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Direct association of Connexin36 with zonula occludens-2 and zonula occludens-3

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Abstract

The gap junction protein connexin36 (C×36) is widely expressed in neurons and was previously shown to interact with the PDZ domain-containing protein zonula occludens-1 (ZO-1). We investigated whether C×36 is also able to interact with other members of zonula occludens family of proteins, namely, ZO-2 and ZO-3, the former of which was reported to be co-localized with C×36 at gap junctions in mouse retina. HeLa cells transfected with C×36 and cultured βTC-3 cells were found to express ZO-2 and ZO-3, and both of these ZO proteins were co-localized with C×36 at gap junctional cell-cell contacts. In lysates of C×36-transfected HeLa cells, ZO-2 and ZO-3 were shown to co-immunoprecipitate with C×36, whereas C×36/ZO-2 association was absent in cells transfected with truncated C×36 lacking its C-terminus SAYV PDZ interaction motif. In vitro pull-down assays revealed that C×36 interacts with the PDZ1, but not with the other two PDZ domains in ZO-2 or ZO-3. Truncated C×36 lacking its PDZ binding motif failed to bind the PDZ1 domain of either ZO-2 or ZO-3. A fourteen amino acid peptide corresponding to the C-terminus of C×36 was also shown to interact with the PDZ1 domains of ZO-2 and ZO-3, and this peptide inhibited the association of C×36 with the PDZ1 domains of these ZO proteins. These results indicate that C×36 associates with the first PDZ domain of ZO-2 and ZO-3 and that this association requires the C-terminus SAYV sequence in C×36. These findings, together with the known association of ZO-2 with a variety of proteins, including transcription factors, suggest that ZO-2 may serve to anchor regulatory proteins at gap junctions composed of C×36.

Keywords

connexin36; zonula occludens-2; zonula occludens-3; PDZ domain; βTC-3 cells

1. Introduction

PDZ domains are widespread protein binding modules that are present in hundreds of mammalian proteins and that generally interact with the last four to six C-terminus amino acids of certain transmembrane and membrane scaffolding proteins (Songyang et al., 1997; Jemth and Gianni 2007). In the central nervous system, PDZ domain-containing proteins play prominent roles in the regulation and formation of chemical synapses (Kim and Sheng

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2004). The family of closely related zonula occludens (ZO) proteins ZO-1, ZO-2 and ZO-3 each contain three PDZ domains (PDZ1, PDZ2, PDZ3) and belong to the membrane-associated guanylated kinase (MAGUK) family. These ZO proteins differentially associate with a number of other proteins, including those at tight junctions, adherens junctions and gap junctions (Gonzalez-Mariscal et al., 2000, 2003; Singh et al., 2005). To date, various approaches have been used to establish that a variety of connexins, including C×30, C×36, C×43, C×45, C×31.9, C×46, C×47 and C×50, interact with ZO-1 (Giepmans and Moolenaar, 1998; Giepmans, 2004; Li et al., 2004a,b,c; 2008; Penes et al., 2005; Song et al., 2006). A regulatory role of connexin/ZO-1 interaction at gap junctions has been indicated by several studies where this interaction was found to be essential for normal gap junctional intercellular communication, gap junction channel accretion and disassembly (Hunter et al., 2005; Laing et al., 2005; Akoyev et al., 2007). Further, C×43 was shown to interact with ZO-1 and ZO-2 in a cell cycle stage-specific manner (Singh et al., 2005). ZO-1 and ZO-2 may serve scaffolding roles at gap junctions, as suggested by their known interaction with a long list of proteins, including association of ZO-1 with the transcription factor ZONAB (Balda and Matter, 2000) and association of ZO-2 with the transcription factors Jun, Fos and C/EBP (Betanzos et al., 2004), the functional importance of which is suggested by the embryonic lethality of ZO knockout mice (Katsuno T, et al., 2008; Xu, et al., 2008). C×36 is a well established gap junction protein whose expression is restricted to neurons in the central nervous system, chromaffin cells in the adrenal gland, beta cells in pancreas and various insulin-producing cell lines (Rash et al., 2000; Serre-Beinier et al., 2000; Martin et al., 2001; Li et al., 2004b; Bavamiab et al., 2007). It was previously demonstrated that mammalian C×36 and its fish ortholog C×35 directly associate with the PDZ1 domain of ZO-1 (Li et al., 2004b,c, 2008; Song et al., 2006; Flores et al., 2008), which is in contrast to the reported interaction of other connexins (C×43, C×31.9, C×46, C×47 and C×50) with the PDZ2 domain of ZO-1 (Giepmans, 2004). The PDZ1 domain of ZO-1 shares relatively high sequence similarity with the first PDZ domains of ZO-2 and ZO-3 (71% and 57%, respectively). Further, we previously found by confocal immunofluorescence analysis that C×36 is co-localized with ZO-2 at neuronal gap junctions in mouse retina (Ciolofan et al., 2006), raising the possibility that C×36 engages in molecular interactions with PDZ domains in ZO-2 and ZO-3. Based on these observations, we used cell transfection, double immunofluorescence labeling, coimmunoprecipitation, gene truncation and in vitro binding assays with fusion proteins and peptides to investigate whether C×36 interacts with ZO-2 and ZO-3 in cultured HeLa cells and βTC-3 cells.

2. Materials and Methods

2.1. Antibodies and peptides

Antibodies against C×36, ZO-1, ZO-2, ZO-3, GAPDH and His were used in this study. Anti-C×36 has been previously characterized (Li et al., 2004b,c). Polyclonal and monoclonal anti-C×36 (Ab51-6300 and Ab37-4600, respectively), monoclonal anti-ZO-1 Ab33-9100, polyclonal anti-ZO-1 Ab61-7300, polyclonal anti-ZO-2 Ab71-1400, and two different polyclonal anti-ZO-3 (Ab36-4000 and Ab36-4100) antibodies were obtained from Invitrogen/Zymed (Carlsbad, CA, USA), monoclonal anti-His antibody (Cat. No. 34698) was purchased from Qiagen Inc. (Mississauga, Ontario, Canada), and polyclonal anti-GAPDH (Ab37168) was obtained from Abcam Inc. (Cambridge, MA,USA). A fourteen amino acid (aa) peptide (biotin-PNFGRTQSSDSAYV) with sequence corresponding to the C-terminus of C×36 and a ten amino acid peptide with the same sequence but lacking the last four SAYV amino acids representing the PDZ domain interaction motif of C×36 (Li et al., 2004c) were obtained from Tufts Protein Chemistry Facility (Boston, MA, USA). Both of these peptides contained a N-terminal biotin label and were HPLC-purified.

2.2. Cell culture and transient transfection

HeLa cells obtained from American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. βTC-3 cells were grown in DMEM supplemented with 12.5% fetal horse serum and 2.5% fetal bovine serum. Lipofectamine™ 2000 reagent (Invitrogen) was used to transiently transfect HeLa cells with pcDNA3 vector, full-length C×36 or truncated C×36 lacking the last four C-terminus amino acids, which was prepared as previously described (Li et al., 2004c). Transiently transfected HeLa cells were taken for analysis 24 h after transfection.

