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Role of the Cytoplasmic Loop Domain of Cx43 in Its Intracellular Localization and Function: Possible Interaction with Cadherin

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Abstract We have previously shown that intracellular trafficking and function of connexin (Cx) 26 and Cx43 are controlled by E-cadherin. In the present study, we attempted to determine which part of Cx43 is involved in this control mechanism. Since Cx26 has a very short C terminus in the cytoplasm, we hypothesized that the C-terminal domain may not be important for this process and, indeed, found that green fluorescence protein (GFP)-tagged Cx43ΔC (deleted from the codon 239) moved to the plasma membrane both in P3/22(E), a mouse papilloma cell line which expresses E-cadherin, and HeLa cells only at high calcium culture conditions. We then found that the GFP-tagged Cx43(CL 26)ΔC mutant, in which the cytoplasmic loop domain of Cx43 was exchanged with that of Cx26, remains in the cytoplasm in HeLa, HeLaCx43 and P3/22(E) cells, suggesting the importance of the cytoplasmic loop domain. In order to determine which part of the cytoplasmic domain plays a key role, we introduced four deletion mutations (deletion of codons 101–111 [mutant D1], 120–130 [D2], 131–137 [D3] or 146–159 [D4]) to the GFP-tagged Cx43ΔC gene. When these mutants were transfected into HeLa cells, D1 and D4 mutants were localized in the cytoplasm, while D2 and D3 were found in the plasma membrane only in high Ca^{2+} medium. However, none of these four mutants recovered gap junctional intercellular communication (GJIC). On the other hand, when these mutants were transfected into HeLaCx43 and P3/22(E) cells (which express functional Cx43), D1, D2 and D3, but not D4, moved to the plasma membrane and colocalized with endogenous Cx43 in high Ca^{2+} medium; all of these mutants showed a dominant negative effect on GJIC in HeLaCx43 cells. Further deletion studies indicated that the critical amino acids involved in this intracellular trafficking of Cx43 lie between codons 100 and 102.

Keywords Gap junction · Cx43 · Connexin · Cadherin · Protein trafficking

Introduction

Adjacent cells directly share ions and molecules of up to 1,000 daltons through intercellular channels called ''gap junctions.'' These intercellular channels span two plasma membranes and result from the docking of two hemichannels, or connexons, each of which is composed of six connexin monomers. So far, at least 21 connexin genes have been identified in the human genome (Willecke et al., 2002; Sohl & Willecke, 2003). All connexins have four transmembrane helices, two highly conserved extracellular loops that are held rigidly together by three disulfide linkages and three highly variable cytoplasmic domains (Milks et al., 1988; Yancey et al., 1989; Yeager & Gilula, 1992).

In the process of gap junction formation, two adjacent cells need to recognize each others as a proper partner. Possible involvement of cadherin in this process has been suggested by several laboratories (Mege et al., 1988; Jongen et al., 1991; Meyer et al., 1992). For example, we have shown that E-cadherin controls intracellular localization and function of connexins. The mouse papilloma cell line P3/22 expresses Cx43 but not E-cadherin, and only when the E-cadherin gene was transfected into these cells did Cx43 move from the endoplasmic reticulum to cell-cell contact areas in high calcium medium and was gap junc-

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Table 1 PCR primers used for preparation of mutant Cx43 cDNAs

