs contain zonula occludens protein 1 (ZO-1), a protein present in tight junctions in epithelial and endothelial cells. Interestingly, ZO-1 interacts with Cx43 [11] and may thus be involved in the localization of Cx43 to intercalated discs in myocytes.

In a search for proteins that bind to the PSD95/Dlg/ZO-1 (PDZ) domains of ZO-1 we identified Cx45 as a second member of the connexin family to associate with ZO-1. In addition to ZO-1, Cx45 also interacts with the PDZ domains of ZO-3, but not with those of ZO-2. Co-precipitation and co-localization experiments confirmed the interaction between ZO-1 and Cx45. Thus, ZO proteins may play a more general role in the assembly of connexons at particular plasma membrane domains and/or in the recruitment of signaling molecules to gap junctions.

2. Materials and methods

2.1. Yeast two-hybrid screen

A yeast two-hybrid screen was performed using the N-terminal three PDZ domains of human ZO-1 (amino acids 1–507) fused in frame to the GAL4 DNA binding domain of the yeast expression vector pGBKT7 (Clontech) as a bait and a pre-transformed mouse 17 day embryo cDNA library fused to the GAL4 transactivator domain in the yeast expression vector pACT2 (Clontech). All experimental details were according to the manufacturer's protocols. Clones obtained in a first screen at low stringency were re-screened at high stringency with dropout media and then tested for β-galactosidase activity. Plasmids from positive clones were isolated and the cDNA inserts sequenced, resulting in the identification of cDNAs encoding the C-terminal region of Cx45. The interaction was retested by transforming the library plasmid encoding amino acids 315–396 of Cx45, or a mutant in which the four C-terminal amino acids (SVWI) were mutated to alanine, with bait vectors carrying the PDZ domains of human ZO-1, canine ZO-2 (amino acids 1–591) or ZO-3 (amino acids1–467). Empty pGBKT7 or a bait plasmid with a laminin cDNA (Clontech) served as negative controls.

2.2. Cell culture and transfection of MDCK cells

Madin–Darby canine kidney (MDCK) strain II cells were cultured and grown on permeable Transwell polycarbonate filter units (Costar) to obtain polarized cell monolayers as described [12]. The full length human Cx45 cDNA (kindly provided by Dr. K. Willecke, Bonn, Germany) was subcloned into the pcDNA3 expression vector and transfected into MDCK cells using Transfast (Promega). Following selection in G418, cells expressing Cx45 were identified by immunofluorescence or Western blotting with a monoclonal mouse anti-human Cx45 antibody (Chemicon; see below).

2.3. Co-immunoprecipitation assays

Control and transfected MDCK cells expressing either wild type or mutant Cx45 were incubated for 30 min at 37°C with 2 μg/ml cytochalasin D (Sigma) and then cooled on ice for 30 min. After washing with cold phosphate-buffered saline (PBS), cells were lysed on ice in lysis buffer containing 0.5% Triton X-100 in PBS supplemented with a protease inhibitor cocktail. The post-nuclear supernatant was pre-cleared and ZO-1 was immunoprecipitated with 3 μg/ml of rabbit anti-ZO-1 antibody (Zymed) and protein G Sepharose (Pharmacia). Immunoprecipitates were washed with lysis buffer, fractionated by SDS–PAGE (10% acrylamide) and blotted onto PVDF membranes. Cx45 was detected using a monoclonal mouse anti-human Cx45 antibody (1:1000; Chemicon), followed by horseradish peroxidase-labeled goat anti-mouse antibody (1:3000; Bio-Rad) and chemiluminescence (Super Signal West Pico, Pierce). Autoradiographs were quantitated by densitometry.

2.4. Immunofluorescence labeling

MDCK cells grown on glass coverslips or 0.4 μm Transwell filters (Costar) were fixed with either cold methanol (2.5 min; −20°C) or paraformaldehyde (PFA) (3.7%; 30 min) and, in the case of PFA fixation, permeabilized with Triton X-100 (0.2% in PBS). After blocking in 10% goat serum (Gibco BRL), cells were incubated with a monoclonal mouse anti-human Cx45 antibody (5 μg/ml; Chemicon) and a rabbit anti-dog ZO-1 (1:200; Zymed) followed by Alexa 488-

and Alexa 594-labeled goat anti-mouse and anti-rabbit antibodies (1:1000; Molecular Probes), respectively. Images were acquired using a confocal laser scanning microscope (Bio-Rad) and IMARIS software (Bitplane).

