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Connexin43 with a cytoplasmic loop deletion inhibits the function of several connexins

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Abstract

Connexins (Cx) form gap junction channels mediating direct intercellular communication. To study the role of amino acids within the cytoplasmic loop, we produced a recombinant adenovirus containing Cx43 with a deletion of amino acids 130–136 (Cx43del_{130–136}). Cx43del_{130–136} expressed alone in HeLa cells localized within the cytoplasm and did not allow transfer of ions, neurobiotin or Lucifer yellow. When co-expressed with wild type Cx43, Cx43del_{130–136} blocked electrical coupling and transfer of neurobiotin or Lucifer yellow. Cx43del_{130–136} and Cx43 co-localized by immunofluorescence and were co-purified from Triton X-100-solubilized cell extracts. Intercellular transfer mediated by Cx37 and Cx45 (but not Cx26 or Cx40) was inhibited when co-expressed with Cx43del_{130–136}. Cx43del_{130–136} co-localized with Cx37, Cx40, or Cx45, but not Cx26. These data suggest that Cx43del_{130–136} produces connexin-specific inhibition of intercellular communication through formation of heteromeric connexons that are non-functional and/or retained in the cytoplasm.

Keywords

Gap junction; Intercellular communication; Adenovirus; Connexin; Heteromeric channels

Gap junctions are membrane specializations containing clusters of intercellular channels that allow intercellular passage of ions and molecules of up to 1000 Da. These channels are oligomeric assemblies of members of a family of related proteins called connexins (Cx). Six connexin monomers assemble to form a hemi-gap junction channel or connexon, which, in turn, forms a complete gap junction channel by docking with a connexon from an adjacent cell.

In a variety of different systems, co-expression of two different connexins within the same cell can have profound effects on the physiological properties of intercellular communication, such as channel conductance [1], permeability [2,3], and regulation by protein kinase dependent pathways [4]. Several disease-associated mutant connexins have been identified that act as dominant negative inhibitors of the function of co-expressed wild type connexins [5–9]. Some dominant negative inhibitors of wild type connexins have been produced through site-directed mutagenesis [10,11]. A wild type connexin, Cx33, may similarly act as an inhibitor of other connexins [12].

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Because intercellular communication is important for the functions of many different tissues, there may be many consequences of manipulation of connexin expression and function. We have recently developed and used recombinant adenoviruses containing wild type connexin sequences to show that expression of Cx37 induces cell death in endothelial cells [13]. The adenoviral vectors can infect many cell types facilitating the high efficiency expression of a variety of proteins.

Several publications have suggested that deletion of portions of the cytoplasmic loop in Cx43 (including amino acids 130–136) produces a dominant negative connexin (Cx43del_{130–136}) which can inhibit the function of co-expressed wild-type Cx43 [14–16], but they do not agree on the mechanisms for its effects nor have they evaluated its effects on other connexins besides Cx43. We prepared a recombinant adenovirus (Ad-Cx43del_{130–136}) containing this mutant and characterized its cellular fate and physiological behavior when expressed alone, together with wild-type Cx43, or with several other connexins. The data assembled suggest that amino acids 130–136 are important in connexin trafficking and suggest that Cx43del_{130–136} can affect intercellular communication mediated by several connexins.

Materials and methods

Connexin adenoviruses

The production of recombinant adenoviruses containing rat Cx43, mouse Cx40, and human Cx37 has recently been described [13]. A DNA fragment containing rat Cx43 with a deletion of amino acids 130–136 (Cx43del_{130–136}) and an epitope tag from the influenza hemagglutinin (HA) attached to its carboxyl terminus was obtained by the polymerase chain reaction. Recombinant adenoviruses were generated with the AdEasy System (Quantum Biotechnologies, West Montreal, Canada) and purified using double cesium chloride gradients. The titers of viral stocks (in infectious units, ifu) were determined by immunostaining HEK293 cells 48 h after infection using antibodies directed against the viral hexon protein.

Cell culture and adenovirus infection

N2A cells stably transfected with Cx43del_{130–136} were isolated and cultured similarly to our previous studies [1].

Stable transfection of HeLa cells with Cx26, Cx40, Cx43, Cx43(His)₆, and Cx45 has been described previously [4,17–19]. HeLa cells were seeded into six-well dish plates or 60 mm culture dishes and cultured for 48 h, at which time they were 70% confluent. Cells were incubated with different dilutions (multiplicity of infection, MOI = 1, 5, 12.5, and 25) of a stock of the recombinant adenoviruses. HeLa cells expressing Cx37FLAG were obtained after infection with a recombinant adenovirus (Ad-Cx37FLAG). Some experiments were also carried out in HeLa cells infected with Ad-Cx40 or Ad-Cx43.