Mutant	Introduced mutation	Primers	
		Forward	Reverse
Cx43ΔC	Stop codon at 239	5′-CTTCTCGAGATGGGTGACTGGA-3′	5′-CCGGATCCTTAACGCCCTTG-3′
Cx43(CL 26)ΔC	Cx43ΔCL	5′-CATATGCCTTCTTGCTGATCCAGTGGTACATC-3′	5′-CACATGGGCCAAGTACAGGAGGGT-3′
	Cx43(CL 26)	5′-TTTAATATACCATATGGCCGGAAGAAGATG-3′	5′-GTATTGCAATACCCAGCTTTGACT-3′
	Cx43(CL 26)ΔC	5′-CTTCTCGAGATGGGTGACTGGA-3′	5′-CCGGATCCTTAACGCCCTTG-3′
D1	del101-111	5′-GGAGCTCAAGGTTGCCCAAA-3′	5′-ATCACATAGAACACATGGGCCAAG-3′
D2	del120-130	5′-3TGAGATAAAGAAGTTCAAGTACGGT-3′	5′-TCAGTTTGGGCAACCTTGA-3′
D3	del131-137	5′-3CGGTATTGAAGAGCATGGTAAGG-3′	5′-ATCTGCTTCAAGTGCATGTCC -3′
D4	del146-159	5′-3TATCCTCTTCAAGTCTATCTT-3′	5′-ACCTTACCATGCTCTTCAA -3′
D1-A	del100-102	5′-3GAAGAGAAGCTAAACAAGAAAGAA-3′	5′-CACATAGAACACATGGGC -3′
D1-B	del103-105	5′-3CTAAACAAGAAAGAAGAGGAGC-3′	5′-CTTCCTCATCACATAGAACACA -3′
D1-C	del108-110	5′-3GAGGAGCTCAAGGTTGC-3′	5′-GTTTAGCTTCTCTTCCTTCCTC -3′

tional intercellular communication (GJIC) ability recovered (Jongen et al., 1991). We further reported that E-cadherin controls intracellular trafficking and function of not only Cx43 but also Cx26 in these cells (Hernandez-Blazquez et al., 2001). It has also been reported that the assembly of N-cadherin precedes the accumulation of Cx43 in cultured adult myocytes (Kostin et al., 1999) and that the localization of Cx43 was determined through Rac1, a downstream signal molecule of N-cadherin, in cardiac myocytes (Matsuda et al., 2006).

While these results strongly indicate that cadherins control intracellular trafficking and function of connexins, it is not known which part of connexins is involved in this control mechanism. In order to approach this problem, we introduced various deletion mutations into the Cx43 gene and examined which part of Cx43 is essential for intracellular localization and function. We first showed that the cytoplasmic loop, rather than the C-terminal, domain is important and further identified more specific sites necessary for this control mechanism.

Materials and Methods

Cell Culture and Cell Lines

All culture media and reagents for cell culture were obtained from Sigma (St. Louis, MO). HeLa cells, which show no detectable level of GJIC or expression of any of the connexin genes examined, and HeLaCx43 cells (stable Cx43 cDNA transfectants) were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. The basal medium for the papilloma-derived cell line (P3/22) was Eagle's minimal essential medium without $CaCl_2$. This medium was modified as indicated by Jongen et al. (1991). This medium was then supplemented with 10% FBS treated with Chelex (Chelex 100 resin; Bio-Rad, Richmond, CA) to remove calcium. The final concentration of Ca^{2+} was obtained by adding $CaCl_2$ to prepare low-calcium (LC) medium (0.05 mM) or high-calcium (HC) medium (1.2 mM). These cells were maintained in LC medium before use in experiments. The P3/22(E) cell line, which expresses endogenous E-cadherin gene, was cloned from the parental P3/22 cells. Cell cultures were maintained in a 37°C incubator under a humidified 5% CO_2 atmosphere and subcultured as required.

