J. Membrane Biol. 207, 45–53 (2006)
DOI: 10.1007/s00232-005-0803-2

The Journal of
Membrane
Biology

Association with ZO-1 Correlates with Plasma Membrane Partitioning in Truncated Connexin45 Mutants

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Received: 24 June 2005/Revised: 13 October 2005

Abstract. Zonula occludens-1 (ZO-1), the most abundant known connexin-interacting protein in osteoblastic cells, associates with the carboxyl termini of both Cx43 and Cx45. To learn more about the role of the cormexin-ZO-1 interaction, we analyzed connexin trafficking and function in ROS 17/2.8 cells that were stably transfected either with full length Cx45 or with Cx45 lacking 34 or 37 amino acids on the carboxyl terminus (Cx45t34 or Cx45t37). All three proteins were transported to appositional membranes in the transfected cells: Cx45 and Cx45t34 displayed a punctate appositional membrane-staining pattern, while Cx45t37 staining at appositional membranes was more linear. Expression of Cx45 decreased gap junction communication as assayed by dye transfer, while expression of Cx45t34 or Cx45t37 increased the amount of dye transfer seen in these cells. We found that Cx43, Cx45 and Cx45t34 co-precipitated with ZO-1 in these cells, while Cx45t37 did not. We also found that Cx45t37 was much more soluble in 1% Triton X-100 than the other connexins examined. In addition, Cx45t37 migrated to a fraction of lighter buoyant density on sucrose flotation gradients than Cx43, Cx45, ZO-1 and Cx45t34. As ZO-1 is an actin-binding protein, this suggested that the differences in Cx45t37 solubility might be due to a difference between the interaction of gap junctions and the actin cytoskeleton in the ROS/Cx45t37 and in the other transfected ROS cells. To examine this possibility, the transfected ROS cells were stained with fluorescently labeled phalloidin and demonstrated that there was a notable loss of actin stress fibers in the ROS/Cx45t37 cells. These findings suggest that association with ZO-1 alters the plasma membrane localization of Cx45 by removing it from a lipid raft compartment and rendering it Triton-insoluble, presumably by promoting an interaction with the actin cytoskeleton; they also suggest that Cx45 has a complex binding interaction with ZO-1 that involves either an extended carboxyl terminal domain or two distinct binding sites.

Key words: Connexin — Cx45 — Cx43 — Gap junction — ZO-1 — Lipid raft — Osteoblastic cells

Introduction

Gap junctions allow the passage of low molecular weight molecules and ions between the cytoplasm of neighboring cells, and are formed by proteins called connexins. There are 21 different connexins in the mouse genome, each of which forms gap junctions with distinct physiological characteristics (Willecke et al., 2002). Osteoblasts express both Cx43 and Cx45 and changes in the relative abundance of these gap junction proteins modulate the permeability of gap junctions in bone cells and alter the expression of osteogenic proteins in osteoblastic cells (Koval et al., 1995; Lecanda et al., 1998). Deletion of the Cx43 gene in mice leads to delayed ossification and a generalized osteoblast dysfunction (Lecanda et al., 2000). Additionally, mutations in human Cx43 result in the developmental disorder oculodentodigital dysplasia, which is characterized by skeletal defects (Paznekas et al., 2003). Gap junctional communication in osteoblasts is modulated by mechanical stress and soluble factors such as parathyroid hormone, which are important regulators of bone remodeling (Ziambaras et al., 1998; Civitelli et al., 1998). These changes may involve altering the amount of Cx43 in gap junctional plaques.

ZO-1 interacts with several different gap junction proteins, including Cx43 (Giepmans and Moolenar, 1998; Toyofuku et al., 1998), Cx31.9 (Nielsen et al., 2002a), Cx36 (Li et al., 2004a), Cx47 (Li et al., 2004b),

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Cx46, and Cx50 (Nielsen et al., 2002b), as well as Cx45 (Kausalya, Reichert & Hunziker, 2001; Laing et al., 2001). ZO-1 binds to a number of different proteins that are associated with tight junctions and adherens junctions. ZO-1 has at least five different domains that mediate protein-protein interactions including three PDZ domains, an SH3 domain, and a catalytically inactive guanylate kinase domain. Cx43 binds to the second PDZ domain in ZO-1; while the Cx45-ZO-1 binding site has not been as thoroughly studied. We recently demonstrated that a fusion protein containing the first 2 PDZ domains in ZO-1 binds to the Cx45 carboxyl terminal polypeptide in a peptide-binding assay (Laing, Chou & Steinberg, 2005). Expression of this fusion protein in ROS cells disrupted the interaction between Cx43 and ZO-1, diminished gap junction formation and function, and altered the plasma membrane localization of Cx43. In contrast, overexpression of ZO-1 in ROS cells increased gap junction formation and gap junctional communication. These studies suggested that ZO-1 plays a critical role in gap junction formation in osteoblastic cells (Laing et al., 2005).

