Direct Association of the Gap Junction Protein Connexin-43 with ZO-1 in Cardiac Myocytes*

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The gap junction protein connexin-43 is normally located at the intercalated discs of cardiac myocytes, and it plays a critical role in the synchronization of their contraction. The mechanism by which connexin-43 is localized within cardiac myocytes is unknown. However, localization of connexin-43 likely involves an interaction with the cytoskeleton; immunofluorescence microscopy showed that in cardiac myocytes, connexin-43 specifically colocalizes with the cytoskeletal proteins ZO-1 and α-spectrin. In transfected HEK293 cells, immunoprecipitation experiments using coexpressed epitope-tagged connexin-43 and ZO-1 indicated that ZO-1 links connexin-43 with α-spectrin. The domains responsible for the protein-protein interaction between connexin-43 and ZO-1 were identified using affinity binding assays with deleted ZO-1 and connexin-43 fusion proteins. Immunoblot analysis of associated proteins showed that the C-terminal domain of connexin-43 binds to the N-terminal domain of ZO-1. The role of this linkage in gap junction formation was examined by a dominant-negative assay using the N-terminal domain of ZO-1. Overexpression of the N-terminal domain of ZO-1 in connexin-43-expressing cells resulted in redistribution of connexin-43 from cell-cell interfaces to cytoplasmic structures; this intracellular redistribution of connexin-43 coincided with a loss of electrical coupling. We therefore conclude that the linkage between connexin-43 and α-spectrin, via ZO-1, may serve to localize connexin-43 at the intercalated discs, thereby generating functional gap junctions in cardiac myocytes.

Gap junctions are aggregates of channels at cell-cell interfaces (1–3). Each channel is formed through the docking of two hemichannels located in opposing cell membranes, and each hemichannel is composed of a connexin homohexamer. By permitting the direct exchange of ions and small molecules between cells, these channels play a major role in a wide variety of cellular processes, including embryogenesis, cellular differentiation and development, and electrical coupling. In heart, gap junctions are a prominent feature of intercalated discs, which connect myocytes in an end-to-end orientation; the coupling provided by gap junctions serves to synchronize the activity of cells, thus providing an asynchrony front for the wave of excitation that sweeps through ventricular muscle (4, 5). How gap junctions are localized at the intercalated discs in cardiac myocytes is unknown, but a component of gap junctions, connexin-43, may interact with specific elements of the cytoskeleton that restrict its diffusion in the plane of the membrane. Recent studies indicate that a number of membrane proteins are anchored by cytoskeletal elements such as PSD-95/SAP90 (6), ankyrin (7), and α-spectrin (8). ZO-1, which has been identified at vertebrate tight junctions (9, 10), is thought to play a role in tissue compartmentalization and in maintaining the apical-basolateral polarity of epithelial cells. In cardiac myocytes, ZO-1 appears at the intercalated discs in the immediate vicinity of the plasma membrane (11–13). Since ZO-1 is tightly associated with α-spectrin (11, 12), which is an actin-linking protein (14), it is possible that ZO-1 acts as an adapter mediating the anchoring of membrane proteins to the cytoskeleton.

We recently demonstrated that when connexin-43 proteins are expressed in HEK293 cells, which otherwise lack gap junctional communication, the cells are able to form gap junctions at cell-cell interfaces (15). This finding serves as the basis for our continued investigation of the functional role of selected protein domains in gap junctional communication as well as the mechanism of gap junction formation. In this study, we examined how the gap junction protein connexin-43 localizes to the intercalated discs in cardiac myocytes. Two assay systems were used to determine the linkage between connexin-43 and cytoskeletal proteins: a biochemical approach involving coprecipitation and binding of tagged proteins and a functional approach involving a dominant-negative assay using a deletion construct of ZO-1.

