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Neuronal connexin36 association with zonula occludens-1 protein (ZO-1) in mouse brain and interaction with the first PDZ domain of ZO-1

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Abstract

Among the 20 members in the connexin family of gap junction proteins, only connexin36 (Cx36) is firmly established to be expressed in neurons and to form electrical synapses at widely distributed interneuronal gap junctions in mammalian brain. Several connexins have recently been reported to interact with the PDZ domain-containing protein zonula occludens-1 (ZO-1), which was originally considered to be associated only with tight junctions, but has recently been reported to associate with other structures including gap junctions in various cell types. Based on the presence of sequence corresponding to a putative PDZ binding motif in Cx36, we investigated anatomical relationships and molecular association of Cx36 with ZO-1. By immunofluorescence, punctate Cx36/ZO-1 colocalization was observed throughout the central nervous system of wild-type mice, whereas labelling for Cx36 was absent in Cx36 knockout mice, confirming the specificity of the anti-Cx36 antibodies employed. By freeze-fracture replica immunogold labelling, Cx36 and ZO-1 in brain were found colocalized within individual ultrastructurally identified gap junction plaques, although some plaques contained only Cx36 whereas others contained only ZO-1. Cx36 from mouse brain and Cx36-transfected HeLa cells was found to coimmunoprecipitate with ZO-1. Unlike other connexins that bind the second of the three PDZ domains in ZO-1, glutathione S-transferase-PDZ pull-down and mutational analyses indicated Cx36 interaction with the first PDZ domain of ZO-1, which required at most the presence of the four c-terminus amino acids of Cx36. These results demonstrating a Cx36/ZO-1 association suggest a regulatory and/or scaffolding role of ZO-1 at gap junctions that form electrical synapses between neurons in mammalian brain.

Keywords

connexins; electrical synapses; gap junction; neurons; retina

Introduction

Gap junctions occur between apposing cells in most tissues and form intercellular gap junctional communication (GJIC) channels that allow direct cell-to-cell exchange of ions and small molecules. These junctions are composed of a large family of connexin proteins with selective cell type expression patterns and distinct junctional permeability and conductance

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properties (Goodenough *et al.*, 1996; Kumar & Gilula, 1996; Veenstra, 1996; Willecke *et al.*, 2002). In the mammalian central nervous system (CNS), connexin36 (Cx36) is expressed selectively in neurons (Condorelli *et al.*, 1998, 2000; Sohl *et al.*, 1998; Teubner *et al.*, 2000) and forms interneuronal gap junctions that mediate neurotransmission at electrical synapses (Rash *et al.*, 2000, 2001). The contribution of electrical synapses to brain circuitry in mammalian CNS is indicated by numerous studies (Galarreta & Hestrin, 1999, 2001a, Galarreta & Hestrin, b; Gibson *et al.*, 1999; Beierlein *et al.*, 2000; Bennett, 2000) and the importance of such synapses composed of Cx36 is supported by reports that transgenic mice lacking the Cx36 gene have functional deficits and aberrant patterns of neuronal activity in various brain regions including retina, cerebral cortex, thalamic reticular nucleus and inferior olive (Deans *et al.*, 2001, 2002; Guldenagel *et al.*, 2001; Hormuzdi *et al.*, 2001; Landisman *et al.*, 2002; Long *et al.*, 2002; Buhl *et al.*, 2003; De Zeeuw *et al.*, 2003).

Gap junctions are highly dynamic structures, and are subject to regulation at levels of connexin trafficking, junction assembly and junctional conductance state (Laird, 1996; Thomas *et al.*, 2002). Among proteins that could contribute to these regulatory processes is the PDZ domain-containing protein zonula occludens-1 (ZO-1). Although ZO-1 was originally identified as a tight junction-associated protein (Stevenson *et al.*, 1986; Mitic & Anderson, 1998; Gonzalez-Mariscal *et al.*, 2003), it was recently found to codistribute with gap junctions at cell-cell contacts, and to interact directly with various connexins, including Cx31.9, Cx43, Cx45, Cx46 and Cx50 (Giepmans & Moolenaar, 1998; Kausalya *et al.*, 2001; Laing *et al.*, 2001; Nielsen *et al.*, 2002, 2003). For those that have been examined, these connexins displayed binding to the second of the three PDZ domains in ZO-1. Several other members of the connexin family encode short 3–6 amino acid C-terminal sequences representing consensus PDZ domain binding motifs (Kausalya *et al.*, 2001; Nielsen *et al.*, 2003), and sequence analysis of Cx36 suggests that it too might contain such a motif. In view of these observations, together with the reported expression of ZO-1 in various cell types that typically do not form tight junctions, including neurons of the CNS (Miragall *et al.*, 1994; Inagaki *et al.*, 2003), we used double immunofluorescence labelling, freeze-fracture replica immunogold labelling (FRIL), coimmunoprecipitation, cell transfection, connexin truncation and glutathione S-transferase (GST)-PDZ pull-down approaches to test the hypothesis that Cx36 interacts with ZO-1 in neurons. In addition, wild-type (WT) and Cx36 knockout (KO) mice were used to establish the specificity of the anti-Cx36 antibodies employed.

Materials and methods

Antibodies

Three anti-Cx36, two anti-ZO-1, one anti-Cx43 and one anti-GST antibodies were used in this study. The same sequence located in the C-terminus region of Cx36 was used to generate polyclonal anti-Cx36 antibody (Ab) 51–6300 and monoclonal anti-Cx36 Ab37–4600, and a different nonoverlapping sequence in the Cx36 C-terminus region was used to generate polyclonal anti-Cx36 Ab36–4600. All anti-Cx36 antibodies, polyclonal anti-ZO-1 Ab61–7300 and monoclonal anti-ZO-1 Ab33–9100, and monoclonal anti-Cx43 Ab13–8300 were obtained from Zymed Laboratories Incorporated (South San Francisco, CA, USA). Rabbit anti-GST Ab06–332 for detection of GST-PDZ domain fusion proteins was obtained from UpState Cell Signalling Solutions (Lake Placid, NY, USA). The specificity characteristics of anti-Cx43 Ab13–8300 and anti-Cx36 Ab51–6300 were described previously (Nagy *et al.*, 1997; Rash *et al.*, 2000, 2001).

Cx36 WT and KO mice

Six Cx36 heterozygous mice (Cx36^{+/-}) (LacZ-neoR^{+/-}) with F2 C57/B6–129vEv mixed background (Deans *et al.*, 2001) were kindly provided by Dr D. Paul (Harvard University,

USA) and the mice were bred in the Central Animal Care Services at the University of Manitoba to generate WT and Cx36 KO mice. To identify heterozygous (Cx36^{+/-}), WT (Cx36^{+/+}) and Cx36 KO (Cx36^{-/-}) mice, genomic DNA was extracted from mouse tail according to standard protocols, and polymerase chain reaction (PCR) was used with specific primers for the Cx36 coding region in exon 2 and for the neomycin resistance gene located in the targeting vector sequence, which were as follows: Cx36 forward primer,

5'-AGCGGAGGGAGCAAACGAGAAG-3';

Cx36 reverse primer,

5'-CTGCCGAAATTGGGAACACTGAC-3';

neomycin forward primer,

5'-CAAGATGGATTGCACGCAG-3';

and neomycin reverse primer,

5'-CATCCTGATCGACAAGAC-3'.

