Mutations in the Second Extracellular Region of Connexin 43 Prevent Localization to the Plasma Membrane, but Do Not Affect Its Ability to Suppress Cell Growth

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Abstract
Connexin 43 (Cx43), the most widely expressed gap junction protein, has a role in regulation of cell growth. In this study, we demonstrate that the point mutations F199L, R202E, and E205R in the second extracellular region of Cx43 prevent localization of the mutant proteins to the plasma membrane. The mutants were aberrantly localized in the cytoplasm if expressed in HeLa cells, which lack Cx43. Coexpression with wild-type Cx43 promoted localization of the F199L and R202E mutant proteins to the plasma membrane. By dye transfer assay, we showed that gap junctional intercellular communication (GJIC) is decreased in cells expressing the mutants, compared to Cx43 wild-type-expressing cells. However, the F199L mutant does not appear to have a dominant-negative effect on GJC. Despite the loss of GJC, the ability of the F199L Cx43 mutant to inhibit growth of either Cx43−/− cells or two cancer cell lines, HeLa and C6 glioma cells, was similar to that of the wild-type Cx43. In addition, we showed that both R202E and E205R Cx43 mutant expressions cause growth retardation of HeLa cells. Therefore, the point mutations in the second extracellular region of Cx43 do not affect the ability of the mutant proteins in vitro to suppress cell growth, although they prevent localization to the plasma membrane. The results support the concept that regulation of cell growth by Cx43 does not necessarily require GJC and suggest that the growth-suppressive properties of Cx43 may be independent of the second extracellular loop.

Introduction
Gap junctional connections are groups of intercellular channels that mediate transfer of ions and small molecules between neighboring cells (1). Gap junctional connections have an important role in essential cellular processes such as development, proliferation, differentiation, and cell death (2). The channels are highly specialized structures, the formation of which requires cooperation between two neighboring cells. In vertebrates, the channels are formed by phosphoproteins called connexins. Six monomers of connexin form half of the channel (hemichannel) in the plasma membrane and interaction of two hemichannels on adjacent cells forms a gap junctional connection (3).

Gap junctional communication (GJC) is regulated by various physiological stimuli, including mitogenic agents (4–8) and growth inhibitory factors (9, 10). GJC is decreased or absent in most tumor cells (11). Products of several oncogenes, such as v-Src (12), v-Mos (13), SV40 T antigen (14), and v-fps (15), can cause a decrease in cell-cell communication. Consistent with these observations, connexin 43 knockout (Cx43−/−) cells have properties similar to those of transformed cells. Cx43−/− cells have a spindle shape and long processes. They grow faster and to higher densities than Cx43+/+ cells. They also adhere less well to the substrate. Cx43−/− cells show greatly reduced GJC, indicating a role for Cx43 in establishing GJC in these cells (16).

Connexin family members have conserved extracellular and transmembrane domains, but are more diverse in their intracellular sequences (17). The extracellular regions of Cx43 are required for docking of connexins from the adjacent plasma membranes during formation of both homo- and heterotypic channels (18). Previous studies of connexin mutants, with mutations in either the extracellular or transmembrane regions, or the COOH-terminal intracellular domain, showed a correlation between aberrant expression and localization of connexins and loss of GJC (19–21). Cx32 mutants with aberrant localization associated with Charcot-Marie-Tooth disease have the E186K mutation in the second extracellular region (21). Studies of patients who suffer from hereditary deafness also showed mutations in the second extracellular region of Cx26 (22–24). These results suggested a role for the extracellular regions of connexin proteins in proper cellular distribution, in addition to interaction of hemichannels.