2.3. Double immunofluorescence

βTC-3 cells and HeLa cells cultured on poly-L-lysine-treated glass coverslips were rinsed with phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer, pH 7.4, 0.9% saline) and fixed in ice-cold 1% formaldehyde for 5 min. Cultures were incubated for 16 h at 4 °C with monoclonal anti-C×36 antibody at 2 μg/ml and simultaneously with either polyclonal anti-ZO-2 or anti-ZO-3 at a concentration of 1 μg/ml in TBST buffer (50 mM Tris-HCl, pH 7.6, 1.5% NaCl, 0.3% Triton X-100) containing 4% normal donkey or normal goat serum. Cultures were then washed four times with TBST for 1 h, and then incubated for 1 h simultaneously with FITC-conjugated horse anti-mouse IgG diluted 1:200 and Cy3-conjugated donkey anti-rabbit IgG diluted 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells on coverslips were then washed for 1 h in TBST and coverslips were mounted on glass slides with antifade medium. Immunofluorescence images of cultured cells were obtained on a Zeiss Axioskop 2 fluorescence microscope using Carl Zeiss Axiovison 3.0 image software (Carl Zeiss Canada, Toronto, ON). The acquired images were assembled using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA), Northern Eclipse 5.0 software (Empix Imaging, Mississauga, ON, Canada) and CorelDraw Graphics Suite 12 (Corel Corporation, Ottawa, ON, Canada).

2.4. Western blotting

Western blotting was performed as previously described (Li et al., 2004c; 2008; Chen et al., 2006). βTC-3 cells and transiently transfected HeLa cells were rinsed briefly with PBS and lysed in immunoprecipitation (IP) buffer containing 20 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride and 5 μg/ml each of the protease inhibitors leupeptin, pepstatin A and aprotinin. Cell lysates were sonicated and briefly centrifuged at 20,000 g for 10 min, followed by protein determination using a kit (Bio-Rad Laboratories, Hercules, CA, USA). Sample protein (20 μg) was separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 7% polyacrylamide gels for immunoblotting of ZO-1, 8% gels for immunoblotting of ZO-2 and ZO-3, and 12.5% gels for immunoblotting of C×36, GAPDH and His-fusion proteins. All samples, except C×36, were boiled for 5 min prior to application on gels. Samples immunoblotted for C×36 were kept either at 20 °C, heated to 60 °C or boiled before application on gels. These differences in sample preparation appeared to influence the extent of C×36 dimerization and the detectability of a C×36 dimer form on immunoblots (Li et al., 2004c). Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) in standard Tris-glycine transfer buffer (pH 8.3) containing 0.5% sodium dodecylsulphate. Membranes were blocked for 2 h at room temperature in TSTw buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl with 0.2% Tween-20) containing 5% non-fat milk powder, briefly washed with TSTw, and then incubated for 16 h at 4 °C with either anti-C×36 (0.25 μg/ml), polyclonal anti-ZO-1 (0.25 μg/ml), polyclonal anti-ZO-2 (0.25 μg/ml), polyclonal anti-ZO-3 (0.25 μg/ml), polyclonal anti-GAPDH (0.1 μg/ml) or anti-His (0.2 μg/ml) in TSTw containing 1% non-fat milk powder. Membranes were then washed four times in TSTw for 40 min, incubated with

horseradish peroxidase-conjugated donkey anti-rabbit IgG or anti-mouse IgG diluted 1:5000 (Sigma-Aldrich Canada, Oakville, ON, Canada) in TSTw containing 1% non-fat milk powder, washed with TSTw four times for 40 min and resolved by chemiluminescence (ECL, Amersham PB, Baie d'Urfe, Quebec, Canada).

2.5. Immunoprecipitation

HeLa cells transiently transfected with full-length C×36 or truncated C×36 lacking its C-terminal four amino acids were homogenized in IP buffer and IP was carried out as previously described (Li et al., 2004c). Briefly, homogenates were sonicated, centrifuged at 20,000 g for 10 min at 4 °C, and 1 mg supernatant protein was pre-cleared for 1 h at 4 °C with 20 μl protein A-coated agarose beads (Santa Cruz BioTech, Santa Cruz, CA, USA) and then centrifuged at 20,000 g for 10 min at 4 °C. Supernatants were incubated separately with 2 μg polyclonal anti-ZO-1, anti-ZO-2 or anti-ZO-3 with shaking for 2 h at 4 °C, followed by 1 h incubation with 20 μl protein-A coated agarose beads, and then centrifuged at 20,000 g for 10 min at 4 °C. The beads were washed vigorously five times with 1 ml of washing buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.5% NP-40). Samples were processed by SDS-PAGE and immunoblot membranes were probed with polyclonal anti-C×36. Control samples were taken through the IP procedure with exclusion of primary antibodies.

2.6. His-PDZ domain fusion proteins and pull-down assays

Constructs of the PDZ1, PDZ2 and PDZ3 domains of ZO-2 and ZO-3 contained in the pET30A vector were kindly provided by Dr. R.A. Hall; Dept of Pharmacology, Emory University School of Medicine, Atlanta (Fam et al., 2005). The pET30A plasmids were transformed into *Escherichia coli* BL21 (DE3) chemically competent cells (Invitrogen Corporation, Carlsbad, CA, USA). Individual clones were selected and grown to 0.8 optical density in LB media supplemented with 100 μg/ml kanamycin at 37 °C, and then supplemented with isopropyl-β-D-thiogalactopyranoside (1 mM) for induction of His-PDZ domain fusion protein expression, followed by a further growth period of 4 h. Bacterial cells were harvested and washed with 50 mM Tris-HCl, pH 7.6, centrifuged at 10,000 g for 20 min, and the pellets were stored at -80 °C.

The His tagged PDZ domain fusion proteins were purified using Ni-NTA agarose beads (Qiagen Inc., Mississauga, Ontario, Canada) and His Bind Buffer Kit (Novagen, Madison, WI, USA). Pellets containing the His-PDZ domains were re-suspended in binding buffer provided with this kit, sonicated and centrifuged for 10 min at 20,000 g, and the supernatant was added to Ni-NTA agarose beads with shaking at 4 °C for 2 h. The mixture was centrifuged at 2000 g for 5 min and the beads were washed five times with PBS buffer containing 1% Triton X-100. The His-PDZ fusion proteins were separated electrophoretically in 12.5% polyacrylamide gels for validation. For in vitro binding assays, Ni-NTA agarose beads separately containing the three PDZ domain fusion proteins of ZO-2 and of ZO-3 were incubated overnight in IP buffer containing lysates of βTC-3 cells, C×36- or truncated C×36-transfected HeLa cells. After washing extensively five times in PBS buffer containing 1% Triton X-100, protein bound to the PDZ domains linked to the beads were eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and analyzed by western blotting with polyclonal anti-C×36. The same membranes were striped and reprobed with anti-His for detection of His-PDZ domain fusion proteins.

