Connexin Family Members Target to Lipid Raft Domains and Interact with Caveolin-1†

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ABSTRACT: Lipid rafts are cholesterol−sphingolipid-rich microdomains that function as platforms for membrane trafficking and signal transduction. Caveolae are specialized lipid raft domains that contain the structural proteins known as the caveolins. Connexins are a family of transmembrane proteins that self-associate to form cell−cell connections known as gap junctions and that are linked to cytosolic proteins, forming a protein complex or Nexus. To determine the extent to which these intracellular compartments intersect, we have systematically evaluated whether connexins are associated with lipid rafts and caveolin-1. We show that connexin 43 (Cx43) colocalizes, cofractionates, and coimmunoprecipitates with caveolin-1. A mutational analysis of Cx43 reveals that the hypothesized PDZ- and presumptive SH2/SH3-binding domains within the Cx43 carboxyl terminus are not required for this targeting event or for its stable interaction with caveolin-1. Furthermore, Cx43 appears to interact with two distinct caveolin-1 domains, i.e., the caveolin-scaffolding domain (residues 82−101) and the C-terminal domain (135−178). We also show that other connexins (Cx32, Cx36, and Cx46) are targeted to lipid rafts, while Cx26 and Cx50 are specifically excluded from these membrane microdomains. Interestingly, recombinant coexpression of Cx26 with caveolin-1 recruits Cx26 to lipid rafts, where it colocalizes with caveolin-1. This trafficking event appears to be unique to Cx26, since the other connexins investigated in this study do not require caveolin-1 for targeting to lipid rafts. Our results provide the first evidence that connexins interact with caveolins and partition into lipid raft domains and indicate that these interactions are connexin specific.

Gap junctions are the sites of intimate cell−cell contact, where intercellular channels mediate the bidirectional passage of ions, second messengers, and other small molecules with a molecular mass below about 1 kDa (i.e., Ca^{2+}, K^+, and IP_3) (*1*−*4*). Each gap junction channel is composed of two symmetrical components, called connexons, which dock head to head with each other to form a hydrophilic pore between the cells (*5*−*8*). Each connexon is formed by six tetraspan membrane proteins called connexins (Cx), which form a pore about 6−7 Å in radius (*9*).

Connexins are a multigene family with a common membrane topology, with intracellular amino and carboxy termini, four transmembrane domains, one intracellular loop, and two extracellular highly conserved loops (*3*, *8*), the latter being important for connexin−connexin noncovalent interactions, which join connexons between cells (*10*−*12*). The most highly divergent amino acid sequences in connexins are within the intracellular C-terminal domain (*3*, *4*, *8*); these sequence differences are likely responsible for many of the connexin-specific functional properties, including sensitivity to various stimuli, such as second messenger molecules, as well as the recruitment of other associated proteins within the Nexus complex (*13*−*15*).

Assembly and disassembly of the connexon, through expression, degradation, and cellular trafficking of connexins are relatively rapid processes, with connexin half-life being only several hours (*16*, *17*). Although connexins may generally assemble into connexons in the ER (*18*) and are trafficked through the Golgi to the plasma membrane (*16*, *19*), certain connexins may take alternative pathways (*20*). Details of possible interactions formed from any of the connexins with additional intracellular compartments are virtually unexplored.

Caveolae are 50−100 nm vesicular invaginations that are sites of transcytosis, potocytosis, and signal transduction (*21*, *22*). They are primarily found in terminally differentiated cells, such as fibroblasts, endothelial cells, and adipocytes. Caveolins-1, -2, and -3, are a multigene family of 21−24 kDa integral membrane proteins. Caveolins-1 and -2 have similar tissue distributions, but caveolin-3 is found primarily in skeletal and cardiac muscle and in the brain (*23*, *24*). Caveolins act as scaffolding proteins to cluster lipids and signaling molecules within caveolae and sometimes regulate those proteins that are targeted to the caveolae (*25*−*31*). In addition to signaling molecules, ion channels have also been localized to lipid rafts and caveolin-enriched caveolae. The voltage-gated K^+ channel, Kv1.5 but not Kv2.1, was found

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to localize with caveolin and to caveolae membranes (*32*). L-type Ca^{2+} channels and plasma membrane Ca^{2+} pumps have been localized to caveolae in smooth muscle cells (*32*, *33*). The Cl^- channel, huH1, was found to colocalize with caveolin in kidney cells (*34*). Finally, caveolin-1 appears to directly regulate the activity of volume-regulated chloride channels, VRACs, which are primarily sensitive to Cl^- (*35*).

Numerous ion channels are localized to lipid rafts/caveolae and interact with and are regulated by caveolins. In addition, both caveolins and connexins are involved in contact inhibition and cellular transformation. Caveolin and connexins are both upregulated in confluent cells and are localized to cell−cell contacts. Therefore, in this study, we investigated the targeting of connexins to caveolae and their interactions with caveolin-1. We show that Cx43 specifically targets to lipid rafts/caveolae and directly interacts with caveolin-1. We also show that Cx32, Cx36, and Cx46, but not Cx50, target to lipid raft membrane fractions and interact with caveolin-1. Interestingly, Cx26 interacts with caveolin-1 but targets only to lipid rafts in the presence of caveolin-1.

MATERIALS AND METHODS

Materials. Antibodies and their sources were as follows: caveolin-1 (2297 and 2234) mouse monoclonal antibodies were the gift of Dr. Roberto Campos-Gonzalez, Transduction Laboratories Inc. Rabbit polyclonal Cx26, rabbit polyclonal Cx32, rabbit polyclonal Cx43, mouse monoclonal Cx43, mouse monoclonal Cx50, and rabbit polyclonal Cx36 were obtained from Zymed Laboratories. Rabbit polyclonal Cx46 was purchased from Alpha Diagnostic, and mouse monoclonal Cx43 was purchased from Chemicon. Additional rabbit polyclonal Cx43 and caveolin-1 (N-20) were purchased from Santa Cruz Biotechnology. All secondary fluorescent antibodies (IgG) were purchased from Jackson Laboratory. The cDNA encoding caveolin-1 was subcloned into the pCB7 mammalian expression vector, as described previously (*24*). cDNAs encoding connexin 26 were obtained from Dr. Bruce Nicholson and subcloned into the PRC vector; connexins 32, 46, and 50 were obtained from Dr. David Paul and subcloned into the pcDNA3 vector; connexin 36 was obtained from Dr. Daniele Conderelli and subcloned into the PCRscript vector; connexin 43 was obtained from Dr. Eric Byer and subcloned into the SG5 vector; and the Cx43 mutations M257, M374, D397Q, D379N, and D379K were obtained from Dr. Mario Delmar and subcloned into the pcDNA3 vector, as previously described (*36*).

Cell Culture. Human embryonic kidney 293T cells and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% donor calf serum (Cellgro), 2 mM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. 293T cells were transfected using the calcium phosphate precipitation method. Cos-7 cells were transfected with Effectene transfection reagent (Qiagen), as suggested by the manufacturer.