The expected length of PCR products for Cx36 and neomycin were 535 and 460 base pairs, respectively. For PCR, DNA was denatured at 94 °C for 3 min, then 30 cycles were run at 94 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s. The PCR products were separated by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Some PCR products were subcloned into a PCR 2.1 vector and sequenced with universal M13 forward and reverse primers for product sequence confirmation. A total of 35 WT and 30 Cx36 KO mice were used according to approved protocols by the Central Animal Care Service, with minimization of the number of animals used. Animals were anaesthetized with equithesin (3 mL/kg) before decapitation or trans-cardial perfusion.

Cx36 and Cx43 expression vectors

Total RNA was isolated from adult mouse brain using guanidine isothiocyanate, and reverse transcription with 500 ng oligo(dT)15 primer (Promega, Madison, WI) was conducted as previously described (Lynn *et al.*, 2001; Li *et al.*, 2002). Oligonucleotide primers, materials for amplifying mouse Cx36 and Cx43 coding sequence, and transfection reagents were purchased from Gibco BRL Life Technologies (Burlington, ON, Canada). Primers chosen for PCR were based on the mouse Cx36 sequence (GenBank accession NM_010290) and rat Cx43 sequence (GenBank accession X06656). These were: full length Cx36 sense primer,

5'-ATGGGGGAATGGACCATC-3';

full length Cx36 antisense primer,

5'-TCACACATAGGCAGAGTC-3';

truncated Cx36 sense primer was the same as that used for full length Cx36;

truncated Cx36 antisense primer for generating Cx36 with deletion of four C-terminal amino acids was:

5'-TCAGTCACTGGACTGAGTC-3';

full length Cx43 sense primer,

5'-ATGGGTGACTGGAGTGCCTTG-3';

full length Cx43 antisense primer,

5'-TTAAATCTCCAGGTCATCAGG-3';

truncated Cx43 sense primer was the same as that used for full length Cx43;

antisense primer for generating Cx43 with deletion of six C-terminal amino acids was:

5'-TTAAGGCCGAGGCCTGCTGC-3'.

For amplification of Cx36 coding cDNA, PCR was conducted in 20 μ L solution containing 2 μ L 10 $\times$ PCR buffer; 0.8 μ L 50 mM MgCl₂; 200 μ M dNTP; 100 ng sense and antisense primers; 1 unit Taq DNA polymerase; and 1 μ L template cDNA. Amplification of Cx43 full length coding cDNA or truncated cDNA was similarly performed, except using 10 ng Cx43 plasmid. For PCR, DNA was denatured at 94 °C for 3 min, and then subjected to 35 cycles at 94 °C for 60 s, 55 °C for 60 s and 72 °C for 90 s; this was followed by a final extension at 72 °C for 10 min for T-A cloning. PCR products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide and purified using a gel purification kit (Qiagen Inc, Mississauga, ON, Canada). PCR products were subcloned into PCR 2.1 vector, digested with *EcoRI*, and ligated into pcDNA3 expression vector using T4 DNA ligase according to the manufacturer's instructions. Recombinant plasmids were extracted, orientation was verified with *BamHI* digestion for Cx36 and *HindIII* digestion for Cx43, and at least two recombinant plasmids were sequenced using T7 universal primer and specific Cx36 or Cx43 primers for confirmation of sequence.

Cell culture and transient transfection

HeLa cells (American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. For transient transfection, HeLa cells were transfected for 24 h with pcDNA3 vector, full length Cx36-pcDNA3, truncated Cx36-pcDNA3, full length Cx43-pcDNA3 or truncated Cx43-pcDNA3 plasmids using LipofectAMINE 2000 reagent as described previously (Li *et al.*, 2002) and harvested for immunofluorescence labelling, Western blotting, immunoprecipitation or *in vitro* pull-down assays.

Western blotting

Cultured HeLa cells were rinsed briefly with phosphate-buffered saline (PBS; 50 mM sodium phosphate buffer, pH 7.4, 0.9% saline) 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 MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, and 5 μ g/mL each of the protease inhibitors leupeptin, pepstatin A and aprotinin. Mouse tissues were collected, rapidly frozen and stored at -80 °C. Tissues were homogenized in homogenization buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 150 mM NaCl, 1% Triton-X 100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride and 2 (μ g/mL pepstatin A, leupeptin and aprotinin). HeLa cell lysates and tissue homogenates were sonicated and briefly centrifuged followed by protein determination using a kit (Bio-Rad Laboratories, Hercules, CA, USA). Sample protein (20–100 μ g) was separated electrophoretically in 12.5% polyacrylamide gels and trans-blotted to polyvinylidene difluoride membranes (Bio-Rad) in standard Tris-glycine transfer buffer (pH 8.3) containing 0.5% sodium dodecylsulphate. Membranes were blocked for 1–2 h at room temperature in TSTw (20 mM Tris-HCl, pH 7.4, 150mM NaCl with 0.2% Tween-20) containing 5% nonfat milk powder, briefly washed with TSTw, and then incubated 16 h at 4 °C with anti-Cx36 antibodies (1–3 μ g/mL) or anti-ZO-1

antibodies (1 µg/mL) in TSTw containing 1 % nonfat 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: 3000-1: 5000 (Sigma-Aldrich Canada, Oakville, ON, Canada) in TSTw containing 1 % nonfat milk powder, washed with TSTw four times for 40 min and resolved by chemiluminescence (ECL, Amersham PB, Baie d'Urfe, Quebec, Canada).

Light microscope immunofluorescence

HeLa cells cultured on poly L-lysine-treated glass coverslips and transiently transfected with full length Cx36, truncated Cx36 cDNA or empty vector were rinsed with PBS buffer and fixed in ice-cold 1–2% paraformaldehyde for 5 min. Cultures were incubated for 16 h at 4°C with polyclonal or monoclonal anti-Cx36 antibodies at a concentration of 1 µg/mL and monoclonal or polyclonal anti-ZO-1 antibodies at 0.5 µg/mL in TEST buffer (50 mM Tris-HCl, pH 7.6, 1.5% NaCl, 0.3 % Triton X-100) containing 4% normal donkey or goat serum (NDS, NGS, respectively). Cultures were then washed four times with TBST for 1 h, then incubated for 1 h simultaneously with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG diluted at 1: 200 and Cy3-conjugated goat anti-mouse IgG diluted at 1: 200, or alternatively, 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). Coverslips were then washed for 1 h in TBST and mounted on glass slides with antifade medium.