Cell toxicity and proliferation assay

Cell toxicity and proliferation were measured using the CellTiter 96 AQ nonradioactive cell proliferation assay (MTS; Promega, Madison, WI). Cells were plated on 96-well plates at a density of 2000 cells/well in 90 µl medium. After 24 h, 10 µl/well of different concentrations of adenovirus was added, and incubation was continued for 24–48 h. Then, 20 µl/well of MTS solution was added. After incubation for 2 h at 37 °C in a humidified 5% CO₂ atmosphere, the absorbance at 490 nm was measured with a microplate reader. Experiments were performed in triplicate.

Electrophysiological methods

Gap junctional currents from cell pairs were analyzed using the double whole-cell patch-clamp technique [1].

Anti-connexin antibodies

Cx43 was detected using mouse monoclonal antibodies directed against amino acids 252–270 (Chemicon, MAB 3068) or against the cytoplasmic loop of Cx43 (Sigma, C 8093) or rabbit polyclonal antibodies directed against a bacterially expressed fusion protein containing the carboxyl terminus of Cx43 and a (His)₆ tag [18]. Cx26 was detected using a mouse monoclonal antibody directed against a segment of the cytoplasmic loop of rat Cx26 (Zymed, 13-8100). Cx37 was detected using an anti-FLAG antibody (Sigma). Cx40 was detected using rabbit polyclonal antibodies directed against a synthetic peptide immunogen corresponding to residues 316–329 [20]. In most experiments, Cx43del_{130–136} was detected using a rabbit polyclonal antibody directed against the HA tag (Zymed; 71-5500).

Immunofluorescent labeling of cells

HeLa cells were cultured in six-well dishes containing glass coverslips, infected with adenovirus, and incubated for another 24 h. Cells were fixed in 4% paraformaldehyde, permeabilized with 1% Triton X-100, and then stained with primary antibodies followed by Cy3- or Cy2-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories). For double-labeling experiments, cells expressing Cx43del_{130–136} and Cx43 were incubated simultaneously with rabbit anti-HA antibodies and the mouse monoclonal antibody to the cytoplasmic loop of Cx43, while cells expressing Cx43del_{130–136} and other connexins were incubated simultaneously with mouse anti-Cx43 monoclonal antibody and rabbit anti-connexin antibodies (or anti-FLAG antibodies in the case of Cx37) followed by Cy2- and Cy3-conjugated secondary antibodies.

Photomicrographs were obtained using a Zeiss Axioplan 2 microscope equipped with a mercury lamp and a digital camera.

Immunoblotting

Proteins (20–100 µg/well) were resolved by electrophoresis through 10% polyacrylamide gels containing SDS, transferred to Immobilon-P membranes (Millipore), incubated with primary antibodies followed by peroxidase conjugated goat anti-rabbit IgG (1:5000 dilution) or donkey anti-mouse IgG (1:4000 dilution) (Jackson ImmunoResearch Laboratories). Binding of secondary antibodies was detected using ECL (Amersham Pharmacia Biotech).

Triton X-100 solubilization

HeLa cell cultures (in 60 mm dishes) were harvested 24–48 h after infection with Ad-Cx43del_{130–136} or Ad-Cx43 in ice-cold PBS containing 1% Triton X-100 with 2 mM phenylmethylsulfonyl fluoride and protease and phosphatase inhibitors. These samples were separated into Triton X-100-soluble and -insoluble fractions by centrifugation at 100,000g for 30 min at 4 °C. Triton X-100-insoluble fractions (pellets) were resuspended in 1× SDS–PAGE sample buffer and sonicated. The Triton X-100-soluble fractions were brought to 1× SDS–PAGE sample buffer with 4× SDS–PAGE sample buffer. Half of the total Triton X-100-insoluble and -soluble fractions were subjected to SDS–PAGE and immunoblotting.

Affinity purification of heteromeric connexons

The 100,000g Triton X-100 supernatant containing solubilized connexins and connexons was used for purification of HA-tagged proteins. Aliquots containing 250 µg of protein were incubated for 30 min at 4 °C with 50 µl anti-HA MicroBeads (µMACS HA isolation kit,

Miltenyi Biotec, Germany) in 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl and 1% Triton X-100. After this time, beads were transferred to columns, placed in a magnetic field, rinsed four times with the same buffer, and rinsed once with buffer containing no NaCl. Bound proteins were eluted with SDS-gel sample buffer at 95 °C. Fractions were analyzed by immunoblotting.