In this study, we analyzed the effect of the mutations in the extracellular region in Cx43 in protein transport, GJC, and cell growth. We isolated a Cx43 mutant with substitution F199L in the second extracellular region. In addition, we created the R202E and E205R mutations in the region that is highly conserved between connexin family members. The F199L Cx43 mutant protein was localized in the cytoplasm, but not the plasma membrane, when expressed in Cx43−/− cells and HeLa cells. We observed similar localization using the R202E and E205R Cx43 mutants. However, in the presence of either endogenous or ectopically expressed wild-type Cx43, the F199L and R202E mutants were localized in both the...
cytoplasm and the plasma membrane. Our results suggest that the second extracellular region of Cx43 may be involved not only in the docking of two hemichannels, but also in transport of Cx43 protein to the plasma membrane. Using double-dye transfer assay and fluorescence-activated cell sorting (FACS) analysis, we found that the F199L, R202E, and E205R mutations decrease the ability of Cx43 to assemble into functional gap junctional channels compared to the wild type. When we analyzed the ability of Cx43 to suppress cell growth, we found that growth inhibition by all three Cx43 mutant proteins was comparable to that of the wild type, despite the inability of the mutants to form functional gap junctions, indicating that regulation of cell growth by Cx43 does not necessarily require GJC.

Results

Establishment of Stable Cx43 Expression in Cx43 Knockout Cells

To obtain uniform expression of Cx43, we infected Cx43-/- cells with viral supernatant from the packaging cell line BOSC 23, transfected with retroviral vector pMSCVCx43. We selected pools of drug-resistant cells that reexpressed either wild-type Cx43 or the F199L mutant. Expression of Cx43 protein was confirmed by immunoprecipitation and Western blot, normalized for total protein concentration. Drug-resistant pools expressed levels of wild-type Cx43 and the F199L mutant that were comparable to each other (Fig. 1A, lanes 2 and 3, respectively). We observed a difference in the pattern of phosphorylated isoforms between the wild type and the F199L mutant. As shown in Fig. 1A, there are two slower migrating bands, designated as P1 and P2, in wild-type Cx43. The P1 form was missing in the F199L mutant lysates (Fig. 1A, lane 3).

To confirm that the difference in slower migrating band pattern was a consequence of phosphorylation, we performed 32P-metabolic labeling and immunoprecipitation. We used HeLa cells expressing either exogenous wild-type Cx43 or the F199L mutant (Fig. 1B), since the infected Cx43-/- cells described above did not show sufficient labeling of Cx43 proteins (data not shown). By analyzing the protein on a 7.5% polyacrylamide gel, we detected three phosphorylated forms of Cx43 wild type (Fig. 1B, lane 2), P0, P1, and P2. The P0 form of the F199L mutant protein was comparable to that of wild type, while Cx43 phosphorylation of forms P1 and P2 was reduced by 34% (Fig. 1B, lane 3).

Localization of Cx43 Wild-Type and Mutant Protein

We next examined localization of the F199L mutant in infected Cx43-/- cells. Almost all cells in the pool of stably infected cells were positive for Cx43 staining as judged by immunofluorescence. No staining was observed in mock-infected Cx43-/- cells (Fig. 2A). As shown in Fig. 2B, wild-type Cx43 was localized in both cytoplasm and in cell-cell contacts. In contrast, the F199L mutant protein accumulated in the cytoplasm and punctate staining at the plasma membrane was not detected (Fig. 2C). Furthermore, a similar intracellular localization pattern for the F199L mutant was found when it was expressed in HeLa cells, which do not express endogenous Cx43 (Fig. 2D). We verified the lack of endogenous Cx43 in HeLa cells by Western blot (see below). These results suggest that the F199L mutant protein fails to properly localize to the plasma membrane in the absence of endogenous Cx43.

The F199L, R202, and E205R Cx43 Mutants Fail to Form Functional Gap Junction Connections in HeLa Cells

We were unable to demonstrate restoration of GJC in Cx43-/- cells expressing wild-type Cx43 after infection, possibly because of a progressive decrease in the levels of Cx43 during drug selection (data not shown). To examine whether the F199L, R202E, and E205R mutants can establish functional gap junctional channels in HeLa cells, we measured GJC in HeLa cells transiently transfected with Cx43 wild type and the mutants using a double-dye transfer assay, as described in “Materials and Methods.”