Immunoblotting. Samples were subjected to SDS−PAGE under reducing conditions and transferred to 0.2 μm pore nitrocellulose membranes (Schleicher and Schuell). The protein bands were stained with Ponceau S (Sigma), washed with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20), blocked with 1% BSA and 1−5% milk for 1 h, incubated with primary antibody for 1 h, washed, incubated with secondary antibody conjugated with horseradish peroxidase (Pierce) for 1 h, washed, and detected using chemiluminescent substrate (Pierce).

Triton Solubility. Thirty-six to forty hours posttransfection, cells were washed three times with ice-cold PBS. Three hundred microliters of cold MBS (25 mM Mes, pH 6.5, 150 mM NaCl) containing 1% Trition X-100 plus protease inhibitors was added to the cells. Following a 30 min incubation without agitation on ice, the soluble fraction was collected. Three hundred microliters of 1% SDS was added to the plate to dissolve the remaining Triton X-100 insoluble fraction and passed through a 26-gauge needle 10 times in order to lower its viscosity. Equal volumes of the Triton X-100 soluble and insoluble fraction were separated by SDS−PAGE and subjected to immunoblotting as described above.

Preparation of Lipid Raft/Caveolae-Enriched Membrane Fractions. Lipid raft/caveolae-enriched membrane fractions were purified as described previously (*37*, *38*). Briefly, 40 h posttransfection, two 100 mm diameter plates were washed three times in cold PBS, scraped into 750 μL of MBS containing 1% Trition 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 4.5 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 200000*g* (44000 rpm in a Sorval rotor, TH-660). Twelve 375 μL fractions were collected, and aliquots of each fraction were subjected to SDS−PAGE and immunoblotting.

Protein Assay. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce) using the protocol described by the manufacturer.

Coimmunoprecipitation. Transfected 293T cells were lysed in a buffer containing 10 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 60 mM *n*-octyl glucoside, and protease inhibitors. Lysates were centrifuged at 20000*g* for 10 min to remove debris. Cell lysates were precleared for 1 h at 4 °C with protein A−Sepharose (Pharmacia Biotech Inc.) and then transferred to a fresh tube containing 30 μL of a 1:1 slurry of protein A−Sepharose beads and buffer. Two micrograms of antibody was added to the mixture. Following a 3−4 h incubation at 4 °C, the mixture was centrifuged, and the immune complexed beads were washed three times with 1 mL of buffer. The samples were boiled with 3× sample buffer and subjected to SDS−PAGE and immunoblotting.

Construction and Purification of GST−Cav-1 Fusion Proteins. The GST−caveolin-1 fusion proteins were constructed and purified as previously described (*39*). Briefly, full-length caveolin-1 (1−178) and selected caveolin-1 regions (1−101, 1−81, 61−101, 135−178) were subcloned into the vector pGEX-4T1. After expression in *Escherichia coli* (BL21 strain; Novagen, Inc.), GST−caveolin-1 fusion proteins were affinity purified on glutathione−agarose beads, using the detergent sarcosyl for initial solubilization.

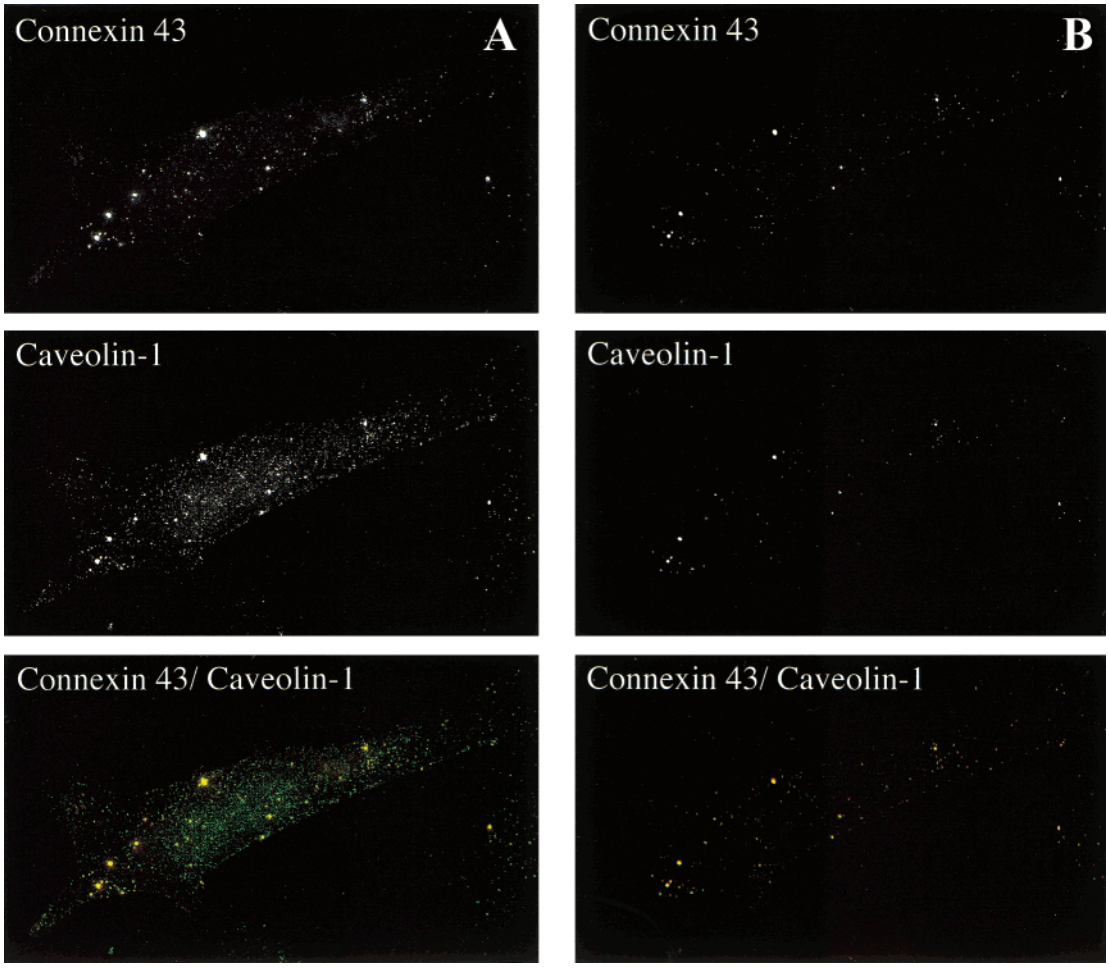

FIGURE 1: Immunofluorescent colocalization of endogenous connexin 43 with caveolin-1. NIH 3T3 cells were labeled with mouse monoclonal Cx43 (Chemicon), rabbit polyclonal caveolin-1 (Santa Cruz), donkey anti-mouse TRITC, and donkey anti-rabbit FITC. The images were captured at 0.5 μm intervals, deconvolved, and recombined to display a two-dimensional image. Panels: upper, Cx43; middle, caveolin-1; lower, merged image (in color, where red is Cx43, green is caveolin-1, and yellow is coincident staining). (A) A 25 μm two-dimensional image of a cell expressing caveolin-1 (green) throughout the cell and Cx43 (red) is shown. (B) A 2 μm section of the same cell showing exact colocalization of caveolin-1 and connexin43 is shown. Note that connexin 43 endogenously colocalizes with a subset of the caveolin-1 expressed in NIH 3T3 cells.