Cell surface biotinylation

HeLa cells were infected with Ad-Cx43del₁₃₀₋₁₃₆ or Ad-Cx43 and cultured for 24 h. Cells were rinsed twice in ice-cold PBS, labeled twice with EZ-Link NHS-LC-Biotin (0.5 mg/ml, Pierce) in PBS at 4 °C for 10 min, and then rinsed five times in cold PBS containing 15 mM glycine to quench the biotinylation reaction. After lysis in 0.5 ml PBS (supplemented with 0.5% SDS, 0.25% Triton X-100, and protease and phosphatase inhibitors), biotinylated proteins were isolated by incubation with ImmunoPure immobilized avidin cross-linked agarose (Pierce) for 30 min at 4 °C. After three rinses in PBS (supplemented with 0.25% Triton X-100 and protease and phosphatase inhibitors) and one rinse in PBS, biotinylated proteins were eluted in 1× SDS-PAGE sample buffer, boiled for 3 min, and subjected to immunoblotting.

Transfer of microinjected tracers

HeLa cells were cultured on glass coverslips and infected with different amounts of adenovirus. After 24 h, intercellular coupling was examined after microinjection of the gap junction tracers, Lucifer yellow (LY; MW 457, net charge -2, Sigma), Lucifer yellow cadaverine biotin-X (LY-873; MW 873.11, net charge -2, Molecular Probes), or neurobiotin (NB; MW 345, net charge +1, Vector Laboratories, Burlingame, CA). After microinjection, cells were fixed with paraformaldehyde, and neurobiotin was detected after staining with streptavidin-Cy3 conjugate (Sigma) [4]. The extent of intercellular transfer was determined by recording the number of cells containing the tracer (excluding the injected cell). Statistical significance was evaluated using Student's *t* test.

Results

Levels, distribution, and function of wild type and mutant connexins in cells infected with connexin adenoviruses

The delivery of connexins to cultured cells by adenoviral infection was performed using HeLa cells because of their low level of endogenous connexin expression and intercellular communication. Since previous studies have shown that introduction of connexins or infection with adenoviruses can affect the growth or survival of various cultured cells, we initially characterized the viability and growth rate of HeLa cells infected with a range of doses of Ad-Cx43 or Ad-Cx43del₁₃₀₋₁₃₆. No difference in growth or viability was observed between infected and uninfected HeLa cells at any of the viral doses tested (data not shown).

The production of connexins after infection of HeLa cells with the recombinant adenoviruses was examined by immunoblotting (Fig. 1). In Ad-Cx43 infected cells, immunoreactive Cx43 was detected as multiple bands of 40–46 kDa (Fig. 1A, lane 1). Several immunoreactive Cx43 bands were also detected after infection of cells with Ad-Cx43del₁₃₀₋₁₃₆, but the slowest migrating forms were less abundant than in homogenates from cells expressing wild-type Cx43 (Fig. 1A, lane 2). Treatment of homogenates with alkaline phosphatase confirmed that the different forms of Cx43 were due to connexin phosphorylation (data not shown). The pattern of Cx43del₁₃₀₋₁₃₆ bands detected with antibodies directed against the HA epitope appeared nearly identical to that detected with anti-Cx43 antibodies (compare Fig. 1A, lane 2 to Fig. 1B, lane 2). As expected, no bands were detected in homogenates of Ad-Cx43 infected HeLa cells using anti-HA antibodies (Fig. 1B, lane 1).

The distribution of connexins following adenoviral infection was assessed by immunofluorescence (Figs. 1C and D). HeLa cells infected with Ad-Cx43 showed staining at appositional membranes and in the cytoplasm as expected for expressed connexins (Fig. 1C). Similarly, HeLa cells infected with adenoviruses containing wild-type Cx37 or Cx40 showed extensive immunoreactive connexin protein at appositional membranes (not shown). In contrast, after infection with Ad-Cx43del₁₃₀₋₁₃₆, immunoreactive Cx43 was only detected within the cytoplasm (Fig. 1D).

The ability of wild type and mutant Cx43-adenoviruses to produce functional gap junction channels was assessed by microinjection of low molecular weight tracers. HeLa cells infected with Ad-Cx43 exhibited intercellular transfer of both Lucifer yellow (Fig. 1E) and neurobiotin (not shown). In contrast, no transfer of these tracers was detected in cells infected with Ad-Cx43del₁₃₀₋₁₃₆ (Fig. 1F and data not shown).