Preparation of Mutant Cx43 cDNAs

The enhanced green fluorescence protein (EGFP)-tagged Cx43ΔC mutant was constructed employing the Cx43ΔC cDNA inserted in the pRC/RSV vector (Omori & Yamasaki 1998). Various mutant Cx43 cDNAs of the cytoplasmic loop domain were prepared from this GFP-tagged Cx43ΔC cDNA. The list of Cx43 mutant cDNAs prepared and the primers used for their preparations is provided in Table 1. For introduction of deletion mutations in the C-terminal region (Cx43ΔC), polymerase chain reaction (PCR; 94°C, 30 s; 62°C, 30 s; 72°C, 60 s; 25 cycles) was performed with the Cx43ΔC cDNA inserted into the pRC/RSV vector as the template with the primers described in Table 1. The PCR products and the pEGFP-N1 vector (Clontech, Mountain View, CA) were digested by *Xho*I and *Bam*HI and ligated using a ligation kit (Nippon Gene, Tokyo, Japan). For the introduction of various deletion mutations in the cytoplasmic loop domain of the Cx43ΔC gene, PCR (94°C, 15 s; 58°C, 30 s; 68°C, 5 min; 30 cycles) was performed with the same template as above using the primers shown in Table 1 and the PCR products were treated with *Dpn*I and inserted into the pEGFP vector.

The chimera Cx43 gene in which the cytoplasmic loop was exchanged with that of Cx26 (Cx43[CL 26]ΔC) was

prepared firstly by deleting the cytoplasmic loop domain of the full-length Cx43 cDNA (Omori & Yamasaki, 1998) using the primers shown in Table 1. Then, the *Nde*I site was inserted using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). For preparation of the Cx26 cytoplasmic domain, the fragment covering the entire coding region of the Cx26 cytoplasmic loop domain was amplified from full-length human Cx26 cDNA (Moriyama et al., 2003) using the primers designed to create an *Nde*I site at both ends (Table 1). The *Nde*I-digested fragments were purified and inserted into the corresponding region of the Cx43 construct without its own cytoplasmic loop domain. The C-terminal domain was deleted from this construct to produce the Cx43(CL 26)ΔC by PCR with the primers listed in Table 1. This construct was then inserted into the pEGFP-N1 vector.

Transient and Stable DNA Transfection

Cells were plated into six-well plates, and transfection was carried out when the culture was 30-50% confluent, employing the DAC-30 kit (Eurogentec, Seraing, Belgium), according to the protocol supplied by the manufacturer (vector 1-2 μg, DAC-30 5-10 μg). These cells were washed and incubated in culture medium for 24–48 h before being fixed with methanol for examination of transient expression. The stable transfectants of HeLa and HeLaCx43 cells were selected by G418, and several stable clones from each transfection were isolated. The results from immunostaining and GJIC measurement confirmed similarities among these clones. Therefore, in the figures, the results from only one clone are presented. When transient transfectants were used, immunostaining results were confirmed by observation from various areas of culture from several culture dishes.

Immunostaining

Cells were grown on a cover glass. At confluence, cultures were washed twice with phosphate-buffered saline (PBS) and then fixed in methanol for at least 30 min. All treatments were done at room temperature. The glass was washed twice with PBS and incubated in 0.2% Triton X-100/PBS for 20 min. Then, the cells were treated with bovine serum albumin in PBS for 1 h and incubated with a mixture of rabbit anti-Cx43 (1:800 dilution; Zymed, San Francisco, CA) and mouse anti-E-cadherin (1:200 dilution, Zymed) in PBS overnight at 4°C. After washing three times with PBS, cells were incubated with the mixture of Alexa-568 anti-rabbit and Alexa-488 anti-mouse secondary antibodies (1:300 dilution; Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing with PBS, the cover glass was mounted on a slide glass with *n*-propylgallate/PBS. Then, the cells were observed under a Bx50 fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of GJIC Ability

GJIC ability was determined by means of microinjection of tracer dye into single cells and measurement of its spread to neighboring cells as described previously (Enomoto et al., 1984). In brief, 5% (w/v) solution of lucifer yellow CH (Sigma) in 0.33 M lithium chloride was transferred to a glass capillary. Individual cells in monolayer cultures were injected under the Olympus Injectoscope (Olympus) with the aid of an Eppendorf automatic microinjector (model 5242; Brinkman Instruments, Westbury, NY); after 5 min, the number of fluorescent neighboring cells was scored under an Olympus fluorescence microscope.