Mice deeply anaesthetized with equithesin were transcardially perfused sequentially with cold (4 °C) solutions of prefixative (50 mM sodium phosphate buffer, 0.1% sodium nitrite and 1 unit/mL of heparin), fixative (0.16 M sodium phosphate buffer, pH 7.6, 0.2% picric acid and 1–2% paraformaldehyde), and then with a sucrose flush (10% sucrose, 25 mM sodium phosphate buffer, pH 7.4). Tissues were removed, stored in cryoprotectant (25 mM sodium phosphate buffer, pH 7.4, 10% sucrose, 0.04% sodium azide) for 24 h, and cryostat sections of retina and brain (10 µm thick) were collected on gelatinized glass slides. Sections were processed for immunofluorescence labelling with all antibodies diluted in TBST containing 10% NGS. In single labelling studies, sections were incubated for 24 h at 4 °C with monoclonal or polyclonal anti-Cx36 antibodies at a concentration of 3 µg/mL. In double labelling studies, sections were incubated simultaneously with monoclonal anti-Cx36 Ab37–4600 (3 µg/mL) and polyclonal anti-ZO-1 Ab61–7300 (0.25 µg/mL) or conversely with polyclonal anti-Cx36 Ab36–4600 (3 µg/mL) and monoclonal anti-ZO-1 Ab33–9100 (4 µg/mL). Sections were washed for 1 h in TBST, then incubated for 1.5h at room temperature simultaneously with FITC-conjugated horse anti-mouse IgG (diluted 1:200) and Cy3-conjugated donkey anti-rabbit IgG (diluted 1:400) or Cy3-conjugated goat anti-mouse IgG diluted 1:400 (Jackson ImmunoResearch Laboratories) and AlexaFluor 488 goat anti-rabbit IgG diluted 1:1000 (Molecular Probes, Eugene, OR, USA).

Immunofluorescence in tissue sections and cultured cells was examined on a Zeiss Axioscop 2 fluorescence microscope and an Olympus Fluoview IX70 confocal microscope. Images were obtained using Carl Zeiss Axiovision 3.0 image software (Carl Zeiss Canada, Toronto, ON) and assembled using Northern Eclipse 5.0, Photoshop 3.0 and CorelDraw 8 software.

Freeze-fracture replica immunogold labelling

Adult rat retinas were obtained by whole-body perfusion with 1% formaldehyde, as described previously (Rash & Yasumura, 1999). The retinas were sliced with a Lancer 1000 vibratome, infiltrated with 30% glycerol as cryoprotectant frozen by rapid contact with a liquid-nitrogen-cooled copper mirror, and freeze-fracture replicated in a JEOL 9010c freeze-fracture device (RMC Products, Tucson, AZ, USA). Before samples were thawed, a gold 'index' grid

containing a suspended droplets of 1.5% Lexan dissolved in ethylene dichloride was placed on the replicated tissue, the solvent was evaporated overnight as the sample temperature was raised from -195°C to -35°C . Samples, with grid firmly attached in Lexan plastic, were thawed, mapped by confocal microscopy, cleaned for 29 h in 1.5–2% sodium dodecylsulphate detergent, rinsed in blocking buffer and immunogold labelled (Rash & Yasumura, 1999; Pereda *et al.*, 2003). Samples were labelled with monoclonal anti-Cx36 (Ab37–4600) and polyclonal anti-ZO-1 (Ab 61–7300) (Zymed Laboratories Inc), rinsed and counter-labelled with goat anti-mouse (6 and 12nm) and goat anti-rabbit (18nm) (Jackson ImmunoResearch Laboratories), as described (Rash & Yasumura, 1999). Labeled replicas were rinsed, air dried, coated on the labelled side with 10–20 nm carbon (to stabilize gold labels and to anneal cracks in the replica), and examined in JEOL 2000 EX-II or JEOL 1200 TEMs operated at 100 kV. Images were photographed as stereo pairs (8° included angle), captured using an ArtixScan 2000f digital scanning device, and prepared using Photoshop 7.01 (Adobe systems). Selected images are presented as stereoscopic pairs (left two images) and reversed stereoscopic pairs (right pair of images).

Immunoprecipitation

HeLa cells transiently transfected with full length or truncated Cx36 and tissues from various brain regions (retina, hippocampus, cerebellum, midbrain, medulla) were homogenized in IP buffer and IP carried out as described (Zhurinsky *et al.*, 2000). Homogenates were sonicated, centrifuged at 20 000 g for 10 min at 4°C , and 2 mg supernatant protein was washed 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 with 2 μg anti-Cx36 antibody or anti-ZO-1 antibody with shaking overnight at 4°C , followed by 1 h incubation with 20 μL protein-A coated agarose beads, and then centrifugation at 20 000 g for 10 min at 4°C . The beads were washed vigorously five times with 1 mL washing buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.5% NP-40) and then incubated at 60°C for 2 min in sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer containing 10% β -mercaptoethanol. Samples were processed by SDS-PAGE and immunoblot membranes were probed with anti-Cx36 or anti-ZO-1 antibodies. Control samples were taken through the IP procedure with exclusion of primary antibody.

Binding assays with GST-PDZ domains of ZO-1

Construction of pGEX-3X plasmids each containing one of the three PDZ domains of ZO-1 linked to GST was previously described (Nielsen *et al.*, 2002, 2003), and constructs were kindly provided to us by Dr B. Giepmans (University of California, San Diego, CA, USA). GST-PDZ domain fusion proteins were produced using standard procedures, and bound to glutathione-agarose beads according to manufacturer's instructions (Sigma, St. Louis, MO, USA). Briefly, the pGEX-3X plasmids were transformed into *Escherichia coli* DH5 α , and individual clones were selected and grown in LB media supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin at 37°C until it reached approximately 0.8 optical density, and then supplemented with isopropyl- β -D-thiogalactopyranoside (1 mM) for induction of GST-PDZ 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 20min, and the pellets stored at -80°C overnight.

Pellets containing GST-PDZ domains were re-suspended in buffer (10 mM sodium phosphate, pH 8.0, containing 150mM NaCl, 1% Triton X-100, 5% glycerol, 1 mM phenylmethylsulphonyl fluoride and 1 mM EDTA, 5 $\mu\text{g}/\text{mL}$ each of aprotinin, pepstatin A, leupeptin), centrifuged for 10 min at 20 000 g, and the supernatant was added to glutathione-sepharose 4B beads with shaking at 4°C for 2h. 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 agarose beads containing PDZ domain fusion proteins were then incubated overnight in IP

buffer containing homogenates of retinal or brain tissue from WT mice, or lysates of HeLa cells transfected with either Cx36 or Cx43. After washing extensively five times in PBS buffer containing 1% Triton X-100, protein bound to the PDZ domains linked to the agarose beads were eluted with SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane and analysed by Western blotting with anti-Cx36 Ab51–6300, anti-Cx43 Ab 13–8300 or anti-GST antibody. After Western blotting, the membranes were stained with coomassie blue.

Results

Characterization of anti-Cx36 and anti-ZO-1 antibodies

Homogenates of retina from WT and Cx36 KO mice, and lysates from Cx36-transfected HeLa cells were used to confirm specificity of anti-Cx36 antibodies by Western blotting. As shown in Fig. 1A, the three anti-Cx36 antibodies Ab37–4600, Ab51–6300 and Ab36–4600 each detected 36 and 80 kDa bands in retina of WT mice, which were absent in retina of Cx36 KO mice. In lysates from HeLa cells transiently transfected with Cx36, the same antibodies recognized a 36 kDa band, which was not detected in empty vector-transfected control HeLa cells (Fig. 1B). In addition, Ab37–4600 detected an 80kDa band in transfected, but not control cells (Fig. 1B, lane 1,2). The absence of the 36 and 80 kDa proteins in Cx36 KO tissue, and lack of the former in control HeLa cells, indicate that these molecular weight species correspond to monomer and dimer forms of Cx36, respectively. For reasons that remain uncertain, not all anti-Cx36 antibodies detected the dimer form of Cx36 in HeLa cells. Bands were detected by Ab37–4600 at about 52, 93 and 100 kDa in retina of both WT and Cx36 KO mice, and some additional bands were evident with Ab51–6300 in control HeLa cells. These appear to represent antibody cross reactions with proteins of unknown identity. Anti-ZO-1 antibody specificity was confirmed using homogenates of retina from WT and Cx36 KO mice, where a band corresponding to the molecular weight of ZO-1 was detected at approximately 220 kDa (Fig. 1C). In view of Cx36/ZO-1 colocalization results presented below, immunoblots were generated to test for possible cross reaction of anti-ZO-1 antibody with Cx36. As demonstrated in Cx36-transfected HeLa cells, polyclonal anti-ZO-1 antibody showed an absence of a band (Fig. 1D, lane 2) corresponding to the migration of monomer Cx36 as shown by detection with anti-Cx36 antibody (Fig. 1D, lane 1). However, anti-ZO-1 did detect a protein migrating at about 30–32 kDa as well as higher molecular weight bands at about 130 kDa, the identity of which remains uncertain.

