Targeted gap junction protein constructs reveal connexin-specific differences in oligomerization

Jayasri Das Sarma, Fushan Wang, and Michael Koval

University of Pennsylvania School of Medicine, Department of Physiology and Institute for Environmental Medicine Philadelphia, PA 19104

Address correspondence to:
Michael Koval
University of Pennsylvania School of Medicine
Department of Physiology
B-400 Richards Building/6085
3700 Hamilton Walk
Philadelphia, PA 19104

215-898-9096, 215-573-5851 (fax), mkoval@mail.med.upenn.edu

Keywords:
endoplasmic reticulum, ERGIC, hemichannel, membrane traffic, protein assembly, di-lysine motif

Running title:
ER/ERGIC targeted connexins
Abstract

To further define the mechanisms of gap junction protein (connexin) oligomerization without pharmacologic disruption, we have examined the transport and assembly of connexin constructs containing C-terminal di-lysine based ER (HKKSL) or ERGIC (AKKFF) targeting sequences. By immunofluorescence microscopy, Cx43-HKKSL transiently transfected into HeLa cells showed a predominantly ER localization, while Cx43-AKKFF was localized to the perinuclear region of the cell. Sucrose gradient analysis of Triton X-100 solubilized connexins showed that either Cx43-HKKSL or Cx43-AKKFF expressed alone by HeLa cells were maintained as apparent monomers. In contrast to Cx43-HKKSL, Cx32-HKKSL was maintained in the ER as stable hexamers, consistent with the notion that Cx32 and Cx43 oligomerization occur in distinct intracellular compartments. Furthermore, Cx43-HKKSL and Cx43-AKKFF inhibited trafficking of Cx43 and Cx46 to the plasma membrane. The inhibitory effect was due to the formation of mixed oligomers between Cx43-HKKSL or Cx43-AKKF and wild type Cx43 or Cx46. Taken together, these results suggest that Cx43-HKKSL and Cx43-AKKFF recirculate through compartments where oligomerization occurs and may be maintained as apparent monomers by a putative Cx43-specific quality control mechanism.
Introduction

Prior to delivery to the plasma membrane and assembly into gap junction channels, connexins are first assembled into hexameric hemichannels (1-5). Most assembly of oligomeric transmembrane proteins occurs in the ER and is a prerequisite for further transport along the secretory pathway (6). However, connexin hemichannel assembly appears to be more complex, since a number of intracellular compartments have been implicated in hemichannel assembly, including the endoplasmic reticulum (ER) (7-9), ER-Golgi intermediate compartment (ERGIC) (7) and trans Golgi network (TGN) (10-12). Defining the intracellular compartments involved in connexin hemichannel assembly is further complicated by the potential for multiple connexin trafficking pathways (10,11,13,14) and the possibility that different connexins, such as Cx32 and Cx43, may have the potential to oligomerize in different compartments (4).

A number of different methods have been used to analyze the itinerary of connexin assembly. Since reticulocyte lysates have the capacity to oligomerize connexins translated in vitro, including Cx32 and Cx43 (9,15-17), it has been suggested that connexin hemichannel assembly occurs in the ER (9). In vitro translation has provided some insights into amino acid determinants which might control connexin oligomerization and the ability of different connexins to form mixed (heteromeric) hemichannels. However, as is the case with many in vitro systems, reticulocyte lysates lack many of the structural elements and compartments present in the intact cell. Thus, oligomerization by microsomes in vitro is not strong evidence that a given process occurs in the ER. For instance, augmentation of microsomes with a liver Golgi fraction stimulates Cx32 oligomerization (18). Also, while Cx43 and Cx46 have the capacity to
intermix when translated in vitro, the ability of these two connexins to intermix is
dependent on cell phenotype and is controlled at the level of Cx46 assembly (11).

Nonetheless, Kumar, et al. (8) found by EM that BHK cells transfected to
overexpress Cx32 had structures resembling gap junction plaques localized to the ER,
suggesting that Cx32 assembly can occur there. In further support of the ER as a site for
Cx32 hemicchannel assembly, mutant forms of Cx32 that are not assembled and which
escape the ER are degraded by a putative quality control mechanism (19).

Previous biochemical analysis of Triton X-100 solubilized extracts from cells
suggests that Cx43 hemicchannel assembly occurs at the TGN (10-12). Musil and
Goodenough used an extensive panel of inhibitors, including BFA, CCCP, 15 ºC
temperature block and CHO cells with a temperature sensitive block in secretory
transport to show that assembly of Cx43 into hexamers required export from the ER (12).
Monensin treatment also caused a 3-4 fold reduction in Cx43 oligomerization in ROS
cells, consistent with the TGN as the site for Cx43 hemicchannel assembly (10). We have
also found that a Cx43-β-galactosidase fusion protein, Cx43/β-gal, which inhibits native
Cx43 trafficking to the TGN also inhibits Cx43 hemicchannel assembly, again consistent
with Cx43 hemicchannel assembly at the TGN (11).

However, we also found that Cx43/β-gal formed sub-hexameric, heteromeric
complexes with native Cx43 and Cx46 in an aspect of the Golgi apparatus, prior to the
TGN (11). George, et al., using cells transfected with connexin-aequorin chimeras, also
found subhexameric assembly intermediates enriched in purified ER, ERGIC and Golgi
apparatus fractions (7). By fluorescence microscopy, most of the Cx-aequorins appear to
be concentrated in the perinuclear region of the cell, with some cell surface expression
Cx26-, Cx32- and Cx43-aequorin all showed similar assembly profiles, with the Golgi apparatus fraction containing the most connexin-aequorin hexamers in each case (7). This, combined with our results using Cx43/β-gal, raised the possibility that native connexin assembly intermediates, such as dimers, might form in an aspect of the secretory pathway prior to the TGN. However, whether these studies reflect intermediates involved in the assembly of native connexins is not definitive, since the constructs used were connexin fusion proteins containing either a ~23 kD aequorin or 180 kD β-gal moiety at the extreme C-terminus. The potential pitfalls of adding a large protein motif to a connexin on oligomerization was illustrated by Lauf, et al. who showed that a red fluorescent protein (DsRed)-Cx43 fusion protein was retained in an intracellular compartment which showed partial co-localization with calnexin, due to oligomerization between DsRed domains to form SDS-resistant tetramers (21). However, when DsRed-Cx43 was co-expressed with native Cx43 or Cx43-EGFP, it was transported to the plasma membrane where it formed heteromeric gap junction channels. This suggests that DsRed-Cx43 and wt Cx43 might have the potential to interact in the ER or another early secretory compartment.