Caveolin-1 GST "Pull-Down" Assay. GST alone or GST−Cav-1 fusion proteins bound to glutathione−agarose beads were extensively washed with TNET buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) and lysis buffer, both containing protease inhibitors. SDS−PAGE followed by Coomassie staining was used to determine the approximate molar quantities of the fusion proteins per 100 μL of packed bead volume. Approximately 100 μL of equalized bead volume was incubated with precleared lysates of 293T cells transfected with connexin 43 at 4 °C for 4 h. After binding, the beads were washed three times with TNET buffer and then resuspended in 3× sample buffer. The samples were subjected to SDS−PAGE and immunoblotting as described above using a polyclonal connexin 43 antibody.

Immunofluorescence. Cos-7 cells were passed 24 h posttransfection onto coverslips and fixed 36 h posttransfection. 3T3 cells were passed directly onto coverslips. Both cell types were fixed with 2% paraformaldehyde for 30 min and washed. The coverslips were quenched using 50 mM NH_4Cl for 10 min and permeabilized for 10 min in PBS with 0.2% bovine serum albumin and 0.1% Triton X-100). Cells were washed in PBS and incubated in primary antibody for 1 h. The cells were washed three times and incubated in secondary antibody conjugated to FITC or TRITC for 30 min. Cells were washed three times in PBS and mounted with Slow-Fade anti-fade reagent (Molecular Probes).

Image Analysis. Cells were imaged using a PlanApo 60×, 1.4 NA objective (Olympus America, Melville, NY) and HiQ-bandpass filters (Chroma Technology, Brattleboro, VT) with a PXL 12-bit, cooled CCD camera (Photometrics, Tuscon, AZ) mounted on an IX70 fluorescence microscope (Olympus) with an electronically controlled illumination shutter (Ludl Electronic Products, Hawthorne, NY) using IPLab Spectrum software (Scanalytics, Fairfax, VA) on a Power Macintosh computer. Deconvolution was performed using IPLab Spectrum software. Three-dimensional data sets were collected at 0.5 μm steps, deconvolved, using an assumed point-spread function, and combined to produce a 2D image. All image acquisition and deconvolution were done at the Analytical Imaging Facility of the Albert Einstein College of Medicine.

RESULTS

Endogenous Colocalization of Connexin 43 with Caveolin-1. Calcium and potassium channels are enriched in lipid

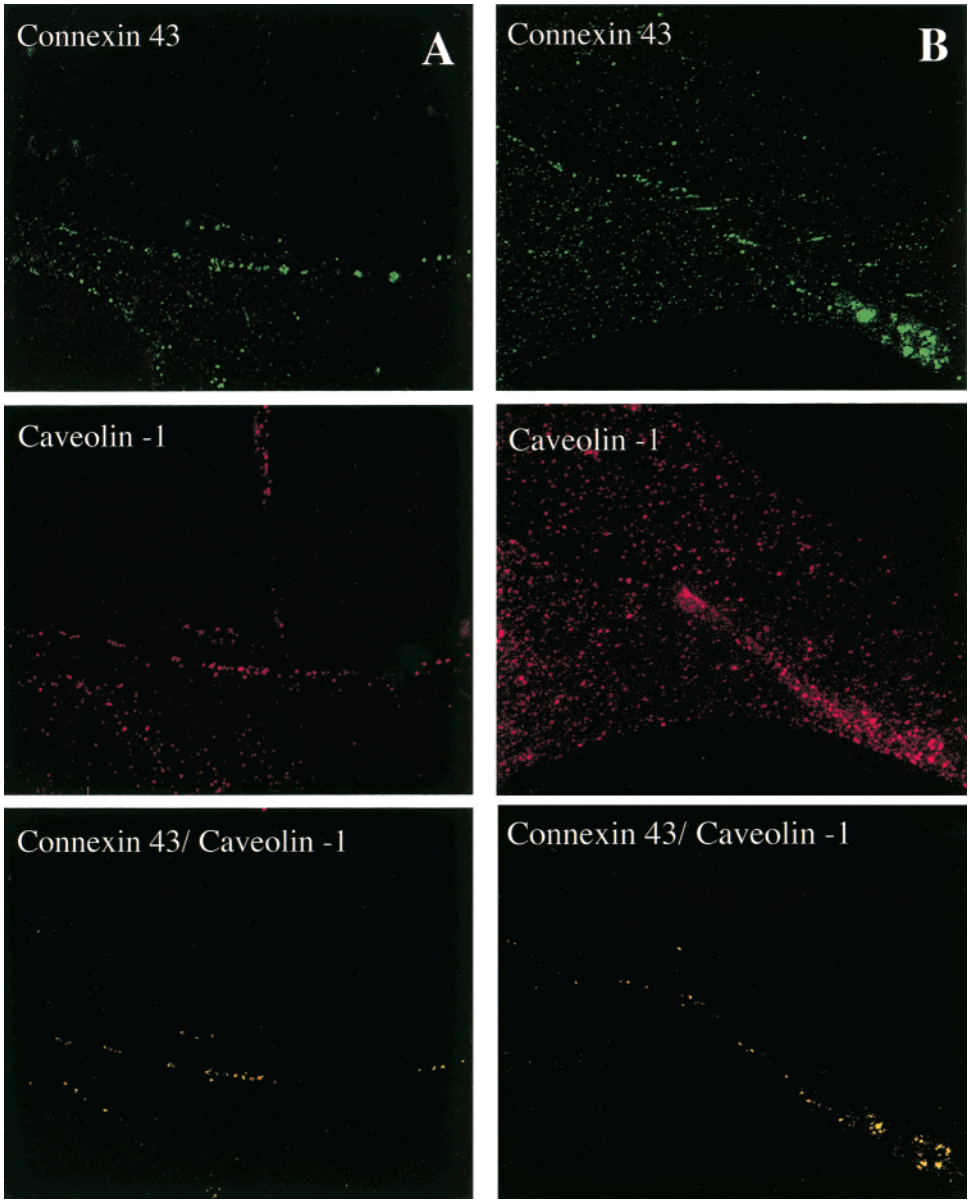

FIGURE 2: Endogenous caveolin-1 and connexin 43 colocalize at areas of cell−cell contact. Double labeling of NIH 3T3 cells to detect endogenous connexin 43, with mouse monoclonal Cx43 (Chemicon), and rabbit polyclonal caveolin-1 (Santa Cruz). Cx43 antibody was detected with an anti-mouse FITC-conjugated secondary antibody, and the caveolin-1 polyclonal was detected with a donkey anti-rabbit TRIC secondary antibody. The images were taken at the focal plane of greatest colocalization and deconvolved. Panels: upper, Cx43 (green); middle, caveolin-1 (red); lower, merged (yellow is coincident staining). In the lower panel all noncoincident staining has been removed to highlight the regions of overlapping labeling. Interestingly, the majority of overlap is found within the regions of cell−cell contact.

raft/caveolae-derived membrane fractions and interact with caveolin-1 (*32*, *34*, *35*). To examine the possible association of gap junctional channels with caveolin-1, we first investigated the localization of connexin 43 and caveolin-1 by immunofluorescence microscopy.