A number of studies have shown that expression of connexin constructs that have blocked or truncated carboxyl termini, which may not interact with ZO-1, form identifiable gap junction plaques in a variety of different cell lines (Bukauskas et al., 2000; Falk, 2000; Windoffer et al., 2000; Hunter et al., 2003). It should be noted that these gap junction plaques are unusually large and studies have indicated most of the channels in these plaques may be inactive (Bukauskas et al., 2000; Hunter, Jourdan & Gourdie, 2003). In this study, we expressed full-length Cx45 and two different carboxyl terminal truncation mutants of Cx45 (that lack the canonical ZO-1 binding peptide site) in ROS cells and examined the trafficking of these mutant proteins and the behavior of the gap junctions formed in these transfected cells.

Materials and Methods

Reagents and Plasmid

Cx43 (Laing et al., 1997) and Cx45 antiserum (Laing et al., 2001) were previously characterized. Polyclonal and monoclonal antibodies directed against ZO-1 were obtained from Zymed (South San Francisco, CA). The monoclonal antibody directed against Cx43 was purchased from Chemicon (Temecula, CA). The cDNA for Cx45 lacking the carboxyl terminal 34 amino acids (Cx45t34) was amplified from chick Cx45 cDNA by PCR and inserted into the pSFFVneo plasmid. Cx45t37-pSFFVneo plasmid was described previously (Koval et al., 1995).

Cell Culture and Transfection

ROS 17/2.8 is an osteosarcoma cell line that expresses Cx43 but not Cx45 (Steinberg et al., 1994). ROS cells were cultured in minimum Eagle's medium containing 10% heat-inactivated bovine calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 5 units/ml penicillin, and 5 μg/ml streptomycin. The Cx45-transfected ROS cells and Cx45t37-transfected ROS cells were characterized previously (Steinberg et al., 1994; Koval et al., 1995). Stably transfected ROS cells were selected and cultured in the same culture media containing 400 μg/ml G418. Clones were screened by immunofluorescence microscopy for cell surface expression of Cx45. We used 4 different ROS/Cx45 clones, 3 different ROS/Cx45t34 clones and 4 different ROS/Cx45t37 clones in the experiments reported in this manuscript.

Immunofluorescence Microscopy

Transfected ROS cells were processed for immunofluorescence microscopy as described previously (Laing et al., 2001). The cells were fixed, permeabilized and stained either with a monoclonal antibody against ZO-1 and a polyclonal antiserum directed against Cx45, a monoclonal antibody against Cx43 and a polyclonal antiserum directed against Cx45 or a polyclonal antiserum directed against Cx45 and the appropriate secondary antibodies. In order to monitor F-actin localization in the transfected cells, the cells were fixed in 1.6% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and stained with Oregon488 phalloidin diluted 1:200 (Molecular Probes, Eugene, OR). These cells were viewed by epifluorescence on a Nikon Eclipse E600FN microscope using a 63 × (numerical aperture 1.4) objective, and the images were processed using Tillvision software (Martinsried, Germany). Confocal images were collected on a Bio-Rad MRC10-24 microscope with LaserShop software, and the images were analyzed using Adobe Photoshop software.

Dye Transfer

Chemical coupling via gap junctions was assessed by a dye injection assay among cells grown in monolayers. Microinjections of Lucifer Yellow (10 mm) were performed using a previously published technique (Steinberg et al., 1994; Koval et al., 1995). Cells were seeded on a 25 mm diameter No. 1 thickness glass cover slip and mounted in a PDMI-2 incubation chamber on a Zeiss Axiovert 35. Lucifer Yellow was injected into single cells with an Eppendorf microinjector and micromanipulator and the number of cells that received dye after 3 minutes was assessed. The data was analyzed by Student's *t*-test.

Immunoprecipitation

The co-immunoprecipitation protocol was adapted from a procedure used to precipitate ZO-1-associated proteins and used previously to show that Cx45 interacts with ZO-1 (Laing et al., 2001). Proteins immunoprecipitated by anti-Cx45 antibodies were transferred to Immobilon-P membranes. The membranes were probed with 1:1000 dilution of monoclonal antibody directed against Cx43 (Chemicon International, Temecula, CA) or ZO-1 (Zymed Laboratories Inc. South San Francisco, CA). All blots were then probed with the appropriate peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals or Jackson Immunoresearch, West Grove, PA) and developed with the SuperSignal West Pico chemiluminescence system (Pierce, Rockford, IL).

Triton X-100 Solubilization

This procedure was adopted from a method published previously (Schubert et al., 2002). Transfected ROS cells were washed three times in ice-cold PBS. Three hundred microliters of cold MBS (25

mM MES, pH 6.5, 150 mM NaCl pH 6.5) containing 1% Triton X-100 and protease inhibitors were added to the cells. Following 30-minute incubation without agitation at 4°C the soluble fraction was collected. Three hundred microliters of 1% SDS were added to the plate to dissolve the remaining Triton X-100 insoluble material, which was passed through a 26 gauge needle 10 times in order to lower its viscosity. Equal volumes of the Triton X-100 soluble material and insoluble material were analyzed by immunoblotting with Cx45 or Cx43 antiserum.

Sucrose Flotation Gradients

Briefly, two 100 mm diameter plates were washed three times in cold PBS, scraped into 750 μl of MBS containing 1% Triton X-100, and passed through a tight-fitting Dounce homogenizer five times. The sample was mixed with an equal volume of 80% sucrose (prepared in MBS lacking Triton X-100), transferred to a 5 ml ultracentrifuge tube, and overlaid with a discontinuous sucrose gradient of 1.5 ml of 30% sucrose and 1.5 ml of 5% sucrose (prepared with MBS lacking Triton X-100), respectively. The samples were centrifuged for 18 h at 200,000 × *g* (44,000 rpm in a Sorvall rotor, TH-660). Eight 550 μl fractions were collected, and aliquots of each fraction were subjected to SDS-PAGE and immunoblotting (Schubert et al., 2002).