In order to further investigate connexin assembly in early secretory compartments, we produced a series of connexin-based constructs containing di-lysine targeting motifs to potentially localize them to early intracellular compartments (22-25). Short di-lysine motifs, oriented toward the cytosol at the extreme C-terminus of a transmembrane protein, enable ER retention by inhibiting clustering into COP II coated secretory vesicles (26,27) and also enable protein retrieval and by interacting with multiple components of COP I coated retrograde transport vesicles (28,29). In contrast
to pharmacologic methods, such as BFA (30), which disrupt cell compartmentation, this approach offers the ability to examine connexins in relatively unperturbed early secretory compartments.

We found that connexin fusion proteins, Cx32-HKKSL and Cx43-HKKSL, were retained in the ER consistent with their retention signals. When expressed alone in cells, the targeted Cx43 constructs were retained as monomers. In contrast, Cx32-HKKSL, which was also retained in the ER, was assembled into hexamers suggesting that Cx32 and Cx43 hemichannel assembly occur in distinct subcompartments. Furthermore, despite the retention of Cx43-HKKSL and Cx43-AKKFF as monomers when expressed alone, these constructs had the capacity to interact with compatible native connexins. Thus, despite the trafficking of Cx43-HKKSL and Cx43-AKKFF through intracellular compartments where oligomerization is initiated, they are maintained as monomers.
Materials and Methods

Antisera and Reagents. Rabbit anti-Cx43 (31) and anti-Cx46 (10) antisera were generated using 6 his-tagged C terminal tail constructs as previously described. Rabbit anti-Cx32, mouse anti-calnexin and anti-β-cop were from Zymed (S. San Francisco, CA). Monoclonal anti Cx43 was from Chemicon (Temecula, CA). Fluorescent and Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Biomag particles coated with goat anti-mouse IgG were from Polysciences (Warrington, PA). Triton X-100 was from Roche Molecular Biochemicals (Indianapolis, IN). Tissue culture reagents were from Life Technologies Inc/GIBCO BRL (Rockville, MD). Unless otherwise specified, all other reagents were from Sigma (St. Louis, MO).

Constructs, transfection and immunofluorescence. Tagged constructs were produced by PCR amplification in a Stratagene Robocycler (La Jolla, CA) using High Fidelity DNA polymerase (Roche), starting with rat Cx43 or Cx32 cDNA as a template. For the Cx43-based constructs, the sense primer was 5’-CGGGGTACCG AATTCCCCAG ACATGGGTGA CTGGA-3’ and the antisense primers were either 5’-CCGCTCGAGT TAGAGTGAAC GTCTTCGTGAAT CTCAGGTCGA TCAGGCC-3’ (HKKSL) or 5’-GGCCTGATGA CCTGGAGATT GCTAAGAAGT TCTTCTAACT CGAGCGG-3’ (AKKFF). Cx32-HKKSL was produced using 5’-CGGGGTACCG AATTCGAATG AGGCAGGATG AACTG-3’ and 5’-CCGCTCGAGT TAGAGTGAAC GTCTTCGTGAAT CTCAGGTCGA TCAGGCC-3’ as the sense and anti-sense primers, respectively. The resulting PCR products were cut with KpnI and XhoI, ligated into a pcDNA3 expression
vector and transformed into bacterial stocks. DNA for transfection was purified from bacteria using the Qiagen Maxiprep kit according to the manufacturers instructions. Prior to transfection, cDNA was purified by EtOH precipitation.

HeLa cells were transiently transfected with either wild type or modified connexin cDNA constructs using Lipofectamine (Life Technologies) at 2 µg/ml DNA using Lipofectamine:DNA ratios of either 3:1 (µl:µg) for 100 mm dishes or 4:1 (µl:µg) for 35 mm dishes and analyzed 48 h after transfection. Transfection efficiencies using this approach were typically greater than 70%. In some experiments, previously generated, stably transfected HeLa/Cx43myc and HeLa/Cx46 cells were transiently transfected in a manner comparable to wild type HeLa cells.

For immunofluorescence, cells plated on glass cover slips were fixed and permeabilized with MeOH/acetone (1:1), then washed 3x with PBS, followed by PBS + 0.5% Triton X-100 and PBS + 0.5% Triton X-100 + 2 % heat inactivated goat serum (PBS/GS). The cells were incubated with primary antisera diluted into PBS/GS for 1 h, washed, and then labeled with secondary antisera diluted into PBS/GS. The cells were then washed with PBS, mounted into MOWIOL and visualized by fluorescence microscopy using an Olympus X-70 microscope system with a 40 x UPlanApo oil immersion objective (1.0 na) with the iris diaphragm partially closed to limit the contribution of out of plane fluorescence and filter packs suitable for green (U-MWIBA BP460-490 | DM505 | BA515-550) and red (U-MNG BP530-550 | DM570 | BA590-800+) fluorescence. Images were acquired with a Hamamatsu Orca-1 CCD camera and Image Pro image analysis software (Media Cybernetics, Silver Spring, MD).
Protein analysis. The techniques outlined below have also been described elsewhere (10,11,32). Cells were washed with PBS, harvested into PBS containing protease inhibitors (10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl chloride, 2 µg/ml leupeptin and 1 µg/ml pepstatin) and phosphatase inhibitors (1mM NaVO₄ and 10mM NaF) then passed through a ball bearing homogenizer 100 times (10,11). The homogenate was centrifuged at 500 x g for 5 min using a IEC CL3R centrifuge and then the resulting post nuclear supernatant was centrifuged at 100,000 x g for 30 min using a Beckman TL-100 ultracentrifuge to obtain a membrane enriched pellet. To analyze total cell connexin expression, this pellet was resuspended in SDS-PAGE sample buffer, resolved by electrophoresis, transferred to PVDF and then detected by immunoblot. Densitometric analysis of non-saturated films was performed using the Kodak IDAS system (Rochester, NY).

