Connexin 43 Interacts with Zona Occludens-1 and -2 Proteins in a Cell Cycle Stage-specific Manner*

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Gap junction channels play an important role in cell growth control, secretion and embryonic development. Gap junctional communication and channel assembly can be regulated by protein-protein interaction with kinases and phosphatases. We have utilized tandem mass spectrometry (MS/MS) sequence analysis as a screen to identify proteins from cell lysates that interact with the C-terminal cytoplasmic region of connexin 43 (Cx43). MS/MS analysis of tryptic fragments yielded several proteins including zona occludens-1 (ZO-1), a structural protein previously identified to interact with Cx43, and ZO-2, a potential novel interacting partner. We confirmed the interaction of ZO-2 with Cx43 by using a combination of fusion protein “pull down,” co-immunoprecipitation, and co-localization experiments. We show that the C-terminal region of Cx43 is necessary for interaction with the PDZ2 domain of ZO-2. Far Western analysis revealed that ZO-2 can directly bind to Cx43 independent of other interacting partners. Immunofluorescence studies indicate that both ZO-1 and ZO-2 can co-localize with Cx43 within the plasma membrane at apparent gap junctional structures. We examined Cx43 interaction with ZO-1 and ZO-2 at different stages of the cell cycle and found that Cx43 had a strong preference for interaction with ZO-1 during G2, whereas ZO-2 interaction occurred approximately equally during G1 and S phases. Since essentially all of the Cx43 in G0 cells is assembled into Triton X-100-resistant junctions, Cx43-ZO-1 interaction may contribute to their stability.

Gap junctions are tightly packed clusters of intercellular channels that directly connect the cytoplasm of adjacent cells. These pathways provide for the cell-to-cell diffusion of small molecules, including ions, amino acids, nucleotides, and secondary messengers (e.g. Ca²⁺, cAMP, cGMP, inositol 1,4,5-trisphosphate) and for the conductance of electrical impulses in excitable cells. There are at least 20 gap junctional protein or connexin family members in humans, many of which have been cloned and characterized in mice (1, 2). Connexin expression is tissue specific, and connexin 43 (Cx43) is the predominant connexin in epithelial and most other tissues. Recent studies utilizing transgenic mice with altered connexin genes and linkage of connexin gene alterations to human disease provide strong support for roles in cell growth control and embryonic development. For example, the homozygous deletion of Cx37, Cx40, Cx43, and Cx45 causes neonatal or embryonic lethality in mice (1–3). In humans, mutations in Cx26, Cx30, Cx31.1, Cx32, Cx43, Cx46, and Cx50 are associated with deafness/hearing loss, skin disorders, Charcot-Marie-Tooth disease, developmental defects, and cataract formation (1, 3).

Recent studies have shown that Cx43 can interact with several different signaling and scaffolding proteins. Perhaps the most well known is the interaction of zona occludens-1 (ZO-1) with the carboxyl terminus of Cx43 (4, 5). Originally identified as a component of tight junctions, ZO-1, ZO-2, and ZO-3 are members of the membrane-associated guanylate kinase family of proteins that each contain at least one PSD95/Dlg/ZO-1 (PDZ) domain, an Src homology 3 domain, and an enzymatically inactive guanylate kinase domain (6–9). PDZ domains are ~90-amino acid protein-protein binding domains that recognize at least a 3-residue peptide motif in the COOH termini of their binding partners (10). PDZ domain-containing proteins like ZO-1 typically act as scaffolding proteins that organize membrane receptors and cytosolic proteins into multimolecular signaling complexes often at the sites of cell-cell contact (11, 12). Divergent roles have been proposed for the interaction of ZO-1 and Cx43 including the control of gap junction formation and localization to gap junction plaques (5), internalization and remodeling of Cx43 in response to intracellular changes (13, 14), and targeting for endocytosis (15). Furthermore, c-Src interaction with Cx43 has been shown to regulate Cx43-ZO-1 interaction (16–18).

Other proteins that have been shown to interact with Cx43 include several kinases and cytoskeletal proteins such as c- and v-Src kinase, protein kinase C, mitogen-activated protein kinase, casein kinase 1, cAMP-dependent protein kinase, receptor protein-tyrosine phosphatase , alpha/beta tubulins, CCN3/NOV, and Drebrin (e.g. see Ref. 19 for review and Refs. 20–22). Other connexins, including Cx31.9, Cx45, Cx46, and Cx50, have also been shown to interact with ZO-1 (19, 23–25). Although several potential functions have been proposed for these protein-Cx43 interactions, the roles that they play in the regulation of Cx43 trafficking, assembly, gating, and turnover are not well understood. We believed that a thorough analysis and classification of Cx43-interacting proteins might elucidate these roles, so we employed a proteomic approach to identify proteins that interact with Cx43 directly or via a protein complex.

kiddgen, CT, carboxyl-terminal; GST, glutathione S-transferase; PBS, phosphate-buffered saline; ZO-1, zona occludens-1; ZO-2, zona occludens-2; MS/MS, tandem mass spectrometry; PDZ, PSD-95/Discs-large/ ZO-1; HA, hemagglutinin.

