Functional domain mapping and selective trans-dominant effects exhibited by Cx26 disease-causing mutations

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Summary

Mutations in Cx26 are a major cause of autosomal dominant and recessive forms of sensorineural deafness. Some mutations in Cx26 are not only associated with deafness but also with skin disease. We examined the subcellular localization and function of two GFP-tagged Cx26 point mutants that exhibit both phenotypes, G59A-GFP and D66H-GFP. D66H-GFP was retained within the brefeldin A-insensitive trans-Golgi network while a population of G59A-GFP was transported to the cell surface. Neither G59A nor D66H formed gap junctions that were permeable to small fluorescent dyes suggesting they are loss-of-function mutations. When co-expressed with wild-type Cx26, both G59A and D66H exerted dominant-negative effects on Cx26 function. G59A also exerted a trans-dominant negative effect on co-expressed wild type Cx32 and Cx43 while D66H exerted a trans-dominant negative effect on Cx43 but not Cx32. We propose that the severity of the skin disease is dependent on the specific nature of the Cx26 mutation and the trans-dominant selectivity of the Cx26 mutants on co-expressed connexins. Additional systematic mutations at residue D66, in which the overall charge of this motif was altered, suggested that the first extracellular loop is critical for Cx26 transport to the cell surface as well as function of the resulting gap junction channels.
Introduction

Gap junctions mediate one method of intercellular communication by allowing for the passage of ions, second messengers, metabolites and other small molecules. By enabling direct communication between cells, gap junctions are thought to be important for cell proliferation, differentiation and maintaining tissue homeostasis (1). Gap junction plaques are composed of a few to hundreds of gap junction channels clustered together. Each individual channel is made up of two hemichannels; one hemichannel (or connexon) is contributed from each of two adjoining cells (2). In turn, each connexon consists of 6 oligomerized polypeptides called connexins (Cx) (2,3). It is well established that the Cx family of genes encode the polypeptide subunits that make up gap junctions and a recent genomic study highlighted the common features of the 19 mouse and 20 human Cx genes (4).

Most tissues express more than one member of the Cx family (3,5). This is particularly important as connexons composed of different members of the Cx family exhibit selective permeability to fluorescent dyes and endogenous metabolites (6-10). Altering the subset of Cxs spatially and temporally expressed may allow cells within a tissue to respond differentially to cell signals leading to specific changes in differentiation. In addition to homomeric connexons, a connexon may be composed of more than one Cx (heteromeric). For example, heteromeric connexons composed of Cx40/Cx43 or Cx26/Cx32 have been characterized and exhibit altered functional properties when compared to their homomeric counterparts (8,11-14). These combinations further expand the potential multitude of signals that may pass between cells and highlight the requirement to characterize the intermixing that may occur between different Cxs co-expressed within a cell population.
Gap junctions are important for keratinocyte growth and differentiation (15). According to a recent study using reverse transcriptase polymerase chain reaction, at least 10 different Cxs are expressed in the skin including Cx43, Cx32, Cx30 and Cx26 (16,17). Interestingly, mutations in Cx26 are responsible for sensorineural deafness and hyperproliferative skin disease in humans (18-21). At least four different mutations have been associated with both hearing loss and skin disease including R75W (20), D66H (18,21), G59A (22), and ΔE42 (23). Specific symptoms associated with these mutations vary in severity suggesting that mutations within various Cx motifs lead to diverse cellular and subcellular phenotypes (24). Recently, experiments using paired oocytes revealed that the ΔE42 mutation was able to exert a dominant-negative effect on wild-type Cx26 function and, surprisingly, a partial trans-dominant effect on Cx43 channel function (23). Since Cx43 and Cx26 are not thought to form functional heteromeric or heterotypic channels (9,25-27), the mechanism by which this effect occurs is not clear. Rouan et al. (2001) further hypothesized that mutant Cx26 causes skin disease by exerting trans-dominant negative effects on other Cxs co-expressed in keratinocytes (23).