Immunofluorescence of Cx36 and ZO-1 in brain

Immunofluorescence of Cx36 with polyclonal Ab36–4600 in WT mouse retina revealed a thin band of sparse punctate labelling, arranged in intermittent clusters, in the outer plexiform layer (OPL), and a far greater density of punctate labelling in the inner plexiform layer (IPL), particularly in the inner portions of this layer where individual puncta were rarely discernible at low magnification (Fig. 2, A1). Confocal microscopy also indicated exclusively punctate Cx36 immunoreactivity (Fig. 2, C1), which precluded identification of Cx36 association with any particular cell type. However, linear arrays of puncta, possibly associated with dendrites, were seen traversing sublayers of the IPL (Fig. 2, C1). Little or no Cx36 was detected in other retinal layers of WT mice, and labelling seen in the OPL and IPL of these mice was absent in retina of Cx36 KO mice (Fig. 2, B1). Similar labelling in retina of WT mice was obtained with Ab37–4600 and Ab51–6300, and this labelling was absent in retina of Cx36 KO mice (not shown). Nonspecific fluorescence staining of cellular nuclei occurred in retina of both WT and Cx36 KO mice, which was evident after primary antibody omission and attributed to nonspecific adherence of FITC-conjugated secondary antibody after weak tissue fixation.

Immunolabelling of ZO-1 with monoclonal Ab33–9100 in retina of WT mice was also largely punctate and localized mainly in the OPL and IPL (Fig. 2, A2 and C2). The intensity of ZO-1 immunofluorescence was slightly but noticeably reduced in these retinal layers of Cx36 KO mice (Fig. 2, B2). Overlay of double-labelled images indicated similar distribution patterns of Cx36 and ZO-1 in the two plexiform layers (Fig. 2, A3), and considerable colocalization of the two proteins at individual puncta as seen by confocal microscopy of the IPL (Fig. 1, C3). However, not all Cx36-immunopositive puncta were labelled for ZO-1 and, conversely, not all ZO-1-immunopositive puncta were labelled for Cx36. Intense staining associated with the outer limiting membrane (OLM) and patches in the OPL and IPL was seen after omission of anti-ZO-1, and was due to nonspecific labelling with Cy3-conjugated secondary antibody.

Immunofluorescence labelling of Cx36 and ZO-1 in the olfactory bulb and inferior olive is shown in Fig. 3. In the olfactory bulb, anti-Cx36 Ab36–4600 and Ab37–4600, generated against different non-overlapping sequences of Cx36, produced identical patterns of relatively intense labelling in the mitral cell layer (MCL; Fig. 3, A1 and B1, respectively), thus providing confidence in specificity of Cx36 detection. This labelling consisted of discrete puncta circularly arrayed apparently around individual mitral cells, with little labelling in immediately adjacent layers. As illustrated with Ab36–4600, Cx36-positive puncta were also seen in synaptic glomeruli (Fig. 3, C1) and in the inferior olive (Fig. 3, D1), and similar labelling was obtained with the other two anti-Cx36 antibodies (not shown). Immunolabelling of ZO-1 with both monoclonal Ab33–9100 and polyclonal Ab61–7300 resembled that of Cx36, but was more widely distributed in all brain regions, including the MCL and adjacent layers (Fig. 3, A2 and B2), synaptic glomeruli (Fig. 3, C2) and inferior olive (Fig. 3, D2). Overlay of double-labelled images indicated substantial Cx36/ZO-1 colocalization in the MCL (Figs 3, A3 and B3), as well as a high proportion of Cx36-positive puncta that were also positive for ZO-1 in olfactory bulb synaptic glomeruli (Fig. 3, C3) and the inferior olive (Fig. 3, D3), but a much lower percentage of ZO-1 -positive puncta that were also positive for Cx36 in these latter structures.

In all areas examined, immunolabelling of Cx36 was absent in mice with Cx36 gene deletion, as demonstrated by comparison of labelling with Ab37–4600 in corresponding fields of the olfactory bulb mitral cell layer of WT (Fig. 3, E1) and Cx36 KO (Fig. 3, F1) mice. In contrast to the conspicuous labelling of Cx36 and ZO-1 in the MCL of WT mice (Figs 3, E2 and E3), the loss of Cx36 in KO animals was accompanied by a large loss of ZO-1 in the MCL (Fig. 3, F2 and F3). As in retina, FITC- and Cy3-conjugated secondary antibodies produced nonspecific labelling cellular nuclei and blood vessels in sections of olfactory bulb processed with omission of anti-Cx36 or anti-ZO-1 primary antibodies, which potentially masked any specific labelling of ZO-1 associated with vascular elements.

FRIL localization of Cx36 and ZO-1 in retina

Immunogold-labelled gap junctions in retina were observed at >50 ultrastructurally defined mixed synapses, as well as at contacts where, because of limited area observed, it was not possible to determine if the gap junction was at a mixed or strictly electrical synapse. ‘Mixed synapses’ were defined by the presence of gap junctions in close association with >25 uniform-diameter vesicles (synaptic vesicles) in nerve terminals (Fig. 4A), active zones in presynaptic plasma membranes, and/or dense clusters of intramembrane particles (IMPs) as postsynaptic densities in postsynaptic plasma membranes (Pereda *et al.*, 2003).

In 52% of neuronal gap junctions (33/64), both in outer plexiform and inner plexiform layers, immunogold beads indicated the presence of both Cx36 and ZO-1-immunoreactivity, with Cx36 indicated by 6 and 12-nm gold beads and ZO-1 indicated by 18-nm gold beads (Fig. 4A and B). These included both conventional ‘plaque-type’ gap junctions (Fig. 4A and B) and ‘string’ gap junctions (not shown). The second population of neuronal gap junctions (25%; 16/64) consisted of large and small ‘plaque-type’ gap junctions that were immunogold-labelled

for Cx36 but not ZO-1 (Fig. 4C; 6 and 12 nm gold beads only). A third population of gap junctions (22%; 15/64) consisted of small or medium-sized plaques that displayed ZO-1-immunoreactivity but no labelling for Cx36 (Fig. 4D and E; 18 nm gold beads). Thus, the three populations of gap junctions were characterized by the differences in sizes of the differentially labelled gap junctions and differences in IMP configurations within the gap junctions.