Results

Importance of the Cytoplasmic Rather than the C-Terminal Domain of Cx43 for Its Ca^{2+}-Dependent Intracellular Trafficking and Function in P3/22(E), HeLa and HeLaCx43 Cells

Our previous studies suggested that Cx43 moves toward the plasma membrane and forms functional gap junctions in P3/22 cells only when functional E-cadherin is present (Jongen et al., 1991; Hernandez-Blazquez et al., 2001). To determine whether the C- terminal domain of Cx43 is necessary for its movement toward the plasma membrane and function, the GFP-tagged Cx43ΔC mutant (deleted after codon 239) was expressed in HeLa, HeLaCx43 and P3/22(E) cells. Cx43ΔC-GFP was transported to the plasma membrane of the cell-cell contact area between adjacent cells in P3/22(E) cells only at high Ca^{2+} conditions (Fig. 1a-1, a-2), suggesting that the C terminus is not indispensable for E-cadherin-dependent intracellular trafficking of Cx43. When transfected into HeLa cells, Cx43ΔC-GFP was also found in the plasma membrane at the cell-cell contact area (Fig. 1a-3). We have recently found that intracellular localization and function of connexins are controlled by N-cadherin in HeLa cells (*unpublished results*). When we cultured GFP-tagged Cx43ΔC-transfected HeLa cells in low calcium conditions, Cx43ΔC-GFP remained in the cytoplam (*data not shown*), suggesting that the C terminus of Cx43 is not necessary for its N-cadherin-dependent intracellular trafficking as well. We also found that Cx43ΔC-GFP mutant and wild-type Cx43 were colocalized at the plasma membrane in HeLaCx43 cells (Fig. 1a-4, a-5, a-6). As shown in Figure 1b, Cx43ΔC-GFP restored GJIC in HeLa cells to a similar level as the wild type. These results suggest that the C-

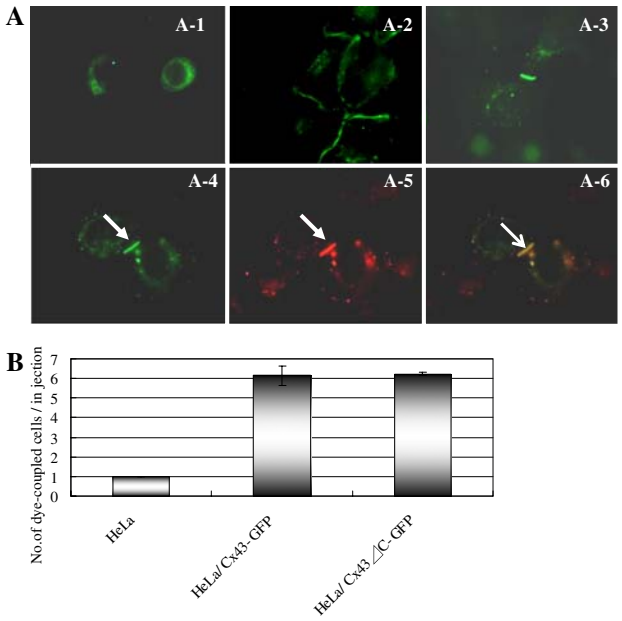

Fig. 1 Cx43ΔC-GFP is localized at the cell-cell contact area in HeLa, HeLaCx43 and P3/22(E) cells at HC and recovers GJIC ability in HeLa cells. (A) Intracellular localization of GFP-tagged Cx43ΔC in P3/22(E), HeLa and HeLaCx43 cells. Endogenous Cx43 was immunostained (*red*). (A-1) P3/22(E) cells in LC medium, (A-2) P3/22(E) cells 24 h after transfer to HC medium, (A-3) HeLa cells, (A-4) HeLaCx43 cells, (A-5) HeLaCx43 cells immunostained for endogenous Cx43, (A-6) merger of a-4 and a-5. *Arrows* indicate Cx43 spots in the plasma membrane. (B) GJIC ability. HeLa cells expressing GFP-tagged full-length of Cx43 and Cx43 truncated C-terminal domain were microinjected with Lucifer yellow, and the number of cells transferred to the neighboring cells was counted.