To estimate transfection efficiency, we transfected cells with the pEGFPN1 plasmid. FACS analysis showed that about 60% of the cells were positive for green fluorescent protein (GFP) (data not shown). Western blot analysis showed higher

![FIGURE 1](http://example.com/fig1.png)

**FIGURE 1.** Stable Cx43 expression in infected Cx43-/- cells and phosphorylation of Cx43. **A.** Immunoprecipitation was performed using affinity-purified rabbit anti-Cx43 antibody and proteins were resolved on a 12% SDS-polyacrylamide gel. Western blot was performed using rabbit polyclonal anti-Cx43 antibody. Cx43 wild type (lane 1), Cx43-/- pMSCV mock-infected cells (lane 2), and Cx43-/- pMSCV F199L Cx43 (lane 3). NP, non-phosphorylated form of Cx43; P1 and P2, slower migrating phosphorylated forms of Cx43. **B.** HeLa cells transiently transfected with Cx43 were metabolically labeled for 4 h. Anti-Cx43 immunoprecipitates were resolved on a 7.5% polyacrylamide gel. Mock transfected (lane 1), Cx43 wild type (lane 2), and Cx43 F199L (lane 3). P0, P1, and P2, phosphorylated forms of Cx43.
levels of Cx43 wild-type and mutant protein expression in transiently transfected HeLa cells, compared to the level of endogenous Cx43 in Cx43+/+ cells (Fig. 3E). As mentioned above, we did not observe endogenous Cx43 in mock-transfected HeLa cells (Fig. 3E). Using the double-dye transfer assay, we measured the percentage of recipient cells that took up dye from donor cells when they were mixed in a 1:1 ratio (see “Materials and Methods”). We detected 95% Cx43+/+ cells that took up dye after 16 h of coculture of donor and acceptor cells (Fig. 3B, Table 1). At the same incubation time, 50% of HeLa cells transfected with wild-type Cx43 took up dye, which may be accounted for by the 60% efficiency of transfection (Fig. 3B, Table 1). In contrast, only 5% of the F199L mutant transfected cells took up dye, a level similar to that of mock-transfected cells (3%) (Fig. 3B, Table 1). The Cx43 R202E and Cx43 E205R mutants showed similar low levels of GJC (4–5%) (Table 1).

We also analyzed the distribution of calcein-AM (the diffusible dye) in the total population of R-18 (the non-diffusible dye)-labeled cells (region R7 in Fig. 3, A and B). As shown in the histogram in Fig. 3D, a fraction of the Cx43 wild-type transfected HeLa cells (presumably those expressing Cx43) had an intensity of calcein-AM in the R7 region, similar to that of Cx43+/+ cells. The mean of calcein-AM in the R7 region for the F199L mutant-expressing cells was similar to that of the mock-transfected control. We also observed a slightly higher calcein-AM fluorescence in the R7 region for mock-transfected cells after 16 h of incubation (mean 17, panel D) compared to time 0 (mean 5, panel C), which is likely to result from slow diffusion of calcein-AM from cells to the culture medium and reuptake of the dye by R-18-labeled cells. These results indicate that the F199L, R202, and E205 mutations prevented establishment of GJC in Cx43-transfected HeLa cells.

**Coexpression of Cx43 Wild Type and Mutants**

We next examined the localization of the F199L mutant protein in cells expressing endogenous Cx43 to test whether the F199L mutant and wild-type Cx43 can form a hemi-channel and localize to the plasma membrane. We constructed a fusion protein with yellow fluorescent protein (YFP) linked to the COOH terminus of the F199L mutant. Previous studies demonstrated that fusing YFP to wild-type Cx43 does not affect the proper localization of Cx43 to the plasma membrane (25). When we transfected the YFP-F199L fusion protein into 293T cells, which express endogenous wild-type Cx43, we observed not only cytoplasmic, but also membrane localization of the mutant in contacts between the cells (Fig. 4B), similar to the YFP-wild-type Cx43 fusion protein (Fig. 4A).