3. Results

3.1. The C-terminus of Cx45 interacts with the PDZ domains of ZO-1 and ZO-3 in a yeast two-hybrid assay

To identify proteins that interact with the PDZ domains of ZO-1, a yeast two-hybrid screen was carried out using a mouse 17 day embryo library. Sequencing of positive clones revealed cDNAs encoding the C-terminal coding region of connexin 45. One Cx45 clone encoding amino acids 315–396 fused in frame to the Gal4 transactivation domain was selected for further characterization. In addition to the PDZ domains of ZO-1, the Cx45 construct interacted with the PDZ domains of ZO-3, but not with those of ZO-2 (Table 1), indicating that Cx45 did not indiscriminately interact with PDZ domains in general. No interactions were detected between the Cx45 construct and either laminin or an empty library vector encoding the Gal4 DNA binding domain alone.

PDZ domains often interact via C-terminal amino acid motifs in transmembrane proteins and the C-terminal SVWI in Cx45 is reminiscent of type-1 PDZ binding motifs [13]. To test whether this motif was required for the interaction with the ZO-1 and ZO-3 PDZ domains, the four C-terminal residues were mutated to alanine. The mutant Cx45 no longer interacted with the ZO-1 PDZ domains in the two-hybrid assay (Table 1), showing that the C-terminal SVWI in Cx45 can bind to PDZ domains and confirming its importance in the interaction with ZO-1 and ZO-3.

Together, these results show that in a yeast two-hybrid assay, the PDZ domains in ZO-1 and ZO-3, but not those in ZO-2, interact with Cx45 via a C-terminal SVWI PDZ binding motif.

3.2. Characterization of epithelial MDCK cells transfected with Cx45 cDNA

To confirm the two-hybrid results, we determined if full length ZO-1 was able to bind to full length Cx45 and whether this interaction occurred in vivo. For this purpose, epithelial MDCK cells, which endogenously express ZO-1, were stably transfected with mouse Cx45 or a Cx45 mutant carrying an inactivated C-terminal PDZ binding motif. Expression of the transfected Cx45 was characterized by Western blot analysis and immunofluorescence using an antibody to mouse Cx45.

Table 1
Interaction of the PDZ domains of ZO-1, ZO-2 and ZO-3 with a construct encoding the C-terminal region of Cx45 or a Cx45 mutant (Cx45mut) in which the SVWI amino acids encoding a putative PDZ binding motif were mutated to alanine

	Cx45	Cx45mut	Library vector	Laminin	T-Ag
ZO-1 PDZ	+++	−	−	−	nd
ZO-2 PDZ	−	−	−	−	nd
ZO-3 PDZ	+++	−	−	−	nd
Bait vector	−	−	−	−	−
p53	−	−	−	−	+++

