The Carboxyl Tail of Connexin32 Regulates Gap Junction Assembly in Human Prostate and Pancreatic Cancer Cells

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Running Title: Carboxyl Tail of Connexin32 and Gap Junction Assembly

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Background: Cytoplasmic tails of connexins are highly divergent yet their role in gap junction assembly remains undefined.

Result: Gap junctions of tail-deleted connexin32 remain small and fail to grow.

Conclusion: The cytoplasmic tail of connexin32 is dispensable for gap junction formation but essential for regulating junction size.

Significance: The cytoplasmic tails of connexins may enhance function of gap junctions by regulating their size.

ABSTRACT

Connexins, the constituent proteins of gap junctions, are transmembrane proteins. A connexin (Cx) traverses the membrane four times, and has one intracellular and two extracellular loops, with the amino and carboxyl termini facing the cytoplasm. The transmembrane and the extracellular loop domains are highly conserved among different Cxs whereas the carboxyl termini, often called the cytoplasmic tails, are highly divergent. We have explored the role of the cytoplasmic tail of Cx32, a Cx expressed in polarized and differentiated cells, in regulating gap junction assembly. Our results demonstrate that compared to the full-length Cx32, the cytoplasmic-tail-deleted Cx32 is assembled into small gap junctions in human pancreatic and prostatic cancer cells. Our results further document that the expression of the full-length Cx32 in cells which express the tail-deleted Cx32 increases the size of gap junctions whereas the expression of the tail-deleted Cx32 in cells which express the full length Cx32, has the opposite effect. Moreover, we show that the tail is required for the clustering of cell-cell channels and that in cells expressing the tail-deleted Cx32, the expression of cell-surface-targeted cytoplasmic tail alone is sufficient to enhance the size of gap junctions. Our live-cell imaging data further demonstrate that gap junctions formed of the tail-deleted Cx32 are highly mobile compared to those formed of full-length Cx32. Our results suggest that the cytoplasmic tail of Cx32 is not required to initiate the assembly of gap junctions but for their subsequent growth and stability. Our findings suggest that the cytoplasmic tail of Cx32 may be involved in regulating the permeability of gap junctions by regulating their size.
INTRODUCTION

Gap junctions are ensembles of cell-cell channels through which molecules up to 1 kDa can directly pass between the cytoplasmic interiors of adjoining cells (1,2). Cell-cell channels are formed of Cxs, which are encoded by a family of 21 distinct genes in humans and are designated according to the molecular mass. Some members of the Cx family are expressed in a tissue-specific manner whereas the expression of others is redundant (2). Knock out studies of Cx genes have unveiled diverse roles of cell-cell communication in maintaining tissue homeostasis (3). Their roles have been substantiated by human genetic diseases, such as ocudental digital dysplasia, palmoplanta keratoderma, and keratitits-icthyosis-deafness syndrome, in which mutations in Cx genes have been detected (3,4). Despite tissue-specific expression of some Cxs, gap junctions in all tissues appear as disc-shaped structures consisting of several regularly spaced particles in freeze-fracture replicas (5). To form a cell-cell channel, six Cxs first oligomerize into a hexamer, called a connexon, which is transported to the cell surface and docks with a connexon in an adjacent cell. A gap junction is formed when several such cell-cell channels cluster (1). Thus, a key step in the assembly of gap junctions is the aggregation of cell-cell channels at one particular spot at areas of cell-cell contact, a process that has not yet been elaborated (6).

A Cx is a transmembrane protein which traverses the membrane four times, and has one intracellular and two extracellular loops, with the amino and carboxyl termini facing the cytoplasm. The transmembrane and the extracellular loop domains are highly conserved among different Cxs whereas the carboxyl termini, often called the cytoplasmic tails, are highly divergent (2,6). The role of the two extracellular loop domains of Cxs in the formation of cell-to-cell channels, as well as of the cytoplasmic tails in regulating the opening and closing of cell-cell channels has been well-documented (5,7-9). The cytoplasmic tails of many Cxs have also been shown to interact directly or indirectly with several proteins (10). While these interactions have been postulated to control the assembly of Cxs into gap junctions, the molecular mechanisms involved have remained unexplored (10). In particular, it is not clear what the exact role of a Cx’s cytoplasmic tail is and at which step it is involved in regulating the assembly of gap junctions. For example, the interaction of a scaffolding protein, ZO-1, which binds to the PDZ-binding domain of several Cxs, facilitates the assembly of Cx43, an ubiquitously expressed Cx, into gap junctions (11,12) yet has an opposite effect on the assembly of Cx50, which is expressed in lens epithelial cells (10,13). Thus, it is highly likely that besides ZO-1 other proteins documented to interact with Cxs might inhibit or enhance gap junction assembly and disassembly in a tissue-specific and cell-context dependent manner. Moreover, most studies with Cx-interacting proteins have been performed with Cx43, which is ubiquitously expressed (6) and not with Cxs whose pattern of expression is restricted to few tissues (2). Tissue-specific as well as redundant expression of some Cxs, combined with the fact that most tissues and cell types express more than one Cx subtype (1,14,15), implies that the assembly of each Cx subtype into gap junctions is likely regulated spatiotemporally in a cell context and physiological state dependent manner by distinct mechanisms to prevent fortuitous gap junction formation and internalization. The cytoplasmic tails of Cxs seem to be the likely targets for such type of regulation as they are the most divergent portions among different members of Cx gene family (2).

Among all the Cxs, the expression of Cx32 is observed in the well-differentiated acinar cells of exocrine glands, such as prostate and pancreas (15). Our previous studies showed that the retrovirus-mediated expression of Cx32 in Cx-null, androgen-sensitive prostate cancer cell line, LNCaP, induced the formation of gap junctions, restored junctional communication, inhibited growth in vitro, and retarded malignancy in vivo (16). We further showed that androgens — the key players that govern prostate morphogenesis and oncogenesis (17) — regulated the formation and degradation of
gap junctions by controlling the expression level of Cx32 posttranslationally (18). In these studies, we had fortuitously observed that the retrovirally-expressed cytoplasmic-tail-deleted Cx32 appeared to assemble into small gap junctions compared to those formed by the expression of the full length Cx32 (18). Moreover, our previous study with cadherin-null human squamous carcinoma cells had also shown that the assembly of Cx32 into gap junctions was facilitated when cells acquired a partially polarized state and that the cytoplasmic tail of Cx32 (abbreviated as Cx32-CT) was required to initiate the formation of a gap junction plaque and/or its subsequent growth in these cells (19). These studies prompted us to explore the role of Cx32-CT in the assembly of gap junctions.

We demonstrate here that compared to the full-length Cx32, the cytoplasmic-tail-deleted Cx32 is assembled into smaller gap junctions despite normal trafficking to the cell surface in human pancreatic and prostatic cancer cell lines. We also document that the expression of the full-length Cx32 in cells stably expressing the cytoplasmic-tail-deleted Cx32 increases the size of gap junctions whereas the expression of the cytoplasmic-tail-deleted Cx32 in cells expressing the full-length Cx32 has the opposite effect. Moreover, our results show that the cytoplasmic tail is required for the clustering of cell-cell channels. Furthermore, we also show that in cells expressing the cytoplasmic-tail-deleted Cx32, the expression of the cell-surface-targeted cytoplasmic tail alone is sufficient to enhance gap junction assembly. In addition, by expressing a series of Cx32 deletion mutants with progressive truncations of the carboxyl tail, our results document that the critical motifs that determine the size of gap junctions reside between residues 230 and 250 of Cx32. Finally, our live-cell imaging data document that compared to the mobility of larger and smaller gap junctional plaques formed of full-length Cx32, the gap junction-like puncta composed of cytoplasmic-tail-deleted Cx32 are highly mobile. Our findings suggest that the cytoplasmic tail is not required to initiate the assembly of Cx32 into gap junctions but for their subsequent growth and stability. These findings suggest that the cytoplasmic tail of Cx32 may be involved in regulating the permeability of gap junctions by regulating their size.

MATERIALS AND METHODS

Cell Culture

The human pancreatic cancer cell line, BxPC3 (CRL-1687), and a prostate cancer cell line, LNCaP (ATCC CRL 1740), were grown in RPMI 1640 and DMEM (GIBCO, Grand Island, NY) containing 7% fetal bovine serum (Sigma Aldrich, St. Louis, MO), respectively, in an atmosphere of 5% CO₂ at 37°C. Stock cultures were maintained weekly by seeding 5x10⁵ cells per 10 cm dish in 10 ml of complete culture medium with a medium change at day 3 or 4 as described (18,20). New stocks were initiated after 10 passages. The two retroviral packaging cell lines, EcoPack and PTi67, were grown as described previously (16,18). BxPC3 and LNCaP cells were infected with various recombinant retroviruses and pooled polyclonal cultures from approximately 2000 colonies were grown and maintained in RPMI containing G418 (200 µg/ml) (see Recombinant DNA Constructs and Retrovirus Production and Infection).