Results

Cx45t34 and Cx45t37 Have Different Patterns of Plasma Membrane Localization

Expression of Cx45, Cx45t34 and Cx45t37 was assessed by immunoblots of solubilized proteins from transfected ROS cells. All three transfected cell lines expressed similar amounts of a Cx45 immunoreactive protein (Fig. 1*A*). Cx45 ran at 46 kDa in these gels, while Cx45t34 and Cx45t37 ran at 41 kDa. The Cx45 antibody also detected a less abundant 46 kDa polypeptide in the ROS/Cx45t34 and ROS/Cx45t37 cells. Immunoblots of parallel samples with a ZO-1 monoclonal antibody detected a 220 kDa and a 210 kDa polypeptide in the ROS cells. They also demonstrated that ROS and ROS/Cx45t34 cells expressed similar amounts of ZO-1, while more ZO-1 was found in the ROS/Cx45 and ROS/Cx45t37 cells (Fig. 1*B*).

In ROS/Cx45 cells, Cx45 staining appeared both in a perinuclear compartment and in a linear punctate pattern at the plasma membranes (Fig. 1*C*). There was a similar staining pattern for Cx45 in the ROS/Cx45t34 cells and in the ROS/Cx45t37 cells. These micrographs showed that there was more Cx45 staining in a perinuclear locale in the ROS/Cx45t34 and ROS/Cx45t37 cells than in the ROS/Cx45cells. Similar results were seen with the other transfected ROS cell clones used in this study.

We next examined the association Cx45 and ZO-1 using double-label immunofluorescence labeling and confocal microscopy (Fig. 2*A*). In ROS/Cx45, as we previously demonstrated, Cx45 colocalized with ZO-1 at appositional membranes, but many regions that had ZO-1 staining did not exhibit Cx45 staining (Laing et al., 2001). Similar results were seen in the ROS/Cx45t34 cells where Cx45t34 colocalized with ZO-1 at appositional membranes. Cx45 staining also colocalized with ZO-1 staining at appositional membranes in the ROS/Cx45t37 cells. These studies also showed that the appositional membrane staining for Cx45 was more punctate in the ROS/Cx45 and ROS/Cx45t34 cells than it was in the ROS/Cx45t37 cells.

We also examined the association of Cx43 and the truncated Cx45 constructs by confocal immunofluorescence microscopy (Fig. 2*B*). In these experiments cells were fixed, permeabilized and stained with Cx43 monoclonal antibody and a Cx45 polyclonal antiserum. Cx45 and Cx43 colocalized in cytoplasmic vesicles and at appositional membranes in the ROS/Cx45 cells, in the ROS/Cx45t34 cells and in the Cx45t37 cells. The appositional membrane staining for Cx45 and Cx43 was more punctate in the ROS/Cx45 and ROS/Cx45t34 cells than it was in the ROS/Cx45t37 cells. These micrographs also demonstrate that there was not as much co-localization of Cx43 and Cx45t37 as there was of Cx43 and Cx45t34 or of Cx43 and Cx45 in these different transfected cells.

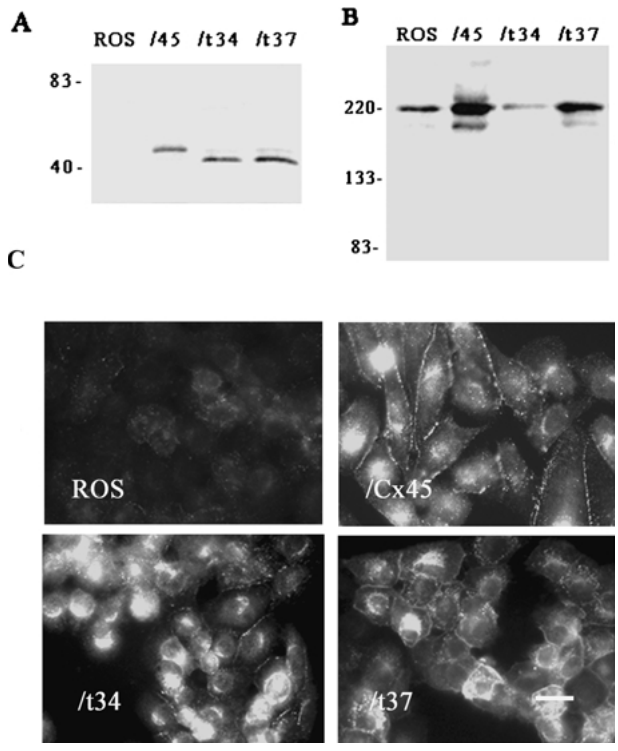

Fig. 1. Cx45 expression in transfected ROS cells. (**A**) and (**B**): Immunoblotting analysis of transfected ROS cells. Transfected ROS cells were harvested and equal aliquots of protein were analyzed by SDS-PAGE and transferred to Immobilon-P. The membranes were probed with rabbit Cx45 (**A**), or ZO-1 antibody (**B**). (**C**). ROS (**a**), ROS/Cx45 (**b**), ROS/Cx45t34 (**c**) and ROS/Cx45t37 (**d**) cells were fixed, permeabilized and stained with a Cx45 antiserum and CY-3-coupled goat anti-rabbit IgG and examined by immunofluorescence microscopy. Bar = 50 μm.