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1 The abbreviations used are: Cx43, connexin 43; NRK, normal rat kidney; CT, carboxyl-terminal; GST, glutathione S-transferase; PBS, phosphate-buffered saline; ZO-1, zona occludens-1; ZO-2, zona occludens-2; MS/MS, tandem mass spectrometry; PDZ, PSD-95/Discs-large/ZO-1; HA, hemagglutinin.
Our mass spectrometry-based approach yielded many potential binding partners (26), and here we report that Cx43 directly binds to ZO-2. We have characterized the interaction of ZO-2 with Cx43 by using a combination of “pull down,” co-immunoprecipitation, Western, and immunofluorescence experiments. We present evidence that the C-terminal tail of Cx43 interacts directly with ZO-2 in vitro, in cultured cells, and in cardiac tissue. Our data suggest that ZO-1 and ZO-2 are key scaffolding proteins that can differentially bind to Cx43 at the membrane junctional complex during the G0 stage of the cell cycle. Our results support that ZO-1 and ZO-2 are karyotyping proteins that can differentially bind to Cx43 at the membrane junctional complex during the G0 stage of the cell cycle.

EXPERIMENTAL PROCEDURES

Plasmids—N-terminal, HA-tagged wild type ZO-2 (HA-ZO-2) was cloned in the cytomegalovirus expression vector GW1 as previously described to generate GWHA1-ZO-2 (27). ZO-2 sequences encoding the PDZ1 (amino acids 4–119), PDZ2 (amino acids 290–371), and PDZ3 (amino acids 492–550) domains were cloned in the PEGX-2T vector to generate glutathione S-transferase (GST)-PDZ1, GST-PDZ2, and GST-PDZ3, respectively. The Cx43 sequence encoding for the carboxyl terminal (CT) amino acids 236–382 of Cx43 was cloned into pGEX-2T or pGEX-2TK vector to generate GST-Cx43CT. CT deletion constructs Δ290 (missing amino acids 280–300), Δ330 (missing amino acids 321–340), and Δ369 (missing amino acids 364–373), T-374 (missing amino acids 375–382), and T-379 (missing amino acids 380–382) were all cloned into the pGEX-2T vector to generate the respective described GST deletion constructs. GST constructs were transformed into DH5α Escherichia coli, and GST fusion proteins were expressed and purified as previously described (28). The recombinant proteins were quantified in gels by Coomassie Brilliant Blue staining using bovine serum albumin as a standard.

Cell Culture and Cell Extracts—Normal rat kidney (NRK) epithelial cells (NRK-E51, American Tissue Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) in a humidified chamber with 5% CO2 at 37 °C. The cells were grown to confluence, washed in PBS, and harvested on ice in 400 μl of radioimmune precipitation assay lysis buffer (25 mM Tris-Cl, 100 mM NaCl, 10 mM EDTA, 50 mM NaF, 500 μM Na3VO4, 0.25% Triton X-100, 0.02% NaN3, 1× Complete protease inhibitors (Roche Applied Science), and 2 μg phenylmethylsulfonyl fluoride, pH 7.2). The lysate was cleared by centrifugation at 13,000 rpm for 2 min. Cell supernatant from NRK cells was precooled with GST bound to glutathione-agarose beads. After washing, Cx43CT and any potential interacting proteins were eluted from the beads using either 6 M urea or cleavage at the thrombin site engineered into the fusion protein. The eluted proteins were digested with trypsin and analyzed by electrospray-MS/MS on a ThermoFinnigan LCQ spectrometer. Tandem mass spectrometry data of individual peptides was analyzed with SEQUEST, an algorithm that correlates the experimental mass spectrometry data with theoretical spectra generated from known protein sequences in a data base and ranks the “fit” with X-correlation and Delta Cn values (30).