Cx43 is the best studied of the gap junction proteins and is highly regulated by signaling molecules. Although most of the connexins have distinct tissue distributions, Cx43 is the most widely expressed family member. Since caveolin-1 and Cx43 (*40*) are endogenously expressed in NIH 3T3 cells, their localization in subconfluent NIH 3T3 cells was determined by immunofluorescence. Cx43 expression was detected with a specific rabbit polyclonal antibody, and caveolin-1 was detected with a mouse monoclonal antibody (mAb cl no. 2234). Images were captured at every 0.5 μm step over a depth of 25 μm on a CCD camera mounted on an inverted microscope with a computer-controlled focusing feature. The images were deconvolved, and each image stack was flattened into a two-dimensional image.

Figure 1 shows that both caveolin-1 and Cx43 are localized throughout the cell membrane in distinctive punctate patterns, with caveolin-1 showing more discrete spots within the cell and Cx43 showing more diffuse overall staining (Figure 1A, top and middle panels). Notably, however, larger spots of immunoreactivity were coincident in these images. Overlay of these flattened image stacks revealed both the coincident and noncoincident staining more clearly (Figure 1A, bottom panel, where green indicates Cav-1, red Cx43, and yellow the colocalization of both antigens). Although most of the small caveolin-1 positive puncta did not overlap with the more diffuse Cx43 immunolabeling, almost all larger spots were strongly immunolabeled with both antibodies.

The extent to which caveolin-1 and Cx43 are colocalized within the cell is better appreciated when staining is evaluated in a thinner (2 μm) section of the same cell, taken at a focal plane of maximal Cx43 expression (Figure 1B, top and middle panels). Note in the overlay of these images (Figure 1B, bottom) that small caveolin-1 and Cx43 puncta are distinctively labeled, whereas there is virtually complete overlap of these antigens in larger immunoreactive spots. We conclude from these experiments that, in NIH 3T3 cells in which both caveolin-1 and Cx43 are endogenously expressed, a subpopulation of caveolin-1 and Cx43 expressed are colocalized in the cell.

Connexin 43 and Caveolin-1 Are Colocalized at Areas of Cell−Cell Contact. To further evaluate the overlap in the distribution of Cx43 and Cav-1, NIH 3T3 cells approaching confluence were coimmunostained with specific antibodies. As is illustrated in Figure 2, immunostaining with both antibodies was particularly strong at junctional regions, although nonjunctional pools of both proteins were detected in these cells. These imaging studies provide an additional indication that subpopulations of these two proteins interact and suggest that a major site of this interaction is within or close to appositional junctional membranes at the cell surface.

Connexin 43 Is Targeted to Lipid Rafts and Interacts with Caveolin-1. Next, we evaluated if connexin 43 is enriched in cholesterol−sphingolipid-rich raft domains. When caveolin-1, a cholesterol-binding protein (*41*, *42*), is expressed, it is targeted to these lipid rafts and induces the formation of morphological caveolae (*39*, *43*, *44*). However, cholesterol−sphingolipid rafts exist in the absence of caveolin protein expression (*38*, *39*, *45*). Interactions between cholesterol and sphingolipids make these plasma membrane microdomains resistant to nonionic detergents at low temperatures, thereby facilitating their rapid purification (*46*−*48*). Likewise, Cx43 becomes Triton insoluble late in its voyage to the plasma membrane, although newly synthesized Cx43 is soluble in Triton (*49*). Figure 3A shows that both caveolin-1 and Cx43 are predominantly Triton insoluble after transient expression in 293T cells. This is consistent with the idea that both caveolin-1 and Cx43 are targeted to lipid raft domains.

To more stringently assess the cofractionation of caveolin-1 and Cx43, cholesterol−sphingolipid rafts/caveolae were purified using an established equilibrium sucrose density gradient system that separates these detergent-resistant membranes from the bulk of cellular membranes and cytosolic proteins (see Materials and Methods) (*28*, *38*, *39*, *50*−*57*). In this fractionation scheme, immunoblotting with anti-caveolin-1 IgG can be used to track the position of lipid rafts/caveolae-derived membranes within these bottom-loaded sucrose gradients (*28*, *38*, *39*, *50*, *52*−*55*). These caveolae-enriched membranes (fractions 4 and 5) exclude >99.95% of total cellular proteins and also markers for noncaveolar plasma membrane, Golgi, lysosomes, mitochondria, and endoplasmic reticulum (retained in fractions 8−12) (*38*, *52*, *53*). Figure 3B shows that caveolin-1 and Cx43 are specifically targeted to these cholesterol−sphingolipid raft domains. Interestingly, apparent mobilities of the Cx43 species detected in the caveolar fractions and the use of antibodies recognizing either all (phosphorylated and nonphosphorylated) or only the dephosphorylated Cx43 isoform indicate that both phosphorylated and nonphosphorylated forms of Cx43 were targeted to lipid rafts/caveolae with similar efficiencies.

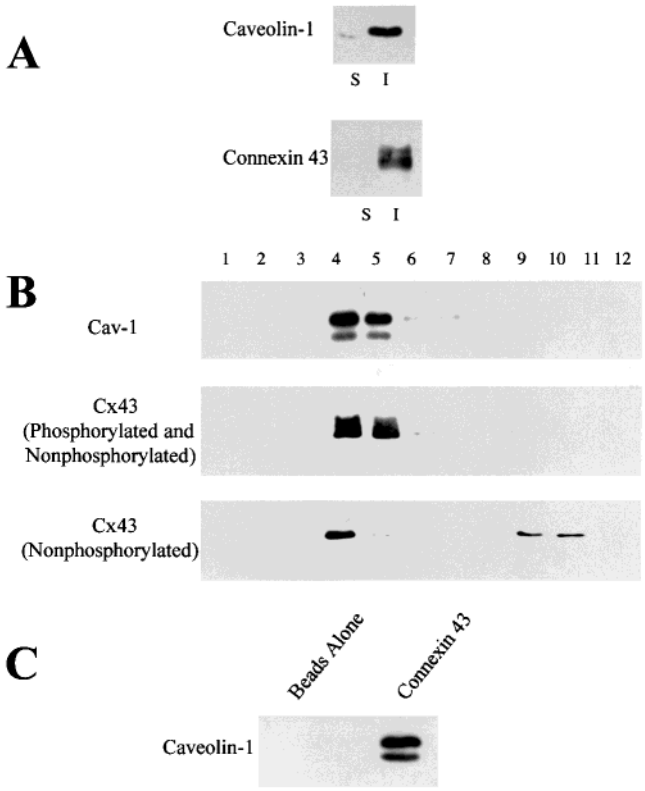

FIGURE 3: Connexin 43 is targeted to lipid rafts and interacts with caveolin-1. 293T cells were transiently transfected with connexin 43 and/or caveolin-1. Thirty-six hours posttransfection, cells were subjected to detailed analyses. (A) Triton solubility. 293T cells were lysed in a buffer containing 1% Triton X-100 to obtain insoluble (I) and soluble (S) fractions. These fractions were adjusted to equal volumes, and an aliquot of each (~20 μL) was analyzed by SDS−PAGE and Western blotting using Cx43 or caveolin-1 specific antibody probes. Note that both caveolin-1 and Cx43 partition predominantly with the Triton-insoluble (I) fraction. (B) Targeting to lipid rafts. 293T cells were homogenized in a buffer containing 1% Triton X-100 and subjected to sucrose density gradient centrifugation. Twelve fractions were collected, and an aliquot of each fraction (~20 μL) was resolved by SDS−PAGE and subjected to immunoblot analysis with anti-caveolin-1 or anti-Cx43 IgG. As expected, recombinant caveolin-1 is highly enriched in fractions 4 and 5 that represent the caveolae-enriched membrane fractions. Note that Cx43 cofractionates with caveolin-1. The distribution of Cx43 was visualized with two distinct antibody probes; one recognizes all forms of Cx43 (Zymed: polyclonal), whereas the other only recognizes the nonphosphorylated form of Cx43 (Zymed: mAb). Similar results were obtained with both of these reagents. (C) Coimmunoprecipitation. 293T cells were lysed and subjected to immunoprecipitation with a rabbit polyclonal antibody directed against connexin 43. After extensive washing, immunoprecipitates were analyzed by SDS−PAGE and Western blotting with mouse mAb directed against caveolin-1. Note that connexin 43 specifically coimmunoprecipitates with caveolin-1.