While the expression of Cx in Xenopus oocytes has provided extensive information on channel conductivity and regulation (28), this system is more restrictive for examining Cx transport and assembly. Consequently, we chose to study two GFP-tagged Cx26 mutations, D66H-GFP and G59A-GFP, in well-understood mammalian cell types. When expressed in gap junctional intercellular communication (GJIC)-deficient cell lines, the D66H-GFP mutant was retained within the trans Golgi apparatus while a population of the G59A-GFP mutant was transported to the cell surface and assembled into structures reminiscent of gap junctions. Both mutants were non-functional with respect to Lucifer yellow dye permeability when directly compared to GFP-tagged wild-type Cx26. When co-expressed with Cx26, the D66H-GFP
mutant was transported to the cell surface but the resulting gap junctions were impermeable to Lucifer yellow, suggesting that this mutant exerted a dominant-negative effect on wild-type Cx26 function. Surprisingly, D66H-GFP was able to exert a limited trans-dominant negative effect on Cx43 but not Cx32. G59A-GFP exerted a dominant-negative effect on Cx26 but also had a trans-dominant negative effect on Cx32 and Cx43 function. Finally, systematic D66 substitutions with neutral and charged residues revealed that this domain is critical for not only Cx26 transport to the cell surface but also for the establishment of functional channels. Overall, our results suggest that the severity of Cx26-linked skin diseases is likely related to selective trans-dominant interactions with other Cxs expressed within the skin.

Materials and Methods

Cell lines

All tissue culture reagents were obtained from Invitrogen (Burlington, ON, Canada). GJIC-deficient HeLa cells and HeLa cells expressing Cx32 (kindly provided by M. Koval, University of Pennsylvania, PA) and normal rat kidney (NRK) cells, were cultured in high glucose DMEM containing 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. The derived cell lines were generated by retroviral infection with viral particles encoding Cx26-GFP, D66H-GFP or G59A-GFP. As a result, Cx26-GFP, D66H-GFP or G59A-GFP was expressed alone in HeLa cells or in combination with Cx32. NRK cells, which express Cx43 endogenously, were retrovirally infected with cDNAs encoding the Cx26 mutants to generate cell lines co-expressing Cx43 and the Cx26 mutants for the microinjection analysis. GJIC-deficient HBL-100 breast tumor cells and derived cell lines (29) were cultured in 10% calf serum supplemented medium. HBL-100 cells expressing Cx26-GFP, D66H-GFP or G59A-GFP alone or in combination with Cx26 were generated by retroviral infection.
Additionally, HBL-100 cells were also generated to co-express Cx43 and D66H-GFP by retroviral infection. Co-expression of Cx30 and D66H-GFP was carried out in HeLa cells by transient transfection.

**Mutant and wild-type connexin cDNA constructs, transfections and infections.**

cDNA encoding Cx43, Cx30, and Cx26 were kindly provided by Dr. C.C.G. Naus (University of British Columbia, Vancouver, BC). Cx43 and Cx26 were cloned into the replication-defective retroviral vector AP2 (30) as previously described (31,32). Human Cx26 mutants, G59A, D66H, D66A, D66E, and D66K, were constructed using the Quik-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per manufacturer’s instructions. The following primer pairs were used to create the Cx26 mutations. The nucleotide change in each case is underlined:

- **D66H** (sense) 5’-GCAAGAACGTGTGCTACCGATCATCACTACTTCCCCCATCTCC-3’
  (antisense) 5’-GGAGATGGGGAAGTAGTGATGAGCACACGTGTTCTTC-3’
- **G59A** (sense) 5’-GCAACACCCCTGCAGCCAGCAGCCTGCAAGAACGTGTC-3’
  (antisense) 5’-GCACACGGTTCTTGCAAGGGGTGTGTTGC-3’.
- **D66A** (sense) 5’-GCAAGAACGTGTGCTACGCTCACTACTTCCCCCATCTCC-3’
  (antisense) 5’-GGAGATGGGGAGTAGTGAGCGTAGACACGTGTTGC-3’
- **D66E** (sense) 5’-GCAAGAACGTGTGCTACGCGGTAGTACACGTTGCTGTC-3’
  (antisense) 5’-GGAGATGGGGAGTAGTGACAGTGGAGACACGTGTTGC-3’
- **D66K** (sense) 5’-GCAAGAACGTGTGCTACGACTACGCTTGGCTGC-3’
  (antisense) 5’-GGAGATGGGGAGTAGTGACAGTGGAGACACGTGTTGC-3’