Interactions were determined by monitoring growth of co-transformed yeast on selective media and β-galactosidase activity. Similar results were obtained under low and high stringency conditions. Empty bait (pGBKT7) and library (pACT2) plasmids and a laminin construct served as negative controls. T-antigen (T-Ag) and p53, known to interact, were used as a positive control. Growth on dropout media and β-galactosidase activities for yeast co-expressing T-Ag and p53 (positive control; +++) were similar for clones co-expressing the C-terminus of Cx45 and the PDZ domains of ZO-1 and ZO-3.
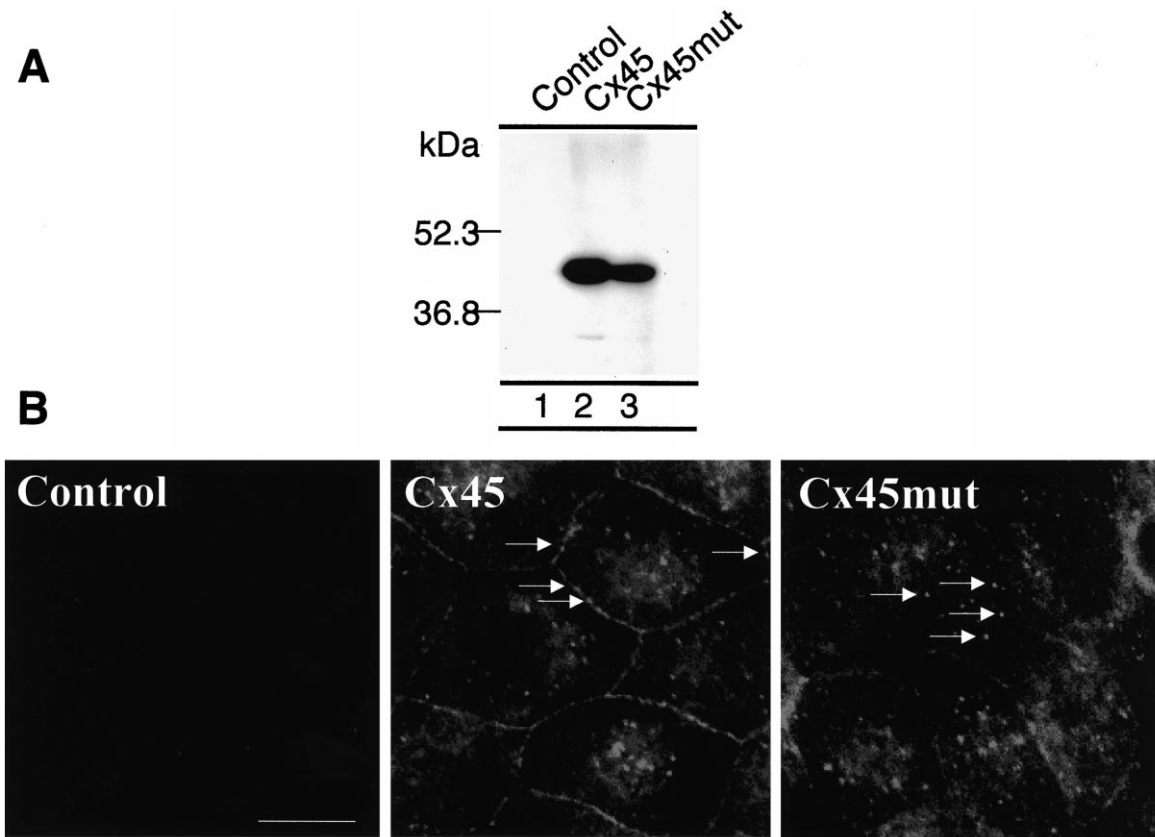
Fig. 1. Characterization of MDCK cells expressing wild type or mutant Cx45. A: Western blot analysis. Lysates of control MDCK cells (lane 1) or cells transfected with a mouse Cx45 cDNA (lane 2) or a construct in which the C-terminal SVWI residues were mutated to alanine (lane 3) were analyzed by Western blot using a monoclonal anti-Cx45 antibody. B: Immunofluorescence analysis. Control cells or cells transfected with wild type (Cx45) or mutant (Cx45mut) Cx45 grown on glass coverslips were fixed, permeabilized and stained with anti-Cx45 and labeled anti-mouse antibodies. Arrows show the accumulation of Cx45, but not Cx45mut, at regions of cell–cell contact. Bar: 10 μm.

As shown in Fig. 1A, a 45 kDa band, corresponding in apparent molecular weight to Cx45, was detected in cells transfected with the wild type (lane 2) or the mutant (lane 3) Cx45, but not in control MDCK cells (lane 1). The expression of Cx45 was confirmed by immunofluorescence microscopy (Fig. 1B). Cells expressing the wild type or the mutant Cx45 showed a punctate staining typical of connexons. Interestingly, the wild type, but not the mutant, Cx45 was concentrated at sites of cell–cell contact (Fig. 1B, arrows).