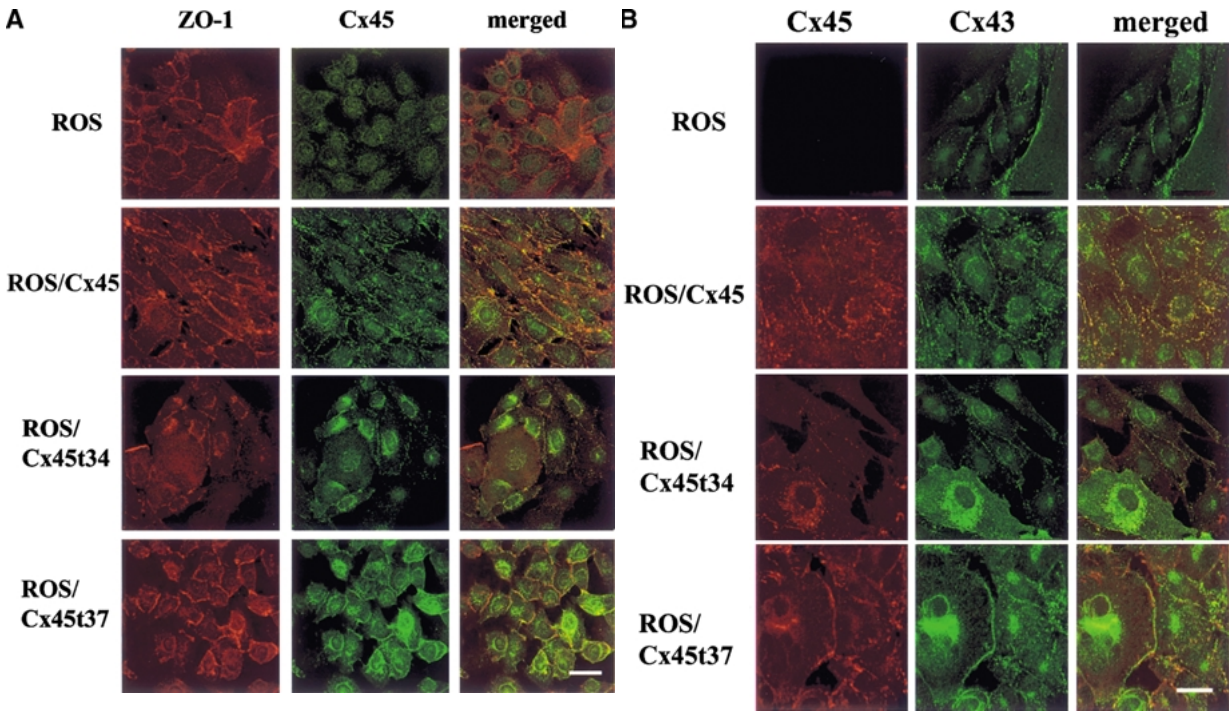

Fig. 2. Truncated Cx45 proteins colocalize with ZO-1 (*A*) and Cx43 (*B*) in transfected ROS cells. Transfected ROS cells were fixed, permeabilized and stained with a polyclonal Cx45 antiserum and a monoclonal ZO-1 antibody. The cells were then stained with an ALEXA 488 goat anti-Rabbit IgG and CY3 goat anti-mouse IgG. (*A*) Cells were stained with a polyclonal Cx45 antiserum and a monoclonal Cx43 antibody. The cells were then stained with CY3 goat anti-Rabbit IgG and ALEXA488 goat anti-mouse IgG (*B*).

Altered Dye Coupling in Transfected Ros Cells

We next examined how the expression of the different Cx45 truncation mutants affected the molecular permeability of gap junctions in ROS cells. Single cells were injected with Lucifer Yellow, and the number of dye-filled cells was counted after 3 minutes. In this experiment Lucifer Yellow passed to 5.5 ± 3.7 cells (n = 29). As can be seen in Fig. 3*A*, expression of Cx45 in ROS reduced the transfer of Lucifer Yellow-filled cells (3.2 ± 2.7 cells (n = 27)). This change in permeability was shown to be statistically significant by Student's t-test ($P < 0.05$). These data are consistent with our previously published studies showing that ROS/Cx45 cells pass Lucifer Yellow about half as efficiently as ROS cells (Koval et al., 1995). In contrast, Cx45t34 and Cx45t37 increased the transfer of Lucifer Yellow (11.8 ± 5 (n = 27) and 10.4 ± 45.5 cells (n = 29)). These changes in permeability were shown to be statistically significant by Student's t-test ($P < 0.01$). Representative micrographs of a dye injection experiment in each cell line are shown in Fig. 3*B*.