For detergent solubilization studies, the membrane enriched pellet was resuspended in PBS + inhibitors containing 1% Triton X-100 and then incubated for 30 min at 4°C. The sample was then centrifuged at 100,000g from 30 min and separated into Triton X-100 soluble supernatant and insoluble pellet fractions. The soluble and insoluble fractions were then resuspended into SDS-PAGE sample buffer, and assayed by immunoblot.

Co-immunopurification was done starting with the Triton X-100 soluble fraction prepared as described above. Magnetic goat anti-mouse IgG coated particles were incubated with the Triton X-100 soluble fraction for 2 h with mouse anti Cx43 antisera in PBS containing 0.25% BSA and 0.2% gelatin. The magnetic particles were then added to the Triton X-100 soluble fraction, incubated for 2 h at 4°C, and isolated using a
ceramic magnet (Stratagene). The particles were washed with cold PBS, resuspended in SDS-PAGE sample buffer and then analyzed by immunoblot.

Sucrose gradient fractionation was done using post nuclear supernatants solubilized in 1 % Triton X-100 and incubated for 30 min at 4°C. The samples were then centrifuged at 100,000 g for 30 min and the resulting Triton X-100 soluble fraction was overlaid onto a 5 - 20% sucrose gradient. The gradient was centrifuged at 148,000 x g for 16h in a Sorvall Ultra Pro 80 centrifuge using an AH-650 swinging bucket rotor. Following centrifugation, 0.5 ml fractions were collected from the bottom of the centrifuge tube at 4°C, and then analyzed by immunoblot.

**Preloading assay.** The preloading dye transfer assay for gap junction formation was done as previously described (32) with modifications. Donor and acceptor HeLa cells were transiently transfected using Lipofectamine with 2 µg/ml of either wt Cx43 or Cx43-HKKSL or Cx43-AKKFF or simultaneously with 1 µg/ml of wt Cx43 plus 1 µg/ml Cx43-HKKSL or 1 µg/ml wt Cx43 plus 1 µg/ml Cx43-AKKFF and allowed to recover for 36 h. Donor cells were incubated overnight at 37°C in tissue culture medium containing 1 mg/ml of Texas Red dextran (mol wt 10,000) as a non-transferable, endocytosed marker. The cells were then washed and incubated in MEM containing 10 µM calcein-AM (Molecular Probes) for 30 min at 37 °C to load the cytoplasm of donor cells with calcein. The donor cells were then washed, trypsinized and then mixed at a 1:10 ratio with unlabeled, trypsinized acceptor cells. The cells were co-cultured for 5 h and the specific transfer of calcein from donor to acceptor cells was scored by fluorescence microscopy using a blinded coding system. Data were expressed as the
fraction of calcein-positive acceptor cells as calculated from at least 150 donor cell-acceptor cell interfaces from 2-4 independent experiments.
Results

Retention of di-lysine targeted Cx43 constructs

Cx43-based cDNA constructs containing two different di-lysine based retention motifs, Cx43-HKKSL and Cx43-AKKFF, were produced as described in Methods. To define the intracellular trafficking of these constructs, Cx43-HKKSL and Cx43-AKKFF were transiently transfected into HeLa cells and then examined by immunofluorescence microscopy. In contrast to wild type Cx43 which showed high levels of transport to the cell surface (Figure 1), both Cx43-HKKSL and Cx43-AKKFF were preferentially retained in intracellular compartments.

We used immunofluorescence co-localization to identify compartments containing the targeted Cx43 constructs (Figure 2). As shown in Figure 2, Cx43-HKKSL showed significant co-localization with the ER marker, calnexin. In contrast, Cx43-AKKFF did not show significant overlap with the ER, consistent with the “weaker” AKKFF ERGIC retention signal. However, both Cx43-HKKSL and Cx43-AKKFF had significant overlap with β-COP (Figure 2). Since di-lysine based motifs interact with coatamer proteins (28,29), this was not unexpected. However, since there are multiple aspects of the Golgi apparatus which co-localize with β-COP in the perinuclear region of the cell at the level of fluorescence microscopy (33), we cannot precisely define the post-ER compartments containing Cx43-HKKSL and Cx43-AKKFF. Note also, that the morphology of the β-COP labeled perinuclear compartment was variable for wild type HeLa cells, as well as transfected HeLa cells expressing di-lysine connexin fusion proteins.
Cx43-AKKFF also occasionally assembled into gap junction plaques localized to the plasma membrane, suggesting that there was some Cx43-AKKFF transport through late elements of the secretory pathway. In contrast, cells transfected with Cx43 HKKSL did not show detectable Cx43-HKKSL at the cell surface by immunofluorescence. To further assess the extent of Cx43 incorporation into plaques, we examined the solubility of Cx43-HKKSL and Cx43-AKKFF in Triton X-100, since Cx43 assembled into gap junction plaques is not soluble in 1% Triton X-100 at 4 °C. As shown in Figure 1d, wild type Cx43 showed both Triton X-100 soluble and insoluble fractions, where the Triton soluble fraction contained only the NP form of Cx43 and the Triton X-100 insoluble fraction contained both non-phosphorylated and phosphorylated forms of Cx43 (10,11,34). Consistent with the relative strength of these retention signals, there was some Cx43-AKKFF in the Triton X-100 insoluble fraction, while nearly all of the Cx43-HKKSL was in the Triton X-100 soluble fraction. This was consistent with functional studies which showed that HeLa cells expressing Cx43-AKKFF were fairly well coupled as opposed to cells expressing Cx43-HKKSL (see below).