Antibodies and Immunostaining

Rabbit polyclonal and mouse monoclonal antibodies against Cx32 and mouse anti-β-catenin, and rabbit anti-β-actin have been described previously (18,19,21). We also used rabbit polyclonal antibodies raised against the carboxyl tail (Sigma; C-3470) and the cytoplasmic loop of Cx32 (Signal, C-3595). For immunostaining 5x10⁵ BxPC3 and 4x10⁵ LNCaP cells were seeded on glass cover slips in six-well clusters and allowed to grow for 3 days, after which they were fixed with 2% paraformaldehyde and immunostained as described (19-21). Anti-rabbit and anti-mouse secondary antibodies, conjugated with Alexa 488 or Alexa 594 (Invitrogen), were used as appropriate. After mounting immunostained cells on glass slides in SlowFade antifade medium (Invitrogen), images were acquired with a Leica DMRIE microscope.
(Leica Microsystems, Wetzler, Germany) equipped with a Hamamatsu ORCA-ER2 CCD camera (Hamamatsu City, Japan) using a 63x oil objective (NA 1.35). Several z-stacked images taken 0.3 μm apart were used to measure colocalization using the commercial image analysis program Volocity 6.0.3 (Improvision, Lexington, MA) as described (19,20).

Recombinant DNA Constructs and Retrovirus Production and Infection

Construction of retroviral vectors containing wild-type rat Cx32 (Cx32-WT) and the cytoplasmic-tail-deleted Cx32 (Cx32Δ220) has been described (18). Briefly, the Cx32Δ220 was engineered by PCR-based cloning technique and cloned into the retroviral vector LXSN. The PCR amplification was with the forward primer: 5’-GCCGAATTCTGAACCTGGACAGGTC-3’, and the reverse primer containing a premature stop codon (TGA, Umber) at amino acid position 221: 5’-CCGGAATTCTCAACGCGGCGACAG-3’.

The mutant was generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The Cx32-CT-Myr construct (containing amino acid residues 220-283) was constructed by PCR cloning as follows: Cx32-CT was first cloned in frame with pmTurquoise-C3 to create pmTurquoiseCx32Δ220. The entire pmTurquoiseCx32Δ220 was amplified by PCR with Nco1 and BamH1 sites added at the 3’ and 5’ ends, respectively. This Nco1 and BamH1 fragment was cloned into a modified pSPUTK vector which added 5’ myristoylation and two Myc tags. The assembled cassette was removed and subcloned in pcDNA3.1 (+) as a HindIII and BamH1 fragment.

Myc-tagged Cx32-WT (Cx32-WT-Myc) and Cx32Δ220 (Cx32Δ220-Myc) were generated by PCR-based cloning. Coding sequence for the Myc tag was incorporated in the reverse primers for Cx32-WT and Cx32Δ220, which also contained the Xho1 site. The primers used were: 5’—ACTCTAGCATCTAGATTACAGGTCTCTCTCG GAGATCGCTTCTGCTCAAGCAGGACAGCAT CGGTC-3’ for Cx32-WT and 5’—

The retroviral vectors were used to produce recombinant retroviruses in EcoPack and PTi67 packaging cell lines as described previously (19-21). The recombinant retroviruses produced from the pooled polyclonal cultures of PTi67 cells were assayed for virus titer by colony forming units as described (22). BxPC3 and LNCaP cells were multiply (2-4 times) infected with the recombinant retroviruses and selected in G418 (400 μg/ml) for 2–3 weeks in complete medium. Pooled cultures from about 2000 colonies obtained from 3-4 dishes were expanded, frozen, and maintained in selection media containing G418 (200 μg/ml). Pooled polyclonal cultures were used within 2-4 passages for immunocytochemical and biochemical analyses.

Detergent Extraction and Western Blot Analysis

BxPC3 (3x10⁶) and LNCaP (2x10⁶) cells, seeded in replicate 10 cm dishes in 10 ml of complete medium, were grown for 72 h. The procedures for cell lysis, detergent-solubility assay with 1% Triton X-100 (TX100), and Western blot analysis have been described previously (18,19,21). Normalization was based on equal cell number for the analysis of detergent-soluble and -insoluble fractions by SDS-PAGE analysis of cell lysates.

Cell Surface Biotinylation Assay

BxPC3 (5x10⁵) and LNCaP (4x10⁵) cells were seeded in 6 cm dishes in replicates and grown to 70-
80% confluence. Biotinylation reaction, using freshly prepared EZ-LinkSulfo-NHS-SS Biotin reagent (Pierce; Rockford, IL) at 0.5 mg/ml in phosphate buffered saline (PBS) supplemented with 1 mM CaCl$_2$ and 1 mM MgCl$_2$ (PBS-PLUS), was performed at 4°C for 1 h. Cells were lysed after quenching the reaction with PBS-PLUS containing 20 mM glycine as described previously (19,21,23). The affinity precipitation of biotinylated proteins was from 200 μg of total protein using 100 µl of streptavidin-agarose beads (Pierce, Rockford, IL) on a rotator overnight at 4°C. SDS-PAGE followed by Western blotting was used to resolve the streptavidin-bound biotinylated proteins after elution as described previously (19,21,23). The kinetics of degradation of cell-surface-associated Cx32-WT and Cx32Δ220 was determined essentially as described previously (20,21). The protein concentration was determined using the BCA reagent (Pierce).

**Cell Transfection**

Twenty-four hours prior to transfection, BxPC3 (1.5x10$^6$) and LNCaP (10$^6$) cells were seeded on glass cover slips in 6-well clusters. Cells were transfected with various plasmids in duplicate using XtremeGENE (Roche Diagnostics) according to the manufacturer’s instructions. We found XtremeGENE to be superior for LNCaP and BxPC3 cells compared to several other lipid-based reagents that we tried. For transfections, 2 μg of plasmid DNA was used per well. For co-transfection of two plasmids, pmCherry (0.5 μg) and the plasmid DNA (1.5 μg), containing the gene whose expression was to be detected, were mixed. Expression was analyzed 24 h post-transfection after fixing and immunostaining cells with the desired antibodies as described (18).

**Immunoprecipitation Assay**

For immunoprecipitation analysis, HEK293T cells were grown to 40% confluence in 10-cm dishes and transfected with 10 μg of Cx32-WT-EGFP, Cx32Δ220-EGFP, Cx32-WT-Myc and Cx32Δ220-Myc either alone or in combination. Twenty-four hrs post-transfection, the cells were harvested and lysed in a non-denaturing lysis buffer containing 1% NP-40, 1% TX-100, 1mM CaCl$_2$, 1mM PMSF, 2mM Na$_3$VO$_4$, 1mM NaF and protease inhibitor cocktail. Following lysis and protein estimation, 500 μg of total protein was mixed with 180 µl of IgG-Sepharose beads (MP Biomedicals, LLC, Santa Ana, CA) pre-incubated with anti-GFP antibody (Clontech) and incubated at 4°C for 2 hrs. Following incubation, the Sepharose-bound immune complexes were washed 5 times with the Wash Buffer (10mM Tris-HCl pH7.5, 150 mM NaCl, and 0.5% Tween-20). The pull-down proteins were eluted with 2x Laemelli’s sample buffer and analyzed by SDS PAGE and Western Blotting with anti-Myc (Covance, Princeton, NJ) and anti-GFP antibodies.

**Communication Assays**

Gap junctional communication was assayed by microinjecting the fluorescent tracers Lucifer Yellow (MW 443 Da; Lithium salt); Alexa 488 (MW 570 Da; A-10436), or Alexa 594 (MW 760 Da; A-10438) (19,21,23,24). Alexa dyes were purchased from Molecular Probes (Carlsbad, CA) and stock solutions (10 mM) for microinjection were prepared in water. Eppendorf InjectMan and FemtoJet microinjection systems (models 5271 and 5242, Brinkmann Instrument, Inc. Westbury, NY), mounted on a Leica DMIRE2 microscope were used to microinject the fluorescent tracers. Junctional transfer of the fluorescent tracers was assessed by counting the number of fluorescent cells (excluding the injected one) either at 1 min (Lucifer Yellow), 3 min (Alexa 488) or 15 min (Alexa 594) after microinjection into test cells as described (16,18,19,25).

**Live-cell Imaging and Fluorescent Recovery after Photo-bleaching**

Bx32-WT-EGFP and Bx32Δ220-EGFP cells were seeded in LabTek FluoroDish culture dishes and allowed to grow to confluence. Confluent monolayers of cells were imaged using a 100 x oil
objective (NA 1.4) and a broad-band GFP filter. Cells were imaged at 37°C in an atmosphere of 5% CO2/95% air in a live-cell imaging chamber mounted on an Olympus IX81 Spinning Disc Inverted Confocal motorized inverted microscope (Olympus America Inc; Center Valley, PA). The microscope was controlled by IX2-UCB U-HSTR2 motorized system with a focus drift compensatory device IX1-ZDC. Images were captured using a Hamamatsu ORCA ER2 CCD camera and processed by imaging software Slidebook version 5.0 (Intelligent Imaging Innovations, Denver, CO). z-stacks of 1 µm were acquired every two minutes for 120 min. The z-stacks (8) were projected into a single Z-projection, which were superimposed on to a phase-contrast image.