Despite the similar results obtained with monoclonal and polyclonal antibodies by immunofluorescence, labelling of retinal neuronal gap junctions by FRIL was obtained with the polyclonal anti-ZO-1, but not with monoclonal anti-ZO-1. Reasons for this discrepancy between results from immunofluorescence and FRIL methods remain uncertain, but could be a result of blockade of the monoclonal anti-ZO-1 epitope by other proteins in the latter but not the former method.

Immunoprecipitation of Cx36 with ZO-1 in retina and brain

To examine molecular association of Cx36 with ZO-1, homogenates of retina and various brain regions of WT mice were taken for IP of ZO-1, and immunoblots of precipitates were probed for Cx36. After IP of ZO-1, immunoprecipitated protein obtained from retinal tissue was consistently found to contain Cx36 (Fig. 5A and B). Confidence in co-IP of Cx36/ZO-1 and in Cx36 detection was provided by the similarity of results obtained in blots probed with two different anti-Cx36 antibodies, by the correspondence of Cx36 migration profile in IP material to that in lysates of Cx36-transfected HeLa cells, and by the absence of Cx36 detection after conducting the IP procedure with omission of anti-ZO-1 antibody. Similar results were obtained after IP of ZO-1 from homogenates of various brain regions, including hippocampus, cerebellum, midbrain and medulla, with lysate from Cx36-transfected HeLa cells shown as a positive control for Cx36 detection (Fig. 5C). Homogenate protein from these brain regions were used at levels optimal for IP and, consequently, levels of Cx36 detected in IP material are not likely to reflect relative levels that occur in these regions *in vivo*.

Interaction of ZO-1 with the c-terminus Cx36

Expression vectors containing full length (FL)-Cx36 and truncated (Tr)-Cx36 cDNA with deletion of four c-terminal amino acids (SAYV) were constructed to examine Cx36/ZO-1 colocalization in HeLa cells transfected with these vectors, and to determine whether the deleted four amino acids constitute a potential PDZ domain binding motif that is responsible for interaction with ZO-1. Cultures of empty vector-transfected HeLa cells were devoid of labelling for Cx36, but displayed labelling for ZO-1 at cell-cell contacts (not shown). In cultures of HeLa cells transfected with FL-Cx36, punctate immunofluorescence labelling for both Cx36 and ZO-1 was distributed around the periphery of cells, and was largely associated with points of contact between cells (Fig. 6A). Cultures displayed a greater abundance of punctate labelling for ZO-1 than Cx36, and overlay of images indicated that whereas nearly all Cx36-positive puncta were colocalized with ZO-1, only a proportion of ZO-1-positive puncta were immune-positive for Cx36. Confocal microscopy revealed puncta at cell-cell contacts to consist of intermittent dots, many of which exhibited a high degree of Cx36/ZO-1 colocalization (Fig. 6B). In HeLa cells transiently transfected with Tr-Cx36, punctate labelling for both Cx36 and ZO-1 was found around the periphery of cells, similar to that seen in FL-Cx36-transfected cells. However, in overlay images shown at low magnification (Fig. 6, C3) and in confocal images of individual puncta (Fig. 6D), Cx36 colocalization with ZO-1 was largely, though not totally, eliminated. Similar immunolabelling results were obtained with all three of the Cx36 antibodies. HeLa cells transfected with FL-Cx36, Tr-Cx36 or empty vector (not shown) displayed a low level of diffuse background fluorescence resulting from nonspecific attachment of primary and secondary antibodies.

Examination of Cx36/ZO-1 association by Western blotting of transfected HeLa cells is shown in Fig. 7. HeLa cells transiently transfected with empty vector were devoid of Cx36 (Fig. 7A, lane 1), whereas robust detection of Cx36 was seen with Ab51-6300 in HeLa cells transfected with FL-Cx36 (Fig. 7A, lane 2; and Fig. 7B, lane 1). HeLa cells transfected with Tr-Cx36 also displayed Cx36 (Fig. 7B, lane 2), indicating that Ab51-6300 used in these studies still recognized the truncated form of Cx36. We further confirmed Cx36 expression by showing that Cx36 immunoprecipitated with antibody Ab51-6300 from HeLa cells transfected with FL-Cx36 (Fig. 7A, lane 3) and Tr-Cx36 (Fig. 7B, lane 3) was detectable in IP material and had a migration profile corresponding to that of Cx36 observed in cell lysates. For comparison with these results, IP of ZO-1 from HeLa cells transfected with FL-Cx36 and Tr-Cx36 followed by probing immunoblots of IP protein with anti-Cx36 Ab51-6300 showed co-IP of Cx36 with ZO-1 from cells transfected with FL-Cx36 (Fig. 7A, lane 4), but not from cells transfected with Tr-Cx36 (Fig. 7B, lane 4). In addition, control IP using lysates of FL-Cx36 transfected HeLa cells with anti-ZO-1 antibody omission showed an absence of Cx36 (Fig. 7A, lane 5). These results indicate that the four carboxy-terminus amino acids of Cx36 are necessary for Cx36 interaction with ZO-1.

Cx36 binds to the first PDZ domain of ZO-1

Recombinant plasmids containing GST linked separately with each one of the three PDZ domains in ZO-1, prepared as previously described (Nielsen *et al.*, 2002), were provided to us and expressed in bacteria. *In vitro* pull-down assays involving incubation of the plasmid-derived fusion proteins with homogenates of retina were conducted to determine which of the three PDZ domains of ZO-1 interacts with Cx36. As a positive control for this procedure, similar assays using homogenate of brain incubated with the three GST-PDZ domains of ZO-1 were performed to confirm the known interaction of Cx43 with the PDZ2 domain of ZO-1 as previously shown (Giepmans & Moolenaar, 1998; Toyofuku *et al.*, 1998). Immunoblots loaded with material eluted from retinal proteins that bound the PDZ1 domain of ZO-1 and probed with anti-Cx36 Ab51-6300 revealed the presence of Cx36 (Fig. 8A, lane 3), with blots of retina and Cx36-transfected HeLa cells serving as positive controls for Cx36 detection (Fig. 8A, lanes 1 and 2). Blots loaded with material bound to the PDZ2 and PDZ3 domains of ZO-1 showed an absence of Cx36 (Fig. 8A, lanes 4 and 5). In contrast, blots loaded with material eluted from brain proteins that bound the PDZ2 domain of ZO-1 and probed with anti-Cx43 Ab13-8300 revealed the presence of Cx43 (Fig. 8B, lane 3), with a blot of brain homogenate serving as a positive control for Cx43 detection (Fig. 8B, lanes 1). Cx43 failed to bind to the PDZ1 or PDZ3 domains of ZO-1 (Fig. 8B, lanes 2 and 4, respectively). Immunoblot membranes stripped and reprobed with anti-GST antibody (Fig. 8C and D) confirmed the presence of GST-PDZ fusion protein with expected molecular weight (40–42 kDa) in material applied to the blots, and its absence in homogenates of Cx36-transfected HeLa cells or retina (Fig. 8C, lane 1 and 2), or brain (Fig. 8D, lane 1). The same membranes stained with coomassie blue indicated that equal amounts of GST-PDZ domain fusion proteins were used in the *in vitro* binding assays (Fig. 8E and F).