Neither Cx43-HKKSL nor Cx43-AKKFF showed phosphorylation to the P1 and P2 forms observed for wild type Cx43. However, Cx43-HKKSL routinely resolved as a doublet by gel electrophoresis (see also Figure 6), with the majority of the protein showing an apparent Mr of 42 kD along with a minor 40 kD Mr band. Since Cx43-AKKFF showed predominantly the 42 kD band, this was suggestive of potential intracellular phosphorylation (35-38). However, samples from Cx43-HKKSL transfected HeLa cells treated with 2 units of calf intestinal alkaline phosphatase for 4 h at 37°C (39) showed little change in the doublet migration pattern (data not shown) suggesting that
this was not the case. The nature of the 40 kD band is not clear at present. One possibility is that this may represent a potential degradation product (19,40).

**Differential oligomerization of Cx43-HKKSL and Cx32-HKKSL**

We then examined the oligomerization of ER-retained Cx43 constructs by sucrose gradient fractionation. As shown in Figure 3a, the Triton X-100 soluble fraction of wild type Cx43 showed the characteristic pattern of two peaks, centered at 8-10 % sucrose and 15-17 % sucrose which correspond to monomeric and oligomerized Cx43, respectively. In contrast, the same fraction isolated from HeLa cells expressing either Cx43-HKKSL or Cx43-AKKFF alone showed only the monomer peak. There was little, if any, oligomerized connexin present in these Triton X-100 soluble fractions. Thus, there were no apparent intracellular Cx43-HKKSL or Cx43-AKKFF oligomers, when they were the only connexin expressed by HeLa cells.

Since it has been suggested that Cx32 hemichannels may be oligomerized in the ER (8), we wanted to determine whether a Cx32-HKKSL construct might be oligomerized. As shown in Figure 4a, Cx32-HKKSL is retained in the ER, showing a similar intracellular distribution to Cx43-HKKSL. However, in contrast to Cx43-HKKSL, virtually all of the Cx32-HKKSL expressed by HeLa cells resolved to a peak at 15 % sucrose in the gradient. This was due to oligomerization, since pre-treatment with SDS caused the Cx32-HHKSL peak to shift to a lighter sucrose density fraction (Figure 4b). Thus, Cx32-HKKSL and Cx43-HKKSL were maintained in the ER in different states of oligomerization, suggesting that Cx32 and Cx43 may oligomerize in distinct intracellular compartments.
**Targeted Cx43 constructs oligomerize with Cx46**

Despite the fact that Cx43-HKKSL and Cx43-AKKFF were not oligomerized, our previous results showing oligomerization of Cx43/β-gal with wild type connexins in a BFA-sensitive compartment (11) raised the possibility that the di-lysine targeted constructs might also interact with wild type connexins. We first examined whether this was the case using HeLa cells stably transfected with Cx46 (HeLa/Cx46 cells). Cx43 and Cx46 are compatible to form mixed gap junction channels in HeLa cells and this offered us the advantage of using two different antibodies to simultaneously localize Cx43 and Cx46 in the same cell (11). As shown in Figure 5, wild type Cx43 and Cx46 co-localize predominantly in gap junction plaques, consistent with co-assembly of these proteins into heteromeric gap junction channels. However, transient transfection of either Cx43-HKKSL or Cx43-AKKFF into HeLa/Cx46 cells caused Cx46 to be preferentially retained in intracellular compartments which co-localized with the targeted Cx43 construct. The ER-retained Cx43-HKKSL was more effective at inhibiting Cx46 transport to the cell surface than the ERGIC retained Cx43-AKKFF construct.

We then defined the assembly intermediates involved in Cx46 retention by targeted Cx43 constructs. By sucrose gradient fractionation, there was good overlap between the migration pattern of the targeted Cx43 constructs and Cx46 (Figure 6). This was due to a direct interaction between Cx43-HKKSL or Cx43-AKKFF and Cx46, since we could specifically co-immunoisolate Cx46 with anti-Cx43 antibodies from HeLa cells expressing both connexins. Thus, despite the fact that Cx43-HKKSL or Cx43-AKKFF expressed alone in HeLa cells did not form stable oligomers, these constructs had the capacity to oligomerize with a wild type connexin. Also, consistent with significant
efflux of Cx43-AKKFF out of the ER as compared to Cx43-HKKSL, more of the Cx43-AKKFF/Cx46 complexes were present as hexamers than was the case for Cx43-HKKSL/Cx46 complexes. The fractionation pattern for Cx43-HKKSL/Cx46 contained complexes which migrated in the region of the gradient between 10 and 14% sucrose, consistent with the notion that there were stable subhexameric complexes formed between Cx43-HKKSL and Cx46. Further, there were Triton X-100 soluble Cx46 complexes which migrated towards the bottom of the sucrose gradient isolated from cells co-expressing Cx46 and Cx43-HKKSL or Cx43-AKKFF. This was not observed for sucrose gradient profiles obtained from control HeLa/Cx46 cells (10) and may reflect saturation of a putative quality control mechanism by the expression of the targeted Cx43 constructs.

**Targeted Cx43 constructs oligomerize with wild type Cx43**

To determine whether targeted Cx43 constructs could interact with Cx43, we examined the effect of Cx43-HKKSL on the trafficking of a myc-epitope tagged Cx43 construct. As shown in Figure 7, transfection of HeLa/Cx43myc cells with wild type Cx43 did not inhibit the trafficking of Cx43myc to the plasma membrane. In contrast, cells expressing Cx43-HKKSL retained Cx43myc in an intracellular compartment which showed partial overlap with the ER. Cx43-HKKSL also changed the assembly state of the Triton X-100 soluble pool of Cx43myc. In control HeLa/Cx43myc cells, the Triton X-100 soluble pool of Cx43myc showed a similar sucrose gradient fractionation pattern to wild type Cx43 (Figure 3a). In contrast, co-expression of Cx43myc with Cx43-
HKKSL caused the Triton X-100 soluble pool of Cx43myc to shift to a mostly oligomerized state.

