The Gap Junction Protein Connexin32 Interacts with the Src Homology 3/Hook Domain of Discs Large Homolog 1*

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Scaffolding of membrane proteins is a common strategy for forming complexes of proteins, including some connexins, within membrane microdomains. Here we describe studies indicating that Cx32 interacts with a PDZ-containing scaffolding protein, Dlg1 (Discs Large homolog 1). Initial screens of liver lysates using antibody arrays indicated an interaction between Cx32 and Dlg1 that was confirmed using coimmunoprecipitation studies. Yeast two-hybrid complementation determined that the Cx32 bound via interaction with the SH3/Hook domain of Dlg1. Confocal microscopy of liver sections revealed that Cx32 and Dlg1 could colocalize in hepatocyte membranes in wild type mice. Examination of levels and localization of Dlg1 in livers from Cx32 null mice indicate that, in the absence of Cx32, Dlg1 was decreased, and the remainder was translocated from the hepatocyte membrane to the nucleus with some remaining in cytoplasmic compartments. This translocation was confirmed by Western blots comparing Dlg1 levels in nuclear extracts from wild type and Cx32 null murine livers. Using SKHep cells stably transfected with Cx32 under the control of a tet-off promoter, we found that acute removal of Cx32 led to a decrease of membrane-localized Dlg1 and an increase in the nuclear localization of this tumor suppressor protein. Together, these results suggest that loss of Cx32 alters the levels, localization, and interactions of the tumor suppressor protein Dlg1, events known in other systems to alter cell cycle and increase tumorigenicity.

Connexins, of which there are more than 20 isoforms in mice and humans, are tetraspan proteins that oligomerize to form hexameric connexons. Connexons contributed by each cell interact head-to-head across the extracellular space, forming channels providing direct cytoplasmic continuity between cells.

Aggregates of these channels, gap junctions, are found in almost all tissues. Connexins have intracellular amino-terminal cytoplasmic loops and carboxyl-terminal domains. Although much of the connexin sequence is highly conserved throughout the protein family, the amino acid sequences of intracellularly localized cytoplasmic loop and carboxyl-terminal domains vary markedly between connexin isoforms, and these regions likely confer isoform specificity. Carboxyl-terminal connexin domains, in particular, contain multiple sites for protein-protein interactions. For example, the major gap junction protein of heart and astrocytes, Connexin43 (Cx43), possesses binding sites for Src homology 3 (SH3), SH2, WW, MAPK (mitogen-activated protein kinase), and PDZ within its carboxyl-terminal domain, and other connexins, although less well mapped, also display potential binding sites. The function of these protein-protein interactions is not well understood, but many of them regulate the function of the gap junction channel (1). Interestingly, analysis of the primary sequence of the α subdivision of connexins (which includes Cx43) for consensus binding sites indicates that many of these connexins have potential PDZ interaction domains, whereas the primary sequences of the β subdivision of connexins (including the major gap junction protein of liver and myelinating glia, Cx32) do not contain these potential interaction sites. This leads to the question of whether the α connexins are scaffolded while the β connexins are not or whether there are alternative binding domains within the PDZ-containing proteins that interact with β connexins.

The PDZ domain-containing proteins, including Discs Large and its mammalian homolog (Dlg1), belong to a family of proteins known as membrane-associated guanylate kinases (MAGUKs). These proteins have multiple functions, including scaffolding of ion channels, heterodimerization to form protein microdomains, and regulation of junctional complexes. Dlg1, in particular, has been shown to be involved in cellular growth control via a nuclear translocation mechanism (2). The PDZ domains are the primary sites of protein-protein interactions with many macromolecular components, but the SH3 region, with its “hook” region also participates in the formation of protein-protein interactions (reviewed in Funke et al. (3)).
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We here report our studies examining the interaction of a β connexin, Cx32, with the MAGUK protein Dlgh1. In normal murine livers, Cx32 and Dlgh1 were found to colocalize at sites of cell-cell contact, whereas in livers of Cx32 null mice and in hepatoma cells, where Cx32 was acutely down-regulated, expression of Dlgh1 was reduced and the remaining Dlgh1 protein was largely localized within the nucleus. As revealed by yeast two-hybrid complementation, the interaction of Cx32 with Dlgh1 is non-canonical, involving the SH3/Hook/GUK region rather than the PDZ domains. When Dlgh1 is localized to cell membranes, it regulates cell growth, blocking cell cycle in the G0/G1 phase. Translocation of hDlg to the nucleus has been shown to increase cell proliferation (3). Loss of Cx32 has been shown to promote the formation of induced hepatic tumors (4, 5). Thus, we propose that one mechanism by which Cx32 may act to control cell proliferation in liver is through maintenance of membrane-associated Dlgh1 in addition to the intracellular communication that gap junctions provide.