2.7. ZO-2 and ZO-3 pull-down with peptide

Aliquots of His-PDZ fusion proteins of ZO-2 and ZO-3 were purified using His Bind Quick Column (Novagen, Madison, WI, USA) according to manufacturer's instruction and taken for pull-down with a biotinylated C×36 14 aa peptide or a biotinylated C×36 10 aa peptide,

as previously described (Li et al., 2004b). Briefly, 300 μl of streptavidin-agarose beads (S-1638; Sigma, ON, Canada) was mixed with 300 μg of peptide in 50 mM Tris-HCl buffer, pH 7.4, the mixture was shaken for 1 h at 4°C, and bead–peptide conjugate was separated from unbound peptide by centrifugation at 10,000 g for 5 min and washing with PBS containing 1% Triton X-100, repeated three times. Aliquots of resuspended streptavidin-agarose beads (50 μl) with bound C×36 peptide were incubated overnight at 4°C in 100 μl of PBS containing 0.5 μg of either the His-PDZ1, His-PDZ2 or His-PDZ3 domains of ZO-2 or ZO-3, followed by centrifugation at 10,000 g for 5 min and washing in PBS containing 1% Triton X-100, repeated five times. His-PDZ fusion proteins bound to peptide were eluted from beads in SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and probed with monoclonal anti-His antibody.

2.8. Peptide competition

Aliquots of Ni-NTA agarose beads each containing 2 μg of the His-PDZ1 domain of ZO-2 or ZO-3 were incubated for 2 h at 4°C with 200 μg lysate protein from HeLa cells transiently transfected with C×36 and simultaneously with various concentrations (0 μM, 10 μM, 30 μM or 100 μM) of C×36 14 aa peptide or C×36 10 aa peptide. After washing extensively five times in PBS containing 1% Triton X-100, proteins bound to the PDZ1 domains were eluted from the beads with SDS-PAGE sample buffer, separated by 12.5% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Membranes were probed with rabbit anti-C×36 Ab51-6300, and then stripped and reprobed with monoclonal anti-His antibody to confirm equal loading of His-PDZ1 of ZO-2 and ZO-3 fusion protein on gels.

3. Results

3.1. ZO-2 and ZO-3 expression in HeLa cells

We first established that C×36-transfected HeLa cells express not only ZO-1 (Li et al., 2004b), but also ZO-2 and ZO-3. The relative levels of these proteins in HeLa cell lysates were compared on immunoblots loaded with 20 μg/lane of protein from each of three cultures separately transfected with C×36 and probed with anti-ZO-1, anti-ZO-2 and anti-ZO-3. Anti-ZO-1 recognized a single band migrating at about 220 kDa (Fig. 1A). Anti-ZO-2 recognized a single protein band migrating at about 160 kDa (Fig. 1B), which corresponds to the predicated molecular weight of ZO-2 (Gonzalez-Mariscal et al., 2000, 2003), and anti-ZO-3 recognized a single protein band migrating at approximately 130 kDa (Fig. 1C), which is consistent with the migration profile of ZO-3 (Inoka et al., 2003). Similar results were obtained after probing for ZO-2 and ZO-3 in lysates of control empty vector-transfected HeLa cells (not shown). Equal loading of proteins from the three separate cultures was shown by probing for GAPDH (Fig. 1D). The levels of each of the ZO proteins detected were consistent from culture to culture, but the density of protein bands revealing ZO-2 and ZO-3 were greater than that of ZO-1, which may reflect either true differences in the levels of expression of these proteins or differences in efficiency of their detection by antibodies. In any case, HeLa cells appeared suitable for in vitro studies requiring endogenous expression of ZO-2 and ZO-3.

3.2. C×36 co-localization with ZO-2 and ZO-3 in HeLa cells and βTC-3 cells

C×36-transfected HeLa cells and βTC-3 cells were taken for double immunofluorescence labeling of C×36 using monoclonal anti-C×36 in combination with either polyclonal anti-ZO-2 or anti-ZO-3. Expression of C×36 in HeLa cells was robust and appeared as fine puncta distributed around the periphery cells, where it was concentrated at cell-cell contacts presumably representing gap junctions (Fig. 2A1 and 2B1). Some diffuse intracellular expression was also evident as deduced from the higher labeling intensity observed in

transfected cells (Fig. 2A1 and 2B1) compared with the lower background fluorescence seen in control empty vector-transfected cells (Fig. 2C1). Immunofluorescence labeling for ZO-2 and ZO-3 also appeared largely punctate and was localized at cell-cell contacts (Fig. 2A2 and 2B2). Overlay of images indicated that the majority of C×36-positive puncta were co-localized with ZO-2-positive puncta (Fig. 2A3) and with ZO-3-positive puncta (Fig. 2B3). In empty vector-transfected HeLa cells, showing an absence of C×36 (Fig. 2C1), punctate labeling of ZO-2 was also localized at cell-cell contacts (Fig. 2C2 and 2C3), and appeared to be of finer grain than that seen in C×36-transfected HeLa cells. Similar results were obtained in cultures of βTC-3 cells, where punctate labeling for C×36 (Fig. 3A1, 3B1), ZO-2 (Fig. 3A2) and ZO-3 (Fig. 3B2) was detected at cell-cell contacts, and C×36 at these contacts was found to be co-localized with ZO-2 (Fig. 3A3, overlay) and ZO-3 (Fig. 3B3, overlay).

3.3. Co-IP of C×36 with ZO-2 and ZO-3

Association of C×36 with ZO-2 and ZO-3 in C×36-transfected HeLa cells was examined by co-IP approaches. Lysates from C×36-transfected HeLa cells were taken for IP with anti-ZO-2 and anti-ZO-3. Controls for co-IP were conducted by omission of anti-ZO-2 or anti-ZO-3 during the IP procedure, and by co-IP of C×36 with ZO-1 from lysates of C×36-transfected HeLa cells, which was previously reported (Li et al., 2004b). Blots of IP materials probed with anti-C×36 showed detection of C×36 after IP of ZO-2 (Fig. 4A, lane 1) and after IP of ZO-3 (Fig. 4B, lane 2). Positive controls indicated detection of C×36 after IP of ZO-1 (Fig. 4A, lane 2; and Fig. 4B, lane 1) and detection of C×36 in lysates of C×36-transfected HeLa cells (Fig. 4A, lane 3; and Fig. 4B, lane 3). Lysates of HeLa cells transfected with truncated C×36, which lacked the C-terminus four amino acid PDZ interaction motif, were taken for IP using anti-ZO-2. Immunoblots of IP material probed with anti-C×36 showed an absence of C×36 (Fig. 4C, lane 2). Positive controls indicated detection of truncated C×36 in lysates of these cells (Fig. 4C, lane 1). C×36 was not detected in IP material after omission of primary antibody (Fig. 4D, lane 2) (i.e., anti-ZO-2 or anti-ZO-3) during the IP procedure, with detection of C×36 from lysates of C×36-transfected HeLa cells serving as a positive control (Fig. 4D, lane 1).