3.3. Cx45 directly associates with ZO-1 in vivo

To analyze if also full length Cx45 and ZO-1 interact in vivo, co-immunoprecipitation experiments were carried out. MDCK cells expressing wild type or mutant Cx45 were lysed and endogenous ZO-1 precipitated using a polyclonal antibody. Immunoprecipitates were then analyzed by Western blot using an antibody to Cx45.

As shown in Fig. 2, Cx45 was co-precipitated with ZO-1 from cells expressing wild type Cx45 (lane 2) but not from untransfected control cells (lane 1). Co-precipitation from two clones expressing different amounts of the mutant Cx45 was greatly reduced as compared to wild type Cx45 (lane 3 and 4), even though expression levels for mutant Cx45 were three- to four-fold higher than for the wild type protein (compare lanes 7 and 8 to lane 6). Quantification of the co-precipitation experiments, taking into account the differences in expression levels, showed that the inactivation of the PDZ binding motif

in Cx45 resulted in a $\sim$20-fold reduction of the Cx45 associated with ZO-1.

While Cx45 co-precipitated with ZO-1, ZO-1 was not readily detected in Cx45 immunoprecipitates, possibly because ZO-1 could mask the C-terminal epitope for the antibody used to immunoprecipitate Cx45.

Thus, full length Cx45 and ZO-1 bind in vivo through a direct interaction that requires the C-terminal PDZ binding motif in Cx45.

3.4. Cx45 co-localizes with ZO-1 in the tight junction region in polarized MDCK cells

Next, we determined if Cx45 and ZO-1 co-localize in fully polarized MDCK cell monolayers. Cells were grown on permeable polycarbonate filters to obtain polarized cell monolayers with well-established tight junctions. Monolayers were fixed, permeabilized, stained with rabbit anti-ZO-1 and monoclonal mouse anti-Cx45 and suitably labeled secondary antibodies, and visualized by confocal microscopy. Images of horizontal sections at the height of tight junctions (Fig. 3A–C) and vertical sections along the apico-basal axis (panels D–F) of the monolayer were acquired.

As shown in Fig. 3, control cells (panels A and D) and cells transfected with wild type (panels B and E) or mutant (panels C and F) Cx45 showed the typical localization of ZO-1 (red color) to tight junctions. No staining for Cx45 (green color) was observed in control cells (panels A and D), whereas cells
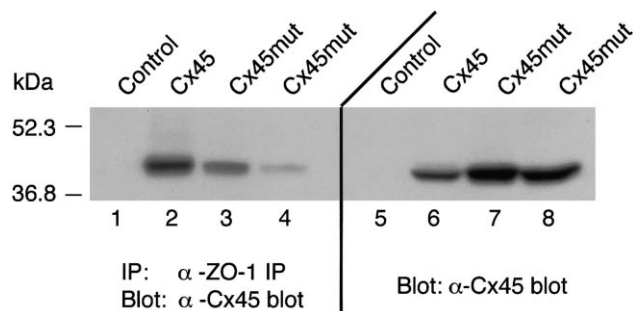
Fig. 2. Cx45 directly interacts with ZO-1 via the C-terminal SVWI. Control MDCK cells (lanes 1 and 5) or cells expressing wild type (lanes 2 and 6) or mutant (lanes 3, 4, 7 and 8) Cx45 were lysed and equal amounts of total protein were used to immunoprecipitate ZO-1 (lanes 1–4). Immunoprecipitates were then analyzed by Western blot to detect Cx45 bound to ZO-1. An aliquot of the cell lysate was directly blotted (lanes 5–8) to determine the amount of wild type and mutant Cx45 present in the cell lysate.

transfected with wild type (panels B and E) or mutant (panels C and F) Cx45 showed a punctate Cx45 staining. A significant degree of co-localization (yellow color) of Cx45 and ZO-1 was observed in cells transfected with wild type Cx45, whereas the localization of the mutant to tight junctions was strongly decreased. Nevertheless, inactivation of the PDZ binding motif in Cx45 did not completely abolish its co-localization with ZO-1 (see panel C, arrows), suggesting that localization of Cx45 to the tight junction area could be mediated by other mechanisms besides a direct association with ZO proteins (see Section 4). Similar results were obtained for co-localization with another tight junction marker, occludin (data not shown).