EXPERIMENTAL PROCEDURES

Liver Lysate Preparation—For antibody arrays (whole lysates from 5 mg of liver tissue), Western (50 μg) and coimmunoprecipitation studies (whole lysates from 5 mg of tissue protein matched across samples). Adult C56B mice were anesthetized using isoflurane and decapitated prior to removal of the liver. Whole liver was extracted, rinsed in phosphate-buffered saline (PBS), pH 7.4, and lysed in complete lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25 mM sodium-deoxycholate, 150 mM NaCl, 2 mM EGTA, 0.1 mM Na3VO4, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, ½ tablet of Complete protease inhibitor (Roche Applied Science). Lysates were sonicated for 30 s, maintained on ice for 30 min, and then triturated and spun at 10,000 revolutions/min for 10 min. Total protein was assessed using the BCA protein assay kit (Pierce). Lysates were then equalized to 50 μg by dilution in lysis buffer. The supernatant was retained for use in arrays and coimmunoprecipitation studies.

Antibody Arrays—Hypermatrix (Worcester, MA) antibody arrays were reacted with liver lysate and probed with HRP-tagged Cx32 antibody according to the manufacturer’s recommendations. In-house antibody arrays were produced on polyvinylidene difluoride membranes and spotted with 32 different antibodies (6). For this, membranes were wet in 100% methanol and rinsed three times in PBS. Antibodies (0.05 μg/spot) were individually spotted onto the membrane using a BioDot array spotter (Bio-Rad). All arrays were made in triplicate for statistical analysis. Following drying of the array, the membrane was blocked for 2 h in 5% skim milk with 0.05% Tween 20, incubated with lysates from whole liver (see above) for 2 h, rinsed three times for 10 min in PBS with 0.05% Tween 20 (PBST), exposed to HRP-conjugated Cx32 antibody (antibody conjugated to HRP using the EZ Link Biotech kit (Pierce catalog number 31489) according to the manufacturer’s instructions for 2 h in 5% skim milk and then rinsed, and HRP was detected using the ECL protein detection kit (Amersham Biosciences). Membranes were exposed to x-ray film and then developed on an Exomat developing system.

Coimmunoprecipitation Studies—Coimmunoprecipitations were performed as previously described (3). Briefly, whole liver lysates were treated as noted above to produce supernatant (50 μg of total protein). Supernatant was precleared by spinning with 50 μl of Sepharose A beads (Amersham Biosciences) in buffer (250 mg of Sepharose A), incubated in 2 ml of lysis buffer on ice for 15 min. The beads were spun at 3000 revolutions/min for 3 min and washed 3 times in lysis buffer. Following the wash, the beads were diluted 1:1 beads/lysis buffer and kept at 4 °C. Following preclear, 1.5 μg of Cx32 antibody was added (Cx32, polyclonal Zymed Laboratories Inc., Temecula, CA), and Dlgh1, monoclonal antibody (Affinity Bioreagents), and supernatant were rotated for 4 h at 4 °C. Thereafter, 100 μl of Sepharose beads was added, and the sample was rotated overnight at 4 °C. The following day, the sample was spun at 5000 revolutions/min for 10 min and supernatant discarded. The beads were rinsed six times in lysis buffer, incubated in 60 μl of loading buffer (2× Laemmli buffer plus dithiothreitol), incubated in 100 °C water for 2 min, and then placed immediately on ice for 2 min. The sample was then spun and the loading buffer removed from the beads for running on Western blots using 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membrane, and Cx32 or Dlgh1 were detected with the appropriate antibodies.

Yeast Two-Hybrid Complementation Assays—The Saccharomyces cerevisiae yeast strain AH109 was cotransformed with the following GAL4ad fusion constructs: GAL4ad-NT (hDlg-(1–233)), GAL4ad-PDZ1-2 (hDlg-(200–463)), GAL4ad-PDZ3 (hDlg-(451–587)), GAL4ad-SH3/Hook (hDlg-(569–764)), and GAL4ad-SV40 Large T antigen (control) or with the following GAL4bd fusion constructs: GAL4bd-Cx32CT (Cx32-(209–283)), GAL4bd-Cx32CL (Cx32-(96–127)), GAL4bd-Cx32NT (Cx32-(1–22)), and GAL4bd-pVA3 murine p53 (control). Cotransformants were assayed for growth on nonselective (+His) and selective (−His) media.