In addition, we examined the phosphorylation state of the YFP-F199L fusion protein to determine whether it is
comparable to that of the YFP-wild-type Cx43 expressed in 293T cells. As shown in Fig. 4C, the F199L mutant was phosphorylated (lane 3), although the level of phosphorylation was reduced by 40% compared to the wild-type Cx43 (lane 2), as might be expected if a fraction of the mutant is retained in the cytoplasm.

To test the effect of coexpressing wild-type and mutant connexins in HeLa cells, we first expressed the YFP-Cx43 F199L, YFP-Cx43 R202E, and YFP-Cx43 E205R fusion proteins in HeLa cells by transient transfection. As shown in Fig. 5, YFP-Cx43 F199L, YFP-Cx43 R202E, and YFP-Cx43 E205R (Fig. 5, A, E, and I, respectively) fusion proteins were localized in the cytoplasm with a staining pattern similar to that shown in HeLa cells transfected with the non-tagged F199L mutant (Fig. 2D). To distinguish Cx43 wild-type and mutant proteins, we created a wild-type Cx43 fusion protein by adding cyan fluorescent protein (CFP) at the COOH terminus of the wild-type Cx43 and used it to transfect HeLa cells. The CFP-wild-type Cx43 fusion protein expressed alone was properly localized in the plasma membrane (data not shown). When we cotransfected HeLa cells with CFP-wild-type Cx43 and the YFP-F199L mutant, WT Cx43 localized at the plasma membrane (Fig. 5B) and the F199L mutant was both in the membrane and the cytoplasm (Fig. 5C). A merged image (Fig. 5D) shows colocalization of the wild-type Cx43 and the F199L mutant in the plasma membrane of HeLa cells. We observed a similar localization pattern using the YFP-Cx43 R202E mutant (Fig. 5, F, G, and H, respectively). However, the YFP-Cx43 E205R fusion mutant protein was localized only in the cytoplasm of HeLa cells even in the presence of the wild-type protein (Fig. 5K).

Taken together, these results show that the YFP-F199L, YFP-R202E, and YFP-E205R Cx43 fusion proteins fail to localize in the plasma membrane when expressed alone. However, both F199L and R202E mutants can localize in the plasma membrane when expressed together with wild-type Cx43. These observations suggest that phenylalanine 199 and arginine 202 are required either for hexamer formation or for targeting of Cx43 to the plasma membrane.

The F199L Mutant Does Not Inhibit GJC When Coexpressed With Wild Type

We tested whether the F199L mutant has a dominant-negative effect on the ability of wild-type Cx43-containing channels to transfer dye. We cotransfected HeLa cells with the F199L mutant and wild-type Cx43, which colocalize in the membrane (above). The cotransfected cells showed comparable levels of communication to HeLa cells expressing only wild-type Cx43 (49% versus 47%) (Fig. 6). These data suggest that the mutant does not have a dominant-negative effect on the function of wild-type Cx43 in GJC.

Regulation of Cell Growth

Expression of Cx43 inhibits the growth of many transformed and tumor cells (11). However, recent data suggest that Cx43 function in growth regulation does not always depend on formation of gap junctional connection (26–30). To examine whether mislocalization of the Cx43 F199L, R202E, and E205R mutants affects their ability to reduce cell growth, we performed retroviral infection of Cx43−/− cells as well as two tumor cell lines, HeLa and C6 glioma, and counted the number of cells daily after infection. Cx43 expression in the stable cell lines is shown in Fig. 7D. As shown in Fig. 7A, we observed significant cell growth inhibition of Cx43−/− cells infected with either wild-type Cx43 or the F199L mutant, compared to mock-infected cells, 5 days after plating. Growth of C6 glioma cells expressing Cx43 or the F199L mutant was also significantly slower than the growth of mock-infected cells 4 days after plating (Fig. 7B). In addition, HeLa cells expressing either wild-type Cx43 or any of the Cx43 mutant proteins showed slower growth rates compared to mock-infected cells (Fig. 7C). These results suggest that the F199L, R202E, and E205R mutant proteins behave similarly to the wild type in their ability to inhibit cell growth.