We then examined the effect of Cx43-HKKSL and Cx43-AKKFF on Cx43-mediated gap junctional communication using the preloading assay. HeLa cells were transiently transfected with either wild type Cx43, Cx43-AKKFF or Cx43-HKKSL and the specific transfer of calcein from double labeled donor cells to unlabeled acceptor cells was assessed by fluorescence microscopy. As a quantitative measure for dye transfer, a coupling index was measured, which is the fraction of donor-acceptor interfaces that mediated specific calcein dye transfer between adjacent cells. The coupling index for cells transiently transfected with wild type Cx43 was high 0.53 ± 0.04 (n = 875 donor/acceptor interfaces), indicating that wild type Cx43 formed functional gap junction channels. Cx43-AKKFF expressing cells also showed high levels of gap junction formation (0.39 ± 0.07, n=602 interfaces)), consistent with the observation that some HeLa cells transfected with Cx43-AKKFF alone showed apparent gap junction plaques (Figures 1, 2). Cells transiently expressing Cx43-HKKSL alone showed low levels of gap junction channel activity using the preloading assays (0.19 ± 0.05 (n = 423 interfaces)), 3-4 fold less than cells transiently transfected with wild type Cx43. Transiently transfecting HeLa cells with twice as much Cx43-HKKSL cDNA (4 µg/ml) did not significantly increase the coupling index (0.25 ± 0.05 (n = 162 interfaces). While the ability to detect gap junction formation by cells transfected with Cx43-HKKSL is likely due to the sensitivity of the preloading assay, this does suggest that there are low levels of Cx43-HKKSL transport to late secretory compartments and the plasma membrane.
Gap junctional communication was then measured with the preloading assay using HeLa cells stably transfected with wild type Cx43 (HeLa/Cx43) and transiently transfected with Cx43-HKKSL cDNA. Cells stably expressing wild type Cx43 showed the expected high levels of coupling with the preloading assay (0.79 ± 0.08 (n = 442 interfaces)). HeLa/Cx43 cells transfected with 0.5 µg/ml or 1 µg/ml Cx43-HKKSL showed little, if any effect on gap junctional communication, with coupling indices of 0.83 ± 0.04 (n = 243 interfaces) and 0.79 ± 0.11 (n = 196 interfaces), respectively. In contrast, HeLa/Cx43 cells transiently transfected with 2 µg/ml Cx43-HKKSL cDNA showed a ~10% decrease in gap junctional communication as compared to HeLa/Cx43 cells, with a coupling index of 0.69 ± 0.03 (n = 159 interfaces). This modest decrease was statistically significant (p < 0.05), consistent with the ability of Cx43-HKKSL to interact with non-tagged, wild type Cx43. Whether this low level of inhibition has physiological consequences seems less likely and remains to be determined.
Discussion

In this study, we found that the HKKSL di-lysine retention/retrieval motif at the extreme C-terminus of Cx43 and Cx32 was able to retain these connexins in the ER, in the case of HKKSL or perinuclear region of the cell, in the case of AKKFF. Using these retention motifs, Cx32 and Cx43 constructs localized to early secretory compartments showed differential states of oligomerization, where Cx32-HKKSL was oligomerized into hexamers while intracellular pools of Cx43-HKKSL and Cx43-AKKFF were maintained as apparent monomers. However, Cx43-HKKSL and Cx43-AKKFF were still able oligomerize with wild type Cx43 and Cx46 when co-expressed in the same cell.

The observation that Cx32-HKKSL and Cx43-HKKSL were in different assembly states, despite both being preferentially localized to the ER, is consistent with the notion that there are at least two connexin oligomerization pathways (Figure 8) (4). Based on dendrogram analysis of amino acid sequences, Cx32 is a beta-class connexin and Cx43 is an alpha-class connexin (2). Whether the results presented here can be generalized to a model where oligomerization of beta connexins occurs in the ER and oligomerization of alpha connexins occurs in post-ER compartments remains to be determined. However, since mixed oligomerization of Cx43 and another alpha connexin, Cx46, depends on cell type (11,41) and since Cx46 retained in the TGN is in an apparent monomeric form (10), there may in fact be more than two connexin oligomerization pathways. This notion of multiple pathways is also suggested by differential sorting of Cx26 and Cx32 (7,42) and by the effect of multiple inhibitors on Cx43 transport to the plasma membrane (13).

While this approach examines the behavior of connexin fusion proteins, these have only five added amino acids to the C-terminus, which is less likely to be disruptive.
than the addition of larger protein moieties, such as β-galactosidase (11). Consistent with this, ER or ERGIC morphology was not perturbed by HKKSL- or AKKFF- tagged connexins. Also, Cx43-HKKSL was not significantly present in a Triton X-100 insoluble fraction (Figure 1), suggesting that it was not misfolded. In addition, the level of Cx43-AKKFF in the Triton X-100 insoluble pool was consistent with the presence of Cx43-AKKFF assembled into gap junction plaques. Using di-lysine tagged connexins also offers the advantage that pharmacologic agents or other approaches were not required to interfere with connexin trafficking. Thus, we are likely to be examining the behavior of Cx32-HKKSL and Cx43-HKKSL in cells which have normally formed and functioning secretory compartments.