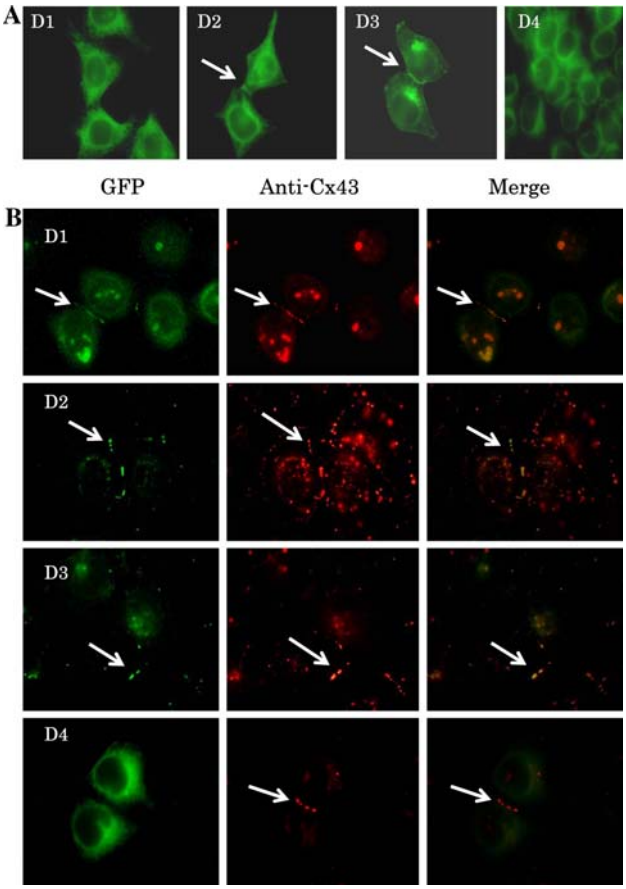

Fig. 2 Localization of the cytoplasmic loop domain deletion mutants of Cx43 (D1-D4) in HeLa (A) and HeLaCx43 (B) cells. *Arrows* indicate Cx43 spots in the plasma membrane

terminal domain is not necessary for its transport to the plasma membrane and function.

To examine whether Cx43 requires its own cytoplasmic loop domain for its intracellular transport and function, we exchanged this domain of the Cx43ΔC gene with that of Cx26 (Cx43[CL 26]ΔC). When the GFP-tagged Cx43(CL 26)ΔC gene was transfected into HeLa, HeLaCx43 and P3/22(E) cells, this mutant remained in the cytoplasm in all of these cell lines (*data not shown*), suggesting that Cx43 must contain its own cytoplasmic loop in order to be at the cell-cell contact area.

Determination of Cytoplasmic Loop Domain Regions of Cx43 Necessary for Its Intracellular Localization and Function

In order to determine which part of the Cx43 cytoplasmic loop domain is necessary for the Cx43 localization and function, we transfected four deletion mutants (D1-D4) into HeLa and HeLaCx43 cells stably (Fig. 2) and into P3/22(E) cells transiently (Fig. 3). We found that mutants D2 ($del_{120-130}$) and D3 ($del_{131-137}$) are localized at the plasma membrane but mutant D4 ($del_{146-159}$) is in the cytoplasm in all of these cell lines in high Ca^{2+} medium. Mutant D1 ($del_{101-111}$) was localized at the cell-cell contact area only in HeLaCx43 and P3/22(E) cells, in both of which wild-type Cx43 is expressed, indicating that this mutant showed different localization in the presence or absence of endogenous Cx43. As expected, when transfected into P3/22 cells, all Cx43 mutants (D1-D4) remained in the cytoplasm (*data not shown*), probably due to the absence of functional E-cadherin.