EXPERIMENTAL PROCEDURES

Construction of Epitope-tagged Connexin-43 and ZO-1 cDNAs—Full-length rat connexin-43 cDNA (16) and mouse ZO-1 cDNA (12) were cloned by reverse transcription-PCR1 using mRNA isolated from rat or mouse heart (see Fig. 1). For immunoprecipitation, cDNAs were modified to encode the epitopes for monoclonal antibodies at the C-terminal ends of the proteins. For the construction of rat connexin-43 cDNA, the sense PCR primer was designed to contain the known sense sequences of connexin-43 and an EcoRI site at the 5′-end. The antisense PCR primer was designed to delete the endogenous stop codon in the cDNA encoding connexin-43 and replace it with a FLAG tag encoding nucleotides with an EcoRI site at the 5′-end. Thus, the sequence at the C terminus of connexin-43 that normally ends as DLEI now became DLEI-GSDYKDDDDK, which binds to a mouse monoclonal anti-FLAG IgG antibody. The PCR product called connexin-43-FLAG was excised by EcoRI and ligated into Bluescript KS+(+) vector sequence was then verified by nucleotide sequencing.

For the construction of mouse ZO-1 cDNA, four domains of ZO-1 (d1, amino acids 1–362; d2, amino acids 358–839; d3, amino acids 836–1257; and d4, amino acids 1254–1745) were first amplified by PCR. The sense PCR primers were designed to contain an EcoRI site at the 5′-end. The antisense PCR primers were, in turn, designed to contain the c-Myc

1 The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione-S-transferase; GFP, green fluorescent protein.
GuK

ZO-1 specifies the three PDZ domains, the SH3 domain, and the gua-
ligated together to produce the full-length ZO-1-Myc cDNA. The map of

1257; and d4, amino acids 1254–1745. Each construct with a c-Myc tag
amino acids 1–362; d2, amino acids 358–839; d3, amino acids 836–
respond to the full-length ZO-1-Myc protein (amino acids 1–1745): d1,

mains (Fig.

teins. The map of connexin-43 specifies the four transmembrane do-
pET28a vector for synthesis of T7-tagged and His-tagged fusion pro-

Each construct was produced by PCR and was ligated in frame into the

To facilitate cloning, cDNAs encoding cytoplasmic domains of

Excision of the nucleotide sequences, the four domains were excised and

pGEX-3X vector for the synthesis of GST fusion proteins. Con-
structs were excised with endogenous restriction endonucleases and
ligated together to produce the full-length ZO-1-Myc cDNA. The map of
ZO-1 specifies the three PDZ domains, the SH3 domain, and the gua-
nylate kinase region (GuK).

tag encoding nucleotides with an EcoRI site at the 5′-end. The sequence at
the C terminus of ZO-1 was GSEQKLISSDE, which binds to a mouse monoclonal anti-c-Myc IgG antibody. The PCR products were
excised with EcoRI and then ligated into Bluescript KS(−). After veri-
fication of the nucleotide sequences, the four domains were excised and
ligated to produce the full-length ZO-1-Myc cDNA using the following
endothelial restriction endonuclease sites: Accl (nucleotide 1075) in d1
and d2, AalI (nucleotide 2510) in d2 and d3, and BamHI (nucleotide 3762)
in d3 and d4.

For the transfection experiment, the full-length cDNA encoding modified
connexin-43-FLAG was ligated into the EcoRI site of a pcDNA3 mammanin expression vector containing a neomycin (G418)-resistant
gene as a dominant selectable marker (Invitrogen). The full-length cDNAs encoding modified ZO-1-Myc and d1 of ZO-1, called ZO-1-d1-
Myc, were ligated into the EcoRI site of a pGEXSV expression vector (Invitrogen) containing a Zeocin-resistant gene as a dominant select-
able marker.

**Construction and Purification of T7-tagged Connexin-43 Fusion Pro-
teins**—To facilitate cloning, cDNAs encoding cytoplasmic domains of
connexin-43 (N, amino acids 1–23; II–III, amino acids 95–150; C1, amino acids 227–382; C2, amino acids 227–302; and C3, amino acids 303–382) were amplified by PCR using sense primers with an

EcoRI site at the 5′-end and antisense primers with an

XhoI site at the 5′-end (Fig.

1). Each fragment was excised with EcoRI and XhoI and then ligated in
frame into a pET28a vector (Novagen). Clones were transfected into

Escherichia coli BL21(DE3) pLysS; overnight cultures were diluted 1:10, incubated for 2 h, and induced for 3 h with 1 mM isopropyl-
β-thiogalactopyranoside. Expressed proteins carrying TF and His epitope
tags encoded in the N-terminal site of the vector were affinity-purified
on His-Bind columns (Novagen) as described in the manufacturer’s
instructions.