3.4. C×36 binding to the PDZ1 domain of ZO-2 and ZO-3

Pull-down assays were performed to examine interaction capabilities of C×36 with the PDZ domains of ZO-2 and ZO-3. Lysates of HeLa cells transfected with C×36 or truncated C×36 were incubated with His-PDZ1, His-PDZ2 and His-PDZ3 domain fusion proteins of ZO-2 and ZO-3, followed by immunoblotting and probing of bound proteins with anti-C×36. As shown in Figure 5, C×36 pull-down occurred with the PDZ1 domain of ZO-2 (Fig. 5A, lane 2) and the PDZ1 domain of ZO-3 (Fig. 5B, lane 2), but not with either of the other two domains of ZO-2 (Fig. 5A, lanes 3 and 4) or the other two domains of ZO-3 (Fig. 5B, lanes 3 and 4). Removal of the C-terminal four amino acids of C×36 rendered truncated C×36 incapable of interaction with the PDZ1 domain of either ZO-2 (Fig. 5A, lane 6) or ZO-3 (Fig. 5B, lane 6). The C×36 bands detected after pull-down with the PDZ1 domains corresponded to co-migrating bands detected in lysates of C×36-transfected HeLa cells (Fig. 5A and B, lane 1) and truncated C×36-transfected HeLa cells (Fig. 5A and B, lane 5), which are shown as positive controls for immunoblotting of C×36. Pull-down with the PDZ1 domain of ZO-2 also resulted in detection of the dimeric form of C×36 migrating at 70-80 kDa (Fig. 5A, lane 2), which was weakly detected after pull-down with the PDZ1 domain of ZO-3, but was not detected in HeLa cell lysates. Immunoblot membranes shown in Figure 5A and 5B were stripped and reprobed with monoclonal anti-His to confirm His-PDZ domain fusion protein loading (Fig. 5C and 5D; lanes 2-4, 6-8). Although loading of His-PDZ3 of ZO-2 (Fig. 5C, lanes 4 and 8) was less than that of His-PDZ1 and His-PDZ2 of ZO-2, separate experiments using a three-fold increase in loading of His-PDZ3 pull-down material still showed an absence of C×36 pull-down (not shown). In these blots, lysates from

C×36- or truncated C×36-transfected HeLa cells show an absence of His-PDZ proteins (Fig. 5C and 5D; lane 1 and 5, respectively), as expected.

Lysates from βTC-3 cells were also taken for pull-down with the three His-PDZ domains of ZO-2. Western blotting of pull-down material probed with anti-C×36 revealed the presence of both the monomeric and dimeric forms of C×36 after pull-down with the PDZ1 domain of ZO-2 (Fig. 6, lane 2), but not after pull-down with the PDZ2 or PDZ3 domains of ZO-2 (Fig. 6A, lane 3 and 4). Lysates from βTC-3 cells, included as a positive control for C×36 detection, showed relatively low levels of C×36 (Fig. 6A, lane 1). A separate blot probed with anti-His monoclonal antibody shows the relative quantities of the His-PDZ1, His-PDZ2 and His-PDZ3 domains of ZO-2 that were used for pull-down of C×36 in Fig. 6A. As expected, His-PDZ protein was not detected in lysates of βTC-3 cells (Fig. 6B, lane 1).

3.5. C×36 14 aa peptide interaction with the PDZ1 domain of ZO-2 and ZO-3

Interaction capability of C×36 with ZO-2 and ZO-3 was examined by using a N-terminally biotinylated C×36 C-terminal 14 aa peptide to pull-down purified His-PDZ domains of ZO-2 and ZO-3. In immunoblots of pull-down material probed with anti-His antibody, the 14 aa peptide linked to streptavidin-agarose beads was found to pull-down the His-PDZ1 domain of ZO-2 (Fig. 7A, lane 1) and the His-PDZ1 domain of ZO-3 (Fig. 7B, lane 1), but not the His-PDZ2 or His-PDZ3 domains of either ZO-2 (Fig. 7A, lanes 2 and 3) or ZO-3 (Fig. 7B, lanes 2 and 3). These pull-down assays were repeated with a N-terminally biotinylated C×36 C-terminal 10 aa peptide lacking the last four C-terminal amino acids that were previously shown to be required for interaction of C×36 with the PDZ1 domain of ZO-1 (Li et al., 2004c). This 10 aa peptide linked to streptavidin-agarose beads failed to pull-down the PDZ1 domain of either ZO-2 (Fig. 7A, lane 4) or ZO-3 (Fig. 7B, lane 4). As in the case of the C×36 14 aa peptide, the 10 aa peptide also lacked pull-down capability of the other two His-PDZ domain of ZO-2 (Fig. 7A, lanes 5 and 6) or ZO-3 (Fig. 7B, lanes 5 and 6).

3.6. Competition of C×36/His-PDZ1 interaction by C×36 peptide

To establish further that C×36 interaction with the PDZ1 domains of ZO-2 and ZO-3 was dependent on the C-terminal region of C×36, pull-down of C×36 from lysates of C×36-transfected HeLa cells by the His-PDZ1 domain of ZO-2 and ZO-3 was conducted in the presence of various concentrations of either C×36 C-terminal 14 aa peptide or the C×36 C-terminal 10 aa peptide devoid of the PDZ1 domain binding motif of C×36 (i.e., SAYV). Immunoblots of C×36 pull-down indicated decreasing levels of C×36 detected with increasing concentrations of C×36 14 aa peptide after pull-down with either the PDZ1 domain of ZO-2 (Fig. 8A1) or the PDZ1 domain of ZO-3 (Fig. 8B1) compared to pull-down with these domains in the absence of the C×36 14 aa peptide (Fig. 8A1, lane 1; Fig. 8B1, lane 1). The C×36 14 aa peptide reduced interaction of the PDZ1 domains with both the monomeric and dimeric forms of C×36. Lower levels of dimeric vs monomeric C×36 seen in these blots at zero peptide concentration may have resulted in the apparent greater competition of peptide with interaction between the PDZ1 domains and the dimeric vs monomeric forms of C×36. Immunoblot membranes in Figure 8A1 and 8B1 were stripped and reprobed with anti-His (Fig. 8A2 and 8B2, respectively) to confirm equal loading of His-PDZ1 domains proteins. Immunoblots of C×36 pull-down with the PDZ1 domain of ZO-2 or ZO-3 in the presence of increasing concentrations of C×36 10 aa peptide indicated an inability of this peptide to compete with pull-down of C×36, as shown by similar levels of C×36 in the absence and presence of this peptide (Fig. 8C1 and Fig. 8D1). Immunoblot membranes in Figure 8C1 and 8D1 were stripped and reprobed with anti-His, confirming equal loading of His-PDZ1 domains of ZO-2 and ZO-3 (Fig. 8C2 and 8D2, respectively).