For FRAP analysis, Bx32-WT-EGFP and Bx32Δ220-EGFP cells were photo-bleached as described (26). For measuring recovery within gap junction plaques, only puncta at the areas of cell-cell contact were photo-bleached whereas recovery in non-junctional areas was determined in single cells in regions of the cells where only diffuse GFP fluorescence was observed, and there were no vesicular puncta. Recovery was measured using a Marianas Live Cell microscopy system (Intelligent Imaging Innovations Inc, Denver, Co.) equipped with a Stanford Research Laser Ablation System (model NL100). After the cells were photo-bleached, we captured z-stack images every 10 seconds for 15-20 minutes. As described in our earlier studies (26), prior to quantitation of relative fluorescence intensity, raw images were deconvolved and collapsed into a projection image. For comparing multiple FRAP experiments, we collected normalized FRAP data using SlideBook (Version 5.0). For determining the relative fluorescence intensity value, we set the pre-bleach and post-bleach values as 100% and 0%, respectively. The graphs shown in Figure 8 represent the plotted relative fluorescence intensity and error bars represent the 95% confidence interval.

Results

Cytoplasmic-tail-deleted Cx32 Assembles into Smaller Gap Junctions

Earlier studies had shown that the truncation of Cx32-CT up to amino acid 219 had no effect on the trafficking of connexons to the plasma membrane (27). We also showed that the cytoplasmic-tail-deleted Cx32, abbreviated as Cx32Δ220, formed smaller gap junctions in human prostate cancer cell line, LNCaP, compared to full-length Cx32, hereafter abbreviated as Cx32-WT (18). Moreover, we further showed that in human squamous carcinoma cell line, A431D, Cx32-WT assembled into gap junctions only when cells acquired a partially polarized state whereas Cx32Δ220 did not (19). These observations hinted that the Cx32-CT was involved in some aspect of gap junction plaque growth and/or initiation.

To define the role of Cx32-CT further, we retrovirally expressed Cx32-WT and Cx32Δ220 in LNCaP and BxPC3 cells in parallel, and obtained pooled polyclonal cultures from each cell line. The pooled polyclonal cultures of LNCaP and BxPC3 cells expressing Cx32-WT and Cx32Δ220 are designated as LN32-WT, Bx32-WT, LN32Δ220 and Bx32Δ220 cells, respectively. Western blot analysis showed that Cx32-WT and Cx32Δ220 were expressed robustly in LNCaP and BxPC3 cells (Figure 1AB). However, immunocytochemical analysis revealed that in both cell types Cx32Δ220 formed only small junctions whereas Cx32-WT formed both large and small gap junctions (Figure 1C; compare the size of gap junction puncta in left panels with the right panels). The small size of gap junctions formed by Cx32Δ220 was not related to the expression level as assessed by analyzing several individual clones of LNCaP and BxPC3 cells that differed in expression level by 2-4 fold (data not shown). We therefore measured the surface areas of 350-500 individual gap junctional puncta in LNCaP and BxPC3 cells expressing Cx32-WT and Cx32Δ220 (Figure 1D). As described earlier, although the limit of resolution of light microscopy cannot distinguish multiple
smaller junctions from a single larger gap junction, we counted each punctum at cell-cell interface, as delineated by E-cadherin or β-catenin staining, as a single gap junction for quantification purposes (19,28). We found that compared to LN32-WT and Bx32-WT cells, LN32Δ220 and Bx32Δ220 cells formed 20-35 fold smaller gap junctions (Figure 1D). Moreover, the frequency of larger gap junctions was decreased in LN32Δ220 cells, with a concomitant increase in the frequency of smaller gap junctions. Furthermore, we found that the number of gap junctions per interface was more in cells expressing Cx32Δ220 compared to cells expressing Cx32-WT (data not shown). In addition, the number of intracellular puncta appeared to be more in cells expressing Cx32Δ220 compared to cells expressing Cx32-WT (Figure 1C, bottom right panel). For example, the mean number of intracellular puncta in LN32-WT and Bx32-WT cells, respectively, was 7 ± 2 (n = 35) and 13 ± 4 (n = 35), whereas the mean number of intracellular puncta in LN32Δ220 and Bx32Δ220 cells was 25 ± 3 (n =15) and 50 ± 10 (n = 17), respectively.

Function and the Detergent-solubility of Gap Junctions Composed of Cx32-WT and Cx32Δ220

To determine if Cx32Δ220 formed functional gap junctions, we microinjected gap junction-permeable fluorescent tracers into LN32-WT and LN32Δ220 cells. We found that the junctional transfer of all fluorescent tracers was reduced in cells expressing Cx32Δ220 compared to cells expressing Cx32-WT (data not shown). In addition, the number of intracellular puncta appeared to be more in cells expressing Cx32Δ220 compared to cells expressing Cx32-WT (Figure 2A; Table 1). To substantiate the immunocytochemical data, we determined gap junction assembly biochemically by measuring the solubility of Cx32-WT and Cx32Δ220 in Triton-X (TX)100 (29). We found that a proportion of Cx32-WT and Cx32Δ220 was detected in detergent-insoluble and -soluble fractions in both LNCaP and BxPC3 cells (Figure 2B). The detergent-solubility of E-cadherin, an adherens junction-associated protein, was used as a control for these experiments. For example, we found that in three independent experiments between 40-50% of total Cx32-WT was in TX100-insoluble fraction in LN32-WT and Bx32-WT cells while this fraction ranged between 20-30% for LN32Δ220 and Bx32Δ220 cells. However, this difference in detergent-soluble and -insoluble fraction between Cx32-WT and Cx32Δ220 in LNCaP and BxPC3 cells was not statistically significant because of large variation in soluble and insoluble fractions.

To assess the relevance of our findings in LNCaP and BxPC3 cells to other cell types, we also retrovirally expressed Cx32-WT and Cx32Δ220 in HEK293T and HeLa cells as well as in another human pancreatic cancer cell line HPAF-II. We found that compared to Cx32-WT, Cx32Δ220 was assembled into smaller gap junction-like puncta in all cell types examined (data not shown). Thus, Cx32Δ220 assembled into smaller gap junction-like puncta in a variety of cell types. Overall, the above data suggest that although the size of gap junction plaques and function composed of cytoplasmic-tail-deleted Cx32Δ220 are compromised, Cx32-CT is not required for initiating the formation of gap junctions.

Cx32-WT and Cx32Δ220 Traffic and Degrade Normally

To determine whether Cx32Δ220 trafficked normally to the cell surface, we used cell surface biotinylation. The data showed that Cx32Δ220 was biotinylated as efficiently as Cx32-WT in both cell types (Figure 3A). For example, in both cell types between 5-10% of input Cx32-WT and Cx32Δ220 was biotinylated (Figure 3A, legend). To investigate if cell surface-associated Cx32Δ220 degrades more rapidly compared to Cx32-WT, we determined their kinetics of degradation after biotinylation. We found that the cell surface-biotinylated Cx32Δ220 degraded with kinetics similar to Cx32-WT (Figure 3B). The data from two independent experiments, which varied by less than 10%, are plotted in Figure 3D. These findings suggest that the assembly of Cx32Δ220 into smaller gap junctions was not caused by its impaired trafficking to the cell surface or its rapid internalization and degradation prior to assembly but by some mechanism that interfered with the
growth of the gap junction plaques after they had been assembled.

**Expression of Cx32-WT Rescues the Small Junction Phenotype of Cx32Δ220**

Because Cx32Δ220 trafficked normally to the cell surface, and degraded with kinetics similar to Cx32-WT, we asked if small junction phenotype of Cx32Δ220 could be rescued by providing the Cx32-CT in Cis (as a part of Cx32-WT). Earlier studies had shown that the defective gap junction assembly of Cx43 in human pancreatic cancer cell lines was restored upon expressing endocytosis-deficient mutants of Cx43 in cells expressing endogenous full-length Cx43, and that restoration was caused by the formation of heteromers between them (20). We rationalized that Cx32-WT, by forming heteromers with Cx32Δ220, might increase the frequency of large gap junctions by providing Cx32-CT in Cis. To test this notion, we transiently expressed Myc-tagged Cx32-WT in LN32Δ220 and Bx32Δ220 cells and, conversely, Myc-tagged Cx32Δ220 in LN32-WT and Bx32-WT cells. Formation of gap junctions was examined immunocytochemically. We found that the expression of Myc-tagged Cx32-WT increased the size of gap junction formed by Cx32Δ220 in both LN32Δ220 and Bx32Δ220 cells (Figure 4AD). Expression of Myc-tagged Cx32A220 in LN32Δ220 and Bx32Δ220 cells had no effect (data not shown). As determined by measuring the areas of 350 gap junction puncta at cell-cell interfaces, we found that the mean surface area of rescued Cx32Δ220 gap junctions was 15-20 fold larger compared to the mean area of gap junctions formed by the Cx32A220 alone (Figure 4D). For comparison, gap junctions formed of Cx32-WT and Cx32Δ220 alone are also shown (Figure 4C).