These results suggest that the deleted region of mutant D1 may be indispensable for the movement into the plasma membrane and that the deleted site of D4 may be indispensable for forming connexon because D4 remained in the cytoplasm even in the presence of wild-type Cx43.

In order to further determine which portion of the deleted site in mutant D1 ($Cx43del_{101-111}$) is necessary for the movement toward the plasma membrane, we constructed three detailed deletion mutants of the D1 region in which three consecutive amino acids were deleted in order. We observed mutant D1-A ($Cx43del_{100-102}$) in the cytoplasm in HeLa cells (Fig. 4a) but at the plasma membrane in HeLaCx43 cells (Fig. 4b), whereas mutants D1-B

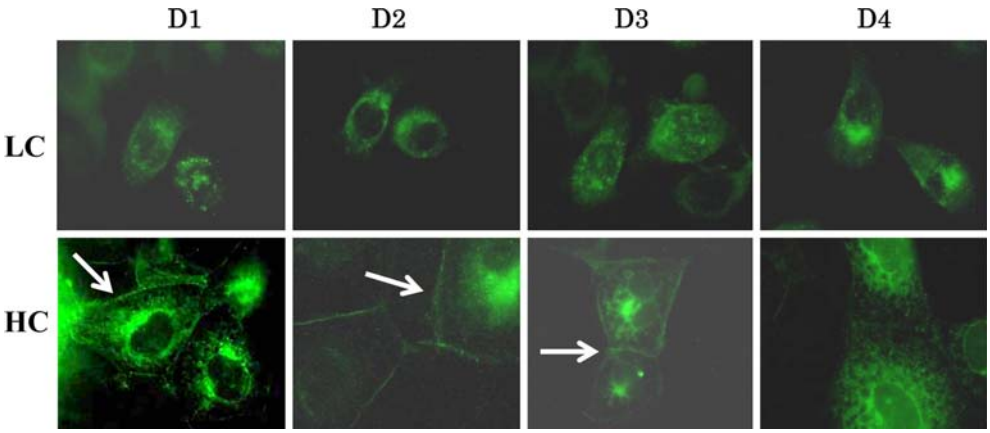

Fig. 3 Localization of Cx43 mutants (D1-D4) in P3/22(E) cells in LC and HC culture conditions. *Arrows* indicate Cx43 spots in the plasma membrane

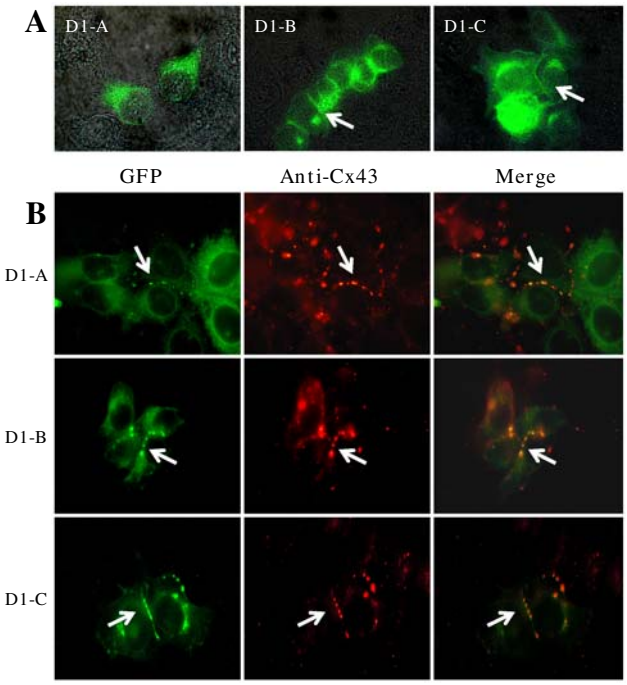

Fig. 4 Localization of D1-A, D1-B and D1-C mutants of Cx43 in HeLa (A) and HeLaCx43 (B) cells. *Arrows* indicate Cx43 spots in the plasma membrane