To further examine the association of Cx43 with caveolin-1, coimmunoprecipitation experiments were carried out using 293T cells cotransfected with Cx43 and caveolin-1. Figure 3C shows that when cell lysates were immunoprecipitated with antibodies directed against Cx43, caveolin-1 is specifically coimmunoprecipitated.

The Carboxyl-Terminal Tail of Cx43 Is Not Required for Its Targeting to Lipid Rafts or for Its Interaction with Caveolin-1. The cytoplasmic COOH-terminal domain of Cx43 (227−382) does not form part of the pore of the gap

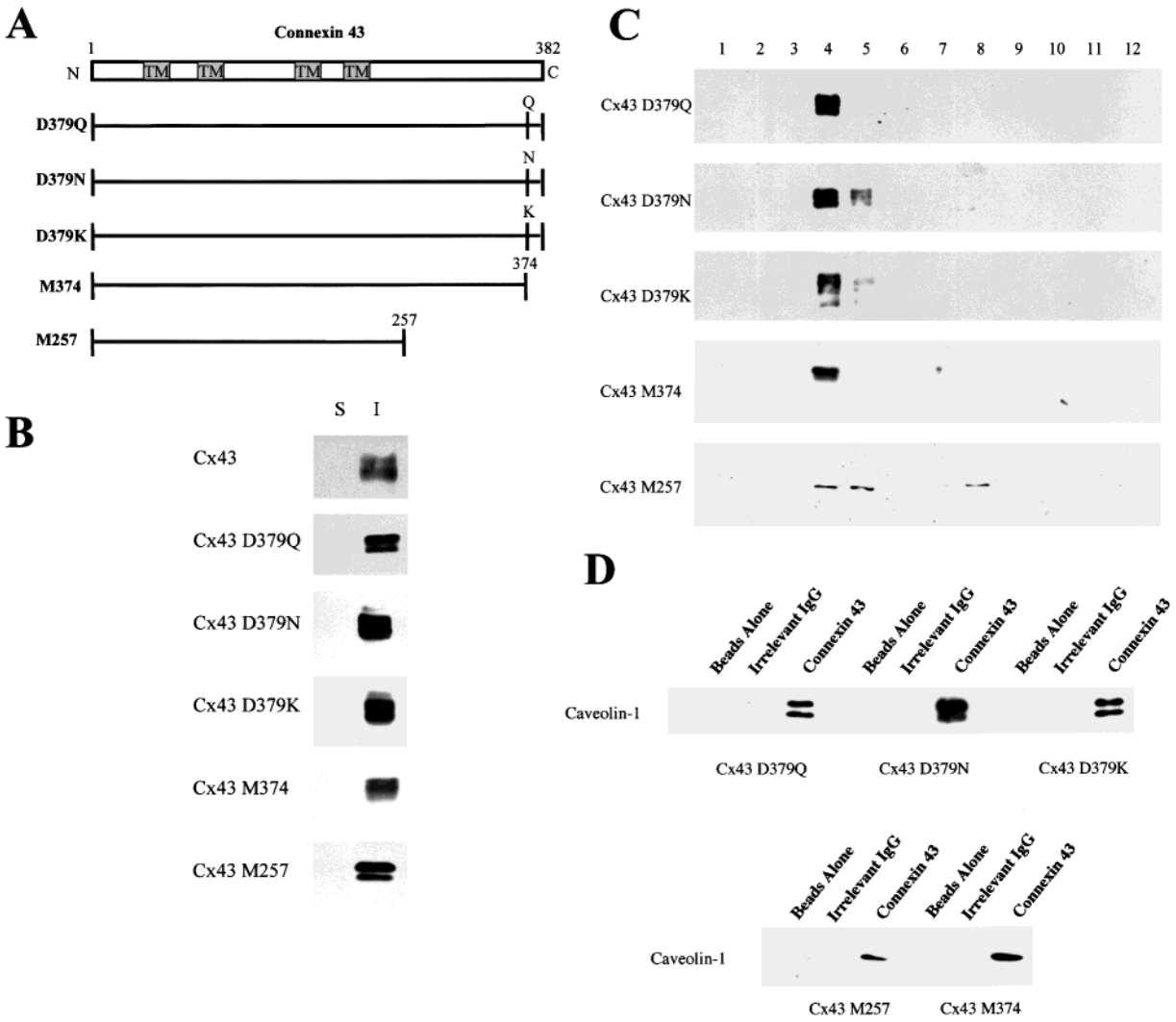

FIGURE 4: The PDZ-binding domain and the C-terminal tail of Cx43 are not required for its targeting to lipid rafts or for its interaction with caveolin-1. 293T cells were transiently transfected with wild-type Cx43 or a variety of Cx43 mutants. Thirty-six hours posttransfection, cells were subjected to detailed analyses. In panel D, cells were cotransfected with caveolin-1. Triton solubility, lipid raft targeting, and coimmunoprecipitation assays were carried out as described in the legend of Figure 3. (A) Cx43 mutants. Schematic diagram summarizing a variety of Cx43 mutants. These Cx43 mutants include three PDZ-binding domain point mutants (D379Q, D379N, and D379K) and two truncation mutants (M374 and M257). (B) Triton solubility. Note that all of the Cx43 mutants examined remain Triton insoluble, behaving like wild-type Cx43. (C) Targeting to lipid rafts. Note that all of the Cx43 mutants examined are correctly targeted to lipid raft domains. However, one mutant (M257) was targeted slightly less efficiently. (D) Coimmunoprecipitation. Note that all of the Cx43 mutants examined still interact with caveolin-1 (Santa Cruz).

junction channel but does play important roles in regulating its opening and closing and most likely also its targeting and retention within the appositional region between cells (*58*). Such regulation is conveyed by numerous signaling molecules, which have been shown to directly interact with the C-terminal regions in vitro and to colocalize with Cx43 in vivo (*59*, *60*). Kinases and phosphatases, as well as protein−protein interactions, affect channel opening and membrane localization of connexons through interactions with the C-terminus of Cx43 (*14*, *59*, *61*−*69*). The inhibition and activation of GJC are caused by different kinases that phosphorylate the C-terminal region of Cx43 (*59*, *70*, *71*). v-Src inhibition of GJC was abolished in cells expressing the C-terminal truncation mutant, Cx43-M256. In addition, after stimulation with PDGF, cells expressing Cx43-M256 inhibited cell growth and motility but did not in cells expressing full-length Cx43 (*72*).