**Construction and Purification of GST-ZO-1 Fusion Proteins**—Four domains of ZO-1-Myc (d1, d2, d3, and d4) in Bluescript KS(−) were
excised using EcoRI and then ligated in frame into pGEX-2X vectors
(Amersham Pharmacia Biotech). After expression in E. coli DH5α,
GST-ZO-1 domain fusion proteins were purified by affinity chromatog-
raphy on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) as
described in the manufacturer’s instructions.

**Cell Culture**—Rat neonatal cardiac myocytes were prepared as de-
scribed previously (17). Briefly, the hearts were isolated from 1-day-old HLA-Wistar rats. The ventricular portions were minced, and the cells were dispersed by digestion with 0.1% collagenase at 37 °C. The dis-
spersed cells were resuspended in Dulbecco’s modified Eagle’s medium
(high glucose) supplemented with 10% fetal calf serum and 10 μg/ml
bromodeoxyuridine and preplanted onto culture dishes for 30 min to
remove fibroblasts. Cells were then plated on glass coverslips to an
initial density of 1 × 10⁶ cells/cm² in 5% CO₂ atmosphere. Gap junction-incompetent HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (high glucose) supplemented with 10% fetal calf serum and penicillin at 37 °C under a 5% CO₂ atmosphere.

**Stable Coexpression of Connexin-43-FLAG and Truncated ZO-1-Myc in HEK293 Cells**—HEK293 cells were transfected with the pCI-N3
vector containing connexin-43-FLAG cDNA using the calcium phos-
phate precipitation technique. For purposes of selection, the transfected

cells were grown in medium containing 800 μg/ml G418. Each of the
clones selected with G418 was further analyzed by Northern blot and immunoblot analyses. Clones expressing connexin-43-FLAG were then
transfected with pZeosV vectors containing either ZO-1-Myc or ZO-1-d1-Myc
cDNAs, and the transfected cells were grown for selection in medium
containing 250 μg/ml Zeocin and 400 μg/ml G418. Each clone selected
with Zeocin and G418 was analyzed by Northern blot and immunoblot
analyses.

**Transient Coexpression of ZO-1-Myc or ZO-1-d1-Myc with Plasmid pGreen Lantern-1 in Cardiac Myocytes**—Cardiac myocytes plated on
coverslips were cotransfected with pGreen lantern-1 (Life

Labs, Inc.) and pZeosV vectors containing either ZO-1-Myc or ZO-1-d1-Myc proteins. Transfected GFP-positive cell pairs were then
subjected to electrophysiological analysis.

**Immunofluorescence Analysis**—Cardiac myocytes were grown on
glass coverslips for 3 days, fixed with 3% paraformaldehyde for 10 min, and then permeabilized with 0.1% Triton X-100 for 10 min. After blocking with 5% bovine serum albumin in phosphate-buffered saline for 30 min, cells were incubated for 2 h with mouse monoclonal anti-

connexin-43 IgG antibodies (Transduction Laboratories, Inc.), rabbit
monoclonal anti-ZO-1 IgG antibodies (Zymed Laboratories Inc.), or
rabbit monoclonal anti-α-spectrin IgG antibodies (Transformation Re-
search, Inc.). Primary antibody-bound connexin-43 complexes were
visualized by incubation with biotinylated anti-mouse IgG antibodies
(Vector Labs, Inc.) for 1 h, followed by incubation with fluorescein
isothiocyanate-conjugated streptavidin (Vector Labs, Inc.) for an addi-
tional 1 h. Primary antibody-bound ZO-1 complexes were visualized
using biotinylated anti-rabbit IgG antibodies (Cappel Inc.), followed by

fluorescein isothiocyanate-conjugated streptavidin. Primary antibody-
bound α-spectrin complexes were visualized using rhodamine-conju-
gated anti-rabbit IgG antibodies (Cappel Inc.). Coverslips were then
mounted in Mowiol 4-88 (Vector Labs, Inc.). The cells were photo-
graphed on an Olympus Provis AX80 microscope fitted with the appro-
priate filters.