Cx43, Cx45 and Cx45t34, but Not Cx45t37, Co-precipitate with ZO-1

We then looked at the interaction between the connexins and ZO-1 in our transfected cells using a co-immunoprecipitation assay. Cx45-associated proteins were analyzed by immunoblotting with an anti-ZO-1 monoclonal antibody. Cx45 immunoprecipitates derived from ROS/Cx45 and ROS/Cx45t34 cell lysates contained ZO-1, but Cx45 immunoprecipitates derived from ROS/Cx45t37 did not (Fig. 4*A*). Similar results were seen in all ROS/Cx45, ROS/Cx45t34 and ROS/Cx45t37 clones in which Cx45 was transported to appositional membranes. In contrast, Cx43 immunoprecipitates derived from ROS, ROS/Cx45, ROS/Cx45t34 and ROS/Cx45t37 cell lysates all contained ZO-1 (Fig. 4*B*). Intriguingly, these blots showed that the 220 and 210 kDa isoforms of ZO-1 were found with the Cx43-associated proteins, while only the 220 kDa polypeptide was detected in the Cx45 immunoprecipitates.

Cx45t37 Exhibits Increased Triton X-100 Solubility and Altered Migration in Sucrose Flotation Gradients

Connexins present in gap junctions at the plasma membrane are considered to be insoluble in 1% Triton X-100. We assessed the Triton X-100 solubility of Cx43 and Cx45 in the ROS cell lines (Fig. 5). Approximately one third of cellular Cx43 was Triton-soluble in ROS cells and the transfected ROS cell lines. Similar proportions of Cx45 and Cx45t34 were insoluble in 1% Triton X-100, 45% and 56% respectively; but a greater

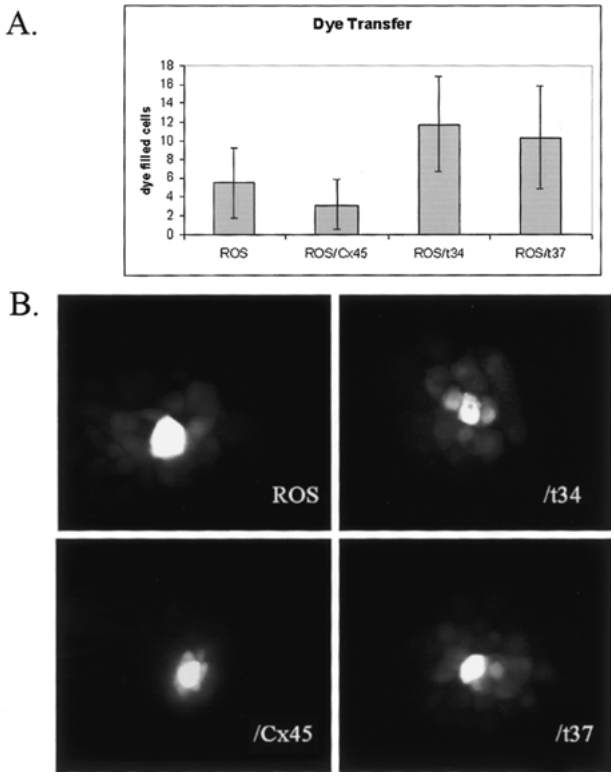

Fig. 3. Lucifer Yellow dye passage is altered in transfected ROS cells. Transfected ROS cells were injected with Lucifer Yellow, and the dye-filled cells were counted. The data are presented as histograms corresponding to the average number of dye-filled cells found after injection in this assay (mean ± standard deviation).

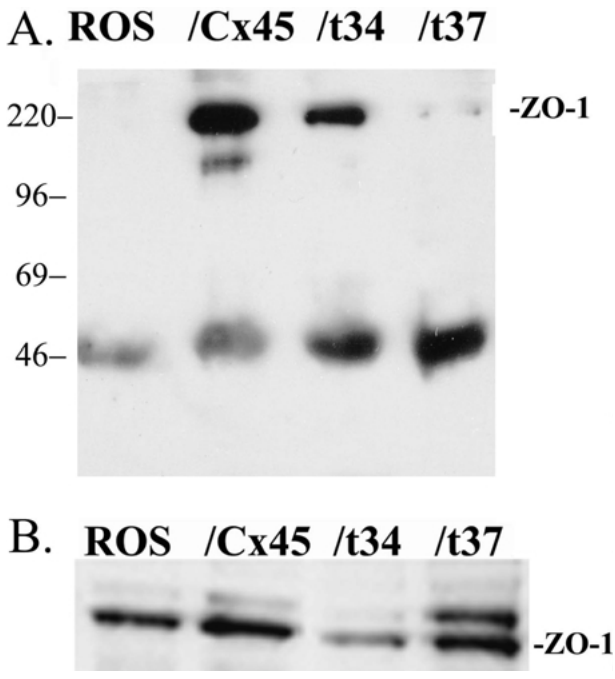

Fig. 4. Truncated Cx45 proteins co-precipitate with ZO-1 and Cx43 in lysates derived from Cx45-transfected ROS cells. Cells were harvested and proteins co-precipitated as outlined in Materials and Methods with a Cx45 antibody (*A*) or a Cx43 antibody (*B*). The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with a monoclonal ZO-1 antibody.

proportion of the Cx45t37 was Triton-soluble than the other connexins examined in this study. Similar results were seen in all of the other stably transfected ROS cell clones examined in this study.