Since connexin 43 is targeted to lipid rafts and coimmunoprecipitates with caveolin-1, we next wanted to determine if Cx43's C-terminal tail is necessary for its interaction with caveolin-1. Using a panel of Cx43 C-terminal mutants (illustrated in Figure 4A), we assessed their (i) Triton solubility, (ii) targeting to lipid rafts/caveolae domains, and (iii) ability to interact with caveolin-1. These Cx43 mutants include three PDZ-domain point mutants (D379Q, D379N, and D379K) and two truncation mutants (M374 and M257).

Interestingly, Figure 4 shows that these five mutants essentially behave as wild-type Cx43 in terms of their Triton solubility (Figure 4B), lipid raft partitioning (Figure 4C), and interactions with caveolin-1 (Figure 4D). Importantly, these results indicate that the PDZ domain and the C-terminal third of the Cx43 molecule are both dispensable for these interactions.

The Scaffolding Domain and C-Terminal Domain of Caveolin-1 Both Recognize Cx43. To further examine the interaction of Cx43 with caveolin-1, we expressed and affinity purified a GST fusion protein carrying the full-length caveolin-1 molecule (GST−Cav-1 FL). GST−Cav-1 FL,

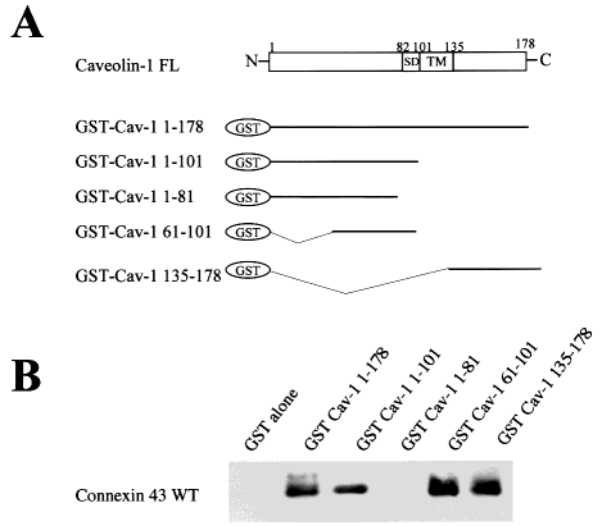

FIGURE 5: The scaffolding and C-terminal domains of caveolin-1 both recognize Cx43. (A) GST−Cav-1 fusion proteins. Schematic diagram summarizing the construction of a panel of GST−Cav-1 fusion proteins. They are as follows: GST−Cav-1 (1−178), GST−Cav-1 (1−101), GST−Cav-1 (1−81), GST−Cav-1 (61−101), and GST−Cav-1 (135−178). (B) Binding of Cx43 to GST−Cav-1. Lysates from 293T cells transfected with the Cx43 cDNA were incubated with a variety of affinity-purified GST−Cav-1 fusion proteins immobilized on glutathione−agarose beads. After extensive washing and elution with reduced glutathione, the eluates were analyzed by SDS−PAGE and subjected to immunoblot analysis with anti-Cx43-specific IgG. Note that GST−Cav-1 (1 − 178) bound Cx43; no binding was observed with GST alone. In addition, GST−caveolin-1 fusion proteins containing the caveolin-scaffolding domain (residues 82−101), i.e., GST−Cav-1 (1−101) and GST−Cav-1 (61−101) retain binding activity. In contrast, a GST−caveolin-1 fusion lacking the scaffolding domain [GST−Cav-1 (1−81)] does not retain any binding activity. An interaction with the caveolin-1 C-terminal domain 1 [GST−Cav-1 (135−178)] is also evident.

attached to glutathione−agarose beads, was incubated with detergent extracts of 293T cells transiently overexpressing Cx43. After extensive washing and elution with reduced glutathione, the samples were separated by SDS−PAGE and subjected to immunoblot analysis with anti-Cx43 IgG. Figure 5 shows that Cx43 bound specifically to full-length caveolin-1, as compared with GST alone.

To identify specific sites within caveolin-1 that recognize Cx43, we next used a panel of GST−caveolin-1 fusions that contain different portions of the caveolin-1 molecule. These fusions are shown schematically in Figure 5A. As shown in Figure 5B, the GST−caveolin-1 fusion proteins containing the caveolin-scaffolding domain (residues 82−101), i.e., GST−Cav-1 (1−101) and GST−Cav-1 (61−101) and the C-terminal domain GST−Cav-1 (135−178), retain binding activity. In contrast, a GST−caveolin-1 fusion lacking the scaffolding domain [GST−Cav-1 (1−81)] does not retain any binding activity. These experiments implicate the caveolin-scaffolding domain and a region of the caveolin-1 C-terminal domain as interaction sites for Cx43. A second interacting region in caveolins (within the C-terminal domain) has been described for the interaction of caveolin-1 with PKA and NOS (*73*, *74*).

Other Connexins (Cx32, Cx36, and Cx46) Are Targeted to Lipid Rafts, While Cx26 and Cx50 Are Specifically Excluded from These Membrane Microdomains. We next explored whether other connexins are targeted to lipid rafts/caveolae and whether they interact with caveolin-1. For this purpose, Cx26, Cx32, Cx36, Cx46, and Cx50 were individually transfected into 293T cells and subjected to the same assays as performed on Cx43. Figure 6A shows that Cx26, Cx32, Cx36, and Cx46 are all Triton insoluble, behaving as expected on the basis of our results with Cx43. In contrast, Cx50 is only partially Triton insoluble.

Figure 6B indicates that connexins 32, 36, and 46 are all efficiently targeted to lipid rafts but that Cx26 and Cx50 are specifically excluded from these membrane microdomains. Thus, although Cx26 is Triton insoluble and Cx50 is partly so (Figure 6A), neither is targeted to lipid rafts, indicating that Cx26 and perhaps Cx50 are targeted to a distinct Triton-insoluble membrane compartment.

To examine their potential interaction with caveolin-1, 293T cells were cotransfected with either connexin 26, 32, 36, 46, or 50 and caveolin-1 and subjected to immunoprecipitation with antibodies directed against a given connexin and immunoblotting with anti-caveolin-1 IgG. Figure 6C shows that caveolin-1 coimmunoprecipitates with Cx26, Cx32, Cx36 and Cx46, but fails to interact with Cx50.

Thus, Cx50 is only partly Triton soluble, is excluded from lipid raft domains, and does not interact with caveolin-1. As such, it is an ideal negative control for these studies and clearly demonstrates the specificity of these interactions.

Targeting of Cx26 to Lipid Rafts Requires Caveolin-1. We chose 293T cells for our recombinant expression studies as they fail to express any known caveolins (*75*). Thus, the targeting of Cx43, Cx32, Cx36, and Cx46 to lipid rafts in these cells is caveolin-independent. However, we made the surprising observation that although Cx26 is excluded from lipid raft domains when it is expressed by itself in 293T cells, it has the ability to form a complex with caveolin-1 when the two proteins are coexpressed.