In the next series of experiments we examined if expression of Myc-tagged Cx32Δ220 in LN32-WT and Bx32-WT cells would attenuate the assembly of Cx32-WT into gap junctions. The results showed that the assembly of Cx32-WT was inhibited upon expressing Cx32Δ220-Myc (Figure 4B). Expression of Myc-tagged Cx32-WT in LN32-WT and Bx32-WT had no effect (data not shown). As assessed visually, we also found that the rescue of Cx32Δ220 gap junctions was accompanied with a concomitant decrease in the number of intracellular puncta (Figure 4A) whereas inhibition of Cx32-WT assembly was accompanied with a concomitant increase in intracellular puncta (Figure 4B). To substantiate these observations, we counted the number of intracellular puncta from 3 independent microscopic fields in 2 independent experiments. We found that the mean number of intracellular puncta in LN32Δ220 and Bx32Δ220 cells was 27 ± 5 (n =28) and 42 ± 8 (n = 35), respectively, which decreased to 17 ± 4 (n =35) and 8 ± 4 (n = 27) in cells expressing Cx32-WT-Myc, respectively. Similarly, the mean number of intracellular puncta in LN32-WT and Bx32-WT cells was 6 ± 4 (n = 27) and 9 ± 4 (n = 27), respectively, which increased to 30 ± 4 (n =15) and 42 ± 4 (n = 15), respectively, in cells expressing Cx32Δ220.

As assessed visually by light microscopy we also found that total Cx32 (WT and Δ220) co-localized nearly completely with the Myc-tagged reciprocal counterparts (Figure 4A). If Cx32-WT and Cx32Δ220 did not form heteromers with Cx32Δ220-Myc and Cx32-WT-Myc, it is less likely that the colocalization would have been as robust. These results suggest that the rescue likely occurs through the formation of heteromers although light microscopy cannot distinguish between gap junctions composed of mixture of homomeric and heteromeric connexons. To test if adding EGFP or Myc tags to Cx32-WT and Cx32Δ220 affects their ability to assemble into gap junctions, we first retrovirally expressed Cx32-WT-EGFP, Cx32-WT-Myc, Cx32Δ220-EGFP and Cx32Δ220-Myc in LNCaP cells. We found that both tags had no discernible effect on the formation of gap junctions (Figure 5A). Hence, to substantiate the immunocytochemical data, we transiently expressed both Cx32-WT-Myc and Cx32Δ220-Myc along with Cx32Δ220-EGFP and Cx32-WT-EGFP, respectively, in HEK293 cells. We then examined if the differentially-tagged proteins could be co-
immunoprecipitated from the detergent-soluble fraction, which likely contains only connexons that are not incorporated into gap junctional plaques (29). We used HEK293 cells for co-immunoprecipitation because of the low transfection efficiencies of BxPC3 and LNCaP. Moreover, HEK293 cells do not express Cx32 endogenously. The results showed that Cx32-WT and Cx32Δ220 reciprocally co-immunoprecipitated each other (Figure 5B).

To test whether the preponderance of Cx32Δ220 or the ratio of Cx32-WT and Cx32Δ220 in a connexon controls gap junction assembly, we transiently expressed Cx32-WT and Cx32Δ220-EGFP at various ratios in HEK293T cells and examined assembly immunocytochemically (Figure 6). For these experiments we used an antibody raised against the Cx32-CT, which would not recognize Cx32Δ220-EGFP. As assessed by an increase in the size of gap junction formed by Cx32Δ220-EGFP and a concomitant decrease in the number of smaller gap junction puncta, we found that the rescue occurred only when the expression of Cx32-WT was high (Figure 6B). Collectively, the data shown in Figures 4, 5 and 6 suggest that Cx32-CT enhances the size of gap junctions.

Expression of Cx32-CT in Trans Affects Cx32Δ220 Junction Assembly

To explore whether expression of Cx32-CT by itself in Trans would modulate the size of gap junctions formed by Cx32Δ220 or Cx32-WT, we rationalized that the Cx32-CT interacts and/or recruits proteins that determine the growth of plaques composed of Cx32Δ220 and that the recruitment and interaction occur at the cell surface. To test this, we engineered a Cx32-CT construct (representing amino acid residues 220-283) in which a myristoylation sequence was added at the amino terminus of Cx32-CT to target it to the plasma membrane; moreover, the Cx32-CT was also tagged with cyan fluorescent protein mTurquoise. This construct is designated as Cx32-CT-TQ-Myr (Figure 7A). As a control, we also engineered an mCherry-Myr (mCH-Myr) construct in which the myristoylation sequence was added to the amino terminus of red fluorescent protein mCherry. Both engineered constructs were also tagged with Myc (Figure 7A). Transient transfection of Cx32-CT-TQ-Myr and mCH-Myr in HEK293 cells, followed by Western blot analysis, showed that both Cx32-CT-TQ-Myr and mCH-Myr migrated at the predicted MW and were appropriately targeted to the cell surface (data not shown).

To test if expression of Cx32-CT in Trans would modulate gap junction size, we transiently expressed Cx32-CT-TQ-Myr and mCH-Myr in LN32-WT, LN32Δ220, Bx32-WT and Bx32Δ220 cells and examined gap junction assembly immunocytochemically. As assessed by the size of the puncta at cell-cell interfaces, we found that while the expression of Cx32-CT-TQ-Myr had no effect on the assembly of Cx32-WT in LN32-WT and Bx32-WT cells, the small gap junction phenotypes of LN-32Δ220 and Bx32Δ220 cells were partially rescued (Figure 7B). When we measured the surface areas of 350-670 individual gap junction puncta in transfected LN32Δ220 and BxΔ220 cells, there was a consistent 2-3 fold increase in the size (surface area) of gap junction puncta in LN32Δ220 and Bx32Δ220 cells expressing Cx32-CT-TQ-Myr compared to those expressing mCH-Myr (Figure 7C). Moreover, we also found that the number of intracellular puncta in Cx32-CT-TQ-Myr expressing cells was significantly decreased (Table 2). These data suggest that Cx32-CT recruits some factor(s) to the cell surface as it traffics along the secretory pathway and/or upon arrival at the cell surface, which facilitate(s) the growth of gap junctions composed of Cx32Δ220.

Carboxyl Tail of Cx32 is Essential for the Clustering of Cell-Cell Channels

Earlier studies had shown that clustering of cell-cell channels composed of Cx43 was induced when intracellular levels of cAMP were elevated (30-33). Moreover, other studies had shown that
elevating intracellular cAMP level enhanced gap junctional communication by stabilizing Cx32 in rat hepatocytes (34,35). These results, combined with our findings, prompted us to investigate whether Cx32-CT is required for the clustering of cell-cell channels. Hence, we expected Cx32Δ220 not to cluster in response to forskolin, which elevates intracellular levels of cAMP by activating adenylyl cyclase (36). Therefore, we treated LN32-WT, LN32Δ220, Bx32-WT and Bx32Δ220 with forskolin for 8 h based on our earlier studies with the other cell lines (37-39). The results (Figure 8) showed the following: 1. Cell-cell channels composed of Cx32-WT clustered and/or redistributed to form large gap junctions in LN32-WT cells (Figure 8, left panels). 2. The channels composed of Cx32Δ220 did not cluster at all in LN32Δ220 cells (Figure 8, right panels). Similar results were obtained with Bx32-WT and Bx32Δ220 cells although the effect was not robust because forskolin was toxic to these cells (data not shown). These results suggest that Cx32-CT is required for the clustering and/or redistribution of cell-cell channels and gap junction assembly.

Domains of Carboxyl Tail of Cx32 that Determine Gap Junction Size

To determine which domain of Cx32-CT determines gap junction size, we engineered a series of Cx32 mutants with progressive truncation of the carboxyl tail (Figure 9A). These mutants were then retrovirally expressed in LNCaP and BxPC3 cells. Western blot analysis showed that the mutants were expressed robustly and migrated at the predicted molecular weight (Figure 9B). Immunocytochemical analysis revealed that the size of gap junctions formed by the mutants Cx32Δ270 and Cx32Δ250 was not discernibly different from those formed by Cx32-WT, whereas a robust decrease in gap junction size was seen with the mutants Cx32Δ230 and Cx32Δ220 (Figure 9C). Taken together, these results suggest that the critical motifs that determine the size of gap junctions composed of Cx32 likely reside between residues 230 and 250 in the Cx32-CT.