Cell Culture—Parental and Cx32-tet-off transfected SkHep cells were plated directly on coverslips and grown until 80–90% confluent. The cells were treated with 30 μM tetracycline (13) for 0, 24, 48, or 96 h and used for immunocytochemistry.

Immunofluorescence Microscopy—Rapidly frozen liver samples were sectioned (15 microns) using a Shandon electronic cryotome. Sections or cultured SkHep1 cells were fixed in 4% paraformaldehyde for 30 min at room temperature and then incubated in 50 mM NH4Cl for 3 h to quench autofluorescence. Following quench, sections were blocked (PBS plus 10% goat serum) for 1 h at room temperature and then incubated with primary monoclonal antibodies directed against Cx32 (Sigma) and polyclonal Dlgh1 SAP97 (Affinity Bioreagents) at 4 °C overnight. Following 30 min of rinsing (3× for 10 min in PBS plus 0.4% Triton X-100) for 1 h at room temperature and then incubated with primary monoclonal antibodies against Alexa Fluor anti-mouse 488 and anti-rabbit 595) for 1 h at room temperature. The slides were rinsed for 50 min (5× for 10 min) and mounted on glass microscope slides with Vectashield anti-fade agent (Vector Laboratories, Burlingame, CA) and examined on a confocal microscope (Olympus BX61WQ Fluoview confocal system). Images were processed using Imaris Image Pro software.

Nuclear Extraction—For SKHep1 cells, at 0, 24, 49, and 96 h time points, 100-mm dishes of confluent SKHep cells were
washed with PBS and prepared for nuclear extraction (Bio-Rad) according to their instructions. Samples were subjected to protein level determination using the BCA protein assay kit (Pierce), run on either 10% (Cx32) or 7.5% (Dlgh1) SDS-polyacrylamide gels, and probed using antibodies against Cx32 and Dlgh1, respectively, as described above.

Segregation of Nuclear and Cytoplasmic Fractions—Isolation and preparation of cells harvested from whole mouse liver. Wild type and Cx32 null mice were anesthetized with isoflurane (inhaled). The left ventricle was located and perfused with an EGTA-containing solution (0.5 mM EGTA, 15 mM HEPES, 130 mM NaCl, 0.5 mM Na2HPO4, 10 mM glucose, 4 mM KCl, 1 mM MgCl2, 5 mM taurine) to remove blood and flush the organs; the right atrium was cut to facilitate solution outflow. The system was perfused until the liver was completely blanched, and the liver was dissected and washed with PBS. To obtain a cell suspension, the liver was ground with a pestle and successively strained through two mesh cell separators of 230 and 80 μm each. The cells were collected by centrifugation, washed twice with PBS, and placed on ice.

Analysis of Cytoplasmic and Nuclear Proteins for Cx32 and Dlgh1—The cytoplasmic and nuclear samples were assayed for protein concentration (Bio-Rad) and their volumes determined. Cytoplasmic (50 μg) and a proportional amount of nuclear protein were loaded in Laemmli buffer for separation by 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Millipore) for Western blotting. The membranes were probed with antibodies to Cx32 and Dlgh1 (Santa Cruz Biotechnology) and visualized on x-ray film using chemiluminescence (Pierce). The purity of the cytoplasmic and nuclear fractions was confirmed by probing the membranes with antibodies to markers of the cell cytoplasm (Rho-GDI, Santa Cruz Biotechnology) and nucleus (Histone H1, NeoMarkers; or Lamin A/C, Santa Cruz Biotechnology). Western blotting was analyzed by densitometry.

Measurements of Junctional Conductance—Cell pairs in cultures seeded at low density were voltage-clamped at a holding potential of 0 mV using patch pipettes connected to an Axc1C patch clamp amplifier; junctional conductance was calculated as the current recorded in one cell divided by a 10 mV step applied to the other cell.

RESULTS

In targeted immunoprecipitation experiments with likely partners, Cx32 has previously been shown to interact with Caveolin1 (7), Cx26 (8), occludin (9), and calmodulin (10). To identify additional binding partners, using an unbiased approach.