**Mycoplasma Detection**—HEK293 cells, overexpressing connexin-43-FLAG or connexin-43-FLAG plus ZO-1-d1-Myc, were immunostained with mouse monoclonal anti-

FLAG IgG antibodies (Eastman Kodak Co.), mouse monoclonal anti-c-Myc IgG antibodies (Calbiochem), or mouse monoclonal anti-N-
cadherin IgG antibodies (Sigma). Anti-FLAG antibody-antigen com-
plexes were visualized using biotinylated anti-mouse IgG antibodies,
followed by fluorescein isothiocyanate-conjugated streptavidin. Anti-c-
Myc and anti-N-cadherin antibody-antigen complexes were visualized
using rhodamine-conjugated anti-mouse IgG antibodies.

**Protein Immunoblot Analysis**—Affinity-purified, T7-tagged connexin-
43 domain fusion proteins and GST-ZO-1 domain fusion proteins
were solubilized in SDS loading buffer, resolved on SDS-polyacrylamide
gels, and transferred to nitrocellulose by electrophoresis. Transfected

HEK293 cells were harvested, pelleted with a microcentrifuge, and
then incubated in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM
phenylmethylsulfonyl fluoride, 10 μM aprotinin, and 10 μg/ml leupi-
ptin). The lysates were then solubilized in SDS loading buffer and

transferred to nitrocellulose. The nitrocellulose blots were incubated
with primary antibodies against target proteins. They were then
subjected to electrophoresis. The gels were blotted to nitrocellulose
film, subjected to electrophoresis, and transferred to nitrocellulose.

**Coimmunoprecipitation Analysis**—Cells overexpressing connexin-
43-FLAG and ZO-1-Myc were collected, washed with phosphate-buff-
ered saline, and incubated in lysis buffer for 30 min at 4 °C. Cell lysates were incubated with 0.1% albumin-coated protein A-Sepharose for 2 h at 4 °C and then clarified by centrifugation at 15,000 × g for 15 min. The supernatants were incubated in a rotating vessel with monoclonal anti-FLAG or anti-c-Myc antibodies bound to protein A-Sepharose for 2 h at 4 °C. After incubation, immunoprecipitates were extensively washed with lysis buffer. Samples were divided into three equal aliquots and subjected to immunoblot analysis with anti-FLAG, anti-c-Myc, or anti-α-spectrin IgG antibodies. ECL and peroxidase-conjugated secondary antibodies against mouse IgG antibodies were used to visualize primary antibody-antigen complexes.

**Affinity Binding Assay—GST or GST-ZO-1 Domain Fusion Proteins (d1, d2, d3, and d4), bound to glutathione-Sepharose 4B beads, were extensively washed with phosphate-buffered saline and then with binding buffer (10 mM Tris (pH 7.5), 150 mM NaCl, 5% bovine serum, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). T7-tagged connexin-43 domain fusion proteins (N, II–III, C1, C2, and C3) were purified on His-Trap columns, concentrated using an Amicon concentrator 10, and re-equilibrated to a concentration of ~1 μg/μl in binding buffer. Approximately 100 μl of GST-ZO-1 domain fusion proteins, bound to glutathione beads, were incubated in a rotating vessel with 100 μl of purified T7-tagged connexin-43 domain fusion proteins for 2 h at 4 °C. The beads were then extensively washed with binding buffer. Finally, associated proteins were eluted with SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. After transfer to nitrocellulose, immunoblot analysis was performed with either anti-T7 tag IgG antibodies against connexin-43 domain fusion proteins or with anti-c-Myc IgG antibodies against GST-ZO-1 domain fusion proteins. Primary antibody-antigen complexes were visualized using ECL and peroxidase-conjugated secondary antibodies against mouse IgG antibodies.