The function of Cx43del₁₃₀₋₁₃₆ was also analyzed using the double whole-cell patch-clamp technique. N2A cells stably transfected with Cx43del₁₃₀₋₁₃₆ showed no detectable junctional conductance in any of 30 N2A cell pairs examined.

The lack of function of Cx43del₁₃₀₋₁₃₆ and its immunolocalization pattern suggested that it might be impaired in trafficking to the plasma membrane or assembly into gap junction plaques. One approach used to examine gap junction plaque formation has been to assess solubility in Triton X-100 [21]. Homogenates from HeLa cells infected with Ad-Cx43 or Ad-Cx43del₁₃₀₋₁₃₆ were treated with 1% Triton X-100 and separated into 100,000g soluble and insoluble fractions. Immunoreactive bands of Cx43 and Cx43del₁₃₀₋₁₃₆ were detected in both the detergent soluble and insoluble fractions (Fig. 2A, lanes 1–4). A similar ratio of insoluble/soluble immunoreactive material was found for cells expressing either wild type Cx43 or Cx43del₁₃₀₋₁₃₆ as determined by densitometry. We also investigated the presence of cell surface connexins by reacting adenovirus infected cells with a biotinylating agent and isolating the material with streptavidin. Immunoblot analysis showed strong immunoreactive bands for biotinylated Cx43 and a weak immunoreactive band for biotinylated Cx43del₁₃₀₋₁₃₆ (Fig. 2B). Thus, some Cx43del₁₃₀₋₁₃₆ was present at the plasma membrane despite being undetectable by immunofluorescence or by function.

Effects of Ad-Cx43del₁₃₀₋₁₃₆ on co-expressed Cx43

To test for possible interactions between Cx43del₁₃₀₋₁₃₆ and Cx43, we first characterized the levels and distribution of mutant Cx43 after infection of untransfected HeLa cells with Cx43del₁₃₀₋₁₃₆ and then, performed similar experiments in HeLa cells transfected with wild-type Cx43 (HeLaCx43). Immunoblots of HeLa cells infected with increasing doses of Ad-Cx43del₁₃₀₋₁₃₆ showed a dose-dependent increase in the intensity of the immunoreactive Cx43del₁₃₀₋₁₃₆ bands detected with anti-Cx43 (Fig. 3A) or anti-HA antibodies (Fig. 3B). Uninfected HeLaCx43 cells contained multiple bands reactive with anti-Cx43 antibodies (Fig. 3C, MOI = 0) consistent with the expected immunoblot pattern of wild type Cx43; they showed no reactivity with anti-HA antibodies (Fig. 3D, MOI = 0). HeLaCx43 cells infected with increasing doses of Ad-Cx43del₁₃₀₋₁₃₆ (MOI = 5–25) showed a dose-dependent increase in the intensity of anti-Cx43 and anti-HA reactive bands similar to that observed in HeLa cells (Figs. 3C and D).

We studied the cellular distribution of wild-type Cx43 and Cx43del₁₃₀₋₁₃₆ by immunofluorescence in uninfected HeLaCx43 cells (Fig. 4A) or Ad-Cx43del₁₃₀₋₁₃₆-infected HeLaCx43 cells (Figs. 4B–D) using an antibody directed against amino acids in the cytoplasmic loop of Cx43 (that reacts with wild-type Cx43, but not Cx43del₁₃₀₋₁₃₆) and anti-HA antibodies (that react only with Cx43del₁₃₀₋₁₃₆). In uninfected HeLaCx43 cells, wild-type Cx43 was localized to discrete puncta at appositional membranes and to a perinuclear,

cytoplasmic area (likely Golgi) (Fig. 4A). In HeLaCx43 cells infected with Ad-Cx43del₁₃₀₋₁₃₆, both Cx43 and Cx43del₁₃₀₋₁₃₆ had very similar distributions (Figs. 4B and C) that showed extensive overlap (Fig. 4D); immunoreactive Cx43 and Cx43del₁₃₀₋₁₃₆ co-localized within the cytoplasm, in a nearly continuous distribution around the cell surfaces, and in a small number of very bright spots at appositional membranes.