FIGURE 1. Antibody array detection of Cx32 interaction with Dlgh1. A, antibody arrays were spotted with different antibodies (Spot a = ANT; Spot b = Claudin-1; Spot c = Dlgh1; Spot d = VDAC), incubated with lysates from murine liver, and then probed with HRP-tagged anti-Cx32 antibodies (Sigma). Although there was slight reaction above background with Spots a, b, and d, a strong HRP signal was seen at Spot c on all arrays. This indicates the presence of Cx32 in the complex captured by the Dlgh1 antibody. B, intensity of interactions was determined by densitometry. Note the high intensity of the interaction of Cx32 with the complex captured by the Dlgh1 antibody, suggesting an interaction between Cx32 and Dlgh1 (n = 3). Confirmation of the interaction between Cx32 and Dlgh1 was done using coimmunoprecipitation studies. C, lysates of murine liver were incubated with antibodies directed against either Cx32 (Cx32IP) or Dlgh1 (Dlgh1IP) and probed for Dlgh1. Note that both the Cx32 antibody as well as the Dlgh1 antibody pulled down a band of 140 kDa labeled with anti-Dlgh1. The reverse coimmunoprecipitation experiment shows that both the Cx32 antibody as well as the Dlgh1 antibody pull a protein that labels with the antibody directed against Cx32. These studies confirm the antibody array data and indicate that Cx32 and Dlgh1 are within the same protein complex in murine liver. Western blot analysis of Dlgh1 was done from wild type and Cx32 null murine livers (C, lanes 1 and 2). The protein levels were normalized against β-actin and GAPDH, and the change in Dlgh1 was quantified. Quantification of total levels of Dlgh1 shows an ~75% decrease in cytoplasmic Dlgh1 protein in the liver from Cx32 null mice (D).
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approach, we initially screened for Cx32 interactions within liver lysates using Hypromatrix antibody arrays, finding a number of novel potential interactions including Dlgh1 (results not shown). These were followed up with in-house antibody arrays in which we spotted membranes with antibodies that we had screened for specificity for the target protein. When incubated with whole liver lysates and probed with HRP-tagged anti-Cx32, the in-house antibody arrays indicated that, within liver, Cx32 interacts with several complexes of proteins, including a complex containing the scaffolding protein Dlgh1 (Fig. 1A). In this illustration, Spot c contained antibodies directed against Dlgh1, and Cx32-HRP binding determined that Cx32 was within the complex of proteins captured at this spot. Densitometric analysis of the spot indicates that the signal was much stronger for the Cx32 interaction with Dlgh1 than with ANT (adenine nucleotide translocator) protein (spot a), Claudin-1 (spot b), or VDAC (voltage-dependent anion-selective channel) (spot d), although for each of these spots the signal was higher than background, presumably indicating weak or higher order interactions with Cx32 (Fig. 1B, n = 3). The Dlgh1-Cx32 interaction was confirmed using coimmunoprecipitation studies (Fig. 1C). The reverse coimmunoprecipitation also indicated an interaction between these two proteins with the Dlgh1 antibody immunoprecipitates containing a 32-kDa protein that was immunoreactive to the Cx32 antibodies. Immunoprecipitation of Dlgh1 from Cx32 null livers indicated that there is no signal for Dlgh1 when Cx32 antibody was used for pulldowns in the Cx32 null background (data not shown). As a control for immunoprecipitation experiments, we performed Western blots of liver homogenate from Cx32 null mice. As shown in Fig. 1C, second lane, Dlgh1 remained in the liver lysate from Cx32 null mice. However, in quantitative experiments in which Dlgh1 levels were normalized against β-actin levels, Dlgh1 abundance was substantially reduced (~4-fold) in livers from Cx32 null mice (1D).