Because the above experiments suggested that Cx45t37 might be in a different plasma membrane compartment than other connexins we next assessed migration of connexins in sucrose flotation gradients. Lysates derived from ROS and transfected ROS cells were fractionated on a 5–30% sucrose flotation gradient. In ROS, ROS/Cx45 and ROS/Cx45t34 cell lines, immunoreactive Cx43, Cx45 and ZO-1 were found in fractions 7 and 8 of these gradients, indicating all three proteins did not enter the sucrose gradients (Fig. 6). However, when lysates derived from ROS/Cx45t37 cells were fractionated on sucrose gradients, most of the Cx45t37 was found in fraction 4 of the sucrose gradients, and most of the ZO-1 was found in fraction 5 of the same sucrose gradients. In contrast, Cx43 was found in fractions 5, 7 and 8 of these gradients.

Lack of Actin Stress Fibers in Ros/Cx45t37 Cells

As ZO-1 is an actin-binding protein, the difference between ROS/Cx45t34 cells and ROS/Cx45t37 cells suggests that there may be a difference between the interaction between actin and the Cx45t37 and the interaction between actin and Cx45t34 in the different transfected ROS cell clones. To determine whether this is due to changes in the actin cytoskeleton in the transfected ROS cells, the cells were stained with fluorescently labelled phalloidin. As can be seen in the resulting micrographs (Fig. 7), phalloidin staining was found both at the cell surface and in stress fibers in the ROS, ROS/Cx45 and ROS/Cx45t34 cells. In contrast, there were significantly fewer actin stress fibers in the ROS/Cx45t37 cells than in the other cell lines studied.

Discussion

These studies demonstrate that truncated Cx45 proteins that differed by three amino acid residues in length differed in their ability to coprecipitate with endogenous ZO-1: Cx45t34 bound ZO-1 in a coprecipitation assay while Cx45t37 did not. The ability to bind ZO-1 correlated with differences in distribution of Cx45 within the plasma membrane. Thus Cx45t34 appeared in a punctate staining pattern similar to that of full-length Cx45, but Cx45t37 displayed a more diffuse appositional membrane-staining pattern. In addition, Cx45t34, like full-length Cx45, was

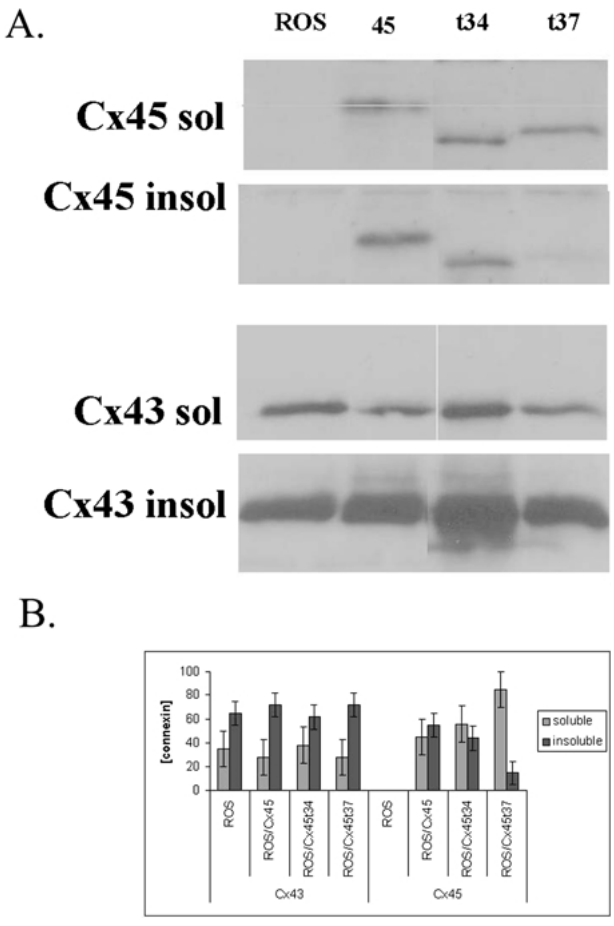

Fig. 5. Solubility of connexins derived from transfected ROS cells in 1% Triton X-100. (**A**) Transfected ROS cells were extracted at 4°C in MBS buffer containing 1 % Triton X-100. The Triton X-100 solution was removed and the extracted cells were scraped in MBS buffer containing 1% SDS. Equal aliquots of the Triton X-100-solubilized material and SDS-solubilized material were subjected to immunoblotting with Cx45 or Cx43 antiserum. (**B**) Results from 3 separate experiments, in which the solubility of Cx43, Cx45, Cx45t34 and Cx45t37 polypeptides in cold 1% Triton X-100 was determined, were analyzed by densitometry and this data is represented as a histogram (mean ± standard deviation).