Discussion

The process of neoplastic transformation is associated with down-regulation of connexins and GJC (11, 31). In this study, we demonstrated that point mutations of conserved residues in the second extracellular region of Cx43 lead to retention of the mutant proteins in the cytoplasm and, consequently, to loss of GJC, without affecting the ability of the mutant proteins to inhibit tumor cell growth in vitro.

We analyzed the Cx43 F199L, R202E, and E205R mutants expressed in cells that lack endogenous Cx43. We found that the mutant proteins were localized in the cytoplasm, but not in the plasma membrane, and did not form functional gap junctional channels in HeLa cells. Most strains of HeLa cells do not express connexins and show reduced GJC (32). The low level of communication that we detected in the cells expressing Cx43 mutants is consistent with the intracellular localization of the mutant proteins as shown by immunofluorescence analysis.

Using CFP and YFP fusion proteins, we showed that the F199L mutant localizes at cell-cell contacts when coexpressed with wild-type Cx43 in HeLa cells or 293T human embryonal kidney cells. We observed similar localization of the R202E mutant coexpressed with the wild type in HeLa cells, but not of the E205R mutant. The cytoplasmic localization of the Cx43 E205R mutant protein in the presence of the wild type in HeLa cells may be explained by the proximity of the E205 residue to the fourth transmembrane domain of Cx43. Our results indicate that insertion of the F199L and R202E mutants into the plasma membrane is promoted by the presence of wild-type Cx43.

Similar conclusions were reached by Krutovskikh et al. (20) who observed that the T145Y mutant was localized in the cytoplasm in A431 cells that lack endogenous connexins, but was correctly localized to the membrane when expressed in rat bladder carcinoma BC31 cells, which, unlike most other tumor cells, express wild-type Cx43 and have functional gap junctions (20). In the case of Cx32, hexameric hemichannel assembly involves formation of dimeric and tetrameric Cx32 intermediates (33). A putative calmodulin-binding site in the COOH terminus of Cx32 is required for formation of hexamers and membrane localization (33). Dimers or tetramers
of wild-type Cx43 may be able to oligomerize with the F199L or the R202E mutant proteins which themselves may lack determinants required for connexon formation or membrane targeting.

The formation of functional Cx43 gap junctional channels requires connexin phosphorylation. If cells are treated with inhibitors of protein trafficking, such as Brefeldin A, Cx43 phosphorylated forms P1 and P2 cannot be detected (34). Our data show that the F199L Cx43 mutant is not phosphorylated to the P2 isoform when expressed in cells without endogenous Cx43, suggesting that cytoplasmic localization prevents the hyperphosphorylation of the mutant protein. The low level of Cx43 phosphorylation in the case of the F199L-YFP fusion protein expressed in 293T cells may be explained by lower phosphorylation of the cytoplasmic form, compared to the less abundant membrane-associated form.

The F199L, R202E, and E205R Cx43 mutants can inhibit cell growth despite their inability to form functional gap junctional channels. We observed a significant reduction of growth in HeLa cells, C6 glioma cells, and in Cx43+/− cells. The level of growth inhibition by the mutants was similar to that by wild-type connexin. The time at which the inhibition by