The co-localization of Cx43 and Ad-Cx43del₁₃₀₋₁₃₆ suggested that Ad-Cx43del₁₃₀₋₁₃₆ may form mixed oligomers with wild-type Cx43. The potential association between Cx43del₁₃₀₋₁₃₆ and Cx43 was examined using an affinity purification strategy similar to those previously used to study formation of heteromeric connexons between Cx43 and other connexins [4,18,19]. HeLa cells stably transfected with Cx43 containing a 6× histidine tag at the carboxyl terminus (Cx43(His)₆) were infected with Ad-Cx43del₁₃₀₋₁₃₆ (HA tagged), and the 100,000g supernatant of a 1% Triton X-100 homogenate prepared from these cells was affinity purified using an HA column. The HA-tag allowed the Cx43del₁₃₀₋₁₃₆ to bind to the column and be eluted. Substantial amounts of both Cx43 and (His)₆ immunoreactivities were detected in the eluted material (Fig. 4E, fraction 8). Because the Cx43(His)₆ did not contain the HA tag, any co-purifying anti-(His)₆ immunoreactivity must have come from Cx43(His)₆ associated with Cx43del₁₃₀₋₁₃₆.

The possible effectiveness of Cx43del₁₃₀₋₁₃₆ as a dominant-negative inhibitor of the function of co-expressed Cx43 was examined in HeLaCx43 cells infected with Ad-Cx43del₁₃₀₋₁₃₆. The extent of intercellular communication was reduced in a dose-dependent manner as assessed by microinjection of three tracers of differing mass and/or charge (Fig. 5A); the greater the dose, the greater the reduction. Bigger reductions were seen for the larger dyes at low doses. Analysis of HeLaCx43 cell pairs using the double whole-cell patch-clamp technique showed a marked (>82%) reduction of electrical conductance after infection with Ad-Cx43del₁₃₀₋₁₃₆ (Fig. 5B).

Effects of Ad-Cx43del₁₃₀₋₁₃₆ on other co-expressed connexins

To examine the possible effectiveness of Cx43del₁₃₀₋₁₃₆ as a dominant-negative inhibitor of other co-expressed connexins, we used Ad-Cx43del₁₃₀₋₁₃₆ to infect HeLa cells expressing Cx26, Cx37, Cx40, or Cx45. The distributions of Cx43del₁₃₀₋₁₃₆ and the co-expressed connexins were studied by double label immunofluorescence (Fig. 6). Cx26 and Cx43del₁₃₀₋₁₃₆ immunoreactivities showed no detectable overlap (Figs. 6A–C). Cx26 was found almost exclusively at appositional membranes, while Cx43del₁₃₀₋₁₃₆ was localized within the cytoplasm. In contrast, Cx43del₁₃₀₋₁₃₆ showed extensive co-localization with co-expressed Cx37 (Figs. 6D–F), Cx40 (Figs. 6G–I), and Cx45 (Figs. 6J–L) in the cytoplasm and at the plasma membrane. A higher proportion of the immunoreactive Cx37, Cx40, and Cx45 localized within the cytoplasm in Ad-Cx43del₁₃₀₋₁₃₆ infected cells compared to uninfected cells.

We also examined intercellular communication after Ad-Cx43del₁₃₀₋₁₃₆ infection of HeLa cells expressing Cx26, Cx37, Cx40, or Cx45. The extent of neurobiotin transfer was dramatically reduced in cells expressing Cx37 or Cx45, but was not affected in cells expressing Cx26 or Cx40 (Fig. 7A). Similarly, Lucifer yellow transfer was not significantly affected by Ad-Cx43del₁₃₀₋₁₃₆ infection of Cx26- or Cx40-expressing cells (Fig. 7B). (Cx37- and Cx45-expressing HeLa cells showed negligible permeability to Lucifer yellow, precluding an assessment of the effects of Ad-Cx43del₁₃₀₋₁₃₆ infection on these connexins with this tracer.) Analysis by double whole-cell patch-clamp showed that Ad-Cx43del₁₃₀₋₁₃₆ infection dramatically reduced gap junctional conductance between HeLaCx45 cells, but it had no significant effect on HeLaCx40 cells (Fig. 7C).

Discussion

The results presented in this study have shown that infection of HeLa or N2A cells (which contain very low levels of endogenous connexins and intercellular communication) with an adenovirus containing Cx43del_{130–136} did not lead to any dye transfer or electrical coupling. Prior studies had suggested that Cx43del_{130–136} does not make functional gap junction channels [14,15]. However, these studies were performed on Cx43-expressing cells. In light of the results presented here, it is likely that the previous conclusion was based on the effect of Cx43del_{130–136} upon endogenous Cx43. It has also been suggested that Cx43del_{130–136} formed functional channels with reduced permeability [16]. However, we have excluded that possibility by showing that infection of cells with Ad-Cx43del_{130–136} not only did not allow transfer of either Lucifer yellow or neurobiotin, but it also did not produce any electrical conductance detectable by double whole-cell patch-clamp.