**Electrophysiology—Gap Junctional Current (Ij) was measured using a Geneclamp 500 amplifier (Axon Instruments, Inc.) and a double whole-cell patch-clamp procedure (15, 18, 19). Pairs of stable HEK293 cells, overexpressing connexin-43-FLAG or connexin-43-FLAG plus ZO-1-d1-Myc, were obtained by freshly dissociating pure populations from confluent cultures and aliquoting them onto 1-cm-diameter glass coverslips. Pairs of cardiac myocytes, overexpressing ZO-1-Myc or ZO-1-d1-Myc (GFP-positive), were selected by fluorescence microscopy. The coverslips were transferred to the stage of a Nikon Diaphot microscope, where experiments were performed at room temperature while exchanging the bath solution (133 mM NaCl, 3.6 mM KCl, 1.0 mM CaCl2, 0.3 mM MgCl2, 16 mM glucose, and 3.0 mM HEPES (pH 7.2)). Patch pipettes were made on a Narishige NA-9 vertical puller and filled with solution containing 10 mM free Ca2+ (135 mM NaCl, 0.5 mM CaCl2, 2 mM MgCl2, 5.5 mM EGTA, and 5.0 mM HEPES (pH 7.2)). High resistance seals (>109 ohms) were formed on each cell with the aid of gentle suction, and access to the cell interior was subsequently obtained by briefly applying strong suction to the patch pipette. Cells were voltage-clamped at holding potentials of ~40 mV, and 10-nV voltage pulses were applied to each cell of the pair. Within each pair of cells, Ij values were measured as the currents recorded from one cell when voltage steps (Vt) were applied to the other cell. Junctional conductance (Gij) was calculated from the following equation: Gij = |Ij|/Vt.

## RESULTS

**Localization of Connexin-43, ZO-1, and α-Spectrin in Cardiac Myocytes—Immunofluorescent Localization of Connexin-43, ZO-1, and α-Spectrin in Cardiac Myocytes.** Isolated rat cardiac myocytes were grown for 3 days and fixed. Fixed cells were stained using monoclonal IgG antibodies against connexin-43 (Cx43), ZO-1, or α-spectrin (right panels) or in the absence of primary antibodies (left panels). Nuclear staining is nonspecific, as it is seen in the absence of primary antibodies. IS, immunofluorescent staining.

**Identification of Associating Regions of Connexin-43 and ZO-1—To define the regions mediating the association between connexin-43 and ZO-1, binding assays were performed using the batch method and bacterially expressed fusion proteins. Purified GST-ZO-1 fusion proteins, encoding distinct domains of ZO-1-Myc (d1, d2, d3, and d4) and coupled to glutathione-Sepharose 4B beads, served as the substrate for binding purified T7-tagged connexin-43 fusion proteins (N, II–III, C1, C2, and C3). After incubation and washing, the proteins associated with ZO-1 fusion or GST beads were resolved by SDS-polyacrylamide gel electrophoresis. Immunoblots of proteins eluted with anti-T7 tag IgG antibodies revealed that the C1 and C3 domains of connexin-43 bind to d1 of ZO-1 (Fig. 4A).

To determine whether the C3 domain is, by itself, sufficient to mediate binding to intact ZO-1, primary cultures of cardiac myocytes were lysed and incubated with either the C3 domain...
and collected in lysis buffer. Cell lysates were immunoprecipitated with overexpressing connexin-43 (Cx43), coimmunoprecipitation of connexin-43-FLAG and ZO-1-Myc. Cells were incubated either with d1 coupled to glutathione-Sepharose 4B beads or with glutathione-Sepharose 4B beads alone, only a 43-kDa protein, which was recognized by anti-connexin-43 IgG antibodies, bound to d1 (Fig. 4C). These findings indicate that the C3 and d1 domains of connexin-43 and ZO-1, respectively, are sufficient to mediate binding between the two proteins. Moreover, d1 does not appear to bind to α-spectrin.

Effects of a Dominant-negative Construct of ZO-1 on Gap Junction Formation—The biochemical approach revealed a direct protein-protein linkage among connexin-43, ZO-1, and α-spectrin, but it left open the question of the functional significance of this interaction. We previously demonstrated that when connexin-43 is expressed in HEK293 cells, gap junctions between transfected cell pairs are reconstituted (15). Using immunoblotting and anti-ZO-1 IgG antibodies, we have now identified endogenous ZO-1 in HEK293 cells (Fig. 5). Therefore, we hypothesized that endogenous ZO-1 may play a role in the clustering of expressed connexin-43 at cell-cell interfaces and in the construction of the functional gap junctions in vivo. To test this idea, we used a dominant-negative approach (20).