Defining the post-ER compartments involved in Cx43 oligomerization is somewhat complex, particularly since there are seven distinct stacks of the Golgi apparatus, including the ERGIC and TGN which are in close apposition in the perinuclear region of the cell (33). Given previous work showing that Cx43/β-gal inhibits wild type Cx43 trafficking by forming a heterodimer with wild type Cx43 in a BFA sensitive perinuclear compartment (11), Cx43 oligomerization may be initiated prior to the medial Golgi apparatus and then completed in the TGN as part of a putative multi-step process for hemichannel formation. Whether this is the case is still a open question, although there is precedence for oligomerization events in the Golgi apparatus including budding of corona viruses from the Golgi apparatus (43) and the kin recognition model for localization of Golgi resident proteins (44-47).

Analysis of cells expressing wild type Cx43 indicates that there are three predominant pools of Cx43: monomeric intracellular Cx43, hexameric intracellular Cx43
and Cx43 assembled into gap junction channels at the plasma membrane (10,12,48). This suggests that there are two rate limiting steps in Cx43 oligomerization, namely, initiation of Cx43 oligomerization and delivery of Cx43 hemichannels to the cell surface for assembly into gap junction channels. Given that Cx43-HKKSL and Cx43-AKKFF hetero-oligomerize with wild type Cx43 and Cx46, the targeted Cx43 constructs must have access to intracellular compartments where oligomerization is initiated (Figure 8). However, this is in apparent conflict with the observation that these di-lysine targeted Cx43 constructs are maintained as apparent monomers when expressed alone by HeLa cells. One possibility is that initiation of oligomerization may be less efficient for Cx43-HKKSL and Cx43-AKKFF due the di-lysine motif itself, although Cx32-HKKSL oligomerization was fairly complete suggesting that this is not the case.

Cx43-HKKSL and Cx43-AKKFF are subject to two competing processes, namely, ER retrieval and initiation of oligomerization (Figure 8). Single Cx43-HKKSL molecules can be retrieved through the di-lysine motif (22,23), however, oligomerization requires two or more Cx43-HKKSL molecules to interact. Given this, ER retrieval is likely to be the preferred pathway for cells expressing Cx43-HKKSL alone, if the level of Cx43-HKKSL monomers available for oligomerization is relatively low. Since cells expressing Cx43-HKKSL and Cx43-AKKFF alone showed intercellular communication using the preloading assay, these connexins do oligomerize on their own. However, stable intracellular hemichannels were not detected when cells expressed Cx43-HKKSL or Cx43-AKKFF alone, consistent with low levels of oligomerization compared to wild type connexins. Once assembled into gap junction plaques at the plasma membrane, the turnover of di-lysine targeted Cx43 constructs is likely to be regulated independently
from the ER retention pathways, instead using previously described internalization pathways used for wild type Cx43 (49,50).

In contrast, when co-expressed with wild type connexins which are readily oligomerizing, hetero-oligomerization of Cx43-HKKSL and wild type connexins can more effectively compete with ER retrieval of monomeric Cx43-HKKSL. This process also could be enhanced by repeated iterations of hetero-oligomers between the ER and post-ER compartments since mixed oligomers with wild type and di-lysine targeted Cx43 are likely to undergo a futile cycle of recycling between the ER and post-ER compartments and thus stabilize the intracellular oligomer pool (Figure 8c). A similar argument can be made for Cx43-AKKFF.

Cx43myc/Cx43-HKKSL and Cx46/Cx43-AKKFF formed hetero-oligomers with a sucrose gradient migration pattern most consistent with hexamers. In both of these cases, the intracellular distribution of these connexins was in a compartment which appeared to be distinct from the ER. In contrast, Cx46 and Cx43-HKKSL, which were more localized to the ER at the level of immunofluorescence oligomerized into subhexameric heteromers. The difference in oligomerization state of Cx43myc/Cx43-HKKSL and Cx46/Cx43-HKKSL might reflect the relative stability of homomeric Cx43 hemichannels, as compared to heteromeric Cx43/Cx46 hemichannels (11,51).

Alternatively, subhexameric Cx46/Cx43-HKKSL oligomers may be more effectively retained in earlier secretory compartments than Cx46/Cx43-AKKFF and Cx43myc/Cx43-HKKSL.

Stable subhexameric oligomers have also been shown to be formed by Cx43-aequorin (7). However, in their system, Cx32- and Cx43-aequorin chimeras showed
similar oligomerization profiles, which initiated in the ER/ERGIC and differed from our observations showing that Cx32-HKKSL and Cx43-HKKSL were in different states of oligomerization. Whether the difference between aequorin and di-lysine connexin chimeras can be attributed to the aequorin moiety remains to be determined. One possibility is that constructs containing a large protein domain, such as aequorin or β-galactosidase, might have near normal rates for initiation of oligomerization and slower rates for completion of oligomerization compared to rates for unmodified connexins, if we assume that initiation and completion of oligomerization are separable events. Whether this is the case remains to be determined.

Di-lysine motifs also help retain proteins in the ER in addition to retrieval from distal secretory compartments. For instance, the di-phenylalanine (FF) domain of the AKKFF motif promotes escape of ERGIC-35 from the ER and enables this protein to recycle between the ER and ERGIC (27). In contrast, a mutant form of ERGIC-35 containing an AKKAA motif now rarely escapes the ER (26). Given the possibility that the di-lysine motif may enhance ER retention, in addition to retrieval, hetero-oligomerization of di-lysine targeted Cx43 with wild type Cx43 may facilitate trafficking of Cx43-HKKSL and Cx43-AKKFF to post-ER secretory compartments, since formation of mixed oligomers could reduce the effectiveness of the di-lysine retention motif. This would mimic a natural role for di-lysine retention signals, since cells use protein oligomerization to mask ER retention motifs in order to regulate assembly of some multimeric proteins, such as the IgE receptor (52).