4. Discussion

The present results demonstrate that βTC-3 and C×36-transfected HeLa cells in culture express ZO-2 and ZO-3, and that these cells display co-association of C×36/ZO-2 and C×36/ZO-3 at points of cell-cell contacts forming gap junction plaques. Further, we demonstrate direct molecular interaction of C×36 with each of these ZO proteins, and show that this interaction occurs by tethering of the PDZ domain binding motif in C×36, represented by the C-terminus SAYV amino acids in this protein, selectively to the PDZ1 but not to the PDZ2 or PDZ3 domains contained in each of ZO-2 and ZO-3. Pull-down of the PDZ1 domains of ZO-2 and ZO-3 with a C×36 14 aa peptide corresponding to the C-terminus of C×36, but not with a C×36 10 aa peptide, and peptide competition of C×36 interaction with these PDZ1 domains further supports the molecular basis for this interaction. In addition, competition results with the C×36 14 aa peptide indicate the potential utility of this peptide in studies aimed to determine the functional consequences of disrupting C×36/ZO protein interaction in situ.

Impetus for the present study was derived in part from our previous observations that ZO-1 is widely associated with C×36-containing gap junctions between neurons in rodent CNS and between some endocrine cells in peripheral tissues, and that C×36 directly interacts with the PDZ1 domain of ZO-1 (Li et al., 2004b,c). These findings have recently been confirmed and extended by demonstrations that C×35, the fish ortholog of mammalian C×36, also interacts with the PDZ1 domain of ZO-1, as shown by surface plasmon resonance (SPR) techniques, and was found to be co-localized with C×35 at interneuronal gap junctions in goldfish brain (Flores et al., 2008). Sequence alignment analysis comparing the PDZ1 domain in ZO-1 with the PDZ1 domain in each of ZO-2 and ZO-3 indicated considerable homology (71% and 57%, respectively), suggesting capability of C×36 interaction with the latter two proteins, as presently confirmed. In addition, it was reported that the claudin family of proteins contain a C-terminus YV amino acid PDZ domain binding motif that interacts with the PDZ1 domains of ZO-2 and ZO-3 (Itoh et al., 1999), which is analogous to the presently found interaction of the C-terminus SAYV PDZ domain binding motif in C×36 with ZO-2 and ZO-3.

Further impetus for the present work originated from our earlier report demonstrating that, in addition to C×36/ZO-1 co-association at neuronal gap junctions in mouse retina, a distinct subpopulation of retinal gap junctions containing C×36/ZO-1 also contained ZO-2 (Ciolofan et al., 2006). While we provided evidence for C×36 association with ZO-2 by showing co-IP of these two proteins from retinal tissue, their remained the possibility of indirect association via a complex of proteins because virtually all C×36-containing retinal gap junctions contained ZO-1, and ZO-1 has been shown to directly interact with ZO-2 (Itoh et al., 1999). Thus, co-IP of C×36/ZO-2 may have resulted indirectly from association of ZO-2 with the ZO-1 that is bound to C×36 at these gap junctions. Although this latter possibility cannot be excluded, the present results indicate the capacity of direct association, either in a competitive manner or perhaps with some connexons or a proportion of connexins within a hexameric connexon interacting with the PDZ1 domain in ZO-1 and another proportion interacting with the PDZ1 domain of ZO-2. It should be noted in relation to observations of ZO-2 localization at gap junctions between neurons in retina that our limited surveys have not revealed ZO-2 association with C×36-containing gap junctions elsewhere in the adult mouse CNS, nor have we detected ZO-3 at such gap junctions. However, more comprehensive analyses of such associations remain to be conducted in adult CNS and peripheral tissues as well as during development when neuronal gap junctions are much more abundant.

Although there have been reports indicating the functional importance of connexin interaction with specifically ZO-1, particularly in relation to the regulation of process associated with gap junctions composed of C×43 (Hunter et al., 2005; Laing et al., 2005; Akoyev et al., 2007), the detailed molecular events governed by this interaction are not yet clear. Adding to the complexity is the variety of so far encountered interactions between connexins and ZO proteins. For example, C×43 was reported to associate only with the PDZ2 domain of only ZO-1 and ZO-2 (Giepmans, 2004; Singh et al., 2005), C×45 only with the PDZ domains of ZO-1 and ZO-3, but not with that in ZO-2 (Kausalya et al., 2001), and C×36 only with the PDZ1 domain of all three of these proteins (Li et al., 2004c; present results). Some clues regarding the functional role of these interactions may be gleaned from consideration of the many known binding partners of ZO proteins and the functions of these binding partners. Besides their PDZ domains, the ZO proteins contain other interaction modules, including an SH3 domain and a guanylate kinase domain, which can collectively recruit a variety of structural and signaling proteins to plasma membrane structures. Thus, in analogy with tight junctions and adherens junctions (Gonzalez-Mariscal, et al., 2003), a concept emerging is that gap junctions also serve as plasma membrane-localized signaling platforms (Giepmans, 2004), where ZO proteins contribute to protein recruitment and scaffolding. For example, we recently demonstrated that individual gap junction plaques in rodent retina co-contain C×36 and C×45, which may be held in a tripartite complex wherein C×36 associates with the PDZ1 domain of ZO-1 and C×45 with the PDZ2 domain of ZO-1 (Li et al., 2008).

Further, ZO-1 and ZO-2 contain nuclear localization and export signals for shuttling between the cytoplasm and nucleus, and they interact with various transcription factors, such as ZONAB in the case of ZO-1 at tight junctions and gap junctions (Balda and Matter, 2000; Ciolofan et al., 2006) and Jun, Fos and C/EBP in the case of ZO-2 in both cell nuclei and tight junctions (Betanzos, et al., 2004; Jaramillo et al., 2004). Some of these ZO-associated transcription factors may regulate connexin gene expression (Piersanti and Lye, 1995; Mitchell and Lye, 2001, 2005), and ZO-2 itself regulates gene expression in epithelial and endothelial cells (Traweger et al., 2003, 2008). In addition, ZO-2 down-regulates cyclin D1 transcription via interaction with an E box element in the cyclin D1 promoter (Huerta et al., 2007). As in the case of other connexins whose expression is under transcriptional control (Oyamada, et al., 2005), C×36 gene expression is also regulated by specific transcription factors. Thus, overexpression of the transcription factor CREB leads to the developmental uncoupling of gap junctions and down-regulation of C×36 (Arumugam et al., 2005) and glucose-mediated transcriptional regulation of the C×36 promoter occurs via a member of cAMP responsive element (CRE) protein family (Allagnat et al., 2005). Further, C×36 gene expression in insulin-producing β-cells is strictly controlled by the transcriptional repressor neuron-restrictive silencing factor (Martin et al., 2003). Although regulatory proteins and transcription factors associated specifically with ZO-2 at C×36/ZO-2-containing gap junctions have not yet been identified, the above wealth of linkages between connexins, ZO proteins and cellular regulatory events influenced by ZO-associated proteins provides a basis for investigations of the regulatory roles of ZO proteins at gap junctions composed of C×36.