Our data suggest that Cx43del_{130–136} exhibits defects in protein trafficking and gap junction plaque formation, because unlike wild-type Cx43, Cx43del_{130–136} showed an intracellular localization and was absent from gap junction plaques. Only a small fraction of the Cx43del_{130–136} reached the plasma membrane where it was accessible to cell surface biotinylation. Thus, our data implicate a role for residues 130–136 in connexin trafficking and gap junction plaque formation. Indeed, amino acids 135 and 136 may be the critical residues, since Seki et al. [22] have recently reported that a mutant of Cx43 containing a deletion of amino acids 130–134 was non-functional, but did form plaques. Moreover, they also reported that the substitution of threonine for isoleucine at position 130 (a mutant found in some patients with oculodentodigital dysplasia) produced a Cx43 variant that was non-functional, but did localize at the plasma membrane [22]. Furthermore, an ODDD-associated mutant in a nearby amino acid (Cx43G138R) is also non-functional, but forms gap junctional plaques [9]. Similarly, a Charcot-Marie-Tooth disease-associated mutant Cx32 with a deletion in the intracellular loop (Cx32del_{111–116}) targeted properly to gap junction plaques, but transferred dye less efficiently than wild-type Cx32 and oligomerized less efficiently than wild-type Cx32 in an in vitro system [23].

Several protein–protein interactions involving the intracellular loop are likely to be important for connexon formation and targeting to gap junctional plaques and for channel function. Duffy et al. [24] have shown that cytoplasmic loop peptides interact with the carboxyl terminus of Cx43 in a pH-dependent manner leading them to propose that the intracellular loop acts as the receptor for a ball-and-chain type of gap junction channel gating. Moreover, the intracellular loop of another connexin, Cx45.6, interacts with aquaporin-0 [25]. Becker et al. [26] found that injection of antibodies against a region of the intracellular loop (including amino acids 130–136) caused a significant decrease in Lucifer yellow transfer between cells of the 8–16-cell mouse embryo. Thus, it is possible that the intracellular loop is involved in several protein–protein interactions, some of which may affect trafficking or clustering of the channels into gap junctional plaques. The carboxyl terminus may also be involved in plaque formation since Cx43 truncated after amino acid 251 is defective at plaque formation (but unlike Cx43del_{130–136} retains function) [27].

Our data showed a comparable 1% Triton X-100 insoluble/soluble ratio for Cx43del_{130–136} and Cx43. Insolubility in Triton X-100 has previously been associated with gap junction plaque formation by Cx43 [21]. However, no gap junctional plaques containing Cx43del_{130–136} were detected by immunofluorescence implying that connexin insolubility in Triton X-100 may not necessarily correlate with presence of connexins in large gap junction plaques.

We found that infection of Cx43-expressing cells (HeLaCx43) with Ad-Cx43del_{130–136} blocked gap junction function as assessed by electrical coupling and by intercellular transfer

of three different tracers. These data confirm and extend the results of other studies, suggesting that Cx43 mutants with deletions of this region act as dominant-negative inhibitors of Cx43 based on inhibition of Lucifer yellow or Alexa 568 transfer and desynchronization of calcium transients [14–16]. Moreover, our data suggest a mechanism for this inhibition: the formation of heteromeric connexons between Cx43del_{130–136} and wild-type Cx43. The colocalization of Cx43 and Cx43del_{130–136} within the cytoplasm and at the plasma membrane suggests that some of the heteromeric connexons remained within the synthetic/secretory pathway while the wild-type Cx43 “rescued” some Cx43del_{130–136} to the plasma membrane. Based on the functional data, however, heteromeric connexons that reached the plasma membrane formed channels that were non-functional. Inhibition of intercellular communication resulting from the formation of nonfunctional heteromeric channels has been suggested by several studies of the co-expression of a wild type connexin with a disease-associated mutant [5–7].

In most reported cases, the dominant negative effects of a disease-associated mutant are restricted to its wild type counterpart (or effects on other connexins were not examined). The effects of Cx43del_{130–136} on other wild type connexins have not been previously reported. Here, we demonstrate that the dominant negative effects of Cx43del_{130–136} were not restricted to wild type Cx43. Infection with Ad-Cx43del_{130–136} blocked or substantially reduced intercellular communication in cells expressing Cx37 or Cx45. Most likely this inhibition also results from the formation of heteromeric connexons containing Cx43del_{130–136} and the wild-type connexin, since there is strong evidence for the formation of heteromeric channels between Cx43 and Cx37 or Cx45 [1,4]. The observation of increased amounts of co-expressed Cx37 and Cx45 inside the cell suggest that interaction of Cx43del_{130–136} with these connexins decreased their trafficking to the plasma membrane. Ad-Cx43del_{130–136} had no significant effect upon intercellular communication in cells expressing Cx26. This result was not surprising, since previous observations suggest that Cx43 does not form heteromeric channels with “beta group” connexins such as Cx32 or Cx26 [19,28], and the data presented here showed no overlap between the Cx43del_{130–136} and Cx26 immunoreactivities with most of the immunopositive Cx26 at gap junctional plaques.