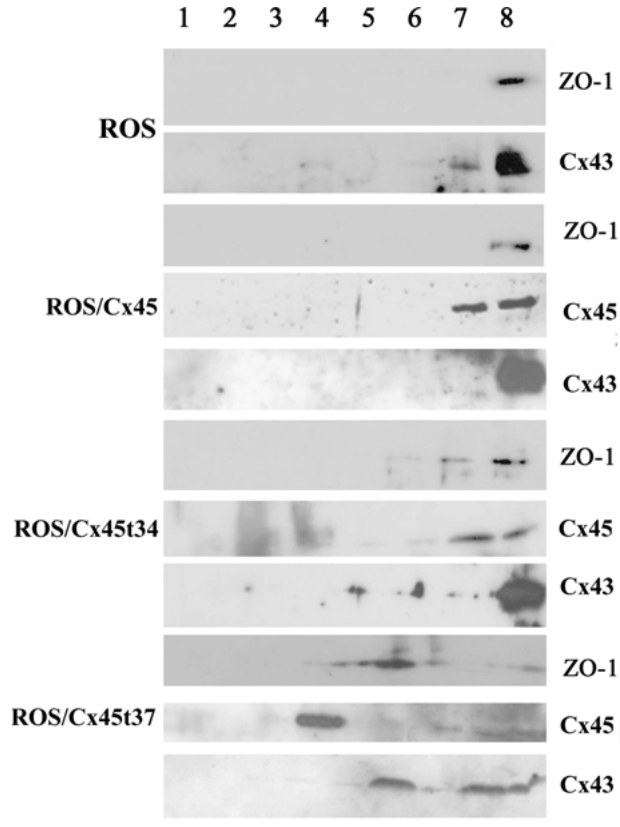

Fig. 6. Expression of the gap junction-associated proteins on sucrose flotation gradients. Transfected ROS cells were harvested in 1% Triton X-100, overlaid with a discontinuous 5–30% sucrose gradient, and centrifuged. Gradient fractions were collected, separated by SDS-PAGE and Cx45, Cx43, and ZO-1 were identified by immunoblotting. Fraction 1 was the top of the gradient, while fraction 8 was the bottom of the gradient.

Triton-insoluble and remained at the bottom of a sucrose flotation gradient, while Cx45t37 was much more Triton-soluble and partitioned into a lipid raft environment on sucrose gradients.

The current data suggest that association with ZO-1 alters the plasma membrane compartment with which connexins are associated (and is consistent with our previous data in which the plasma membrane localization of Cx43 was similarly altered when the interaction between Cx43 and ZO-1 was disrupted by expression of a fusion protein containing the Cx43 binding domain of ZO-1 in ROS cells), diminished the formation of new gap junction plaques and reduced gap junctional communication in the transfected cells. Furthermore, expression of the ZO-1-derived fusion protein causes the redistribution of Cx43 from a non-raft-associated pool of proteins into a lipid raft-associated pool of protein. In contrast, overexpression of ZO-1 in ROS cells increases gap junction permeability and gap junction formation (Laing et al., 2005). Toyofuku and colleagues had previously used this approach to disrupt gap junction formation in cardiac myocytes and in HEK cells (Toyofuku et al., 1998).

ZO-1 bound to a truncated Cx45 protein that lacked the canonical PDZ binding domain-binding present at the carboxyl terminus (Cx45t34). We have previously demonstrated that ZO-1 could specifically bind to peptides consisting of the last 10 amino acids in Cx45 (Laing et al., 2001); the current result suggests that Cx45 may have a large and complex binding site for ZO-l, one that incorporates the residues between amino acid 357 (the carboxyl terminal of Cx45t37) and the Cx45 carboxyl terminus. In support of this notion, a recent study demonstrated that the second PDZ domain of ZO-1 binds to the last 21 amino acids in the carboxyl terminal of Cx43 (Sorgen et al., 2004). An alternative possibility is that there are two distinct binding sites for ZO-1 in Cx45,

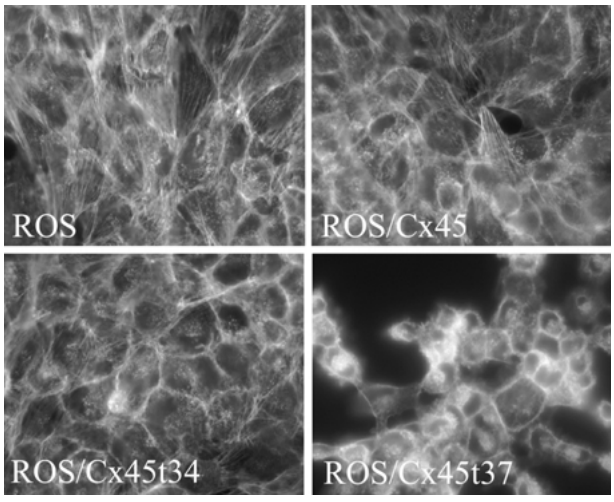

Fig. 7. Lack of actin stress fibers in ROS/Cx45t37 cells. Transfected ROS cells were fixed, permeabilized, and stained with Oregon 488 Phalloidin.

one that involves the carboxyl terminus and one that involves the amino acids proximal to amino acid 360. Mapping studies indicated that the interaction between the tight junction protein, AF-6, and ZO-1 involves 2 distinct domains within AF-6 (Yamamoto et al., 1997). We cannot presently exclude the possibility that we have created an artifactual ZO-1 binding site at the carboxyl terminus of Cx45t34. It is also possible that this interaction between Cx45t34 and ZO-1 could be indirect, as Cx45t34 associates with Cx43, which also binds to ZO-1. This notion is supported by the observation that Cx45 and Cx45t34 co-purify with Cx43 and ZO-1 on a sucrose flotation gradient, while Cx45t37 does not. Nonetheless, these two truncated proteins, Cx45t34 and Cx45t37, differ by three amino acids and in their ability to interact with ZO-1, which allows us to gain insights into the connexin-ZO-1 interaction in these cells.