Movement of Gap Junction Plaques Composed of Cx32-WT and Cx32Δ220

Next we investigated the dynamic behavior of gap junctions composed of Cx32-WT and Cx32Δ220 in LNCaP and BxPC3 cells. To test this we retrovirally expressed Cx32-WT-EGFP and Cx32Δ220-EGFP in LNCaP and BxPC3 cells. Like their untagged counterparts, Cx32-WT-EGFP and Cx32Δ220-EGFP were assembled into large and small gap junctions, respectively (Figure 10, see also Figure 5). Live-cell imaging was performed on confluent cells to allow unambiguous detection of gap junction plaques at the areas of cell-cell contact as well as to minimize artifacts that might result from the movement of cells during imaging (see Materials and Methods). For most studies related to live-cell imaging, we utilized BxPC3 cells because they were easier to image compared to LNCaP cells. The results showed that gap junction plaques composed of Cx32Δ220-EGFP were highly dynamic compared to those composed of Cx32-WT-EGFP (Movies S1 and S2). By individually tracking the movement and disappearance of puncta at cell-cell contact areas, we found that most Cx32Δ220-EGFP puncta moved and disappeared within 10 min whereas most Cx32-WT-EGFP gap junction puncta moved slowly and did not disappear even up to 2 h (Figure 10, movies S1 and S2). For example, the average speed of Cx32Δ220-EGFP puncta was twice that of Cx32-WT-EGFP puncta (Figure 11A). We also measured the average speed of small puncta formed by the Cx32-WT-EGFP, comparable in size to those formed of Cx32Δ220-EGFP, and found that their speed was not significantly different from those formed by Cx32-WT-EGFP puncta (Figure 11A). By analyzing the behavior of both large and small puncta of Cx32-WT-EGFP and several puncta of Cx32Δ220-EGFP, we found that Cx32Δ220-EGFP puncta moved abruptly whereas the movement of large as well as small Cx32-WT-EGFP puncta was saltatory (Movies S1 and S2, Figure 11B). Moreover, the puncta we have imaged are less likely to be vesicles because their size was 2-3 times larger than the size...
of the vesicular puncta in the cytosol. Furthermore, most intracellular vesicular puncta lay at a different Z-plane and not only their spatio-temporal pattern of movement was different from those at cell-cell contact areas but also they could not be imaged together. Finally, it seems highly improbable that the EGFP-tagged cytoplasmic-tail-deleted connexin32 aggregated nonspecifically while the full-length connexin32 continued to assemble into gap junctions.

To assess if the mobility of Cx32Δ220-EGFP at the plasma membrane was different from Cx32-WT-EGFP, we photobleached gap junction plaques (contacting cells) and non-junctional areas (single cells) and measured recovery by FRAP analysis (Figure 11CD, see Materials and Methods). The results showed that the mobile fraction of Cx32-WT-EGFP and Cx32Δ220-EGFP in gap junction plaques was low and was not different from each other (Figure 11C). Moreover, the mobile fraction of both WT and cytoplasmic-tail-deleted Cx32 in non-junctional areas was comparable to that observed in gap junction plaques (Figure 11D). Earlier live-cell imaging studies had indicated that the smaller gap junction-like puncta could fuse to give rise to larger puncta (40). Therefore, we also examined if larger plaques arose by fusion of the smaller plaques, a process that could be facilitated by the Cx32-CT. As assessed by analyzing the behavior of several large and small puncta composed of Cx32-WT-EGFP and Cx32Δ220-EGFP, we found that the small puncta rarely fused with one another (Table 3, movies S1 and S2). Collectively, these data suggest that the Cx32-CT is not required for the mobility of Cx32 both in the plaques and in non-junctional regions.

Discussion

The cytoplasmic tails of Cxs are highly divergent in amino acid composition, and phosphorylation of specific serines or tyrosines by a variety of kinases in the tails has been documented to be important in regulating the permeability of gap junctional channels (41). The cytoplasmic tails have also been shown to interact with many cytoskeletal and cell-junction-associated proteins (10). It is thought that, besides regulating the permeability of channels, these interactions govern the assembly of Cxs into gap junctions either directly or indirectly (10). Evidence favoring the role of cytoplasmic tails in controlling gap junction assembly has been obtained through colocalization and coimmunoprecipitation studies (10), and most studies have been performed with Cx43, which is ubiquitously expressed (2,6). The extent to which the assembly of a particular Cx into gap junctions upon arrival at the cell surface is determined by factors intrinsic to the Cx itself or by extrinsic factors, such as the direct or indirect interaction of a Cx’s cytoplasmic tail with the cytoskeleton or the cell-surface-associated proteins, has not yet been explored mechanistically for many other Cxs (4,10). The first major conclusion of our study is that the size of gap junction plaques composed of cytoplasmic-tail-deleted Cx32Δ220 is drastically diminished, and that the tail is not required for initiating the formation of a gap junction plaque but for its subsequent growth. The second major conclusion of our studies is that gap junctions composed of cytoplasmic-tail-deleted Cx32 are highly unstable and that the tail of Cx32 is able to fine-tune the growth of a gap junction plaque when provided either in Cis as a part of a connexon or in Trans as a separate molecular entity targeted to the cell surface. Our findings suggest that the cytoplasmic tail is not required to initiate the assembly of Cx32 into gap junctions but for their subsequent growth and stability.

Our results showed that Cx32Δ220 not only trafficked normally to the cell surface but also degraded with kinetics similar to Cx32-WT (Figure 3). Despite this, it formed 20-35 fold smaller gap junctions (Figure 1CD). These data rule out impaired trafficking and/or enhanced degradation as one possible mechanism for the decrease in the size of gap junctions composed of Cx32Δ220. Moreover, gap junctions composed of Cx32Δ220 were also functional and detergent-resistant (Figure 2, Table 1). These results suggest that the connexons formed of cytoplasmic-tail-deleted connexin32 aggregated nonspecifically while the full-length connexin32 continued to assemble into gap junctions.
Cx32Δ220 were able to dock and assemble into miniscule functional gap junctions and that the formation of nascent plaques is not dependent on the cytoplasmic tail, while the subsequent growth of the plaques is tail-dependent. Furthermore, though not directly demonstrated through electron microscopic studies, the puncta we have imaged at the site of cell-cell contact represent detergent-resistant functional gap junctions. Thus, these results strongly imply that the distinct puncta seen at the light microscopic level at the cell-cell contact sites likely represent gap junctional plaques rather than aggregates of undocked connexons (Figure 1C). In this regard, it is noteworthy to mention that the cytoplasmic-tail-deleted Cx43 mutants, Cx43Δ244 and Cx43Δ258, were also assembled into gap junctions; however, the plaque growth composed of these mutants was not affected and compromised (42,43). In fact, the average diameter of the plaques formed by the cytoplasmic-tail-deleted Cx43Δ258 was larger than those formed by the full-length Cx43 (42). Given the fact that the expression of Cx32 is restricted to the polarized and differentiated cells of the exocrine glands (15), our results imply that the assembly of gap junctions composed of Cx32 is regulated differently from those composed of Cx43.

A salient as well as an intriguing aspect of our data is that the size of gap junctions composed of Cx32Δ220 was also increased 2-3 fold by expressing the membrane-targeted form of Cx32-CT (Cx32-CT-TQ-Myr) in Trans (Figure 7). Moreover, increase in size of Cx32Δ220 puncta upon expressing Cx32-CT-TQ-Myr also decreased the number of intracellular puncta (Table 2). One possible explanation for these data is that Cx32-CT-TQ-Myr either sequesters protein(s) at the cell surface that inhibit plaque growth and/or recruits additional proteins that facilitate growth. The latter possibility is supported by the Cx32-mediated recruitment of disc large protein, Dlg, to the cell surface in hepatocytes possibly through direct or indirect interaction (44,45). Lack of robust effect of Cx32-CT-TQ-Myr alone in incrementing Cx32Δ220 plaque growth compared to that observed upon expressing Cx32-WT may be due the time required by Cx32-CT-Myr, which is delivered randomly to the cell surface, to arrive near the connexons or plaques as compared to when Cx32-CT is available as a part of the connexon itself.

Earlier live-cell imaging studies had indicated that the smaller gap junction-like puncta could fuse to give rise to larger puncta (40). Therefore, we also rationalized that the larger plaques arose from the fusion of the smaller plaques, a process that could be facilitated by the Cx32-CT. However, our live-cell imaging data revealed that this mechanism was less likely as small puncta were rarely seen to fuse with one another (Movies S1 and S2, Table 3). Moreover, while our live-cell imaging data showed substantially decreased mobility of larger plaques formed of Cx32-WT-EGFP, we also found that gap junction-like puncta composed of Cx32Δ220-EGFP were highly mobile and moved faster and abruptly (Figure 11B). Furthermore, even small gap junction-like puncta formed by the Cx32-WT-EGFP, comparable in size to those formed by Cx32Δ220-EGFP, were as stable and sessile as larger puncta (Figure 11A, Movies S1 and S2). In addition, our FRAP analysis showed that the mobility of both Cx32Δ220-EGFP and Cx32-WT-EGFP within gap junctional plaques (in contacting cells) and in non-junctional membranes (in single cells) was not discernibly different (Figure 11CD). These results rule out differential diffusion of Cx32-WT and Cx32Δ220 connexons at the cell surface as one mechanism to account for the drastic difference in the size of gap junction plaques. In this regard it is noteworthy to mention that the mobility of gap junction-like puncta composed of cytoplasmic tail-deleted Cx43 was not different from Cx43-WT (43).