Thus, one viable hypothesis is that Cx26 requires caveolin-1 for its targeting to lipid rafts, via a "piggy-back" targeting mechanism. To test this hypothesis directly, we expressed Cx26 alone or in combination with caveolin-1 and subjected these cells to fractionation to separate lipid raft domains from the bulk of cellular membranes.

Figure 7A shows that, as expected, Cx26 alone is excluded from lipid rafts. In striking contrast, coexpression of Cx26 with caveolin-1 efficiently recruits Cx26 to lipid raft membranes. In accordance with these observations, when Cx26 and caveolin-1 are coexpressed, they are precisely colocalized as seen by confocal microscopy (Figure 7B). Thus, these results indicate that, for Cx26 to be targeted to lipid rafts, it requires coexpression and interaction with caveolin-1. This trafficking event appears to be unique to Cx26 since the other connexins investigated in this study do not require caveolin-1 for targeting to lipid rafts.

DISCUSSION

The findings in this paper indicate that caveolin-1 and the gap junction protein Cx43 are at least partially colocalized in cells where they are endogenously expressed. Such colocalization is most prominent at junctional membranes in contacting cells and in large spots that may represent either delivery or retrieval vesicles in sparse cell cultures. Like caveolin-1, Cx43 was found to be largely Triton-insoluble in 293T cells transiently cotransfected with caveolin-1 and Cx43, and both proteins were found in the lipid raft domains

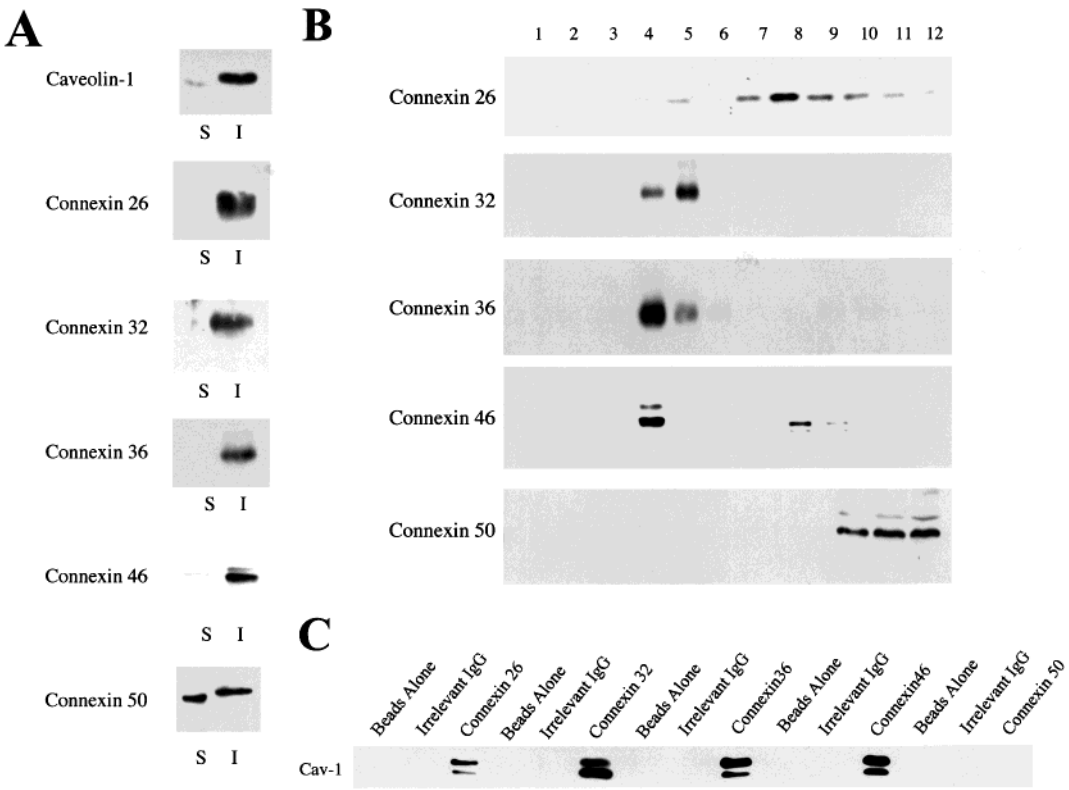

FIGURE 6: Other connexins (Cx32, Cx36, and Cx46) are targeted to lipid rafts, while Cx26 and Cx50 are specifically excluded from these membrane microdomains. We next explored whether other connexins are targeted to lipid rafts/caveolae and whether they interact with caveolin-1. For this purpose, connexins 26, 32, 36, 46, and 50 were individually transfected into 293T cells and subjected to the same assays as performed on Cx43. Triton solubility, lipid raft targeting, and coimmunoprecipitation assays were carried out as described in the legend of Figure 3. In panel C, cells were cotransfected with caveolin-1. (A) Triton solubility. Note that connexins 26, 32, 36, and 46 are all Triton insoluble, behaving as expected on the basis of our results with Cx43. In contrast, Cx50 is only partially Triton insoluble. (B) Targeting to lipid rafts. Note that connexins 32, 36, and 46 are all efficiently targeted to lipid rafts, but that Cx26 and Cx50 are specifically excluded from these membrane microdomains. Thus, although Cx26 is Triton insoluble (panel A), it is not targeted to lipid rafts, indicating that it is targeted to a distinct Triton-insoluble membrane compartment. (C) Coimmunoprecipitation. Note that caveolin-1 coimmunoprecipitates with Cx26, Cx32, Cx36, and Cx46 but fails to interact with Cx50. Thus, Cx50 is excluded from lipid raft domains and does not interact with caveolin-1. As such, it is an ideal negative control for these studies and clearly demonstrates the specificity of these interactions.

following sucrose gradient centrifugation. These findings are largely consistent with previous studies showing that Cx43 becomes Triton-insoluble as it matures and reaches the junctional membrane (*49*). Moreover, the demonstrated association with the cholesterol−sphingolipid raft domains is consistent with reports of the lipid composition within junctional membranes (*76*, *77*) and with the demonstrated colocalization of gap junctions with the cholesterol marker filipin within certain subcellular compartments, although perhaps not within the appositional gap junction itself (*78*, *79*). Such differences in lipid composition of junction-containing membranes (see above and ref *80*) may provide an explanation for the altered detergent solubility of connexins within the plasma membrane.

Perhaps surprisingly, mobilities of Cx43 isoforms detected with a polyclonal antibody recognizing all phosphorylation states spanned a wide range, suggesting that both phosphorylated and nonphosphorylated Cx43 proteins are within the lipid rafts, and the presence of the nonphosophorylated Cx43 form was confirmed by immunoblotting with the Zymed monoclonal antibody, which recognizes primarily this isoform (*81*). This finding provides additional evidence that junctional membranes contain a mixture of Cx43 phosphorylation isoforms; such heterogeneity is the presumed substrate for the modulation of coupling strength provided by kinases and phosphatases (see refs *82* and *83*).