RESULTS

Cx43 Associates with ZO-2—The C-terminal region of Cx43 contains multiple putative protein interaction motifs and several phosphorylation sites that have been shown to regulate protein function. We employed tandem mass spectrometry (MS/MS) as a screen to identify Cx43-interacting partners in a pull down experiment. The C-terminal tail (amino acids 246–382) of Cx43 fused to a GST tag (Cx43CT) was incubated with NRK cell lysates, and bound proteins were precipitated, washed, eluted, trypsinized, and subjected to MS/MS. The mass spectra were analyzed using SEQUEST, an algorithm that correlates the experimental mass spectrometry data with theoretical spectra generated from known protein sequences in a data base and ranks the “fit” with X-correlation and Delta Cn values (30).
Our mass spectrometry-based approach identified numerous proteins that potentially interact with the C-terminal tail of Cx43. In a preliminary report associated with the 2003 International Gap Junction Conference, we identified 19 possible interacting proteins (26). Four have been reported to interact with Cx43, including protein kinase Ca, casein kinase 1, tubulin, and ZO-1 (4, 5, 16, 28, 35). Another ZO family member, ZO-2, was identified with the highest degree of certainty of the group. Two distinct ZO-2 peptides were identified, STGDITAAGYTEANKPR (P1) and VVDTLYDGK (P2), with high X-correlation (3.53 and 2.54) and Delta Cn values (0.58 and 0.47), respectively. These sequences are absent in other ZO proteins; sequences C-terminal to the 3-amino acid PDZ domain-binding motif can influence binding to PDZ domains (10).

We next determined which region of ZO-2 bound to full-length Cx43. Lysates of NRK cells were incubated with GST fusions of the three separate PDZ1-2, -3 regions of the ZO-2 molecule bound to glutathione beads (Fig. 1B). After extensive washing of the beads, bound proteins were eluted in sample buffer, and Western analysis for Cx43 was performed. Connexin signal was only detected in the lane where the fusion protein containing PDZ2 was used and not with PDZ1, PDZ3, or GST alone (Fig. 1B).

Cellular Cx43 and ZO-2 Interact—We sought to determine whether cellular Cx43 complexes with endogenous ZO-2 in NRK cells. We immunoprecipitated ZO-2 from NRK cell lysates using an anti-ZO-2 antibody and immunoblotted for Cx43 (Fig. 2A). Cellular Cx43 appears as several bands. The bands of lower electrophoretic mobility correspond to phosphorylated forms of Cx43 and are often labeled as P1 and P2, whereas species that co-migrate with the nonphosphorylated band have the highest mobility (P0). Unidentified Cx43 phosphorylation events have been associated with the formation of gap junction plaques (36, 37). A strong Cx43 signal was observed in lanes representing whole cell lysate, ZO-1 immunoprecipitate (which served as a positive control) and the ZO-2 immunoprecipitate. No signal was observed when a control antibody (to Myc) or protein A beads alone were incubated with the cell lysate. We also immunoprecipitated Cx43 and immunoblotted for ZO-2 (Fig. 2B). ZO-2 was detected in the whole cell lysate and when Cx43 antibody was used for immunoprecipitation but not when the Myc antibody or Protein A beads alone was used.

ZO-2 Co-localizes with Cx43 in NRK Cells and Heart—To show whether ZO-2 could interact with Cx43 prior to cellular lysis, we performed co-immunolabeling using confocal microscopy. As shown in Fig. 3, ZO-2 and Cx43 are predominately localized to the plasma membrane in NRK cells and were frequently but not exclusively co-localized at cell-cell contacts in punctuate staining patterns reminiscent of gap junctions (shown in all three dimensions by superimposing the signal for Cx43 and ZO-2 as shown in the Overlay panel).

We also performed high resolution immunofluorescence to determine whether Cx43 and ZO-2 can co-localize in the intercalated disk region of heart tissue (Fig. 3). Distinct co-localization of Cx43 and ZO-2 was frequently demonstrated in the x-, y-, and z-planes. In both NRK cells and heart tissue, com-
Cx43 Interacts with ZO-2

Fig. 3. Confocal immunofluorescence analysis of the distribution of ZO-2 and Cx43 in NRK cells and heart. Immunostained NRK cells and heart tissue were imaged and are presented in x-y (0.8 μm thick), x-z, and y-z dimensions (vertical cross-sections) for the x-z and y-z were obtained at the positions indicated by the dashed lines in the x-y panel. Both individual channels (black and white) and merged images resulting from the combination of fluorescent signal from Cx43 (green) and the ZO proteins (red) channels are shown (bar, 10 μm). The yellow areas indicate co-localization.

Fig. 4. Cx43 binds directly to ZO-2. Whole cell lysates from 293-T cells transfected with the GW1HA-ZO-2 construct (+) or untransfected (−) were run on a SDS-PAGE gel and transferred to nitrocellulose. The blot was probed with a radiolabeled Cx43CT in a far Western (FW) assay (left panel) and then with anti-ZO-2 (IB side, lower panel), followed by anti-ZO-1 (IB side, upper panel) in an immunoblot (IB).