Acknowledgments

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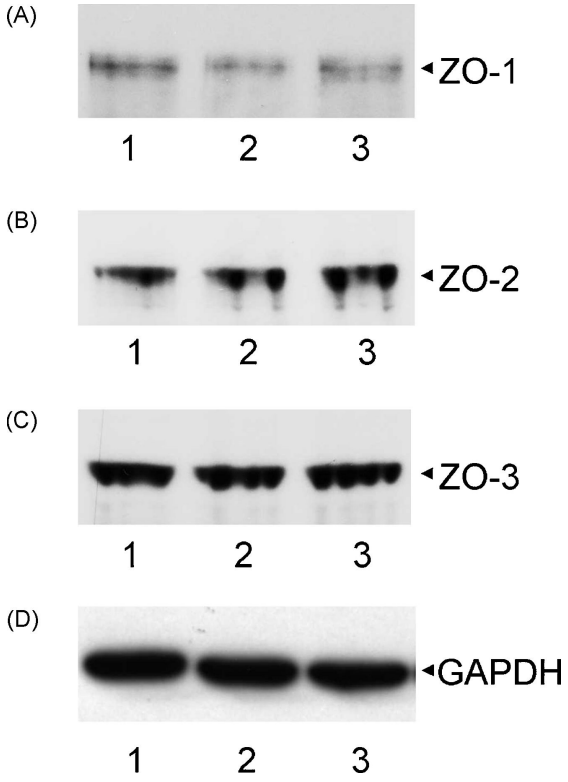

Fig. 1.

Western blots showing detection of ZO-1, ZO-2 and ZO-3 in C×36-transfected HeLa cells. A-C: Immunoblots showing equal volumes of cell lysates probed for either ZO-1, ZO-2 or ZO-3. The three lanes in each blot represent separate transiently transfected cultures of HeLa cells, and show a ZO-1 band migrating at about 220 kDa (A, lanes 1-3), a ZO-2 band migrating at 160 kDa (B, lanes 1-3) and a ZO-3 band migrating at 130 kDa (C, lanes 1-3). D: Immunoblot of lysates from the three C×36-transfected cultures probed for GAPDH, showing equal protein loading from the separate cultures (lanes 1-3).

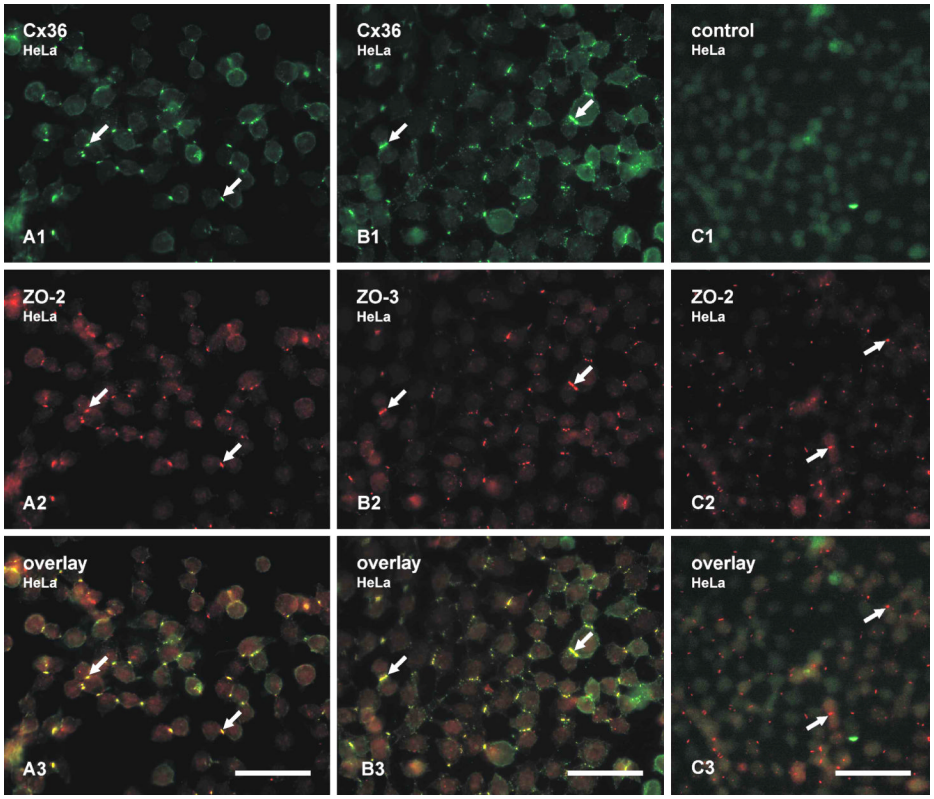

Fig. 2.

Double immunofluorescence labeling of C×36 with ZO-2 and ZO-3 in C×36-transfected HeLa cells. A: Punctate labeling for C×36 (A1, arrows) and ZO-2 (A2, arrows) in the same field, with co-localization of labeling seen as yellow in overlay (A3, arrows). B: Punctate labeling for C×36 (B1, arrows) and ZO-3 (B2, arrows) in the same field, with co-localization of labeling seen as yellow in overlay (B3, arrows). C: Double immunofluorescence of control empty vector-transfected HeLa cells showing absence of C×36 (C1) and labeling for ZO-2 at cell-cell contacts (C2, C3, arrows). Scale bars: 50 μm.

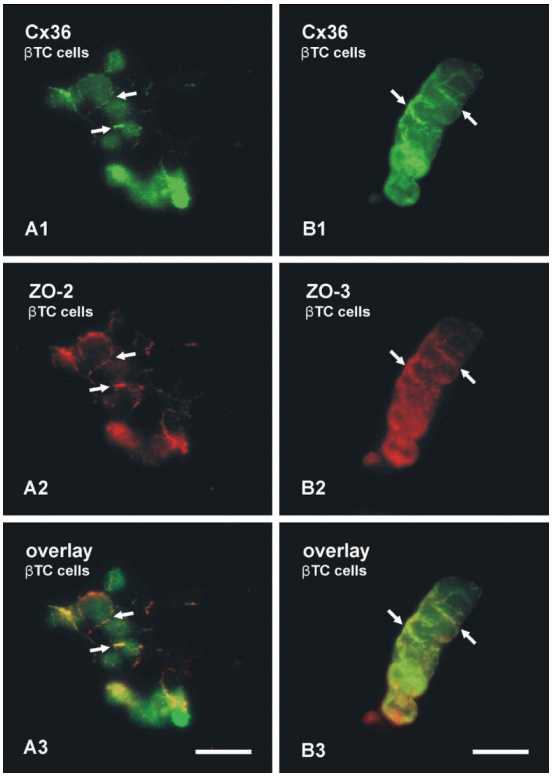

Fig. 3.

Double immunofluorescence labeling of C×36 with ZO-2 and ZO-3 in cultured βTC-3 cells. A: Punctate labeling for C×36 (A1, arrows) and ZO-2 (A2, arrows) in the same field, with co-localization of labeling seen in overlay (A3, arrows). B: Punctate labeling for C×36 (B1, arrows) and ZO-3 (B2, arrows) in the same field, with co-localization of labeling seen in overlay (B3, arrows). Scale bars: 50 μm.