To establish by *in vitro* pull-down assays that the c-terminus amino acids of Cx36 were responsible for mediating Cx36 interaction with the PDZ1 domain of ZO-1, these assays were repeated using lysates of HeLa cells transfected with FL-Cx36 or Tr-Cx36 lacking four c-terminal residues. As a positive control for the effect of C-terminus amino acid deletion on connexin interaction with ZO-1, HeLa cells transfected with FL-Cx43 or Tr-Cx43 lacking six C-terminal amino acids were also examined for binding with the three PDZ domains of ZO-1. Immunoblots loaded with HeLa cell lysate protein that bound to, and was eluted from, the three ZO-1 domains confirmed FL-Cx36 interaction with PDZ1 of ZO-1, as shown by detection of Cx36 with anti-Cx36 Ab51-6300 (Fig. 9A, lane 3), and FL-Cx43 interaction with PDZ2 of ZO-1, as shown by detection of Cx43 with anti-Cx43 Ab13-8100 (Fig. 9B, lane 4). In contrast,

Tr-Cx36 failed to bind the PDZ1 domain (Fig. 9A, lane 7), and Tr-Cx43 failed to bind the PDZ2 domain (Fig. 9B, lane 8) of ZO-1. HeLa cell lysates from FL- and Tr-Cx36 (Fig. 9A, lanes 2 and 6, respectively), and FL- and Tr-Cx43 (Fig. 9B, lanes 2 and 6) transfected cells served as positive controls for connexin detection, and empty vector-transfected HeLa cells were devoid of Cx36 (Fig. 9A, lane 1) and Cx43 (Fig. 9B, lane 1). Immunoblot membranes stripped and reprobed with anti-GST antibody (Fig. 9C and D), and then stained with coomassie blue (Fig. 9E and F) showed the presence and equal loading of GST fusion proteins derived from the pull-down assays, and their absence in lysates of empty vector-transfected (Fig. 9C and D, lane 1) or connexin-transfected (Fig. 9C and D, lane 2) HeLa cells.

Discussion

We demonstrated by immunofluorescence that Cx36 is colocalized with ZO-1 in rodent brain and in HeLa cells transfected with Cx36, and by FRIL that ZO-1-immunoreactivity is associated with small gap junction plaques and with string gap junctions containing Cx36. Using biochemical and molecular approaches, we established that Cx36 directly interacts with the first PDZ domain of ZO-1, and that this interaction is mediated by at most the four c-terminal amino acid residues of Cx36. Based on widespread Cx36/ZO-1 colocalization, and with Cx36 now an adequate marker for neuronal gap junctions, it appears that ZO-1 is ubiquitously expressed by neurons forming electrical synapses via gap junctions composed of Cx36, and that ZO-1 is a major structural and/or regulatory component in one large subpopulation of these synapses.

Cx36 localization in brain

Immunofluorescence labelling for Cx36 shown here is consistent with reports of Cx36 immune-localization in retina, olfactory bulb and inferior olive (Guldenagel *et al.*, 2000, 2001; Rash *et al.*, 2000; Teubner *et al.*, 2000; Deans *et al.*, 2001; Feigenspan *et al.*, 2001; Hidaka *et al.*, 2002; Meier *et al.*, 2002; De Zeeuw *et al.*, 2003), but was substantially more robust in various brain regions than previously described, particularly in the mitral cell layer of the olfactory bulb (Belluardo *et al.*, 2000; Zhang & Restrepo, 2003). Although requiring ultrastructural confirmation, the striking circular patterns of Cx36-positive puncta we observed in the mitral cell layer appear to be localized to mitral cells, and likely correspond to gap junctions formed by these cells. This possibility is supported by reports of Cx36 mRNA expression in mitral cells (Condorelli *et al.*, 1998; Belluardo *et al.*, 2000), by the identification of gap junction plaques on mitral cell bodies and gap junctions between mitral cells and other types of olfactory bulb neurons (Pinching & Powell, 1971; Landis *et al.*, 1974; Paternostro *et al.*, 1995; Miragall *et al.*, 1996), and by observations of gap junction-mediated dye-coupling between mitral cells and between mitral cells and granule cells (Paternostro *et al.*, 1995). The specificity of labelling that we observed in the olfactory bulb and in other brain regions with anti-Cx36 antibodies was confirmed by demonstrations of similar immunolabelling and Western blotting results with three different anti-Cx36 antibodies raised against two different epitopes in Cx36, and by an absence of Cx36 detection in brain tissue of Cx36 KO mice and nontransfected HeLa cells. Based on the high quality and reliability of Cx36 detection with these antibodies, it is now possible to map the distribution of Cx36 in the mammalian brain, with the aim of gaining an appreciation of sites and relative densities of electrical synapses containing Cx36.

ZO-1 localization in brain

ZO-1 has been found in association with tight junctions in a wide variety of tissues (Stevenson *et al.*, 1986; Gonzalez-Mariscal *et al.*, 2003), but it also occurs in certain cell types lacking tight junctions, and is known to be a component of adherens junctions (Itoh *et al.*, 1991, 1993; Howarth *et al.*, 1992) as well as gap junctions (Thomas *et al.*, 2002). In the CNS, ZO-1

has been studied extensively in relation to its association with tight junctions of vascular endothelial cells (Wolburg & Lippoldt, 2002; Vorbrodt & Dobrogowska, 2003), but less so in non-circumventricular regions of brain parenchyma, where ZO-1 immunohistochemical detection was reported to be sparse or absent (Howarth *et al.*, 1992; Smith & Shine, 1992; Petrov *et al.*, 1994). However, ependymal cells and developing neuroepithelial cells, which either lack or possess minimal tight junctions, contain an abundance of ZO-1 (Dermietzel & Krause, 1991; Petrov *et al.*, 1994; Aaku-Saraste *et al.*, 1996; Saitou *et al.*, 1997). ZO-1 also was found in olfactory sensory neurons, as well as in the olfactory bulb mitral cell layer, where it was suggested to be associated with oligodendrocytes (Miragall *et al.*, 1994). More recently, ZO-1 was found localized at puncta adherentia between mossy fibre terminals and pyramidal cell dendrites in hippocampus, indicating that both granule cells and pyramidal cells express ZO-1 (Inagaki *et al.*, 2003). Notwithstanding these few reports, our results indicate that ZO-1 expression in the CNS is far more extensive than recognized previously. This apparent discrepancy could be explained by our finding that very weak tissue fixations are required for adequate immunolabelling of ZO-1.

Given the widespread and exclusive neuronal expression of Cx36 in the CNS (Condorelli *et al.*, 1998, 2000; Rash *et al.*, 2000, 2001; Teubner *et al.*, 2000), together with extensive Cx36/ZO-1 colocalization, ZO-1 is likely to be expressed in a large subset of neurons that form gap junctions composed of Cx36. Consequently, the association of ZO-1 with Cx36 may be a common feature of electrical synapses between these neurons. In some cases, neuronal expression and/or targeting of ZO-1 to plasma membranes may even be dependent on the presence of Cx36 in gap junction plaques, as suggested by the substantial reduction of punctate labelling for ZO-1 that we observed in the olfactory bulb mitral cells layer of Cx36 KO mice. Such an association, however, might not be restricted to neurons, as we have found similar Cx36 association with ZO-1 in some peripheral tissues, including pancreatic islets and adrenal gland (unpublished observations). Since it is well established that neurons do not form tight junctions, additional sites of ZO-1 expression that we detected may include adherens junctions, tight junctions within myelin, and neuronal and/or glial gap junctions consisting of connexins other than Cx36. The latter possibility is suggested by our FRIL results in retina, where ZO-1-immunoreactivity was found in association with some neuronal gap junctions lacking Cx36. Conversely, the absence of ZO-1 in some Cx36-positive retinal junctions may reflect a dynamic regulatory role of ZO-1 involving only transient association with Cx36.