FIGURE 3. GJC. The level of cell-cell communication was measured by double-dye transfer assay. HeLa cells were transiently transfected with either vector alone, Cx43 wild type, or the F199L Cx43 mutant. A population of acceptor cells was labeled with membrane-bound dye R-18 (panels A and B, UL, upper left quadrants) and a population of donor cells was labeled with calcein-AM (LR, lower right quadrants). The two populations were mixed in ratio 1:1 either just before analysis (time 0, panels A and C) or mixed and cultured overnight (time 16 h, panels B and D). The resulting populations of R-18-labeled cells that received calcein-AM are shown in the upper right quadrants, R7 (panels A and B). R7 is the region containing all R-18-labeled cells. Panels C and D show histogram analysis of calcein-AM distribution in the R7 region. FL1 is the intensity of calcein-AM fluorescence. These data are quantified in Table 1. Western blot analysis showed comparable levels of Cx43 wild type and the F199L mutant protein in HeLa cells, higher than level of endogenous Cx43 in Cx43+/+ cells (panel E).
the mutant or the wild-type occurred was similar as well. These results suggest that the functional gap junctional connections established in cells infected with wild-type Cx43 did not enhance the reduction of growth rate.

Inhibition of cell growth by expression of Cx43 has been well documented. There is accumulating evidence that GJC is not the sole mechanism of inhibition. For example, the growth of C6 glioma cells is inhibited by conditioned medium from cells transfected with Cx43, suggesting secretion of growth inhibitory factors into the medium (26). In addition, Cx43 can decrease the expression of growth-promoting factors such as milk fat globule epidermal growth factor 8 (27) or monocyte
The mutations in Cx43 analyzed in this study have their counterparts in other connexins that are associated with human diseases. For example, the E205R Cx43 mutation corresponds to the R202E in Cx32, which correlates with hereditary deafness (Fig. 8). The substitution E183K in Cx31 is associated with deafness (29). Another study demonstrated that Cx43 inhibits breast cancer growth in vivo regardless of insufficient rescue of GJC (30). Our results further support the notion that Cx43 can inhibit cell growth by a mechanism that does not require functional gap junctional channels.

Phenylalanine 199, arginine 202, and glutamic acid 205 are conserved amino acid residues in the second extracellular loop of various connexin family members (Cx43, Cx32, Cx26, Cx46, Cx50, Cx45, Cx30). They are proximal to the fourth transmembrane region (39). The extracellular regions are involved in hemichannel docking (40) and determination of compatibility between different connexin family members during formation of heterotypic gap junctional channels (41). In addition, the cysteine residues in the extracellular regions allow intramolecular interactions (42). The two regions appear to have specific functions. While the first extracellular loop is important for the channel conductance and selectivity (43–44), the second extracellular region seems to be involved in targeting of the hemichannels to the plasma membrane.

The mutations in Cx43 analyzed in this study have their counterparts in other connexins that are associated with human diseases. For example, the E205R Cx43 mutation corresponds to the E186K mutation in Cx32 (Fig. 8) which causes cytoplasmic localization of Cx32 and is found in patients affected by Charcot-Marie-Tooth disease (21, 45). The substitution E183K in Cx31 is associated with deafness (46). In addition, there are several mutations in the second extracellular loop of Cx26, the most abundant connexin of the cochlea, which correlate with hereditary deafness (Fig. 8). The R184Q Cx26 substitution that corresponds to the R202E in Cx43 is a dominant mutation (22). There are several reported recessive mutations in Cx26, such as substitutions R165W, W172stop, P175T, V178A, R184P, or R184W (http://www.crg.es/deafness/). The mechanisms underlying these GJC impairments are not completely clear. Martin et al. showed that a 12-amino acid sequence starting from position 207 in Cx32 is crucial for oligomerization (47), which precedes insertion of a hemichannel to the membrane. If the corresponding motif in Cx43 is required for its oligomerization, then the F199L, R202E, or E205R mutations could modify protein structure in that region and prevent the proper folding and protein-protein interactions.

In summary, our results show that three independent point mutations in the second extracellular loop of Cx43 have similar effects on cellular localization and growth inhibition by Cx43. The results support the notion that growth inhibition by Cx43 does not necessarily require functional gap junctional channels, and suggest that growth suppression is independent of the second extracellular loop of Cx43.