It was of interest to determine whether the interaction between Cx32 and Dlgh was via a PDZ domain, particularly because the Cx32 carboxyl-terminal domain does not contain a canonical PDZ interaction site. Thus, we performed a yeast two-hybrid assay to identify which of the Cx32 and Dlgh1 domains physically associate in vivo (Fig. 2). The CT, CL, and NT domains of Cx32 and the NT, PDZ1–2, PDZ3, and SH3/Hook domains of Dlgh1 (see “Experimental Procedures”) were coexpressed in AH109 yeast strain AH109 was cotransformed with GAL4ad fusion constructs GAL4ad-NT (hDlg-(1–233)), GAL4ad-PDZ1–2 (hDlg-(200–463)), GAL4ad-PDZ3 (hDlg-(451–587)), GAL4ad-SH3/Hook (hDlg-(569–764)), and GAL4ad-SV40 Large T antigen (control) or with GAL4bd fusion constructs GAL4bd-Cx32CT (Cx32-(96–127)), GAL4bd-Cx32CL (Cx32-(128–163)), and GAL4bd-pV5A mouse p53 (control). Cotransformants were assayed for growth on nonselective (+His) and selective (~His) media. ad, activation domain; bd, DNA-binding domain. B, schematic representation of the interaction between Cx32 and Dlgh1. The exact amino acid sequences of the Cx32 are as yet unknown.

FIGURE 2. Characterization of the hDlg and Cx32 interaction by the yeast two-hybrid assay. The S. cerevisiae yeast strain AH109 was cotransformed with GAL4ad fusion constructs GAL4ad-NT (hDlg-(1–233)), GAL4ad-PDZ1–2 (hDlg-(200–463)), GAL4ad-PDZ3 (hDlg-(451–587)), GAL4ad-SH3/Hook (hDlg-(569–764)), and GAL4ad-SV40 Large T antigen (control) or with GAL4bd fusion constructs GAL4bd-Cx32CT (Cx32-(96–127)), GAL4bd-Cx32CL (Cx32-(128–163)), and GAL4bd-pV5A murine p53 (control).
signal seen in the nuclear component, whereas Cx32 was absent in the nuclear fraction, we found that Cx32 was primarily present in the cytoplasmic fraction and histone to determine the purity of the nuclear fraction. Using Rho-GD1 to determine the purity of the cytoplasmic fraction, liver nuclear extracts from both wild type and Cx32 knock-out mice were blotted for Cx32 and Dlgh1 (K). Histone was used to determine the purity of the nuclear fraction, whereas Rho-GD1 was used to determine the purity of the cytoplasmic fraction. The nuclei of wild type animals (n = 3) showed little Cx32 but did contain a small amount of Dlgh1, whereas nuclei from Cx32 knock-out mice (n = 3) showed no Cx32 but had a large increase in the levels of Dlgh1 (K) within these purified nuclear extracts (L). There were no changes in overall cadherin levels (M), and localization of cadherin in the Cx32 null hepatocytes (O) was identical to that seen in the wild type hepatocytes (N).

Cx32 null liver sections using the Imaris colocalization algorithms (11). Regions of the wild type liver were selected in which colocalization profiles of Cx32 with Dlgh1 were characterized (Fig. 4, high magnification image in upper right panel), and regions of Cx32 null liver sections were selected for calculating colocalization of Dlgh1 with DAPI-stained nuclei (Fig. 4, high magnification image in upper right panel). Wild type liver exhibited regions along cell membranes where Cx32 and Dlgh1 were colocalized within the entire confocal stack (Fig. 4, lower right panel). In contrast, the localization profile of Dlgh1 showed strong colocalization with the nuclei in Cx32 null livers throughout the confocal stack (Fig. 4, lower right panel). Chronic long term loss of Cx32, as is seen in the Cx32 null mouse, might be expected to trigger alterations in cell function that subsequently lead to a loss of Dlgh1 at cell membranes. Thus, the loss of Cx32 might not be a primary step in the Dlgh1 alterations seen in the Cx32 null livers. To determine whether similar changes occurred with acute loss of Cx32, we used the

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Using Rho-GD1 to determine the purity of the cytoplasmic fraction and histone to determine the purity of the nuclear fraction, we found that Cx32 was primarily present in the cytoplasmic fraction of cells from wild type livers, with little signal seen in the nuclear component, whereas Cx32 was absent from both liver fractions from Cx32 null mice. The small amount of Cx32 seen in the nucleus in wild type liver likely corresponds to a small cytoplasmic contaminant evidenced by a low but visible Rho-GD1 signal in the wild type liver nuclear fraction. Dlgh1 was found in the cytoplasm and at low levels in the nucleus of the hepatocytes from wild type livers. However, there was a large increase in the levels of Dlgh1 within the nuclear fraction of the Cx32 null livers (Fig. 3, K and L).