Biochemical assays had already revealed that the N-terminal region of ZO-1 (d1) binds to connexin-43, but not to α-spectrin (Fig. 4). In an attempt to disrupt the interaction between endogenous ZO-1 and expressed connexin-43, we generated stable HEK293 cells that overexpressed connexin-43-FLAG alone or in combination with ZO-1-d1-Myc (Fig. 5). The effect of ZO-1-d1-Myc on gap junctions reconstituted from expressed connexin-43-FLAG was then analyzed. Immunoblots showed that connexin-43-FLAG proteins were expressed to almost the same degree in both cell lines and that ZO-1-d1-Myc proteins were expressed in larger amounts than endogenous ZO-1 (Fig. 5A). The distributions of connexin-43-FLAG and ZO-1-d1-Myc were then localized by immunofluorescence using anti-FLAG and anti-c-Myc IgG antibodies, respectively. In cells not expressing ZO-1-d1-Myc, the connexin-43-FLAG proteins were distributed at cell-cell interfaces (Fig. 5B) but were not affected by ZO-1-d1-Myc. These results indicate that ZO-1-d1-Myc lacks the ability to localize at the cell-cell interfaces, and moreover, it inhibits the transport of connexin-43-FLAG to the cell membrane.

Effects of Dominant-negative Constructs of ZO-1 on Gap Junctional Conductance—To assess the effect of ZO-1-d1-Myc on the conductance properties of connexin-43-mediated gap junctions, whole-cell voltage-clamp recordings were obtained either anti-FLAG or anti-c-Myc IgG antibodies bound to protein A-Sepharose. These immunoprecipitates were subjected to immunoblot analysis with anti-c-Myc (top panels), anti-FLAG (middle panels), or anti-α-spectrin (bottom panels) IgG antibodies. Note that connexin-43-FLAG, ZO-1-Myc, and α-spectrin were specifically associated in vitro. The band at 55 kDa (arrowhead in the middle panels) represents immunoglobulin heavy chains. IP, immunoprecipitation. The mobility of molecular mass markers is shown on the right.
Association of Connexin-43 with ZO-1

from pairs of transfected HEK293 cells (Fig. 6A). Repetitive 10-mV pulses were applied to pairs of voltage-clamped cells, and \( G_f \) was measured as described under “Experimental Procedures” (19). In control HEK293 cells, \( G_f \) was typically below the level of detectability (<20 picoSiemens). However, expression of connexin-43-FLAG markedly increased \( G_f \) to 45.9 ± 18.6 nanosiemens (n = 16), which is compatible with the reported conductance of connexin-43-expressing SHCep1 cell pairs (21, 22). Exposing voltage-clamped cell pairs to solution containing octanol, an inhibitor of gap junctional communication (19), reduced the junctional conductance. Thus, the gap junctions reconstituted from expressed connexin-43-FLAG proteins appear to be functional. In contrast, when ZO-1-d1-Myc was co-expressed with connexin-43-FLAG, \( G_f \) was decreased to control levels (Fig. 6A).

We next examined the role of ZO-1 in gap junction formation in cardiac myocytes (Fig. 6B). Because we were unable to generate stable cultures of cardiac myocytes overexpressing dominant-negative ZO-1-d1-Myc, a transient expression strategy was used. Cardiac myocytes were cotransfected with plasmid pGreen Lantern-1 and ZO-1-d1-Myc cDNA at a ratio of 1:5. This meant that cells transfected with ZO-1-d1-Myc could be easily identified using fluorescence microscopy. Subsequently, pairs of the GFP-positive cells were subjected to electrophysiological analysis. In control cells and in cells transfected with ZO-1-Myc, \( G_f \) was measured to be 126.6 ± 67.3 nanosiemens (n = 9) and 118.4 ± 92.4 nanosiemens (n = 13), respectively. These values are similar to the conductance previously reported for rat cardiac myocyte pairs (23). In contrast, \( G_f \) measured in cardiac myocytes transfected with ZO-1-d1-Myc was only 34.2 ± 20.3 nanosiemens (n = 16). Thus, dominant-negative ZO-1-d1-Myc markedly decreased \( G_f \) in cardiac myocytes. Taken together with the immunocytochemical results from transfected HEK293 cells, these electrophysiological findings confirm the notion that overexpression of ZO-1-d1-Myc inhibits gap junction formation by connexin-43-FLAG.