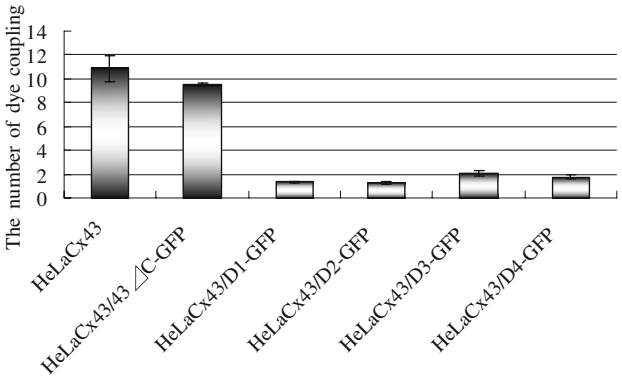

Fig. 5 Dominant negative effect of Cx43ΔC and Cx43 cytoplasmic loop domain mutants (D1-D4) on GJIC in HeLaCx43 cells. GJIC ability of HeLaCx43 cells expressing GFP-tagged Cx43ΔC and D1-D4 Cx43 mutants was determined as described in Materials and Methods

(Cx43del$_{103\text{-}105}$) and D1-C (Cx43del$_{108\text{-}110}$) are localized at the cell-cell contact zone in HeLa and HeLaCx43 cells (Fig. 4). These results indicate that three amino acids (100-102) deleted in the D1-A mutant are important for proper intracellular localization.

Cytoplasmic Loop Domain Deletion of Cx43 Exerts a Dominant Negative Effect on GJIC Ability of Endogenous Cx43 in HeLaCx43 Cells

When the cytoplasmic loop domain deletion mutants were transfected into HeLa cells, none of them recovered GJIC ability, reinforcing the importance of the cytoplasmic loop domain for Cx43 function (*data not shown*). In order to examine whether these mutants show a dominant negative effect on endogenous Cx43, we examined effects of mutants D1-D4 on the GJIC ability of HeLaCx43 cells. As shown in Figure 5, all deletion mutants exhibited a marked dominant negative effect over endogenous Cx43.

Discussion

We have shown that the cytoplasmic loop, rather than the C-terminal region, of Cx43 is important for its intracellular movement toward the plasma membrane. This detailed deletion study indicates that codons 101-111 may be the region responsible for this control. This was found both in HeLa cells and in mouse papilloma P3/22(E) cells. We previously reported that the triggering of movement of Cx43 and Cx26 from the endoplasmic reticulum to the plasma membrane via the Golgi apparatus is dependent on E-cadherin in mouse papilloma cells (Hernandez-Blazquez et al., 2001). In HeLa cells, our recent results suggest that

endogenously expressed N-cadherin is responsible for the control of intracellular localization and function of connexins, including Cx43 (Yamasaki et al., *unpublished results*). Therefore, we consider that the cytoplasmic loop domain of Cx43, especially the part containing codons 101-111, may be an important region for cadherin-mediated control of intracellular trafficking. However, since there is no evidence that cadherins and connexins directly interact with each other, it is likely that some other proteins are involved in this control mechanism. In fact, we have recently identified the proteins which bind to the cytoplasmic loop of Cx26 and Cx43, AP26 and AP43, by the yeast two-hybrid method (Ise et al., 2004; Kawasaki, Miyoshi & Yamasaki, 2004). Such proteins may be good candidates which link the functions of cadherins and connexins.