The findings that the small size of gap junctional plaques composed of Cx32Δ220 could be rescued upon co-expressing Cx32-WT and vice versa (Figures 4, 6), combined with the fact that the tail had no effect on the mobility of Cx32, raise intriguing possibilities with regard to the molecular mechanisms involved in the genesis of larger plaques and the role played by the Cx32-CT in
plaque growth. Evidence to date supports the notion that once a plaque has been nucleated, the subsequent growth of a plaque occurs upon recruitment of connexons to its periphery either by diffusion (43,46-48) or upon direct delivery of connexons to its vicinity (49). Moreover, it is highly likely that the docking of connexons to form functional cell-cell channels occurs either in the vicinity of the plaque with a concomitant incorporation of the channels into the plaque or concurrently (12,50). What might then be the possible explanation for the failure of Cx32Δ220 to assemble into larger gap junctions?

The molecular collisions among several transmembrane proteins have been postulated to be regulated by the partitioning of the entire plasma membrane by the actin-based membrane cytoskeleton and its associated proteins into several subdomains (51). Anchoring of transmembrane proteins to the cytoskeleton proteins either directly or indirectly via a scaffolding protein is likely to hinder or prevent their diffusion in the plane of the plasma membrane compared to proteins that are not anchored (52-54). Thus, tethering of a Cx’s cytoplasmic tail to the cytoskeleton directly or indirectly could attenuate the delivery of connexons to the plaque by hindering mobility on the plane of the membrane. Alternatively, tethering could stimulate plaque growth by anchoring connexons near the plaque to increase the probability of docking of connexons to form cell-cell channels with concomitant incorporation of the channels into the plaque. A similar mechanism may be evoked to explain the increased motility of small gap junctions composed of Cx32Δ220 and increased stability of plaques composed of Cx32-WT. In addition, the cytoplasmic tail might recruit some factors to the sites of nascent plaques to stabilize membrane subdomains near its vicinity to permit docking of connexons and the subsequent incorporation of channels in the plaque.

Several lines of evidence provide support for this notion. For example, earlier studies showed that when interaction between the cytoplasmic tail of Cx43 and ZO-1 — a scaffolding protein that links Cx43’s cytoplasmic tail to the cytoskeleton — was disrupted, connexon recruitment and/or incorporation to the plaque periphery was facilitated, leading to an increase in the size of gap junction plaque (12). On the other hand, deletion of the PDZ-binding domain of Cx50, a Cx expressed in the lens epithelial cells and shown to interact with ZO-1(55), inhibited gap junction assembly (13). Hence, it is possible that the cytoplasmic tail is required for anchoring Cx32 to cytoskeletal proteins near the plaque to facilitate docking of connexons between the contiguous cells and the subsequent incorporation of the channels into nascent plaque. Support for this line of thought comes from our data showing increase in the size of gap junctions composed of Cx32Δ220 upon transient expression of Cx32-WT (Figure 4A), decrease in the size of gap junctions composed of Cx32-WT upon transient expression of Cx32Δ220 (Figure 4B), and failure of Cx32Δ220, but not Cx32-WT, to cluster to form larger gap junctions upon elevation of intracellular levels of cAMP (Figure 8). In this regard, it is noteworthy to mention that in contrast to ubiquitously expressed Cx43, only few proteins have been shown to interact with the cytoplasmic tail of Cx32. These proteins included SAP97/Dlg (44,45), calmodulin (56,57), ZO-1 (58), caveolin-1 (59) and occludin (60). However, in LNCaP and BxPC3 cells, none of these proteins, except caveolin-1, co-localized with Cx32 as assessed by immunocytochemical analysis (unpublished observations) and hence their involvement in regulating the assembly of Cx32 into gap junctions is less likely. Our future studies will explore the role of caveolin-1 in regulating the assembly of Cx32 into gap junctions.

Based on the above arguments, we propose that the cytoplasmic tail of Cx32 is essential for the stability and subsequent growth of the plaque but is not required to initiate the formation of a gap junction plaque. Once the plaque has been nucleated, the tail permits the linkage of Cx32 to the cytoskeleton by mass action, which stabilizes the plaque. This permits more channels to be recruited and incorporated in the plaque. The nascent
plaques formed by the cytoplasmic-tail-deleted Cx32Δ220 remain unstable because they fail to establish a linkage with the cytoskeleton. The Cx32Δ220 plaques remain small because they are dislodged before a significant increment of connexons or channels occurs at the plaque periphery. This explanation is in accord with the increased stability of both the small and the large plaques formed of Cx32-WT. We also propose that the mobility of Cx32-WT in non-junctional areas away from the plaque is not dependent on the anchoring of the tail to the cytoskeleton. Earlier studies showed that in pig thyrocytes, which expressed endogenous Cx43 and Cx32, as well as in Cx43-expressing human embryonic kidney cells in which Cx26 was transiently expressed, while both Cxs were assembled into gap junctions the plaques composed of each Cx subtype resided in two different plasma membrane sub-domains (61,62). Our results with Cx32Δ220 may provide rational explanation for the segregation of plaques composed of Cx43 and Cx32 in different subdomains of the plasma membrane as well as for the increased motility of Cx26-GFP, a tail-less Cx, in gap junction-like clusters (47). Altogether, our results suggest that the assembly of Cx32 is regulated differently than that of Cx43 and substantiate the conclusion drawn from earlier studies, which showed that the assembly of Cx32 and Cx43 into gap junctions was differentially regulated in cadherin-null human squamous carcinoma cells (19).

What might be the possible physiological relevance of cytoplasmic-tail-mediated facilitation of gap junction assembly with regard to the growth of a plaque? The number of cell-cell channels in a given gap junction plaque varies substantially and may range from 50 to over 10,000; moreover, there is a wide range of variation in the size of gap junctions formed between the two contiguous cells (5,63). Furthermore, hormone-induced clustering of cell-cell channels to increase the size of gap junctions is frequently observed (15,64). Clustering of cell-cell channels is a prerequisite for the opening of cell-cell channels and that the larger gap junctional plaques have more open channels compared to the smaller plaques (50). It is tempting to speculate that while post-translational modifications in the cytoplasmic tail of a Cx are used to acutely regulate gating of cell-cell channels, the engagement and the disengagement of a Cx’s tail with the cytoskeletal proteins are used for the chronic regulation of permeability of channels with slower, spatio-temporal characteristics. Alternatively, the cytoplasmic-tail-mediated increase in gap junction plaque size and stability may facilitate the assembly of other junctional complexes, which are required to maintain the polarized and differentiated state of epithelial cells (65,66). More elaborate studies are needed to assess further the physiological significance of our findings with respect to functional role of gap junctional communication.

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19
Figure Legends

Figure 1: Cytoplasmic-tail-deleted Cx32 assembles into small gap junctions.

LNCaP and BxPC3 cells were infected with the control and Cx32-harboring recombinant retroviruses and Cx32 expression level was determined by Western blot (AB) and immunocytochemical analyses (CD). A. Note that only cells infected with Cx32-harboring retroviruses express Cx32-WT (32-WT) and Cx32Δ220 (32Δ220) in LNCaP (A) and BxPC3 (B) cells. The numbers on the left indicate the position of the molecular weight markers. An upward shift in the β-actin band in (B) is likely due to the presence of air bubbles at the interface of the gel and the running buffer. C. Cells expressing Cx32-WT and Cx32Δ220 were immunostained for Cx32 with antibody raised against the cytoplasmic loop of Cx32. Note that, as indicated by arrows, Cx32-WT expressing LNCaP (upper row, left panels) and BxPC3 (lower row, left panels) cells form large gap junctions (red) whereas Cx32Δ220 expressing LNCaP (upper row, right panels) and BxPC3 cells (bottom row, right panels) form miniscule gap junctions (red). The enlarged images of the boxed areas in upper and lower panels are shown towards left. Immunostaining for E-cadherin (E-Cad, green) was used to delineate the cell-cell interfaces. The nuclei (blue) were stained with DAPI. Bar = 10 μm. D. Gap junction areas of LNCaP and BxPC3 cells expressing Cx32-WT and Cx32Δ220. Areas (Mean ± SE) of 350 distinct gap junction puncta, from 3 single optical sections from 3 independent experiments after iterative volume de-convolution of the captured images, were determined using the measurement module of Volocity. The area is represented in μm² (see Materials and Methods). Note the 20-35 fold decrease in the mean gap junction plaque area in cells expressing Cx32Δ220. The mean surface area of gap junctions composed of Cx32-WT was 58.5 ± 7.3 μm² and 56.4 ± 9.3 μm² for LNCaP-32 and Bx32-WT cells, respectively. The differences in areas between Cx32-WT and Cx32Δ220 expressing cells were statistically highly significant (P ≤ 0.001). E. Topology of Cx32. The site at which Cx32 was truncated is indicated by the arrow. TM1-TM4 = Four transmembrane domains. EC1 and EC2 = Extracellular loop 1 and 2. CL = Cytoplasmic loop. NH₂ and COOH are the amino and the carboxyl termini.