Cadherin, the functional component of adherens junctions, functions to maintain normal cell-cell contact. To determine whether there were changes in cadherin expression that might alter the ability of cells to adhere and form proper cell-cell contacts in the Cx32 null livers, we evaluated cadherin levels using Western blot analysis and immunostaining. Using a pan-cadherin antibody, which identifies both the 135-kDa isoform as well as a 120-kDa isoforms, we found no significant difference between the levels of cadherin in hepatocytes from Cx32 null livers (Fig. 3M). In conjunction with the Western blot analysis, immunostaining studies of cadherin revealed no difference in the staining pattern for cadherin in wild type and Cx32 null liver sections (Fig. 3, N and O). Co-localization analysis of Cx32, Dlgh1, and nuclei were done on confocal stacks of images from both wild type and

(FIG. 3K). Using Rho-GD1 to determine the purity of the cytoplasmic fraction and histone to determine the purity of the nuclear fraction, we found that Cx32 was primarily present in the cytoplasmic fraction of cells from wild type livers, with little signal seen in the nuclear component, whereas Cx32 was absent from both liver fractions from Cx32 null mice. The small amount of Cx32 seen in the nucleus in wild type liver likely corresponds to a small cytoplasmic contaminant evidenced by a low but visible Rho-GD1 signal in the wild type liver nuclear fraction. Dlgh1 was found in the cytoplasm and at low levels in the nucleus of the hepatocytes from wild type livers. However, there was a large increase in the levels of Dlgh1 within the nuclear fraction of the Cx32 null livers (Fig. 3, K and L).

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normally connexin-deficient SkHep1 cell human hepatoma cell line transfected with Cx32 driven by a tet-off promoter (12). In the absence of tetracycline, Cx32 is robustly expressed in these cells (Fig. 5A), and Dlgh1 is prominent at cell membranes (Fig. 4). Overlay shows regions of overlap of these two proteins. Dual whole cell voltage clamp studies on cell pairs indicate that these cells are highly coupled (Fig. 5M). After 24 h of tetracycline treatment, Cx32 was no longer seen at cell membranes (Fig. 5D); Dlgh1 was still present, although its localization at the membrane was patchy, generally not fully encircling the cells (Fig. 5E). Overlay of these images shows substantially less colocalization of these two proteins than before tetracycline treatment (Fig. 5F). Junctional conductance was reduced by >10-fold in cells treated for 24 h with tetracycline (Fig. 5M). Extending the tetracycline treatment to 48 h further decreased Cx32 levels (Fig. 5G), and Dlgh1 was primarily located in the cytoplasm rather than at the membrane (Fig. 5H). Junctional conductance remained very low (Fig. 5M). Overlay of the images indicates no colocalization of Cx32 and Dlgh1. By 96 h of treatment, complete loss of Cx32 protein occurred (Fig. 5J), and Dlgh1 was then seen only localizing with cell nuclei (Fig. 5K, overlay 5L).

**DISCUSSION**

Initial studies showing interaction of connexins with scaffolding proteins have begun to find multiple interacting proteins with a small subset of connexins. The studies reported here are the first to show interaction of Cx32 with such binding partners. Although connexins turn over in the plasma membrane rather rapidly (13), scaffolding proteins remain. Thus, although the connexins may aid in the organization of the microdomain, the scaffolding protein is likely to be the more stable link. This would set up a “target” for cycling connexins that may underlie how connexins are added to the plaque along the outer edges of the plaques (14). Cross-talk between these proteins could then allow for a symbiotic relationship where, at least in this case, Cx32 may “signal” where the microdomain forms, and once recruited, Dlgh1 is needed to form a stable platform. Thus, we

**FIGURE 5.** Confocal studies of SkHep cells transfected with tet-off Cx32. SkHep cells were immunostained for either Cx32 (green) or Dlgh1 (red) and examined for the amount of overlay of the two proteins. A shows images of non-tetracycline-treated SkHep cells, where Cx32 can be seen at sites of cell-cell contact, and as is common with connection-transfected cells, perinuclearly. Dlgh1 can be seen around the periphery of the cells outlining the cell membranes (B). Overlay images show that these two proteins show areas of colocalization in these untreated SkHep cells (C). Following 24 h of tetracycline treatment, Cx32 was reduced to the perinuclear fraction (D), whereas the Dlgh1 was still seen on parts of the membrane, although slightly decreased as compared with the untreated cells (E). Overlay images show less colocalization of Cx32 with Dlgh1 following the 24 h treatment period (F). G shows that 48 h of tetracycline treatment obliterates almost all Cx32, and Dlgh1 is then found to be almost exclusively nuclear or perinuclear (H). Overlay images show no colocalization between the two proteins (I). By 96 h of tetracycline treatment, there was no Cx32 expression (J), the Dlgh1 was localized within the nuclei (K), and again, no colocalization of the two proteins was seen (L). Electrophysiological studies indicate that the Cx32 forms functional channels in these cells when tetracycline is absent, but coupling is lost as early as only 24 h after treatment and remained low for the entire treatment period (M).
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hypothesize that, when Cx32 is initially removed from the cells, the Dlg1 remains for a while, but when newly translated Cx32 does not insert into the plasma membrane, the organizational signal is lost and the Dlg1 is no longer directed to the plasma membrane but rather to the cell nucleus as a portion of the signal that indicates loss of cell-cell coupling. The remaining Dlg1 is degraded over time, causing a delay between the loss of Cx32 and translocation of Dlg1.