Following sequencing to verify mutation and to ensure no other mutations were introduced, PCR was used to add XhoI and EcoRI restriction sites to the 5’ and 3’ ends.
respectively of Cx26, D66H or G59A and the resulting cDNAs were cloned into the pEGFP-N1 vector (BD-Biosciences Clontech, La Jolla, CA) and sequenced for verification. A 17 amino acid linker sequence separates the Cx26 and GFP moieties of the resulting fusions Cx26-GFP, D66H-GFP, G59A-GFP, D66E-GFP, D66K-GFP, and D66A-GFP. Next Cx26-GFP, D66H-GFP and G59A-GFP cDNAs were inserted into the XhoI and NotI sites of the replication-defective AP2 retroviral expression vector (30). Supernatants containing retroviral particles encoding the Cx26, Cx43, Cx26-GFP, G59A-GFP and D66H-GFP were generated as described elsewhere (31,32). Following 3 rounds of retroviral infection and several medium changes and cell passages, at least 80% of cells were found to stably express the appropriate complement of Cxs (31). These cells were subsequently used for immunofluorescent and dye coupling experiments as described below. Studies on the subcellular localization and function of D66E-GFP, D66A-GFP and D66K-GFP were carried out using HeLa cells transiently transfected with these three D66 mutations as described below.

Cx30 cDNA, encoded within the pBluescript vector, was digested with XhoI and NotI and cloned into the XhoI/NotI sites of pEGFP-N1 vector. This combination of restriction enzymes remove the EGFP cDNA from the vector. Co-expression of Cx30 and D66H-GFP and expression of D66A-GFP, D66E-GFP and D66K-GFP was carried out by transient transfection using LipofectAMINE 2000 according to the manufacturer’s protocol (Invitrogen). For co-expression of Cx30 and D66H-GFP, HeLa cells were grown to 60% confluency on 12 mm coverslips and co-transfected with 1 µg of cDNA encoding Cx30 and 1 µg of cDNA encoding D66H-GFP. Expression of D66A-GFP, D66E-GFP or D66K-GFP was carried out similarly. The cells were fixed 48 hours later and immunolabeled as appropriate.
**Triton-X-100 extraction**

Triton X-100 soluble components of HBL-100 cells expressing Cx26-GFP, G59A-GFP or D66H-GFP were extracted according to a modified procedure of Musil et al. (1991) (33). Cells were grown on glass coverslips until reaching 70-80 confluency. Following two rinses in serum-free medium, cells were incubated in 1% Triton-X-100 diluted in serum-free medium for 15 minutes. Cells were then quickly rinsed in PBS, fixed in 3.7% formaldehyde in PBS for 15 minutes and immunolabelled with antibodies specific for giantin and LAMP2 as described below.

**Inhibition of degradation, immunocytochemistry and confocal microscope imaging**

To inhibit lysosomal or proteasomal degradation, cells were incubated in the presence of 100 µg/ml leupeptin or 10 µM lactacystin, respectively for 6 hrs. Following retroviral infection, treatment or transient transfection, cells grown on 12 mm glass coverslips were fixed in a solution of ice cold 80% methanol and 20% acetone for 10 minutes followed by rinses with PBS and then processed for immunolabeling as previously described (34). Cx expression was analyzed using a mouse anti-Cx32 (1:1500 dilution, HAM8, generously provided by Y. Fujikura) (35), mouse anti-Cx26 (1:200 dilution, Zymed Laboratories, San Francisco, CA), rabbit anti-Cx43 (1:500 dilution, Sigma-Aldrich), and rabbit anti-Cx30 (1:200 dilution, Zymed Laboratories). Rabbit polyclonal anti-giantin was purchased from Bio/Can Scientific (Mississauga, ON) and used at a 1:500 dilution. Secondary antibodies conjugated to Texas Red or FITC were purchased from Jackson Immunoresearch Laboratories (Westgrove, PA). Following immunolabelling, coverslips were mounted and images were collected on a Zeiss LSM 410 confocal microscope. Final images were prepared using Corel Draw 10 software.
Visualization of the Golgi apparatus using BODIPY-TR ceramide

To visualize the subcellular localization of D66H-GFP more closely, HBL-100 cells expressing D66H-GFP were treated with 5 µg/ml brefeldin A (BFA) for up to 3 hrs. Cells on coverslips were fixed at various intervals during BFA treatment and the Golgi apparatus was labelled post-fixation with BODIPY-TR ceramide (Molecular Probes, Leiden, Netherlands) that was prepared as per manufacturer’s instructions. Following fixation in ice cold 80% methanol, 20% acetone solution, cells on 12 mm coverslips were incubated with BODIPY-TR ceramide for 1 hour, washed 3 times with 1X PBS and mounted in Airvol on microscope slides.