Figure 2: Cx32-WT and Cx32Δ220 are assembled into detergent-insoluble functional gap junctions in LNCaP and BxPC3 cells.

A. LNCaP cells expressing Cx32-WT and Cx32Δ220 were microinjected with Alexa 488 (Green, MW 570) and Alexa 594 (red, MW 760). Note extensive transfer of both tracers in LNCaP cells expressing Cx32-WT and weaker transfer in cells expressing Cx32Δ220. The microinjected cells are marked by the arrows. Bar = 20 μm. B. Cells expressing Cx32-WT and Cx32Δ220 were grown in 10 cm dishes for 5-7 days. TX100-solubility assay was used to measure the assembly of Cx32-WT and Cx32Δ220 into gap junctions. TX100-solubility of E-cadherin (E-Cad) was used as a control. Note that both Cx32-WT and Cx32Δ220 are found in TX100-soluble and TX100-insoluble fractions.
Figure 3: Cx32-WT and Cx32Δ220 traffic and degrade normally in LNCaP and BxPC3 cells.

A. The cell surface proteins of LNCaP and BxPC3 were biotinylated and pulled down by immobilized streptavidin and immunoblotted for Cx32. Biotinylation of E-cadherin (E-cad) was used as a positive control. A non-biotinylated dish was kept as a control (-). For input, 10 µg of total cell lysate was used. Note that both Cx32-WT and Cx32Δ220 and E-cad are efficiently biotinylated. B. Cell surface associated Cx32-WT and Cx32Δ220 degrade with similar kinetics in LNCaP and BxPC3 cells. Cells were biotinylated at 4°C, and were incubated for various times at 37°C before streptavidin pull-down and Western blotting (Materials and Methods). Representative blots for Cx32-WT and Cx32Δ220 from LNCaP (B) and BxPC3 (C) cells are shown. D. The blots were quantified using C-digit and the values from 2 independent experiments were plotted graphically with Sigma plot. Note that cell surface associated biotinylated Cx32-WT and Cx32Δ220 (Cx32T220) degrade with similar kinetics in both cell lines. The values in the two independent experiments varied by ≤10%.

Figure 4: Cytoplasmic tail of Cx32 controls gap junction size.

A. Myc-tagged Cx32-WT (Cx32-WT-Myc) was transiently expressed in LN32Δ220 (top row) and Bx32Δ220 (bottom row) cells, which express Cx32Δ220 stably. After 24 h, cells were immunostained for Myc (green) and Cx32 (red) using antibody raised against the cytoplasmic loop of Cx32 (amino acids 115-124). Note the formation of large gap junction plaques in cells expressing Cx32-WT-Myc compared to control cells expressing only Cx32Δ220 (C, right panels). B. Myc-tagged Cx32Δ220 (Cx32Δ220-Myc) was transiently expressed in LN32-WT (top row) and Bx32-WT (bottom row) cells expressing Cx32-WT stably. After 24 h, cells were immunostained for Myc (red) and Cx32 (green) using antibody raised against the carboxyl tail of Cx32, which does not recognize Cx32Δ220. Note loss of gap junction plaques in cells expressing Cx32Δ220 compared to control cells expressing Cx32-WT alone (C, left panels). C. Gap junction size in cells expressing Cx32-WT (Cx32-WT, green) and Cx32Δ220 (Cx32Δ220, red) in LNCaP (top row) and BxPC3 (bottom row) cells transiently transfected with pmCherry and pAcGFP (not shown). D. Mean surface area of the rescued gap junctions in LN32Δ220 and Bx32Δ220 cells in A. Areas (Mean ±SE) of 350 distinct gap junction puncta, from 3 single optical sections from 3 independent experiments after iterative volume de-convolution of the captured images, were determined using the measurement module of Volocity. The area is represented in µm² (see Materials and Methods). Note the 15-20 fold increase in the mean gap junction plaque area in cells expressing Cx32-WT-Myc.

Figure 5: Cx32-WT and Cx32Δ220 tagged with Myc or EGFP are assembled into gap junctions and co-immunoprecipitate each other.

A. Cx32-WT-Myc and Cx32Δ220-Myc, Cx32-WT-EGFP and Cx32Δ220-EGFP were retrovirally expressed in LNCaP cells and pooled polyclonal cultures were immunostained for Cx32, Myc, β-catenin (β-cat). Top panels: Cx32 (green) and β-cat (red). Middle panels: Myc (green) and β-cat (red). Bottom panel: β-cat (red) alone. Note that tagged Cx32-WT and Cx32Δ220 are assembled into gap junctions like their untagged counterparts and addition of tags has no effect on gap junction size. Bar = 10 µM. B. HEK293T cells were
transfected with Cx32-WT-EGFP and Cx32Δ220-EGFP either alone or in combination with Cx32Δ220-Myc and Cx32-WT-Myc, respectively. EGFP-tagged proteins were pulled down with immobilized anti-GFP antibody and immunoblotted for anti-Myc and anti-GFP antibodies. Note that Cx32-WT-Myc co-immunoprecipitated with Cx32Δ220-GFP and Cx32Δ220-Myc with Cx32-WT-EGFP, indicating that both Cx32-WT and Cx32Δ220 form heteromers with each other. The arrows indicate the position of Cx32-WT and Cx32Δ220 on Western blots for pull-down and input lanes.

**Figure 6: An optimal expression of Cx32-WT and Cx32Δ220 is required to restore gap junction assembly.**

**A.** HEK293T cells were transiently transfected with Cx32-WT and Cx32Δ220-EGFP at different ratios as indicated by the numbers on right. Transfected cells were fixed and immunostained for Cx32 (red) using antibody against the Cx32-CT. Note that cells expressing high levels of Cx32-WT are assembled into larger gap junctions. **B.** Surface areas (Mean ± SE) of gap junctions in cells transfected with Cx32-WT and Cx32Δ220-EGFP at the indicated ratios. The means represent areas from 100-150 individual puncta from 100-200 transfected cells. The asterisk (*) indicates P value of ≤ 0.005 and asterisks (**) indicate P value of ≤ 0.0001. A two tailed Student’s t test was used to calculate P value assuming unequal variance.

**Figure 7: Cx32-CT partially restores gap junction assembly.**

**A.** A schematic diagram of the membrane-targeted mCherry and Cx32-CT constructs. Note that the membrane targeted Cx32-CT construct contains monomeric Turquoise instead of mCherry (see Materials and Methods). **B.** LN32-WT, LN32Δ220, Bx32-WT and Bx32Δ220 were transiently co-transfected with mCherry, mCherry-Myr (mCH-MYR), and Cx32-CT-Turquoise-Myr (32-CT-TQ-MYR). Cells were fixed and immunostained for Cx32 (green) 24 h after transfection. Note that only the expression of Cx32-CT-TQ-Myr increases the size of gap junction-like puncta. The color of the blue channels indicating the expression of Cx32-CT-TQ-MYR was converted to red to simplify the figure. **C.** Areas (Mean ±SE) of 350-670 distinct gap junction puncta, from 3 single optical sections from 3 independent experiments in LNCaP cells after iterative volume de-convolution of the captured images, were determined using the measurement module of Volocity. The area is represented in µm² (see Materials and Methods). Note the 2-3 fold increase in the mean gap junction plaque area in cells expressing Cx32-CT-TQ-MYR. The mean surface area of gap junctions composed of Cx32-WT was 37 ± 1.2 µm². The mean size of gap junctions composed of Cx32Δ220 was 1.5 ± 0.94 µm² for LN-32Δ220 cells transfected with mCH-MYR.

**Figure 8: Cx32Δ220 fails to cluster in response to forskolin.**

**A.** LN32-WT and LN32Δ220 cells were treated with forskolin (+ Forskolin; 10 µM) for 8 h and gap junction assembly was examined immunocytochemically. Controls (-Forskolin) were treated with 0.05 % DMSO. Some gap junctions are indicated by the white arrows. Note that forskolin increased the size of gap
junctions of Cx32-WT but not of Cx32Δ220 puncta. Bar = 10 μm. **B.** Gap junction areas of Cx32-WT and Cx32Δ220 expressing LNCaP cells treated with forskolin as in **A.** Surface area (Mean ± SE) of gap junctions composed of Cx32-WT (blue) and Cx32Δ220 (red) were determined with the measurement module of Volocity. Areas (Mean ± SE) of 75-80 distinct gap junction puncta, from 15 single optical sections from 2 independent experiments after iterative volume de-convolution of the captured images, were determined using the measurement module of Volocity. The area is represented in μm² (see Materials and Methods). Note the 3-6 fold increase in the mean area of gap junctions composed of Cx32-WT (blue) in cells treated with forskolin. Note lack of effect for gap junctions composed of Cx32Δ220 (red).