Previous reports suggest that, in addition to providing direct intracellular communication between coupled cells, connexins (either alone or as components of the Nexus (15) complex) might be involved in other processes, including growth control (16–18), migration (19), intercellular and cell-matrix adhesion (20), and signaling through intracellular space (20–22). There are also numerous studies reporting that manipulation of gap junction gene expression modifies the expression of other genes (23). There are at least two distinct mechanisms by which expression of other genes may be modified by expression of connexins, channel-dependent mechanisms and channel-independent mechanisms. Channel-dependent mechanisms that have been proposed include the direct transfer of genetic information, a concept that was attractive in the early days of gap junction research but turned out to be artifactual (24). However, this possibility has recently reappeared with the demonstration that RNA interference-sized oligonucleotides may pass from cell to cell (25) and by a report that gap junctions may permit passage of large peptides, permitting “cross-presentation” of antigens (26). Moreover, it has been proposed that intracellular communication provided by Cx43 can modulate gene transcription in osteocytes by differential recruitment of Sp1/Sp3 transcription factors to the osteocalcin promoter (27, 28). In this scenario, the effectors are believed to be gap junction-permeant second messenger molecules that may act through increasing or decreasing the phosphorylation state of transcription factors. Connexin-dependent but channel-independent mechanisms for the control of gene expression include the possibility that the carboxyl-terminal domain of Cx43 can translocate to the nucleus, acting there as a transcription factor (29), or that connexins might bind to molecules with both membrane or cytosolic localization and also with transcriptional activity; in this scenario, varying connexin abundance would lead to changes in the expression of other genes by shifting the equilibrium between the connexin-bound pool and a nonbound pool that could translocate to the nucleus. The first evidence for this mechanism was the study of Wnt-1 regulation of Cx43 expression and function in cardiac myocytes (30); β-catenin was shown to interact directly with Cx43 and also to translocate to the nucleus when activated by Wnt-1 signaling. β-catenin transactivated Cx43 transcription, providing a mechanism whereby Cx43 binding to another molecule, independent of its channel function, could regulate its own transcription. A similar mechanism has been recently proposed involving the growth regulator CCN3 (NOV) (31, 32), in which NOV is localized to Cx43 at the membrane. When Cx43 is down-regulated, NOV translocates to the nucleus producing a transformed phenotype with increased growth rate. Similarly, Penes et al. (33) have reported that the transcription factor ZO-1-associated nucleic acid-binding protein (ZONAB) is colocalized and coimmunoprecipitates with ZO-1 and connexins in astrocytes and oligodendrocytes, providing an additional substrate for the regulation of transcription factor availability by the abundance of connexins. Nuclear localization of members of the MAGUK family of proteins has been reported for both ZO-1 and CASK (calcium/calmodulin-dependent serine kinase) with nuclear translocation causing alterations of cell proliferation, activation of the TCF/LEF family of transcription factors and changes in the levels of cell cycle regulators such as Cyclin D and c-Myc (34). Our finding that, in response to cellular perturbation, Dlg1 also translocates to the nucleus expands the repertoire of regulatory roles of MAGUK proteins. Regulation of this translocation may be one mechanism by which Cx32 regulates cell proliferation.

In summary, we describe here the novel interaction of Cx32 and the scaffolding protein Dlg1 by a noncanonical interaction of the SH3/Hook domain of Dlg1 and the Cx32 cytoplasmic loop and carboxyl tail. Further research is needed to determine the functions of this interaction and will likely yield interesting information on the role of connexins in macromolecular complexes.

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