Comparison of the individual channels (i.e., Cx43 and ZO-2) indicates that they often coincidentally increase in intensity (i.e., signal strength and shape for each channel are related), indicating bona fide co-localization. However, fluorescence co-localization studies are limited by the resolution of light, so we cannot tell whether the apparent interaction is direct or not.

ZO-2 Can Bind Directly to Cx43—ZO-1 is known to form independent complexes with ZO-2 and ZO-3 at tight junctions (38), and thus, ZO-2 could be interacting indirectly with Cx43 via its interacting partner ZO-1. To investigate the specificity of the ZO-Cx43 interaction further, we employed a far Western analysis. Western blots of lysates from GW1HA-ZO-2-transfected and untransfected 293-T cells were incubated with a radiolabeled Cx43CT probe. The Cx43CT probe bound to a 160-kDa protein only in the lane of the 293-T cells transfected with HA-ZO-2 (Fig. 4, FW panel, + lane). Western analysis of the same blot using ZO-2 antibodies indicated that this band corresponds to the exogenously expressed ZO-2 (IB panel, lower portion). Untransfected 293-T cells contain only low levels of intrinsic ZO-1 (IB panel, upper portion) and ZO-2 (IB side, lower panel, − lane), so essentially no detectable radiolabeled probe was bound. Thus, Cx43 only bound to ZO-2 that was ectopically expressed in the 293-T cells. This confirmed that the binding of Cx43 with ZO-2 was specific and direct.

ZO-1 and ZO-2 Bind Differentially to Cx43 during the Cell Cycle—Since both ZO-1 and ZO-2 can bind to the same region of Cx43, we compared their co-localization at different stages of the cell cycle to potentially elucidate distinct roles. As shown in Fig. 5A, Cx43, ZO-1, and ZO-2 interact extensively during G0 (Cx43 (green) + ZO-1 (blue) + ZO-2 (red) = white in the overlay), whereas green (Cx43) and yellow (Cx43 + ZO-2) were more apparent and independent of ZO-1 in S phase (Fig. 5B). Calculation of the percentage of co-localization (see “Experimental Procedures”) indicated that significantly more (p < 0.03) Cx43 co-localized with ZO-1 in G0 phase (~60%) than in S phase (~30%) cells (Fig. 5C). Cx43 binding to ZO-2 appeared to be independent of cell cycle stage, with ~45% co-localization at either stage (Fig. 5C). Higher magnification of the indicated junctional regions (marked with parallel white lines) in the G0 and S phase images (Fig. 5, A and B) showed more apparent green (Cx43) and yellow (Cx43 + ZO-2) in the S phase (Fig. 5E) compared with G0 (Fig. 5D) images consistent with the co-localization values. Line scan analysis of the indicated regions often showed very good concordance of all three proteins in G0 cells (Fig. 5D; regions with good overlay are indicated by an asterisk), but S phase cells showed less alignment of Cx43 and ZO-1, so Cx43-ZO-2 association was more apparent (Fig. 5E). Since co-localization studies are limited by the resolution of light and both Cx43 and the ZO proteins are present in the plasma membrane, the percentage of co-localization calculated above may overestimate the true extent of interaction. However, these analyses confirm that the signal from Cx43, ZO-1, and ZO-2 channels can appear proportionally in strength and shape. Therefore, bona fide co-localization can occur, and the relative levels change during the cell cycle.

In order to get a more direct measurement of interaction, we also examined whether ZO-1 bound directly to Cx43 in a cell cycle-dependent manner via far Western analysis. Western blots containing cell lysates from G0, G1, S, and G2/M were analyzed (Fig. 6) via far Western analysis with radiolabeled Cx43CT (Fig. 6A) followed by reprobing of the same blot for ZO-1 (Fig. 6B), ZO-2 (Fig. 6C), and Cx43 (Fig. 6D) using multiple antibodies and the Li-Cor imaging system. In G0/M cells, Cx43 migrated as multiple bands, including an M phase-specific band (P3), which has reduced mobility compared with P1 and P2 (39). Comparison of the extent of Cx43CT binding with the amount of ZO-1 protein present (Fig. 6E) showed that Cx43 bound significantly more extensively (p < 0.01) to ZO-1 present in G0 cells than in either G1, S, or G2/M cells. The level of binding of Cx43CT to ZO-2 was lower than ZO-1 and not significantly different at any of the cell cycle stages. Thus, cell cycle-dependent post-translational modification of ZO-1 may affect its interaction with Cx43. However, one caveat with these experiments is that although the gel-fractionated proteins are full-length and presumably appropriately post-translationally modified, the radiolabeled Cx43CT probe is not, so judging relative affinities of ZO-1 and ZO-2 is not possible with this method. However, both the far Western and immunofluorescence studies indicate that Cx43 interacts most extensively...
with ZO-1 when cells are quiescent and that ZO-2 may play a more predominant role during the cell cycle.