**Figure 9:** Identification of the critical domain that determines gap junction size. **A.** Diagram showing Cx32 deletion mutants. The numbers on the right correspond to the number of the amino acid residue at which the truncation was introduced. **B.** Western blot analysis of various mutants after transient transfection in HEK293T cells. The numbers on the left correspond to the position of the molecular weight markers. **C.** The mutants shown in A were retrovirally expressed in LNCaP cells and pooled polyclonal cultures expressing the mutants stably were immunostained for Cx32 using antibody raised against the cytoplasmic loop of Cx32. Note that only cells expressing Cx32Δ220 and Cx32Δ230 form small gap junctions. **D.** Surface area (Mean ± SE) of 135-150 gap junctions composed of Cx32-WT, Cx32Δ270, Cx32Δ250, Cx32Δ230 and Cx32Δ220 were measured from 3 single optical sections from 3 independent experiments. The area is represented in μm². Note, there is a significant decrease in surface area of gap junctions composed of truncation mutants Cx32Δ230 and Cx32Δ220. The asterisks (**) indicate P value of ≤ 0.0001. A two tailed Student’s t test was used to calculate P value assuming unequal variance. Bar = 10 μm.

**Figure 10:** Gap junctions composed of Cx32Δ220 are more mobile than those formed of Cx32-WT.

Selected time lapse images of BxPC3 cells expressing EGFP-tagged Cx32-WT (Bx32-WT) and Cx32Δ220 (Bx32Δ220) are shown. The tracked gap junction puncta are indicated by the yellow arrows. The numbers in white indicate lapsed time in minutes. Note the rapid disappearance of Cx32Δ220 puncta compared to Cx32-WT puncta. Bar = 10 μm.

**Figure 11:** Mobility of gap junctions and connexons composed of Cx32-WT and Cx32Δ220.

**A.** Average (Mean ± SE) speed of gap junction puncta composed of EGFP-tagged Cx32-WT and Cx32Δ220 in BxPC3 cells (n=10). **B.** Motile behavior of gap junction puncta composed of EGFP-tagged Cx32-WT and Cx32Δ220 in BxPC3 cells as determined by tracking individual puncta (n=5; only two are shown). Note that the average speed of gap junction puncta composed Cx32Δ220 is nearly twice that of puncta composed of Cx32-WT. Note also that Cx32-WT puncta comparable in size to Cx32Δ220 puncta also move slower. **CD.** Analysis of mobility fraction of Cx32-WT and Cx32Δ220 (Cx32-T220) by FRAP within gap junctional plaques in contacting cells (**C, Gap Junctional**) and in single cells (**D, non-junctional**). Note that there is no difference in the mobile fraction of Cx32-WT and Cx32Δ220 in gap junction plaques and at non-junctional regions. In (**C**), Cx32-T220 = Cx32Δ220.
**TABLES**

**Table 1:** Junctional transfer of fluorescent tracers in LN32-WT and LN32Δ220 cells

| Junctional Tracer | Experiment Number | Junctional Transfer | LN32 | LN32Δ220 |
|-------------------|------------------|---------------------|------|----------|
| Lucifer Yellow    | 1                | 26±4(18)            | 7±2(14) |
|                   | 2                | 29±6(16)            | 6±2(13) |
| Alexa 488         | 1                | 22±5(16)            | 6±2(14) |
|                   | 2                | 23±6 (14)           | 8±3(18) |
| Alexa 594         | 1                | 13±3 (14)           | 4±1(22) |
|                   | 2                | 11±4(17)            | 3±1(29) |

Cells, seeded in 6 cm dishes in replicate, were grown for 4-7 days to 70% confluence. Junctional transfer was measured after microinjecting fluorescent tracers (see Materials and Methods).

**a:** The number of fluorescent cell neighbors (mean ± SE) 1 min (Lucifer Yellow), 3 min (Alexa 488) and 15 min (Alexa 594) after microinjection into test cell. The total number of injection trials is shown in the parentheses.
Table 2. Expression of membrane-targeted Cx32-CT decreases number of intracellular puncta.

| Transfection | LN32-WT  | LN32Δ220 | Bx32-WT  | Bx32Δ220 |
|--------------|----------|----------|----------|----------|
| mCherry-Myr  | 10.7±4.1 (30) | 18.7±3.3 (25) | 18.2±3.8 (25) | 18.6±3.6 (42) |
| Cx32-CT-TQ-Myr | 3.3±1.0 (57)  | 8.1±3.6 (62)  | 7.0±4.3 (62)  | 6.2±3.8 (57)  |

LNCaP and BxPC3 cells stably expressing Cx32-WT (LN32-WT, Bx32-WT)) and Cx32Δ220 (LN32Δ220, Bx32Δ220) were transfected with membrane-targeted mCherry-Myr or Cx32-CT-TQ-Myr. The number of intracellular puncta was determined 24 h after transfection as described in Materials and Methods. Numbers represent the Mean ± S.E. In parenthesis is the number of transfected cells counted. Transfected cells were from 10 different fields in two independent experiments.
Table 3. Larger gap junctions do not arise from the fusion of microscopically detectable small gap junctions

| BxPC3 Cells Expressing | # of Puncta Tracked | # Puncta Never Fused (%) | # Puncta Fused but Separated | # Puncta Fused |
|------------------------|---------------------|--------------------------|-----------------------------|---------------|
| Bx32-WT-EGFP           | 205                 | 188 (92)                 | 8 (4)                       | 17 (8)        |
| Bx32Δ220-EGFP          | 193                 | 186 (96)                 | 5 (2.6)                     | 7 (3.6)       |
| LN32Δ220-EGFP          | 105                 | 101 (96)                 | 6 (5.7)                     | 8 (7.6)       |

Live cell imaging of BxPC3 and LNCaP cells expressing EGFP tagged Cx32-WT and Cx32Δ220 was performed and puncta that appeared at the cell-cell contact were individually tracked as described in Materials and Methods. For Bx32-WT-EGFP and Bx32Δ220-EGFP the data were obtained from 5-8 independent live-cell imaging experiments. Numbers in parenthesis represent % of Total.
Figure 2

A

Alexa-594

CX32-WT

CX32Δ220

Alexa-488

CX32-WT

CX32Δ220

B

|          | LN32-WT | LN32Δ220 |
|----------|---------|----------|
|          | T       | S       | I       |
| CX32-WT  |         |         |         |
| CX32Δ220 |         |         |         |
| E-Cad    |         |         |         |

|          | Bx32-WT | Bx32Δ220 |
|----------|---------|----------|
|          | T       | S       | I       |
|          |         |         |         |
|          |         |         |         |

Figure 2
Figure 3

A

| BIOTIN | LN32-WT | LN32Δ220 | Bx32-WT | Bx32Δ220 |
|--------|---------|----------|---------|----------|
| -      | +       | +        | -       | +        |

- Cx32 Pull
- E-cad Pull
- Cx32 Input

B

| Hrs Post-Biotinylation |
|------------------------|
| 0 1 2 3 4 5 6          |

LN32-WT

- Pull
- Input

LN32Δ220

- Pull
- Input

C

| Hrs Post-Biotinylation |
|------------------------|
| 0 1 2 3 4 5 6          |

Bx32-WT

- Pull
- Input

Bx32Δ220

- Pull
- Input

D

- LN32-WT
- LN32Δ220
- Bx32-WT
- Bx32Δ220

Degradation vs. Time(hrs)
Figure 5
Figure 6
Figure 7

A

N  mCherry  C
N  Myr  Myr  Myr  mCherry  C
N  Myr  Myr  220  Cx32-CT  283  mTurquoise  C
N  Myr  Myr  32-WT

C

Surface Area (µm²)

32-WT  mCH-MYR  32-CT-MYR  32Δ220  mCH-MYR  32-CT-MYR
NS  NS  NS  *P=0.0148  NS  *P=0.0124

B

Cx32/mCherry  Cx32/mCH-MYR  Cx32/32-CT-TQ-MYR

LN32-WT

LN32A220

Bx32-WT

Bx32A220
Figure 8

A

Cx32-WT/E-Cad  Cx32A220/E-Cad

-Forskolin

+ Forskolin

B

![Graph showing surface area comparison between Cx32-WT and Cx32A220 with and without forskolin.](http://www.jbc.org/)

*p = 0.018

NS
Figure 9

A

B

C

Cx32-WT/β-cat  Cx32Δ270/β-cat

Cx32Δ250/β-cat  Cx32Δ230/β-cat

Cx32Δ220/β-cat

D

Surface Area (µm²)

Cx32-WT  Cx32Δ270  Cx32Δ250

Cx32Δ220  Cx32Δ220

**
Figure 10
Figure 11

A

Particle Tracking

B

A B

C

D

Normalized Fluorescence

Normalized Fluorescence

Gap Junctional

Non-Junctional

Cx32-WT

Cx32-A220

Δ Cx32-WT

Δ Cx32-T220

Δ Cx32-WT

Δ Cx32-T220

0.000 0.002 0.004 0.006 0.008 0.010

Average Speed (micron/sec)

0.000 0.010

* p = 0.015718

* p = 0.00468

NS, p = 0.205626

1

2

1

2

0 100 200 300 400 500 600 700

Time (sec)

Normalized Fluorescence

0 0.1 0.2 0.3 0.4

Normalized Fluorescence

0 0.1 0.2 0.3 0.4

Time (sec)