The lack of influence of Ad-Cx43del_{130–136} upon Cx40 function was relatively unexpected since co-expressed Cx40 and Cx43del_{130–136} co-localized in the cytoplasm and at gap junction plaques. Because of the resolution of the immunofluorescence, co-localization could result from the presence of closely adjacent homomeric connexons at either cellular location; however, the presence of Cx43del_{130–136} at gap junction plaques (Figs. 6G–I) suggests that Cx40 “rescued” the trafficking of Cx43del_{130–136} through an interaction within the synthetic/secretory pathway. This interpretation is in agreement with the reported formation of heteromeric connexons between Cx40 and Cx43 [18,29]. The lack of effect on function is consistent with our previous suggestion that most Cx40–Cx43 heteromeric channels are dead or inefficiently functional, since only a rather limited variety of sizes of single channel events have been observed in co-expressing cells [18].

Ad-Cx43del_{130–136} may be a useful tool for manipulating intercellular communication; it may have distinct advantages over currently available reagents including specificity for gap junction channels, lack of toxicity, and connexin selectivity. A variety of pharmacologic agents (including medium chain alcohols, volatile anesthetics, glycyrrhetic acid derivatives, and quinine derivatives) have been used as inhibitors of gap junction intercellular communication, but they have a variety of side effects due to inhibition of other channels or cellular processes [30,31]. The dominant-negative Cx43 characterized in this study should avoid most of these issues. Unlike previous reports that suggested that introduction of wild type or mutant connexins (including Cx43del_{130–136}) might affect cell proliferation or induce cell death [13, 14,32], we found no evidence that expression of either Cx43del_{130–136} or Cx43 altered the growth or viability of our HeLa cell cultures. Additional experiments will be required to

reconcile these results, but it is possible that all cell types are not similarly affected by these connexins. The selective inhibition of only certain connexins by Cx43del_{130–136} may be advantageous for manipulation of intercellular communication and clarification of the roles of different connexins in cells that contain both sensitive and insensitive connexins such as atrial myocytes (which abundantly express both Cx43 and Cx40) or endothelial cells (that differentially express Cx40, Cx37, and Cx43 depending on the vascular bed).

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Fig. 1.

Cx43del₁₃₀₋₁₃₆ produced in adenovirus infected HeLa cells localizes in the cytoplasm and does not support dye transfer. (A,B) Cell lysates from HeLa cells infected with Ad-Cx43 (lane 1) or HA-tagged Ad-Cx43del₁₃₀₋₁₃₆ (lane 2) were analyzed by immunoblotting with antibodies directed against Cx43 (A) or against the HA epitope (B). (C,D) HeLa cells infected with Ad-Cx43 (C) or Ad-Cx43del₁₃₀₋₁₃₆ (D) were fixed and permeabilized, and immunoreactive Cx43 was detected by indirect immunofluorescence. (E,F) Fluorescence micrographs obtained after microinjection of Lucifer yellow into a single cell within a cluster of HeLa cells infected with Ad-Cx43 (E) or Ad-Cx43del₁₃₀₋₁₃₆ (F).

Fig. 2.

Cx43del₁₃₀₋₁₃₆ is partially soluble in 1% Triton X-100, but little is available for cell surface biotinylation. (A) HeLa cells infected with Ad-Cx43 (lanes 1 and 2) or Ad-Cx43del₁₃₀₋₁₃₆ (lanes 3 and 4) were homogenized and incubated with 1% Triton X-100. The homogenates were separated into soluble (lanes 1 and 3) and insoluble (lanes 2 and 4) fractions by ultracentrifugation. (B) HeLa cells infected with Ad-Cx43 (lane 1) or Ad-Cx43del₁₃₀₋₁₃₆ (lane 2) were incubated with a biotinylating reagent and lysed. Biotinylated proteins were affinity-purified by incubation with avidin–Sepharose beads and subjected to immunoblotting with anti-Cx43 antibodies.