In conclusion, Cx45 is present together with ZO-1 in the tight junction region of MDCK cells and this localization is mediated by the PDZ binding motif of Cx45.

4. Discussion

Using a yeast two-hybrid assay, we show that the C-terminus of Cx45 interacts with the PDZ domains of ZO-1 and ZO-3, but not those in ZO-2. Furthermore, full length Cx45 and ZO-1 interact in vivo in epithelial MDCK cells based on co-immunoprecipitation and co-localization experiments. The C-terminus of Cx45 resembles classical PDZ binding motifs [14] and mutational analysis confirmed the requirement of the SVWI motif for the interaction with the PDZ domains of ZO-1 and ZO-3, both in the yeast two-hybrid assay and with full length ZO-1 in vivo. MDCK cells were chosen since, in contrast to the broad distribution throughout the area of cell–cell contact in osteoblasts or cardiac myocytes, ZO-1 is restricted to discrete tight junctions, facilitating the interpretation of co-localization experiments.

Although mutation of the SVWI dramatically reduced the fraction of Cx45 that co-precipitated with ZO-1, the interaction was not completely abolished, suggesting that mutant Cx45 may be able to associate with ZO-1 via an alternative mechanism. Since different connexins can assemble into a single connexon, Cx45 may be incorporated into heteromeric connexons containing a second connexin able to bind ZO-1. MDCK cells express Cx43 [15], which forms heteromeric connexons with Cx45 [16] and also interacts with ZO-1 [11,17]. Indeed, Cx45 has recently been detected in a complex with Cx43 and ZO-1 [18], although this study did not conclusively demonstrate a direct interaction between Cx45 and ZO-1. Thus, while mutation of the PDZ binding motif in Cx45 abolishes its direct interaction with ZO-1, it may still associate
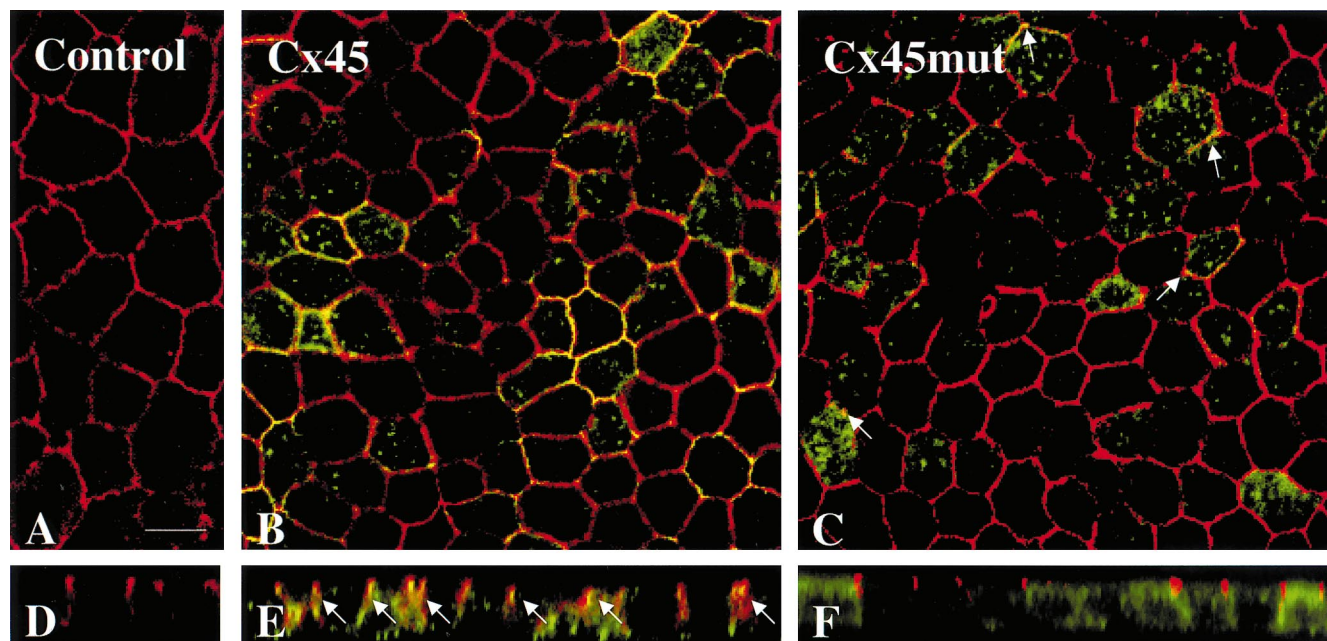