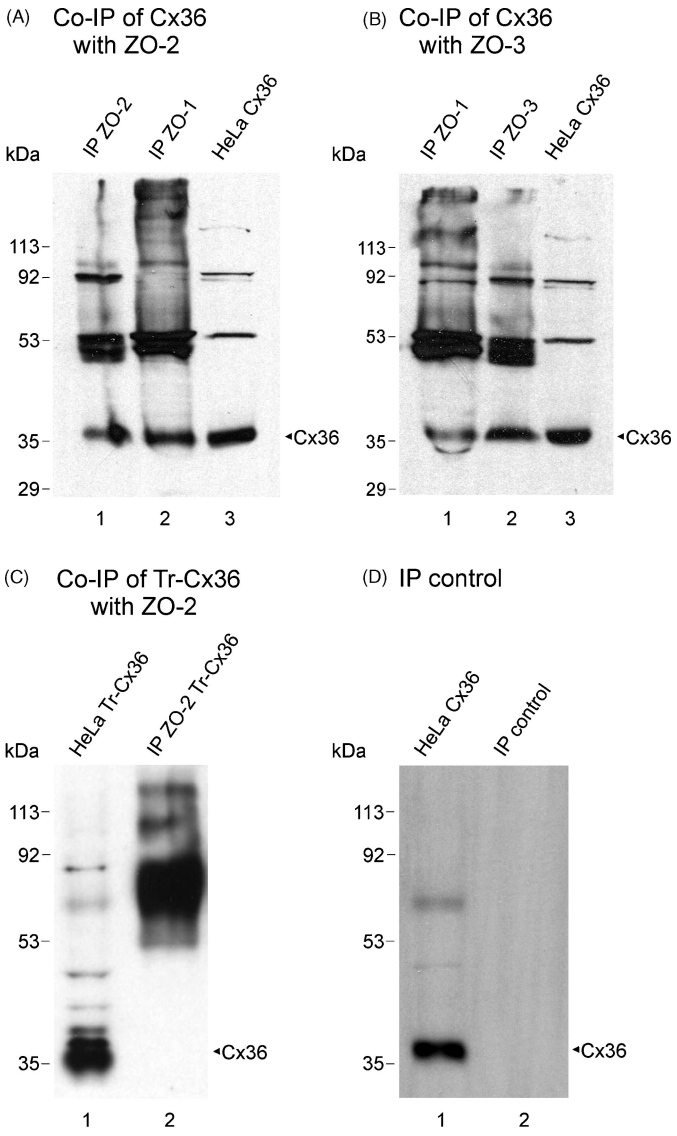

Fig. 4.

Co-IP of C×36 with ZO-2 and ZO-3 from C×36-transfected HeLa cells. Lysates of cells were taken for IP with antibodies against ZO-1, ZO-2 and ZO-3, and blots of IP materials were probed with anti-C×36. A: Blot showing detection of C×36 after IP of ZO-2 (lane 1). IP of ZO-1 (lane 2) and lysates of C×36-transfected cells (lane 3) were used as positive controls. B: Blot showing detection of C×36 after IP of ZO-3 (lane 2). IP of ZO-1 (lane 1) and lysates of C×36-transfected cells (lane 3) were used as positive controls. C: Blot showing absence of C×36 detection after IP of ZO-2 from HeLa cells transfected with truncated C×36 lacking the C-terminus PDZ domain interaction motif (lane 2). Detection of truncated C×36 (lane 1) is shown as a positive control. D: Blot showing absence of C×36 detection in control IP conducted with omission of primary antibody (i.e., anti-ZO-2 or ZO-3) (lane 2), with detection of C×36 in C×36-transfected cells shown as positive control (lane 1).

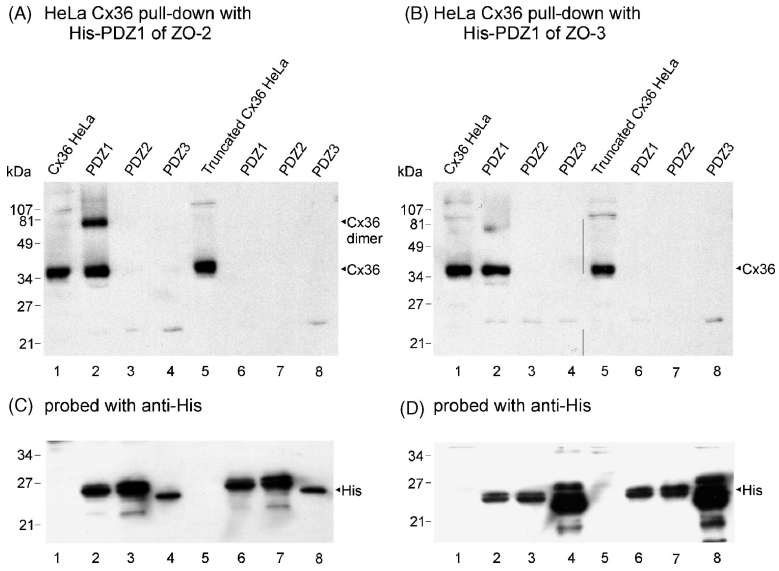

Fig. 5.

Pull-down of C×36 with the PDZ1 domains of ZO-2 and ZO-3 from C×36-transfected HeLa cells. Lysates of HeLa cells transfected with C×36 or truncated C×36 were incubated with the His-PDZ1, His-PDZ2 or His-PDZ3 domains of ZO-2 or ZO-3, followed by immunoblotting and probing of bound proteins with anti-C×36. A: Blot showing binding of C×36 to PDZ1 (lane 2), but not to the other two PDZ domains of ZO-2 (lanes 3 and 4), and absence of truncated C×36 binding to any of the PDZ domains of ZO-2 (lanes 6, 7 and 8). B: Blot showing binding of C×36 to PDZ1 (lane 2), but not to the other two PDZ domains of ZO-3 (lanes 3 and 4), and absence of truncated C×36 binding to any of the PDZ domains of ZO-3 (lanes 6, 7 and 8). Blots also show positive controls for anti-C×36 detection of C×36 (A, lane 1; B, lane 1) and truncated C×36 (A, lane 5; B, lane 5) in transfected HeLa cells. C,D: Membranes from A and B were stripped and reprobed with monoclonal anti-His (C and D, respectively), and show detection of His-PDZ domain fusion proteins (C and D, lanes 2-4, 6-8), and absence of His in HeLa cells transfected with C×36 (C and D, lane 1) or truncated C×36 (C and D, lane 5).