**DISCUSSION**

In this study, biochemical analysis demonstrated that connexin-43 is directly linked to ZO-1 in cardiac myocytes and that the linkage involves an interaction between the C-terminal domain of connexin-43 and the N-terminal domain of ZO-1. Furthermore, dominant-negative assays using a deletion construct of ZO-1 showed that overexpression of the N-terminal domain of ZO-1 inhibits localization of connexin-43 at cell-cell interfaces and that there is a loss of electrical coupling between transfected cell pairs. We therefore propose that the linkage between ZO-1 and connexin-43 may serve to localize connexin-43 at the intercalated discs, thereby generating functional gap junctions in cardiac myocytes.

Cytoskeletal proteins play a major role in the regional localization of membrane proteins (6, 8). The N-terminal domain of ZO-1, which participates in the protein-protein interaction with connexin-43, contains repeats of the 90-amino acid PDZ motif (24, 25). PDZ domains have been detected in enzymes and structural proteins concentrated at specialized cell-cell junctions, such as neural synapses and epithelial tight junctions; they were found to bind to a variety of receptors, ion channels, and signaling proteins, anchoring them at their target sites (26–32). A C-terminal (T/S)XV consensus motif in the target protein has been identified as one of the interacting sites for the PDZ domain of PSD-95/SAP90 (26–28, 32). However, several studies, including this one, have shown that the interacting sites of the PDZ domain proteins are not restricted to the (T/S)XV consensus motif (29–31, 33). X-ray crystallography analysis indicated that all PDZ domains may share certain structural elements (25). Nevertheless, binding studies with oriented peptide libraries revealed that different PDZ domains...
display preferences for distinct targets (34). In this regard, it is noteworthy that the various PDZ domains of multivalent proteins are specialized for distinct functions by associating with different target proteins (32, 33). In this way, the various components of signaling cascades are organized into distinct physical and functional units by multivalent PDZ domain proteins, such as ZO-1, which assemble their target proteins at specialized sites and in close proximity.

The dominant-negative assay using the deletion construct of ZO-1 provides an insight into the functional role of ZO-1 in gap junction formation. Assembly of gap junctions is a multistage process (35, 36). The first step is the synthesis of connexin. Next, connexons are formed by the oligomerization of six connexin monomers during their transport from the endoplasmic reticulum to the Golgi apparatus; this step is thought to be critical for gap junction formation. Finally, a connexon in the plasma membrane of one cell docks with a connexon in an opposing plasma membrane to produce intercellular channels. In this study, overexpression of the N-terminal domain of ZO-1,
which lacked the ability to localize at cell-cell interfaces, disrupted the transport of connexin-43-FLAG to the target site. The precise reason why the N-terminal domain of ZO-1 is not localized at cell-cell interfaces remains unknown; perhaps it lacks the ability to interact with cytoskeletal proteins such as α-spectrin. Whatever the reason, overexpression of the N-terminal domain of ZO-1 inhibits the linkage between endogenous ZO-1 and connexin-43 apparently by dominantly forming complexes with connexin-43 in cytoplasmic structures. We therefore conclude that the transport of connexin-43 to regions of cell-cell interface is achieved by translocation of associated ZO-1 into close proximity with the cell surface. Furthermore, the cell adhesion molecule cadherin has also been shown to participate in gap junction formation (37, 38). Taken together with the findings of a recent study showing that translocation of ZO-1 to the cell surface is regulated by cadherins (39), it seems likely that the subcellular targeting of connexin-43 may be regulated by a cadherin-mediated signaling pathway.

In conclusion, we showed that ZO-1 functions as an adapter for the transport of connexin-43 in cardiac myocytes. Although occludin has been demonstrated to be another target protein of ZO-1 at the tight junction of epithelial cells (13), it is not yet known how the specificity of ZO-1 for different target proteins is determined in different cell types. The identification of all ZO-1 target proteins as well as the corresponding PDZ domains should provide an experimentally tractable system with which to define the structural basis for the interactions between ZO-1 and its target proteins in vivo.

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