Fig. 3. Cx45 co-localizes with ZO-1 to the tight junction region in MDCK cells. Control cells (A,D) and cells transfected with wild type (B,E) or mutant (C,F) Cx45 were grown on polycarbonate filters to obtain polarized cell monolayers. Cells were fixed, permeabilized and stained for Cx45 (green) and ZO-1 (red). Confocal images corresponding to horizontal sections at the height of tight junctions (A–C) or to vertical sections along the apico-basal axis of the monolayer (D–F) were acquired. Cx45 extensively co-localizes with ZO-1 (yellow; arrows) at the level of tight junctions (B,E). Although the co-localization of the mutant Cx45 with ZO-1 is strongly reduced (D,F), occasional co-localization is observed (C, arrows). Bar: 10 μm.

indirectly via Cx43. Mixed Cx43/Cx45 connexons are therefore likely to account for the occasional, albeit dramatically reduced, detection of mutant Cx45 in the tight junction region.

Cx43 interacts with the second PDZ domain of ZO-1, also via a C-terminal PDZ binding motif [17]. Several other members of the connexin family encode putative PDZ binding sequences at their C-termini [14], raising the possibility that other connexins besides Cx43 and Cx45 also bind to ZO proteins. In this case, different connexins could compete for a particular PDZ domain, interact with different PDZ domains in one ZO protein, or bind to different ZO proteins. Whether Cx43, like Cx45, interacts with other ZO proteins is not known.

The functional relevance of the association between connexins and ZO proteins is still unclear. Connexins and ZO-1 co-localize at sites of cell-cell contact in a variety of cell types, including cardiac myocytes (Cx43 [11]), fibroblasts (Cx43 [17]), osteoblasts (connexin 43 and 45 [19]), kidney epithelial cells (Cx45, this report) and hepatocytes (Cx32 [20]). ZO-1 or other ZO proteins could therefore play a role in targeting or localizing connexins to specialized plasma membrane domains. Indeed, different connexins are restricted to specialized plasma membrane domains in many cell types, including Schwann cells [9,10] and cardiac myocytes [11]. In addition to osteoblasts and myocytes, Cx45 is expressed in endothelial cells, which can establish ZO-1-containing tight junctions. Interestingly, mice deficient for Cx45 show severe defects in blood vessel maturation [21].

ZO proteins may also play a role in connexon assembly and stability. The close opposition of the plasma membranes of adjacent epithelial cells at tight junctions, for example, may facilitate the formation of gap junctions between neighboring cells. Alternatively, binding to ZO proteins could determine which connexin types co-assemble into a particular connexon. Interestingly, over-expression of Cx32 in hepatocytes increases the number and complexity of tight junction strands and small gap junction plaques are observed between strands [20]. Although Cx32 is associated with occludin [20], it is not known if this interaction is direct or via a ZO protein. An increased turnover rate observed for a Cx43 mutant that no longer binds ZO-1 [22] also supports a role of ZO proteins in stabilizing connexons, possibly by tethering them to the actin cytoskeleton.

The observation that ZO-1 only co-localizes with a subset of the total cellular connexin protein implicates the presence of distinct connexon populations in a single cell, which could differ in function or mode of regulation. Recent evidence supports a role for ZO proteins in signal transduction events, possibly as platforms to assemble signaling molecules [23]. Thus, ZO proteins associated with gap junctions could serve to recruit signaling molecules involved in the regulation of intercellular communication.

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