This suggests an alternative model, where the level of wild type Cx43 expression in the ER itself might be high enough to initiate hetero-oligomerization with Cx43-
HKKSL or Cx43-AKKFF and subsequent escape from the ER. However, transfection with high levels of Cx43-HKKSL (4 µg/ml) did not increase the level of gap junction formation, arguing against the notion that increased levels of Cx43-HKKSL expression can drive ER oligomerization. The notion of Cx43 assembly at the ER was also suggested by Lauf, et al. (21), who showed that DsRed-Cx43 expressed alone by HeLa cells aggregated and was retained in a perinuclear compartment which partially co-localized with the ER marker calnexin by immunofluorescence microscopy. Co-expression with wild type Cx43 enabled DsRed-Cx43 to be further transported along the secretory pathway, due to the formation of DsRed-Cx43/wild type Cx43 hetero-oligomers. Although the initiation of oligomerization involving DsRed-Cx43 may occur in the ER, it is also possible that DsRed can escape from the ER, is misfolded and then retrieved to the ER for degradation, as is the case for some misfolded Cx32 mutants (19) and other misfolded proteins (53-55). Consistent with this, DsRed-Cx43 was only localized to a subset of the ER, as opposed to Cx43-HKKSL which showed extensive overlap with calnexin in a compartment which resembled unperturbed ER. Results obtained with DsRed-Cx43 are also complicated by the ability of DsRed to oligomerize on its own, a point of concern when analyzing connexin chimeras with β-gal (11) and aequorin (7).

Our results are also consistent with the possibility that partially oligomerized Cx43-HKKSL or Cx43-AKKFF is retrieved to the ER and then actively disassembled by a putative quality control apparatus or set of chaperones. The idea of connexin chaperones stems from observations that Cx43 and Cx46 hemichannel assembly occurs in post-ER compartments (10-12). In order for this to occur, monomeric Cx43 (and
Cx46) must remain stably integrated in the membrane of the ER and other secretory compartments. Since charged amino acid residues which normally line the aqueous gap junction channel pore are likely to be exposed to the hydrophobic core of the lipid bilayer when connexins are monomeric, it seems plausible that putative transmembrane chaperones may exist to shield these charged residues. To date, such chaperones have not been identified, however, our results with targeted Cx43 constructs are consistent with this possibility. Whether this is the case will require identifying components of this potential quality control pathway.
Acknowledgments

Thanks to Elliot Hertzberg, Paul Lampe and Cecilia Lo for helpful discussions and to Michael Beers for a critical reading of the manuscript. This work was supported by an American Heart Association Grant in Aid and NIH GM61012 to MK.
References


Figures

Figure 1. Targeted Cx43 constructs are retained in intracellular compartments.

HeLa cells transiently transfected with either wild type Cx43 (a), Cx43-HKKSL (b) or Cx43-AKKFF (c) were fixed, permeabilized and then immunostained with rabbit anti-Cx43 antibodies and Texas Red goat anti-rabbit IgG. Wild type Cx43 showed punctate cell surface labeling while Cx43-HKKSL and Cx43-AKKFF were retained in intracellular compartments, although Cx43-AKKFF occasionally showed a localization pattern consistent with gap junction plaques (arrowhead). Bar = 20 µM. d. Membrane preparations from HeLa cells transfected with either wild type Cx43 (lanes 1,2), Cx43-HKKSL (lanes 3,4) or Cx43-AKKFF lanes (5,6) were incubated in 1% Triton X-100 at 4°C for 30 min and then centrifuged at 100,000 g to separate Triton X-100 soluble (lanes 1, 3, 5) and insoluble fractions (lanes 2, 4, 6), which were resolved by SDS-PAGE and detected by immunoblot. There were barely detectable levels (<8% total) of Cx43-HKKSL present in the Triton X-100 insoluble fraction (lane 4), in contrast to cells expressing wild type Cx43 (lane 2) or Cx43-AKKFF (lane 6). Note the presence of 44 kD and 46 kD phosphorylated species of wild type Cx43 in the Triton X-100 insoluble fraction (lane 2) and the 40 kD + 42 kD Cx43-HKKSL doublet (lane 3). The position of the 48 kD Mr marker is shown.

Figure 2. Intracellular localization of targeted Cx43 constructs. HeLa cells were transiently transfected with Cx43-HKKSL (a-c, g-i) or Cx43-AKKFF (d-f, j-l) fixed, then double label immunolabeled using mouse anti-Cx43 (a,d,g,j) and rabbit anti-calnexin (b,e) or rabbit anti-β-cop (h,k), which were stained with Texas Red goat anti-mouse IgG
and FITC goat anti-rabbit IgG secondary antibodies. Merged images are shown in (c,f,i,l). Cx43-HKKSL showed extensive co-localization with the ER marker calnexin (arrows). When compared to β-cop, Cx43-HKKSL showed partial co-localization (arrowheads), while Cx43-AKKFF showed nearly complete co-localization with β-cop. Bar = 20 µM.

**Figure 3. Intracellular, targeted Cx43 constructs expressed alone by HeLa cells are monomeric.** Post nuclear supernatants from HeLa cells transfected with wild type Cx43 (a), Cx43-HKKSL (b) or Cx43-AKKFF (c) were solubilized in 1% Triton X-100 and then analyzed by sucrose gradient fractionation as described in Methods. The peak at 5 - 11% sucrose corresponds to Cx43 monomers while oligomerized Cx43 sedimented at 13 - 20% sucrose (panel a). Neither Cx43-HKKSL nor Cx43-AKKFF expressed alone by wt HeLa cells showed stable intracellular oligomers when analyzed by sucrose gradient fractionation.

**Figure 4. Cx32-HKKSL is ER retained and oligomerized.** a. HeLa cells were transfected with Cx32-HKKSL and then fixed, permeabilized and then immunostained with rabbit anti-Cx32 antibodies and Texas Red goat anti-rabbit IgG. Note the immunofluorescence localization pattern similar to Cx43-HKKSL (see Figures 1,2). b. HeLa cells transfected with Cx32-HKKSL were solubilized in 1% Triton X-100 in either the absence (●) or presence (■) of 0.2 % SDS and then analyzed by sucrose gradient...
fractionation. In contrast to Cx43-HKKSL, Cx32-HKKSL was oligomerized into SDS-sensitive complexes.