PDZ binding motif of Cx36 and ZO-1 interaction

PDZ domain-containing proteins interact with short binding motifs located at the c-terminus of their target proteins (Sheng & Sala, 2001). ZO-1 contains three PDZ domains, one SH3 domain and one guanylate kinase domain (Gonzalez-Mariscal *et al.*, 2003). The C-terminal YV residues of some members of the claudin protein family interact with the first PDZ domain in each of the three tight junction-associated proteins ZO-1, ZO-2 and ZO-3 (Itoh *et al.*, 1999). Similarly, an identical YV motif at the c-terminus of Cx36 likely mediates Cx36 interaction with the first PDZ domain of ZO-1, as supported by the elimination of this interaction after deletion of the SAYV residues of Cx36. This contrasts with the binding of Cx43, Cx31.9, Cx46 and Cx50, as well as Cx47 (unpublished observations) to the second PDZ domain in ZO-1 (Giepmans & Moolenaar, 1998; Toyofuku *et al.*, 1998; Nielsen *et al.*, 2002, 2003). The significance of connexin/ZO-1 interaction and the differential mode of their binding to ZO-1 at the first or second PDZ domain remain to be determined. It appears, however, that interaction with ZO-1 is not a strict requirement for assembly of connexins into gap junctions, as junctions were still formed by various connexins, including Cx36, tagged with myc or fluorescent protein at their c-terminus (Jordan *et al.*, 1999; Martin *et al.*, 2001; Zoidl *et al.*, 2002), which eliminates connexin interaction capability with ZO-1 (Giepmans & Moolenaar, 1998; Nielsen *et al.*, 2002). Similarly, deletion of the SAYV c-terminal sequence of Cx36 did not prevent

presumptive gap junction formation in HeLa cells transfected with Tr-Cx36. Nevertheless, there are beginning to emerge some functional deficits that may reflect a role of ZO-1 at these junctions. Thus, mutations that prevent Cx43, Cx45 and Cx50 C-terminus interaction with ZO-1 resulted in either reduced capacity for gap junction formation, altered connexin turnover rate or formation of junctions with loss of pH sensitivity (Kausalya *et al*, 2001; Toyofuku *et al*, 2001; Xu *et al*, 2002).

Our results showing persistence of low levels of ZO-1 at gap junctions composed of Tr-Cx36 lacking ZO-1 binding ability suggests association of ZO-1 with other proteins at gap junctions, which is consistent with the presence of multiple protein binding modules in ZO-1 that may serve as scaffolding for recruiting structural and/or signalling proteins to plasma membranes. A large number of ZO-1 interacting proteins with diverse functions include the G-protein α_{12} , the serine kinase ZAK, the signalling-related protein Neph1, and the phosphoprotein VASP; and ZO-1 has been implicated in transcriptional regulation (Balda *et al*, 1996, 2003; Gottardi *et al*, 1996; Comerford *et al*, 2002; Meyer *et al*, 2002; Huber *et al*, 2003). ZO-1 also interacts with cytoskeletal components, and the cellular cytoskeleton is functionally linked to gap junctions, such that cytoskeleton integrity is required for gap junction stability (Wang & Rose, 1995; Johnson *et al*, 2002; Thomas *et al*, 2002). In analogy with these studies in other cell systems, identification of the binding partners of ZO-1 at interneuronal gap junctions formed by Cx36 is expected to provide insight into the regulation of electrical synapses in the mammalian CNS.

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Abbreviations

Ab	antibody
CNS	central nervous system
Cx36	connexin36
FITC	fluorescein isothiocyanate
FL	full length
FRIL	freeze-fracture replica immuno-gold labelling
GJIC	gap junction intercellular communication
GST	glutathione S-transferase
IP	immunoprecipitation
IPL	

	inner plexiform layer
KO	knockout
MCL	mitral cell layer
NGS	normal goat serum
OLM	outer limiting membrane
OPL	outer plexiform layer
PDZ	postsynaptic protein PSD-95/Drosophila junction protein Disc-large/tight junction protein ZO-1
TBST	50mM Tris-HCl, pH7.6, 1.5% NaCl, 0.3% Triton X-100
Tr	truncated
TSTw	20 mM Tris-HCl, pH7.4 150 mM NaCl with 0.2% Tween-20
WT	wild-type
ZO-1	zonula occludens-1

Fig. 1.

Specificity of anti-Cx36 and anti-ZO-1 antibodies. (A) Immunoblots show monomer and dimer forms of Cx36 detected with three different anti-Cx36 antibodies (indicated above blots) in retina of WT mice (lanes 1,3,5), and absence of Cx36 detection in retina of Cx36 KO mice (lanes 2,4,6). (B) Blots show Cx36 detected with three anti-Cx36 antibodies (indicated above blots) in HeLa cells transfected with Cx36 (lanes 1,3,5), and absence of Cx36 in control vector-transfected cells (lanes 2,4,6). (C) Blot showing anti-ZO-1 antibody detection of ZO-1 in retina of WT (lane 1) and Cx36 KO (lane 2) mice. (D) Control blot of Cx36-transfected HeLa cells probed with anti-Cx36 (lane 1) and anti-ZO-1 (lane 2) showing lack of polyclonal anti-ZO-1 antibody cross-reaction with Cx36 in these cells.

Fig. 2.

Double immunofluorescence labelling of Cx36 and ZO-1 in mouse retina. (A) Retina from WT mouse showing punctate labelling of Cx36 in the outer plexiform layer (OPL) and the inner plexiform layer (IPL) (A1). Little or no labelling is seen in the inner nuclear layer (INL) or outer nuclear layer (ONL). In the same field, punctate labelling of ZO-1 (A2) is codistributed with Cx36 (yellow in overlay, A3). (B) Retina from Cx36 KO mouse showing absence of labelling for Cx36 (B1), persistence of labelling for ZO-1 (B2), and absence of yellow in overlay (B3). (C) Confocal double immunofluorescence showing punctate labelling for Cx36 (C1, arrows) and ZO-1 (C2, arrows) in the IPL layer of retina from WT mouse, and considerable, though not total, colocalization of Cx36 with ZO-1 (yellow in overlay, C3, arrows). Scale bars, 50 μ m (A and B); 10 μ m (C).

Fig. 3.