We found that the C-terminal domain (after codon 239) is not indispensable for transport to the plasma membrane and function of Cx43. These results are consistent with our previous report that not only Cx43 but also Cx26, which has almost no cytoplasmic C-terminal tail, moved to the plasma membrane after the calcium shift in P3/22(E) cells. These data, in turn, suggest that phosphorylation of the C-terminal tail of Cx43 is not essential for N- and E-cadherin-mediated transport toward the plasma membrane. These findings are consistent with several other reports which showed that elimination of a large portion of the C-terminal domain does not inhibit Cx43 transport to the plasma membrane and function (Maass et al., 2004; Moreno et al., 2002). Since the Cx43 C-terminal domain has been associated with several proteins, including ZO-1 (Toyofuku et al., 1998; Giepmans & Moolenar, 1998) and CCN3(NOV) (Fu et al., 2004) and since this domain is often phosphorylated (*reviewed by* Laird, 2005), the C-terminal tail may be important for channel regulation, rather than for its constitutive function.

When we further deleted the C-terminal domain before codon 239, we found that the minimum amino acid residues necessary for localization in the plasma membrane exist between codons 233 and 237 (*data not shown*). This is consistent with the report by Kumar (1999), who showed that the Cx43 C-terminal mutant truncated before amino acid 231 accumulated within the intracellular compartment and reduced the delivery of wild-type Cx43 to the cell surface. However, we cannot exclude the possibility that the deletion beyond amino acid 233 simply caused conformational hindrance which disturbed proper insertion into the plasma membrane.

When codons 146-159 were deleted from the cytoplasmic loop domain (mutant D4), Cx43 was unable to move toward the plasma membrane even in the presence of wild-type Cx43, i.e., in HeLaCx43 and P3/22(E) cells. Since other deletion mutants of this region moved to the plasma membrane in the presence of wild-type Cx43, presumably forming heteromeric connexons between mutant and wild-type Cx43 molecules, it is possible that this region is important for assembly of connexins into connexons. The assignment of such an important role to this region is consistent with the fact that the deleted amino acid sequence of mutant D4 is homologous among almost all of the connexins.

We also found that none of the cytoplasmic loop domain deletion mutants (D1-D4) was able to recover GJIC when transfected into HeLa cells. Moreover, all of them exerted a dominant negative effect on wild-type Cx43, suggesting importance of each portion of the cytoplasmic loop domain of Cx43 for its normal function. The functional loss and dominant negative effect of some of these mutations can be related to physiological disorders exerted by those connexin gene mutations found in hereditary human diseases. For example, Cx43 gene mutations in the region corresponding to our D1 and D3 mutants were found in oculodentodigital dysplasia (Paznekas et al., 2003). Furthermore, the deleted region of mutant D1 is homologous between Cx26 and Cx43 and a mutation of this region in the Cx26 gene was found in a hereditary asymptotic deafness family (Kelsell et al., 1997; White, 2000; Bruzzone et al., 2001; Mese et al., 2004). In addition, the deleted region of mutant D2 has high homology between Cx43 and Cx32 and a mutation of the Cx32 gene in this region has been reported in an X-linked Charcot-Marie-Tooth syndrome family (Nelis, Haites & Van Broeckhoven, 1999; Bruzzone et al., 1994; Ressot et al., 1998; Wang et al., 2004). The dominant negative effects of D1, D2 and D3 are presumably caused by heteromeric connexon formation by wild-type and mutant connexins since they were in the cytoplasmic membranes. However, mutant D4 connexins exerted a dominant negative effect in spite of the fact that they were in the cytoplasm, while the endogenous wild-type Cx43 was still at the cell-cell contact area. Thus, this dominant negative effect cannot be due to the formation of heteromeric connexons with wild-type Cx43, and we speculate that partner proteins necessary for proper function of Cx43 might be sequestered by mutant D4. Further studies aimed at defining the role of the cytoplasmic loop domain of connexins may reveal whether and how such proteins regulate the function of connexins.

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