**Figure 5. Targeted Cx43 constructs alter the intracellular distribution of Cx46.** HeLa/Cx46 cells were transiently transfected with either wild type Cx43 (a-c), Cx43-HKKSL (d-f) or Cx43-AKKFF (g-i). 48 hrs after transfection the cells were fixed, permeabilized and then double label immunostained using mouse anti-Cx43 (a, d, g) and rabbit anti-Cx46 (b, e, h) which were detected using Texas red goat anti mouse IgG and FITC goat anti rabbit IgG as secondary antibodies. Merged images are shown in (c,f,i). HeLa/Cx46 cells expressing wild type Cx43 showed Cx43 and Cx46 co-localized at the plasma membrane (arrows). In contrast, expression of Cx43-HKKSL or Cx43-AKKFF, caused Cx46 to be retained in intracellular compartments (arrowheads). Note, the presence of Cx43-AKKFF co-localized with Cx46 at the plasma membrane in some cells (arrow).

**Figure 6. Co-assembly of targeted Cx43 constructs and Cx46 into oligomeric complexes.** a, b. Post nuclear supernatants from HeLa/Cx46 cells transiently transfected with either Cx43-HKKSL (a) or Cx43-AKKFF (b) were solubilized in 1% Triton X-100 and then analyzed by sucrose gradient fractionation as described in Methods. The dashed line (■) corresponds to Cx46 and the solid line (●) corresponds to either Cx43-HKKSL (a) or Cx43-AKKFF (b). c. Membrane preparations from HeLa/Cx46 cells transfected with either Cx43-HKKSL (lanes 1, 4), Cx43-AKKFF (lanes 2, 5) or non-transfected controls (lanes 3, 6) were solubilized in 1% Triton X-100 and then incubated with mouse
anti-Cx43 IgG and goat anti mouse IgG conjugated magnetic beads to magnetically isolate targeted Cx43 constructs. The proteins were solubilized in sample buffer, resolved by SDS-PAGE and then immunoblotted using rabbit antisera which recognizes either Cx43 (lanes 1-3) or Cx46 (lanes 4-6), which was then detected by enhanced chemiluminescence using HRP-conjugated Goat anti-rabbit IgG. Note that Cx46 specifically co-immunisolated with Cx43-HKKSL and Cx43-AKKFF (lanes 4, 5). Shown are the migration positions for 93 kD, 48 kD, 35 kD Mr markers.

**Figure 7. Cx43-HKKSL alters the intracellular distribution and assembly of Cx43myc.** HeLa/Cx43myc cells were transiently transfected with either wild type Cx43 (a-c) or Cx43-HKKSL (d-f). 48 hrs after transfection the cells were fixed, permeabilized and then double label immunostained using mouse anti-Cx43 (a, d) and rabbit anti-myc (b, e) which were detected using Texas red goat anti mouse IgG and FITC goat anti rabbit IgG as secondary antibodies. Merged images are shown in (c,f). HeLa/Cx43myc cells expressing wild type Cx43 showed Cx43 and Cx43myc labeling at the plasma membrane (arrows) and, to a limited extent, intracellular compartments (arrowheads). In contrast, expression of Cx43-HKKSL caused Cx43myc to be retained in intracellular compartments (arrowheads). g. Post nuclear supernatants from HeLa/Cx43myc cells transiently transfected with either wild type Cx43 or Cx43-HKKSL were solubilized in 1% Triton X-100 and then analyzed by sucrose gradient fractionation as described in Methods. The solid line (■) corresponds to fractions containing Cx43myc from HeLa/Cx43myc cells expressing wild type Cx43. The dashed line (●) corresponds to fractions containing Cx43myc from HeLa/Cx43myc cells expressing Cx43-HKKSL.
Note the shift in the Cx43myc peak from predominantly monomers to predominantly oligomers when co-expressed with Cx43-HKKSL.

**Figure 8. Models for oligomerization of di-lysine retained connexins.**

a. Cx32-HKKSL is oligomerized in the ER retained in early secretory compartments using the ER retrieval pathway. 

b. In contrast to Cx32-HKKSL, Cx43-HKKSL (or Cx43-AKKFF) oligomerization is initiated in later secretory compartments. In this model, free monomers are preferentially retrieved to the ER. However, once oligomerization is initiated, it may either progress further to generate hemichannels or oligomers might be retrieved to the ER and disassembled. 

c. When co-expressed with wild type connexins (dark ovals), this helps drive oligmerization. However, heteromeric oligomers also have the capacity to be retrieved to the ER as part of a futile trafficking cycle which, in turn, may enrich the oligomer content of the intracellular connexin pool. The intracellular compartments involved in each of these processes in this model are denoted by the lines above, although there are alternate possibilities.
Das Sarma, et al. Figure 1
Das Sarma, et al. Figure 2
Das Sarma, et al.  Figure 3

a. wt Cx43
b. Cx43-HKKSL
c. Cx43-AKKFF

% sucrose
Das Sarma, et al. Figure 6
Figure 7
Das Sarma, et al. Figure 8

Diagram:

- **a**
  - ER/ERGIC: 0 → 0 → 0 → PM
  - Cx32-HKKSL: ER retrieval
  - Golgi/TGN: ER retrieval

- **b**
  - Cx43-HKKSL: ER retrieval (slow ER efflux/disassembly?)
  - PM

- **c**
  - Cx43-HKKSL + wtCx43: ER retrieval (rapid ER efflux)
  - PM

\[\bigcirc = \text{wt Cx} \quad \bigotimes = \text{dilysine Cx}\]
Targeted gap junction protein constructs reveal connexin-specific differences in oligomerization
Jayasri Das Sarma, Fushan Wang and Michael Koval

J. Biol. Chem. published online April 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M111498200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2002/04/02/jbc.M111498200.citation.full.html#ref-list-1