**DISCUSSION**

Multiple laboratories have used the C-terminal region of Cx43 in a pull-down approach to screen for possible interacting proteins (20, 26, 40). Two of these studies utilized SDS-PAGE separation and sequencing to identify specific, prominent bands (20, 40). Tubulin was detected in all three studies, and Drebrin interaction was detected in one (20). Our study utilized direct detection by mass spectrometry and yielded many additional possible interacting proteins including HSP71, 40S ribosomal protein S7, serine/threonine protein phosphatase 2A, POU domain class 3 transcription factor 4, SNIP, interleukin-12α, fibrillin-2, α-tubulin, vimentin, γ-aminobutyric acid receptor, Lin-7, Discs-large, band 4.1(L), casein kinase Iα, vascular protein-tyrosine phosphatase 1, BIG-2, cyclic nucleotide-gated channel, ZO-1, and protein kinase Cα (26). Although this technique is quite sensitive, false identifications are likely, and thus any identification has to be considered preliminary until it is confirmed via other methods. However, four of these were known to be or have turned out to be reported as Cx43-interacting proteins.

As is the case for several highly regulated membrane proteins, Cx43 can interact with multiple scaffolding and signaling molecules. However, in many cases, these interactions were detected by immunofluorescence or co-precipitation studies performed in a manner that did not distinguish whether the interactions were direct or indirect. Interaction of ZO-1 with Cx43 has been clearly documented by multiple laboratories (e.g. see Refs. 4 and 5). Here we present several lines of evidence that indicate that the PDZ2 domain of ZO-2 interacts with Cx43: (a) a GST fusion containing the PDZ2 portion of ZO-2 can pull down Cx43; (b) an intact C-terminal region of Cx43 is necessary for the interaction; (c) an antibody to ZO-2 can co-precipitate Cx43, and vice versa; (d) Cx43 and ZO-2 can co-localize in the plasma membrane of NRK cells and at intercalated disks in heart cells. As has been previously reported for Cx43 interaction with ZO-1 in cardiac tissue, co-localization of Cx43 and ZO-2 was primarily at the plasma membrane and was extensive but not complete in the intercalated disk region (13).

Since ZO-1 and ZO-2 have been shown to interact with each other, it was possible that Cx43 might interact with ZO-2 indirectly via ZO-1. Furthermore, given the direct binding of ZO-1 to ZO-2, the resolution of light microscopy and even
immunoelectron microscopy or fluorescence recovery after photobleach could not definitively distinguish between direct and indirect interaction between these molecules and Cx43. Here we present evidence that ZO-2 can interact directly with Cx43, including the following: (a) Cx43 binds to ZO-2 in a far Western overlay assay independent of ZO-1; (b) analysis of confocal images indicates that the level of interaction of Cx43 with ZO-1 and ZO-2 changes independently during the cell cycle.

The functional significance of ZO-1 or ZO-2 interaction with Cx43 is not as yet well defined. PDZ-containing proteins often play an important role in the clustering of transmembrane receptors (e.g. the spatial organization of ion channels) (11). Most proteins with PDZ domains are membrane-associated and organize specialized membrane domains at synapses, junctions, and apical basolateral interface regions (12). In mouse epithelial cells lacking any ZO-1 expression, there is retarded gap junctional communication, also appears to negatively affect the stability of gap junctions and explain why different studies have come to different conclusions about the role of the interaction of Cx43 and ZO-1.

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43. Solan, J. L., and Lampe, P. D. (2005) The method for evaluating the role of Cx43 in the context of ZO-1 expression is described.

**FIG. 6. Far Western and immunoblot analysis of Cx43 binding to ZO-1 and ZO-2 during different stages of the cell cycle.** The same blot was probed with radiolabeled Cx43CT (A) followed by antibodies to ZO-1 (B), ZO-2 (C), and Cx43 (D). In E, the relative levels of Cx43CT bound to the ZO-1 normalized to the levels of ZO-1 shown in the immunoblot (FW/IB) are shown for each stage of the cell cycle. Triplicate experiments were used to calculate error bars (± S.D.).