### Materials and Methods

#### Cell Culture

Cx43−/− and Cx43+/+ (derived from wild-type littermates) were kindly provided by Dr. Alan Lau (University of Hawaii at Manoa). 293T human embryonic kidney cells and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (Intergen, Purchase, NY) at 37°C in 10% CO₂/90% air humidified atmosphere.

#### Plasmids

Full-length wild-type Cx43 cDNA was inserted into retroviral plasmid pMSCV, and into plasmid pcDNA3. The pMSCV and pcDNA3 Cx43 mutants were created by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA).

### Table 1. GJC Measured by Double-Dye Transfer Assay

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Note: Table shows percentage of GJC between HeLa cells transfected with Cx43, calculated from FACS analysis data (see Fig. 3). Percentage of communication = Upper Right Quadrant/(Upper Left Quadrant + Upper Right Quadrant). Results from two independent experiments are shown.

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**FIGURE 4.** The F199L mutant localizes to the plasma membrane in 293T human embryonic kidney cells that express low levels of endogenous Cx43. Cells were transiently transfected with YFP-Cx43 fusion protein and fixed 24 h after transfection. A. YFP-wild-type Cx43. B. YFP-F199L Cx43. Arrows indicate Cx43 in contacts between cells. C. Phosphorylation of YFP-Cx43 fusion protein expressed in 293T cells. Cells were metabolically labeled for 4 h and Cx43 immunoprecipitated using anti-GFP polyclonal antibody; mock transfected (lane 1), YFP-wild-type Cx43 (lane 2), and YFP-F199L Cx43 mutant (lane 3).
Cx43-CFP and Cx43-YFP cDNA Constructs

Cx43 cDNA lacking the stop codon was PCR amplified by using a 5′ primer with an EcoRI site and a 3′ primer with a BamHI restriction site. The PCR products were fused in frame with CFP or YFP to plasmids pECFP-N1 and pEYFP-N1 (Clontech, 6900-1, 6006-1, Palo Alto, CA).

Stable Expression of Cx43

The BOSC23 or 293 GP packaging cell line was transiently transfected with pMSCV Cx43 using standard calcium-phosphate precipitation. After 48 h, cells were infected with viral supernatants collected from packaging cells as described (48). The pMSCV plasmid carries a Hygromycin B resistance gene (49). The infected cells were incubated in the presence of 400 μg/ml of Hygromycin B 72 h after infection to select cells expressing the Hygromycin B resistance gene of the plasmid. Drug-resistant cells were cultured as a pool and uniform expression of Cx43 in the population was confirmed by immunofluorescence.

Transient Transfections

Human 293T embryonal kidney cells and HeLa cells were transiently transfected by calcium-phosphate precipitation and with Effectene reagent (Qiagen, Valencia, CA), respectively. Cell lysates were prepared by scraping the cells in NP40 buffer [20 mM Tris (pH 8), 150 mM NaCl, 50 mM NaF, 1% NP40, 1% Na-deoxycholate], with addition of 160 μM Na3VO4 and protease inhibitors. Western blot analyses of whole cell lysates were performed as in immunoprecipitation experiments (below). For immunofluorescence analysis, the cells were transiently transfected for 48 h.

Cell Lysis, Immunoprecipitation, Gel Electrophoresis, and Western Blotting

Cx43 expression was analyzed either in stably infected cells or 48 h after transient transfection. Cell lysates were prepared by scraping cells in 1% Triton X-100 buffer [20 mM Tris (pH 7.5), 5 mM EDTA, 100 mM NaCl, 1 mM phenylmethlysulfonyl fluoride, 1% Trasylol, 50 mM β-glycerophosphate, 5 mM NaF, 1 mM...
Na$_3$VO$_4$, 1 mM DTT]. For immunoprecipitation, 1 mg of total protein was incubated with a rabbit anti-Cx43 antibody that recognizes amino acid residues 368–382 of rat heart Cx43 (12) and 30 µl of Protein A beads (Repligen, Needham, MA). The immune complexes were washed three times with the lysis buffer and resolved by SDS-PAGE on a 12% polyacrylamide gel and transferred to Immobilon P membrane (Millipore, Bedford, MA). Western blotting was performed using the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ) as described previously (21).