Double immunofluorescence labelling of Cx36 and ZO-1 in olfactory bulb and inferior olive. (A) Olfactory bulb showing punctate labelling of Cx36 with polyclonal antibody 36–4600 (A1, arrows) and monoclonal anti-ZO-1 (A2, arrows) around mitral cell bodies, and overlap of Cx36/ZO-1 (A3, arrows). (B) Same as A, except double-labelled with monoclonal anti-Cx36 37–4600 (B1, arrows) and polyclonal anti-ZO-1 (B2, arrows), showing similar Cx36/ZO-1 colocalization around mitral cells (B3, arrows). (C) Olfactory bulb synaptic glomeruli showing punctate labelling for Cx36 (C1) and ZO-1 (C2), and colocalization in overlay (C3). (D) Confocal double labelling of Cx36 (D1) and ZO-1 (D2) in the inferior olivary nucleus, with colocalization shown by yellow in overlay (D3). (E and F) Double labelling in olfactory bulb

of WT mouse shows similar distributions of Cx36-positive (E1, arrows) and ZO-1-positive (E2, arrows) puncta in the mitral cell layer (E3, overlay). A similar field of olfactory bulb from Cx36 KO mouse, with orientation matching that in A, shows absence of Cx36 labelling (F1, arrows), and reduction of ZO-1 labelling (F2, arrows) in the mitral cell layer (F3, overlay). Scale bars, 50 μm (A and B); 10 μm (C and D); 100 μm (E and F; shown in F).

Fig. 4.

Freeze-fracture replicas of gap junctions in the inner plexiform layer (IPL) of adult rat retina after simultaneous double-labelling for Cx36 (6 and 12 nm gold beads) and ZO-1 (18 nm gold beads). (A and B) Overview image of 'mixed' (chemical plus electrical) synapses in the IPL. The nerve terminal contains abundant, uniform-diameter synaptic vesicles (S V). The nerve terminal plasma membrane E-face (E) contains one small gap junction (inscribed area and 4B) that is labelled by five 6 nm and three 12 nm gold beads (Cx36) and two 18 nm gold beads (ZO-1). Note the very low nonspecific background. P, P-face; *, postsynaptic P-face. (C) Gap junction plaques with gold beads for Cx36 (6 and 12 nm) but with no gold beads for ZO-1. At this magnification, 6 nm gold beads are difficult to discern beneath IMP's (left pair), but are

easily resolved in reverse stereoscopic images (right pair). (D and E) Neuronal gap junctions labelled for ZO-1 (18 nm gold beads). Labeling for Cx36 (6 and 12 nm gold beads) was absent from approximately 25% of plaque-type gap junctions in IPL that were, nonetheless, well labelled for ZO-1, thereby revealing a previously undetected population of interneuronal gap junctions. Scale bars, 0.1 μm .

Fig. 5.

Co-IP of Cx36 and ZO-1 from various brain regions. (A and B) Immunoblot detection of Cx36 with polyclonal (A, lane 1) and monoclonal (B, lane 1) antibodies after IP of ZO-1 from retina with monoclonal anti-ZO-1. Cx36 is absent after omission of anti-ZO-1 antibody during IP (A, B, lane 3). Blots of Cx36-transfected HeLa cells (A and B, lane 2) are shown for comparison of Cx36 migration. (C) Immunoblots of Cx36 after IP of ZO-1 from the brain regions indicated, with lysate from Cx36-transfected HeLa cells as positive control (lane 1).

Fig. 6. Double immunofluorescence of Cx36 and ZO-1 in HeLa cells transfected with full length (FL)-Cx36 and truncated (Tr)-Cx36 lacking four carboxy-terminus amino acids. (A and B) Low magnification micrographs of the same field (A1 and A2) and confocal immunofluorescence of the same field (B1 and B2) showing colocalization of FL-Cx36 (A1 and B1) with ZO-1 (A2 and B2) around the periphery of cells, as seen by yellow in overlay (A3 and B3). (C and D) Low magnification micrographs of the same field (C1 and C2) and confocal immunofluorescence of the same field (D1 and D2) showing reduced colocalization of Tr-Cx36 (C1 and D1) with ZO-1 (C2 and D2), as seen by reduced yellow in overlay (C3 and D3). Scale bars, 50 μ m (A and C); 10 μ m (B and D).

Fig. 7.

Coimmunoprecipitation of Cx36 and ZO-1 from full length (FL)-Cx36-transfected and truncated (Tr)-Cx36-transfected HeLa cells. (A) Immunoblot showing absence of Cx36 in control vector-transfected HeLa cells (lane 1), and presence of Cx36 in lysate (lane 2), in anti-Cx36 IP protein (lane 3) and in anti-ZO-1 IP protein (lane 4) from FL-Cx36-transfected HeLa cells. Cx36 is absent in control IP with anti-ZO-1 omission (lane 5). (B) Immunoblot showing Cx36 in FL-Cx36-transfected HeLa cells (lane 1), and in lysate (lane 2) and anti-Cx36 IP protein (lane 3) from Tr-Cx36-transfected HeLa cells. No Cx36 is detected after IP of ZO-1 from Tr-Cx36-transfected HeLa cells (lane 4).

Fig. 8.

Analysis of ZO—1 interaction with Cx36 from retina and Cx43 from brain. (A) Retina homogenate incubated with fusion protein GST-PDZ1, GST-PDZ2 or GST-PDZ3 domain of ZO-1, followed by blotting of bound protein with anti-Cx36 antibody (51–6300) shows Cx36 binding to PDZ1 (lane 3), but not to PDZ2 or PDZ3 (lanes 4 and 5). Blots of Cx36-transfected HeLa cells (lane 1) and retina (lane 2) are shown as positive controls for Cx36 detection. (B) Brain homogenate incubated with each of the three PDZ domains of ZO-1, followed by blotting of bound protein shows Cx43 binding to PDZ2 (lane 3), but not to the other two domains (lanes 2 and 4). Blot of brain is shown as positive control for Cx43 detection (lane 1). (C–F) Membranes from A and B stripped and reprobed with anti-GST antibody (C and D) and stained

with coomassie blue (E and F) show equal loading of GST-PDZ fusion proteins. Cx36-transfected HeLa cells and retina (C, lanes 1, 2), and brain (D, lane 1) show absence of GST.

Fig. 9.

Analysis of ZO-1 PDZ domain 1,2 and 3 binding to full length (FL)-Cx36, truncated (Tr)-Cx36, FL-Cx43 and Tr-Cx43. (A) HeLa cell lysates incubated with fusion protein GST-PDZ1, GST-PDZ2 or GST-PDZ3 domain of ZO-1, followed by blotting of bound protein show FL-Cx36 binding to PDZ1 (lane 3), but not to the other two domains (lanes 4 and 5), and absence of Tr-Cx36 binding to PDZ1 (lane 7). Blots of HeLa cells probed with anti-Cx36 51-6300 show positive controls for Cx36 detection in cells transfected with FL-Cx36 (lane 2) or Tr-Cx36 (lane 6). Vector-transfected HeLa cells show negative control for Cx36 detection (lane 1). (B) HeLa cell lysates incubated with fusion protein GST-PDZ1, GST-PDZ2 or GST-PDZ3 domain of ZO-1, followed by blotting of bound protein show FL-Cx43 binding to PDZ2 (lane 4), but not to the other two domains (lanes 3 and 5), and absence of Tr-Cx43 binding to PDZ2 (lane 8). Blots probed with anti-Cx43 antibody 13-8300 show FL-Cx43-transfected (lane 2) and Tr-Cx43-transfected (lane 6) HeLa cells as positive controls for Cx43 detection, and nontransfected cells (lane 1) as a negative control. (C-F) Membranes from A and B stripped and reprobed with anti-GST antibody (C and D) and stained with coomassie blue (E and F) show equal loading of GST-PDZ fusion proteins. Control (C and D, lanes 1) and transfected (C and D; lanes 2, 6) HeLa cells show absence of GST.