Single cell microinjection

After a fresh medium change, cell lines stably (or transiently in the case of D66A-GFP, D66E-GFP and D66K-GFP) expressing wild-type or mutant Cxs (minimum of two cell passages post-infection to ensure stable expression and removal of all viral particles) were pressure microinjected with 5% Lucifer yellow (Molecular Probes) using an Eppendorf microinjection system and a Zeiss inverted epifluorescent microscope. Digital images capturing the dye spread were acquired with a CCD Sensicam 1 minute after microinjection and the number of neighbouring cells receiving dye was counted. At least 20 cells were microinjected for each Cx expression complement. Results are expressed as the mean number of cells receiving dye per microinjection +/- standard error of the mean (SEM). The results were analyzed by non-parametric ANOVA using the statistical analysis software Instat 3.0.

Determination of the relative stability of Cx26-GFP, G59A-GFP and D66H-GFP

HBL-100 cells expressing Cx26-GFP, G59A-GFP and D66H-GFP were incubated in culture medium containing 20 µg/ml cycloheximide (CHX) (Sigma, Oakville, ON). Cells were
solubilized in lysis buffer (36) at 0, 0.5, 1.5, 3, and 5 h after the addition of CHX. Following SDS-PAGE and transfer of proteins to nitrocellulose membranes, non-specific binding was blocked by incubating the membranes in 5% milk in PBS containing 0.05% Tween-20 for 30 minutes. Western blotting was carried out as described (36) with an anti-GFP monoclonal antibody (Chemicon, Temecula, CA, 1:3000 dilution). Following detection of GFP-tagged connexins, membranes were stripped using re-blot mild stripping solution (Chemicon) as per manufacturer’s directions and re-probed with an anti-vimentin antibody (Chemicon, 1:5000 dilution). To estimate the relative stability of the wild-type and mutant Cx26 proteins, blots were scanned and densitometry was performed using Scion image software. The integrated band intensities of GFP-tagged connexins were standardized using the vimentin signal as a control.

**Results**

**Subcellular localization of Cx26 mutants G59A and D66H**

In order to determine whether wild-type Cx26 and G59A and D66H mutants formed gap junctions at the cell surface, these Cxs were expressed in GJIC-deficient HBL-100 cells (32). Cx26-GFP was efficiently transported to the cell surface and formed gap junction plaques in HBL-100 cells (Fig. 1A, arrows). The G59A-GFP mutant was localized inside the cell within a wide array of punctate structures and at the cell surface where it appeared in gap junction-like plaques (Fig. 1D, arrows). The D66H-GFP mutant, however, was primarily retained within a distinct juxtanuclear intracellular compartment (Fig. 1G, double-arrow). To determine the localization of intracellularly-retained wild-type or mutant Cx26, HBL-100 cells were immunolabeled for a constituent resident of the Golgi apparatus, giantin. A small population of both Cx26-GFP (Fig. 1A-C, double-arrows) and G59A-GFP (Fig. 1D-F, double-arrows)
exhibited some cellular localization in the general proximity of giantin. The D66H-GFP mutant, on the other hand, co-localized almost completely with giantin (Fig. 1G-I, double-arrows).

Musil et al. (1991) reported that upon channel clustering at the cell surface, gap junctions were resistant to extraction by Triton-X-100 in situ (33). We treated live HBL-100 cells expressing Cx26-GFP, G59A-GFP or D66H-GFP with Triton-X-100 and found that a significant number of detergent-resistant structures containing Cx26-GFP (Fig. 2B, arrows) or G59A-GFP (Fig. 2D, arrows) were present at the cell surface. The intracellular-retained D66H-GFP mutant, however, was completely detergent-sensitive (Fig. 2F). As controls, immunolabeling studies revealed that both giantin and LAMP-2 (lysosomal associated membrane protein-2) were sensitive to Triton-X-100 extraction (data not shown).

To more precisely identify the Golgi compartment that housed the D66H-GFP mutant, the cis Golgi network, cis, medial and trans compartments of the Golgi apparatus were disrupted with brefeldin A (BFA) (34) and BFA-treated cells were examined to determine whether the D66H-GFP mutant resided in the BFA-resistant trans Golgi network (TGN). When D66H-GFP expressing HBL-100 cells were treated with BFA for 30 min, a significant population of D66H-GFP did not redistribute into the endoplasmic reticulum (ER) while giantin was found to completely redistribute within the ER (Fig. 3A-D). However, when D66H-GFP expressing HBL-100 cells were labelled with BODIPY ceramide, which labels all compartments of the Golgi apparatus, a significant population of BODIPY did not redistribute upon BFA-treatment and the remaining perinuclear BODIPY staining co-localized extensively with the BFA-insensitive population of D66H-GFP (Fig. 3E-H). Collectively, these results strongly suggest that D66H-GFP is retained within the TGN and that a mutation at residue D66 has dramatic effects on the ability of Cx26 to continue to be transported to the cell surface.
**Gap junctions formed by G59A-GFP are not permeable to Lucifer yellow**