Metabolic Labeling

HeLa cells or human 293T embryonal kidney cells were transiently transfected as described above. At 48 h after transfection, the cells were incubated in medium containing 4% dialyzed calf serum, at 37°C for 30 min, followed by incubation with 1 mCi/ml of $[^{32}P]P_i$ (ICN Pharmaceuticals, Irvine, CA) at 37°C for 4 h.

The cells were rinsed with cold Tris buffer, lysed in 1% Triton X-100 buffer, and lysates were clarified by centrifugation. Cx43 proteins were immunoprecipitated and resolved by SDS-PAGE on a 7.5% polyacrylamide gel and transferred to Immobilon P membrane (Millipore) as described above. The phosphoproteins were detected by exposure to Kodak X-OMAT AR film (Eastman Kodak Company, Rochester, NY).

Double-Dye Transfer Cell-Cell Communication Assay

Two populations of each cell line to be analyzed were separately plated at a density of $5 \times 10^5$ cells in 6-cm dishes. After 24 h of incubation, one population was labeled with 2.5 µg/ml of membrane-bound dye, octadecyl rhodamine B chloride (R-18, Molecular Probes, O-246, Eugene, OR) in culture medium at 37°C for 30 min. The other population was labeled with 0.5 µg/ml of gap junctional channel permeable dye, calcine AM (Molecular Probes, C-1430) in calcine loading buffer [10 mM glucose, 30 mM HEPES (pH 7.0), 0.1% BSA, in
PBS] at room temperature for 30 min. Cells were trypsinized, washed two times with PBS, and resuspended in 2 ml of culture medium. Cells labeled with R-18 were mixed with calcein AM-labeled cells at a ratio of 1:1 (1 ml each) and plated in a well of a six-well plate. The remaining 1 ml of either calcein AM or R-18-labeled population were plated in two separate wells which contained 1 ml of culture medium and mixed just before analysis as a negative control for dye transfer. Cells were incubated overnight, washed three times with PBS, trypsinized, resuspended in calcein loading buffer, and analyzed by FACS. The calcein-AM-labeled cells that establish gap junctional channels with R-18-labeled cells will transfer dye and such R-18-labeled cells will show calcein fluorescence. We used the fraction of acceptor cells that took up dye as a measure of intercellular communication.

Immunofluorescence

Cx43+/− cells were cultured on coverslips. After washing with PBS (pH 7.4), cells were fixed in 3.7% formaldehyde, washed in 0.1 mM glycine/PBS, permeabilized, and blocked with 0.4% Triton X-100 and 2% normal goat serum for 15 min. After three washes in 0.2% BSA, 0.2% Triton X-100/PBS cells were incubated with affinity-purified rabbit anti-Cx43 antibody (above), in 0.1% Triton X-100 solution/PBS, at 4°C overnight. The cells were then incubated with goat anti-rabbit-Texas red (Southern Biotechnology Associates, Inc., Birmingham, AL) at a dilution of 1:200 at room temperature for 2 h, followed by two washes in PBS, 0.2% BSA, 0.2% Triton X-100/PBS. The coverslips were mounted with a Gel/ Mount self-polymerizing mounting solution (Biomeda, Foster City, CA) and analyzed by fluorescence microscopy. GFP, YFP, and CFP fusion protein-expressing cells were prepared for microscopy by washing with PBS, and fixing in 3.7% formaldehyde.

Cell Growth Assay

Cells infected with retroviral vectors expressing Cx43 were plated at a density of 10^5 per 60-mm dish (Cx43+/− and C6 glioma cells) or 10^3 per well of a 24-well plate (HeLa cells) in DMEM medium supplemented with 10% fetal bovine serum containing Hygromycin B to maintain drug selection, as described above. Cells were fed every 3 days and counted daily after infections.

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References


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