As recent work has shown that many connexins may be targeted to lipid raft domains by binding to caveolin-1, it was rather surprising that both Cx43 and Cx45 were not associated with lipid rafts in the transfected ROS cells (Guan & Ruch, 1996; Schubert et al., 2002; Lin et al., 2003). The association of a membrane protein with a lipid raft was defined experimentally as a protein that is insoluble in cold 1% Triton X-100, and floats in a 5–30% sucrose flotation gradient (Chamberlain, 2004). Our data showed that Cx45t37 was solubilized by cold 1% Triton X-100 but was associated with a lipid raft as judged by flotation in a 5–30% sucrose flotation gradient (Chamberlain, 2004). This suggests that Cx45t37 may be associated with a lipid domain that is more soluble in non-ionic detergents than a classically defined lipid raft. Recent work has indicated that there may be a great deal of heterogeneity in lipid rafts found in cells, thus the detergent solubility of all the lipid raft-associated proteins may not be equivalent (Chamberlain, 2004). Indeed, the insulin receptor, a caveolae-associated protein, is soluble in 0.1% Triton X-100 but can float in sucrose gradients (Gustavsson et al., 1999). The data presented in this study further indicate that the solubility of the lipid rafts that connexins associate with may be further altered by an interaction with ZO-1.

Our studies showed that there was a striking lack of actin stress fibers in the ROS/Cx45t37 cells as compared to the ROS, ROS/Cx45 or ROS/Cx45t34 cells. A loss of stress fibers was seen in epithelial cells that were transfected with an amino terminal fusion protein derived from ZO-3, as well as a loss of tight junction complexes. These changes correlated with a loss of Rho activity in these cells. (Wittchen, Haskins & Stevenson, 2000, 2003). Recent studies have demonstrated that actin stress fibers are regulated by changes in Rho and Rho kinase activity and that changes in Rho kinase activity alter junctional stability (Sahai & Marshall, 2002). This suggests that there may be less activated Rho kinase activity in the ROS/Cx45t37cells, a possibility that should be determined by further experimentation. It is also possible that expression of Cx45t37 alters the disposition of actin stress fibers in these cells by other, more indirect means.

Taken together, our studies suggest that although ZO-1 is not required for connexin trafficking to the plasma membrane, it may play an important role in the compartmentalization of connexins within the plasma membrane. An interaction between ZO-1 and F-actin could cross-link lipid rafts into larger and heavier membrane domains, which do not enter our sucrose gradient. This effect may also be dependent on the presence of actin stress fibers in the ROS cells, as demonstrated by the altered mobility of Cx43 and ZO-1 on the ROS/Cx45t37 sucrose flotation gradients. This indicates that there may be two distinct molecular mechanisms involved in defining the physicochemical properties of the membrane domains with which osteoblastic connexins are associated, association with lipid rafts and association with the actin cytoskeleton. Recent studies have shown that the compartmentalization of a number of membrane proteins, including N-cadherin and αIIbβ3 integrin, can be regulated by interaction of both lipid rafts and the actin cytoskeleton (Holowka, Sheets & Baird, 2004; Bodin et al., 2005; Causeret et al., 2005).

This study showed that expression of Cx45 decreased gap junctional coupling in ROS cells, while expression of Cx45t34 and Cx45t37 increased gap junctional communication in ROS cells. This suggests that all three proteins alter gap junction communication in the transfected ROS cells, and that elimination of both the last 34 to 37 amino acids in Cx45 make gap junction channels that are more permeable to Lucifer Yellow. We have previ-

ously demonstrated that Cx45 and Cx45t37 interact with Cx43 in transfected ROS cells, in a manner that indicates that these proteins may interact with Cx43 gap junction channels (Koval et al., 1995). It is still not clear why expression of a truncated Cx45 construct increases the permeability of ROS gap junction channels.

Cx43 and Cx45t37 polypeptides are biochemically separable, suggesting that these channels are physically distinct. The similarity in Lucifer Yellow transfer in the ROS/Cx45t34 and the ROS/Cx45t37 cells suggested both truncated proteins may alter dye coupling through a similar mechanism and that this effect was not dependent upon an interaction of Cx45 with ZO-1. We do not know if the gap junctions formed by either Cx45t34 or Cx45t37 would be permeable to Lucifer Yellow.

In summary, these studies demonstrate that altering the Cx–ZO-1 interaction by removing the ZO-1 binding site of Cx45 alters the distribution of Cx45 within the plasma membrane. They also suggest that in the absence of ZO-1, connexins reside within a Triton-soluble lipid raft environment at the plasma membrane, and that ZO-1 serves to recruit connexins to or stabilize connexins within a more discrete gap junction environment, possibly by interacting with the actin cytoskeleton. Thus, the connexin–ZOl interaction may be an important regulator of gap junction formation and maintenance.

This work was supported by the American Heart Association Scientist Development Grant 990013N (to J.G.L, and National Institutes of Health Grants DK-46686 and GM-54660 (to T.H.S.).

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