To determine whether the mutations in Cx26 led to loss-of-function gap junction channels, HeLa cells expressing Cx26-GFP, G59A-GFP, or D66H-GFP were microinjected with Lucifer yellow and the number of neighbouring cells receiving dye was counted. While microinjected Lucifer yellow spread to an average of 6.5 +/- 0.8 Cx26-GFP-expressing cells (Fig. 4A-C, J) only 0.3 +/- 0.1 G59A-expressing cells received dye (Fig. 4D-F, J). Not surprisingly, since D66H-GFP did not form detectable gap junction plaques, HeLa cells expressing D66H-GFP only transferred dye to 0.5 +/- 0.1 cells (Fig. 4G-I, J). These studies were repeated in HBL-100 cells with virtually identical results (Fig. 4J). Both the G59A and D66H mutants were also found to be impermeable to the fluorescent dye, calcein AM, confirming that they were loss-of-function mutations (data not shown).

**Connexin-specific differential rescue of D66H-GFP transport to the cell surface and gap junction plaque formation.**

To assess whether specific Cxs could rescue the transport of TGN-retained D66H-GFP to the cell surface, we examined HeLa cells that co-expressed Cx32 or Cx30 in addition to D66H-GFP and HBL-100 cells that co-expressed Cx26 or Cx43 in addition to D66H-GFP. To distinguish between wild-type rat Cx26 and the human D66H-GFP Cx26 mutant in co-expression studies, we immunolabeled cells with an antibody from Zymed that reacted with rat Cx26 but, in our immunofluorescence studies, did not cross-react with the human Cx26-GFP or the Cx26 mutants.

When co-expressed with wild-type rat Cx26, a significant population of D66H-GFP was transported to the cell surface and assembled into gap junction plaques (Fig. 5A-C, arrows), without any apparent increase in retention of wild-type Cx26 within the Golgi apparatus. When
D66H-GFP was co-expressed with Cx32, Cx32 was also able to rescue the assembly of a population of D66H-GFP into gap junction plaques (Fig. 5D-F, arrows). Likewise, the co-expression of Cx30 and D66H-GFP resulted in D66H-GFP transport and assembly into gap junction plaques (Fig. 5J-L, arrows). However, when co-expressed with Cx43, D66H-GFP was still retained within the Golgi apparatus (Fig. 5G-I, arrows) and Cx43 distribution at the cell surface did not appear to be affected. These results indicate that D66H-GFP is capable of being transported to the cell surface and assembled into gap junctions when co-expressed with specific members of the Cx family of proteins. As controls, when wild-type Cx26, Cx32 or Cx43 was expressed alone in HeLa or HBL-100 cells, typical gap junction plaques were assembled (Fig. 5M, N, O, arrows).

**Inhibition of lysosomal and proteasomal degradation fails to rescue D66H-GFP transport to the cell surface**

Since D66H-GFP was retained within the Golgi apparatus and co-expression with specific Cxs was able to rescue D66H-GFP transport to the cell surface, we investigated whether the failure to detect D66H-GFP at the cell surface was a result of premature targeting of the mutant Cx26 to lysosomes or proteasomes for degradation. In the presence of the lysosomal inhibitor, leupeptin, there was an increase in intracellular Cx26-GFP (Fig. 6C) and D66H-GFP (Fig. 6D) when compared to non-treated HBL-100 cells expressing Cx26-GFP (Fig. 6A) or D66H-GFP (Fig. 6B). The proteasome inhibitor, lactacystin, did not noticeably affect the distribution of either Cx26-GFP or D66H-GFP (Fig. 6E, F). Since there was no apparent rescue of D66H-GFP to the cell surface in the presence of either lysosome or proteasome inhibitors, these results suggest that the defect in D66H-GFP trafficking is not the result of premature degradation.
D66H-GFP has a prolonged half-life

To determine whether either of the Cx26 mutants exhibited altered turnover kinetics, we treated cells with cycloheximide to inhibit protein synthesis and examined the levels of Cx26 and Cx26 mutants over time by Western blots (Fig. 7A). Both Cx26-GFP and G59A-GFP had similar turnover kinetics while the D66H-GFP mutant had a statistically slower rate of degradation (Fig. 7B). To assure equal gel loading these data were normalized to the long lived protein, vimentin.