Coimmunoprecipitation experiments indicate that Cx43 interacts with caveolin-1. Direct interactions between Cx43 and the tight junction-associated protein zonula occludens-1 (ZO-1) (*13*, *14*), v- and c-Src (*67*), and β-catenin (*84*) have been demonstrated using similar techniques, and these findings suggest that Cx43 is part of a complex, the Nexus, that may include both signaling and scaffolding molecules. Although the connexin−protein interactions shown previously appear to involve the carboxyl terminus of Cx43, the results obtained in our studies with five Cx43 mutants, including two that severely truncate the carboxyl-terminal domain, indicate that they behave quite similarly to wild-type Cx43 with regard to Triton solubility, lipid raft partitioning, and interactions with caveolin-1. To determine which domains of caveolin-1 are responsible for the Cx43 binding, pull-down experiments were performed with GST fusion constructs of caveolin-1 domains. These experiments implicate both the caveolin-scaffolding domain and a region of the caveolin-1 C-terminal domain as interaction sites for Cx43.

Studies on additional connexin types indicate that several (Cx32, Cx36, Cx46) behave similarly to Cx43 with regard to detergent solubility and lipid raft partitioning in the absence of caveolin-1, although some connexins (Cx26, Cx50) behave differently with respect to one or more of these parameters. Thus, while Cx26 was Triton insoluble, and

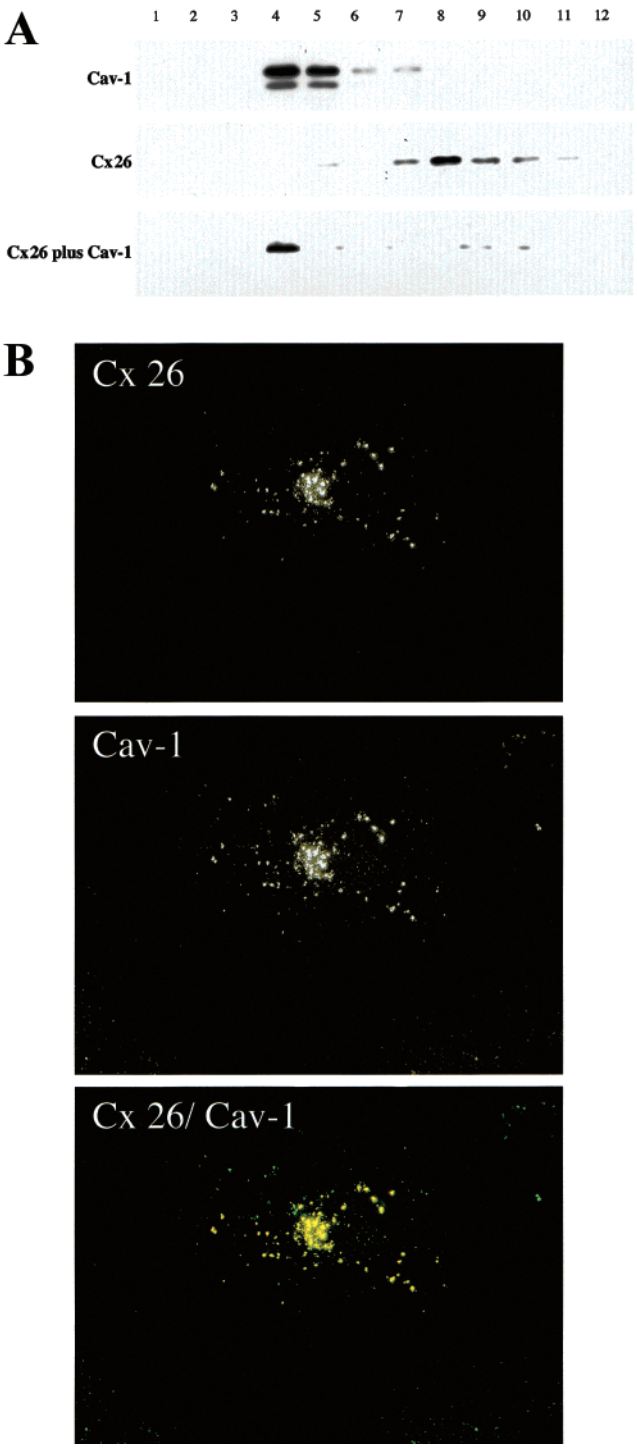

FIGURE 7: Targeting of Cx26 to lipid rafts requires caveolin-1. 293T cells were transiently transfected with wild-type Cx26 in the absence or presence of caveolin-1. Thirty-six hours posttransfection, cells were subjected to detailed analyses to evaluate the effect of caveolin-1 coexpression on the distribution of Cx26. (A) Targeting to lipid rafts. Note that Cx26 alone is excluded from lipid rafts/caveolae. In striking contrast, coexpression of Cx26 with caveolin-1 efficiently recruits Cx26 to lipid raft membranes. (B) Colocalization of Cx26 with caveolin-1. In accordance with the subcellular fractionation data presented in panel A, when Cx26 and caveolin-1 are coexpressed, they precisely are colocalized as seen by confocal microscopy. Thus, these results indicate that for Cx26 to be targeted to lipid rafts, it requires coexpression and interaction with caveolin-1. This trafficking event appears to be unique to connexin 26, since the other connexins investigated in this study do not require caveolin-1 for targeting to lipid rafts.

Cx50 was partially so, neither was incorporated into the lipid rafts, indicating the existence of a spatially distinct Triton-insoluble compartment. Nevertheless, Cx26 was incorporated into the lipid rafts in the presence of caveolin-1, and these two proteins were found to be largely colocalized within cotransfected cells.

Although the implications of differences in connexin−lipid raft associations for physiological processes remain unclear, it is intriguing to suggest that the anomalous behavior of Cx26 may in part explain the alternative trafficking pathway followed by this connexin in cells such as hepatocytes where it is coexpressed with another gap junction protein, in this case Cx32. In differentiated cultured hepatocytes, Cx32 trafficking to junctional domains is sensitive to block by brefeldinA, whereas Cx26 is only partially affected (*85*), and the pathways are hypothesized to be only partly overlapping (*20*).

The unusual behavior of Cx50 compared to most other gap junction proteins, and in particular to Cx46, with which it is tightly coexpressed in lens fiber cells, suggests the possibility that the two coexpressed connexins may reach the cell surface through distinct pathways. Alternatively, the formation of heteromeric connexons, in which Cx46 and Cx50 coexist within the same hemichannel (*86*), may provide a mechanism whereby a largely Triton-insoluble connexin may be incorporated into lipid rafts. Interestingly, although expression of Cx46 and Cx50 overlaps quite extensively in normal lens fiber cells, mice lacking one or the other of these connexins exhibit different phenotypes (*87*, *88*). Although both Cx46- and Cx50-deficient mice exhibit juvenile-type cataracts, the Cx50 knockout lenses are smaller, perhaps reflecting an inability of this connexin to partition into the appropriate membrane environment at an early developmental stage.

Future studies will have to address the role of caveolin-2 in the targeting of connexins to lipid rafts and caveolae. Unfortunately, when caveolin-2 is expressed alone, it is retained intracellularly in the ER/Golgi compartments and is degraded by the proteasome, as it requires coexpression with caveolin-1 for its transport to the plasma membrane and caveolae (*89*−*91*). As such, it would be difficult to assess whether the effects of caveolin-2 expression are due to caveolin-2 itself or to caveolin-1. Thus, the analysis would be confounded. Fortunately, caveolin-1 does not require coexpression with caveolin-2 for any of its known functions.

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