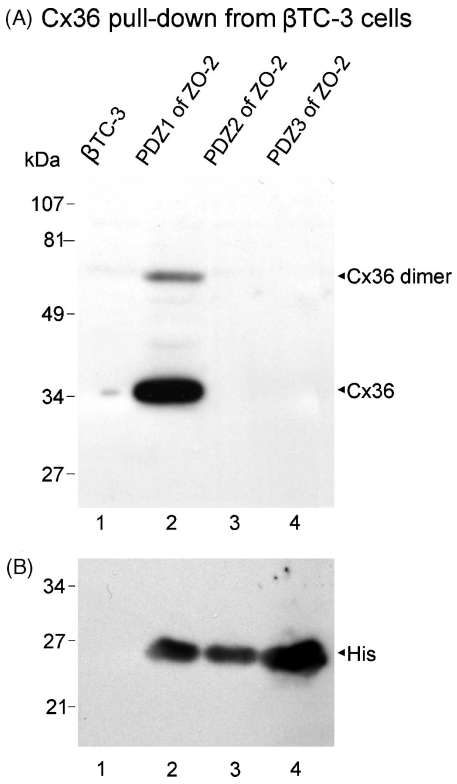

Fig. 6.

Pull-down of C×36 with the PDZ1 domain of ZO-2 from βTC-3 cells. A: Lysates of βTC-3 cells were incubated with the His-PDZ1, His-PDZ2 or His-PDZ3 domains of ZO-2, followed by immunoblotting and probing of bound proteins with anti-C×36. Blot shows binding of C×36 to the PDZ1 (lane 2), but not to the other two PDZ domains of ZO-2 (lanes 3 and 4), and the presence of relatively low levels of C×36 in lysates of βTC-3 cells (lane 1). B: Blot probed with anti-His, showing detection of the His-PDZ1 (lane 2), His-PDZ2 (lane 3) and His-PDZ3 (lane 4) domains of ZO-2 that were used for pull-down in A, and absence of His in βTC-3 cells (lane 1).

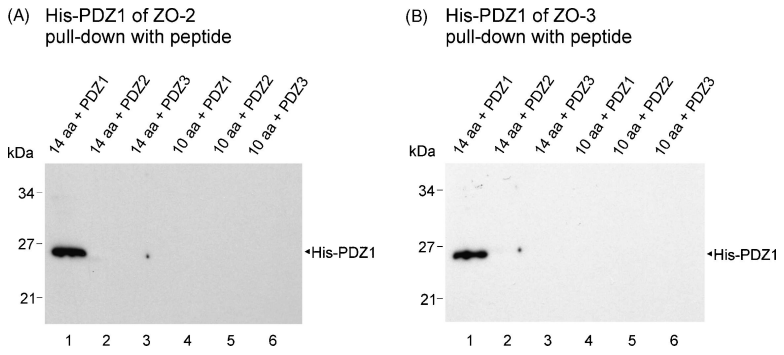

Fig. 7.

Pull-down of His-PDZ1 domains of ZO-2 and ZO-3 with C×36 peptide. A,B: Aliquots of each of the three His-tagged PDZ domains of ZO-2 and ZO-3 were incubated with streptavidinagarose beads linked to either C×36 C-terminal 14 aa or 10 aa peptide and pull-down material was probed with anti-His. The immunoblots show pull-down of the His-PDZ1 domain of ZO-2 (A, lane 1) and His-PDZ1 domain of ZO-3 (B, lane 1) with C×36 14 aa peptide, and lack of pull- down of the other two PDZ domains of ZO-2 (A, lanes 2 and 3) or ZO-3 (B, lanes 2 and 3) with this peptide. The C×36 10 aa peptide lacking the C-terminus PDZ interaction motif of C×36 fails to pull-down the His-PDZ1 domain of either ZO-2 (A, lane 4) or ZO-3 (B, lane 4). Control lanes show lack of pull-down of the other two PDZ domains of the ZO proteins with the C×36 10 aa peptide (A and B, lanes 5 and 6).

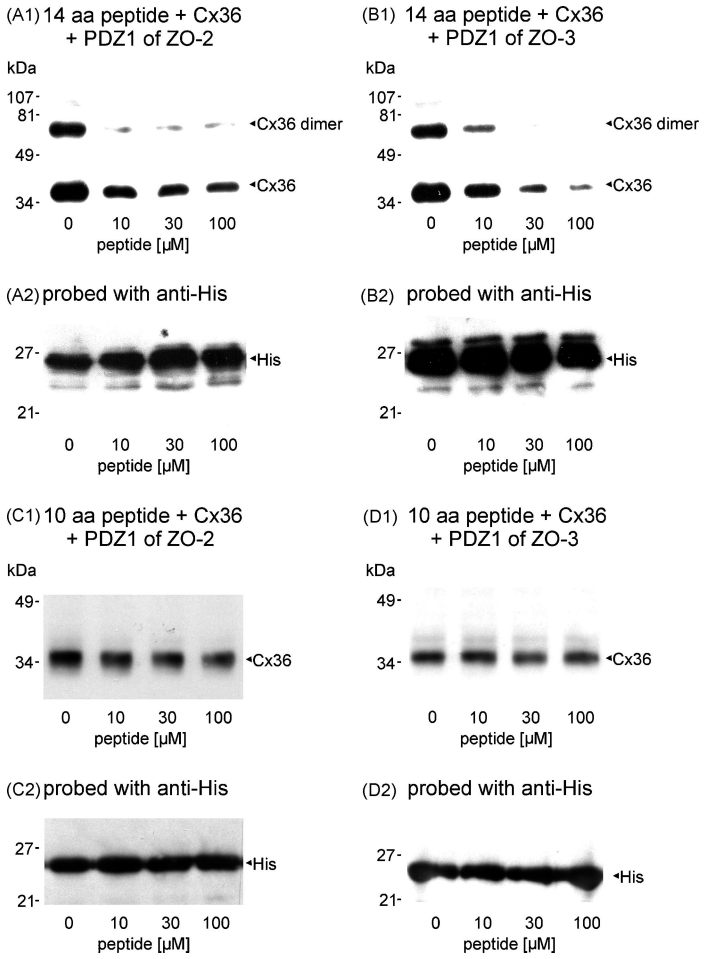

Fig. 8.

Competition of C×36 interaction with His-PDZ1 of ZO-2 and ZO-3 by C×36 14 aa peptide. A,B: Pull-down of C×36 from lysates of C×36-transfected HeLa cells with the His-PDZ1 domains of ZO-2 (A1) and ZO-3 (B1) was conducted in the absence of C×36 14 aa peptide (A1 and B1, lane 1) or in the presence of various concentrations of this peptide as indicated, followed by probing of pull-down material with anti-C×36. Blots show decreasing detection of C×36 with increasing concentration of peptide. Blots in A1 and B1 were stripped and reprobed with anti-His, confirming the loading of His-PDZ1 domains of ZO-2 (A2) and ZO-3 (B2). C,D: Pull-down of C×36 from lysates of C×36-transfected HeLa cells with the His-PDZ1 domains of ZO-2 (C1) and ZO-3 (D1) was conducted in the absence of C×36 10 aa peptide (C1 and D1, lane 1) or in the presence of various concentrations of this peptide as indicated, followed by probing of pull-down material with anti-C×36. Blots show similar levels of C×36 detection with increasing concentration of peptide. Blots in C1 and D1 were stripped and reprobed with anti-His, confirming the loading of His-PDZ1 domains of ZO-2 (C2) and ZO-3 (D2).