Fig. 3. Dose-dependent production of Cx43del₁₃₀₋₁₃₆ after adenoviral infection. HeLa (A,B) or HeLaCx43 (C,D) cells were infected with various doses of Ad-Cx43del₁₃₀₋₁₃₆. After 24 h, cells were lysed and proteins were resolved by SDS-PAGE and subjected to immunoblotting using anti-Cx43 (A,C) or anti-HA (B,D) antibodies.

Fig. 4.

Cx43del₁₃₀₋₁₃₆ co-localizes and forms heteromeric connexons with wild-type Cx43. (A–D) Double label immunofluorescence analysis of uninfected HeLaCx43 cells (A) or HeLaCx43 cells after infection with Ad-Cx43del₁₃₀₋₁₃₆ at a MOI = 5 (B–D) using anti-Cx43 (A,B) and anti-HA (C) antibodies. The overlap of anti-Cx43 (B) and anti-HA (C) immunoreactivities appears yellow in (D). (E) Co-purification of Cx43 and Cx43del₁₃₀₋₁₃₆ by affinity chromatography. HeLa cells stably transfected with Cx43(His)₆ were infected with HA-tagged Ad-Cx43del₁₃₀₋₁₃₆. Cells were lysed, incubated with 1% Triton X-100 and a supernatant fraction was obtained after ultracentrifugation. Aliquots of this fraction were incubated with anti-HA microbeads. After thorough rinsing, bound proteins were eluted. The fractions were analyzed by immunoblotting with anti-Cx43 or anti-(His)₆ antibodies. Lane 1, cell extract; lane 2, flow through; lanes 3–7, rinses; lane 8, eluate.

Fig. 5.

Ad-Cx43del₁₃₀₋₁₃₆ infection blocks gap junctional intercellular communication in HeLaCx43 cells. (A) Dose-dependent block of intercellular transfer after Ad-Cx43del₁₃₀₋₁₃₆ infection of HeLaCx43 cells. HeLaCx43 cells were infected with different doses of Ad-Cx43del₁₃₀₋₁₃₆. Twenty four hours after infection, intercellular communication was assessed by microinjection of neurobiotin (●), Lucifer yellow (▲), or Lucifer yellow 873 (■) and quantified by counting the number of cell neighbors to which the tracer transferred. Values represent the average $\pm$ SEM ($n \geq 8$ for each dye at all doses). (B) Block of gap junction conductance after Ad-Cx43del₁₃₀₋₁₃₆ infection of HeLaCx43 cells. HeLaCx43 cells were infected with Ad-Cx43del₁₃₀₋₁₃₆ at an MOI of 12. Twenty four hours after infection, intercellular conductance was determined by double whole-cell patch-clamp. Values represent the average $\pm$ SEM ($n = 12$), * $p < 0.001$.

Fig. 6.

Ad-Cx43del₁₃₀₋₁₃₆ shows different degrees of co-localization with different co-expressed connexins. HeLa cells expressing Cx26, Cx37, Cx40, or Cx45 were infected with Ad-Cx43del₁₃₀₋₁₃₆. Twenty four hours after infection, cells were fixed, permeabilized, and reacted with mouse anti-Cx43 antibody and rabbit anti-connexin (or anti-FLAG for Cx37-FLAG) antibodies followed by Cy2- and Cy3-conjugated anti-mouse and anti-rabbit secondary antibodies. The cellular distribution of the wild-type connexins are shown in red (A, D, G, and J), the distribution of Cx43del₁₃₀₋₁₃₆ (detected with anti-Cx43 antibodies) is shown in green (B, E, H, and K), and the overlap of the two signals is seen yellow (C), (F), (I), and (L).

Fig. 7.

Effects of infection with Ad-Cx43del₁₃₀₋₁₃₆ upon intercellular communication mediated by different co-expressed connexins. HeLa cells expressing Cx26, Cx37, Cx40, or Cx45 were infected with Ad-Cx43del₁₃₀₋₁₃₆. Twenty four hours after infection, intercellular communication was quantified by counting the number of tracer-filled neighboring cells after microinjection of neurobiotin (A) and Lucifer yellow (B) and or by determination of gap junction conductance using the double whole-cell patch-clamp technique (C). The values represent means $\pm$ SEM in uninfected (black bars) or infected (gray bars) cells. For the tracer injection experiments, $n > 9$ for all determinations; * $p < 0.05$ infected vs. uninfected cells. For the gap junction conductance measurements, $n = 12$ for Cx40 and $n = 8$ for Cx45; * $p < 0.001$.