Cx26 mutants exhibit both dominant and trans-dominant negative properties

As described earlier, neither G59A-GFP nor D66H-GFP formed functional gap junctions as determined by their permeability properties to fluorescent dyes. We then assessed whether these mutants were capable of inhibiting channels containing wild-type Cx26 in a dominant negative fashion. HBL-100 cells stably expressing both wild-type Cx26 and D66H-GFP exhibited a 90% reduction in Lucifer yellow dye transfer compared to the parental cells that expressed untagged wild-type Cx26 alone or Cx26 in combination with Cx26-GFP (Fig. 8A). Likewise, co-expression of Cx26 and G59A-GFP led to a similar decrease in dye permeability. Our results strongly suggest both D66H-GFP and G59A-GFP exhibit a dominant-negative effect on wild-type Cx26 function.

To test whether D66H-GFP or G59A-GFP had a trans-dominant effect on other members of the Cx family that are co-expressed in the skin we examined the effect of expressing these mutants on the function of both Cx32 and Cx43. Interestingly, the D66H-GFP mutant did not have a significant effect on Cx32 function whereas G59A-GFP caused a 50% reduction in dye transfer compared to wild-type Cx32 alone or cells that expressed both Cx32 and Cx26-GFP (Fig. 8B). A similar reduction in dye transfer was observed in cells co-expressing Cx43 and
G59A-GFP while D66H-GFP exerted a modest 30% decrease in Cx43 channel permeability (Fig. 8C). These results suggest that both D66H-GFP and G59A-GFP exert strong dominant-negative effect on wild-type Cx26 and even partial, Cx-specific, trans-dominant effects on wild-type Cx43 and Cx32.

**Charge and the side chain structure at amino acid position 66 are critical for both Cx26 transport to the cell surface and functional channel formation**

The D66H mutation is a substitution of a positively charged for a negatively charged amino acid. To assess whether the charge at this amino acid is important for aiding in efficient transport of Cx26, 3 other mutations were generated: D66A (neutral amino acid), D66E (negatively charged amino acid), and D66K (positively charged amino acid). Both D66A-GFP and D66E-GFP were transported to the cell surface and formed gap junction plaques when expressed in HeLa cells (Fig. 9A and B, arrows). The D66K-GFP, however, was predominantly retained within the cell (Fig. 9C, double-headed arrow) similar to the D66H-GFP mutant. This result suggests that the charge at residue 66 is important for normal transport to the cell surface. We next determined whether D66A-GFP or D66E-GFP was functional. Interestingly, neither mutants formed gap junction plaques that were permeable to Lucifer yellow (Fig. 10). Thus, although a change in the side chain at residue 66 can result in apparent normal transport of mutant Cx26 to the cell surface, even minor changes at this residue appear to affect the function of the resulting gap junctions.

**Discussion**

Several studies have reported on the expression of multiple connexins in rodent and human epidermis (17,26,37-41). Furthermore these studies identified differences in the expression and abundance of Cx family members in the stratified layers of the skin. Di et al.
(2001) reported protein expression of at least Cx26, Cx30, Cx31, Cx32, Cx40 Cx43, and Cx45 and mRNA expression of Cx30.3, Cx31.1 and Cx37 in human skin (16). Cx26 is normally absent from interfollicular skin and is restricted to hair follicles and eccrine sweat glands (40). Numerous studies, however, suggest that Cx26 is involved in the differentiation accompanying hyperproliferation that occurs in response to different stimuli such as the wound healing response (37,42-45). Cx26 knockout mice die around E11 due to a placental developmental defect (46) and thus these animals do not lend themselves well for examining skin differentiation and diseases. Humans with loss-of-function mutations in Cx26 survive but suffer from hearing loss and a variety of skin diseases that vary in severity (47-49).

Mutations in Cx26 are responsible for a large proportion of human autosomal dominant and recessive hearing loss cases (50,51). Cx26 is localized to the cochlea and supporting cells and is thought to function by buffering K+ and recycling ions within the endolymph for proper function of inner hair cells (48). Recently, a targeted mouse knockout of Cx26 in the inner ear showed these mice have extensive hearing loss which was correlated with an increased apoptosis of inner hair cells (52). Furthermore, Bakirtzis and colleagues (53) have constructed the first transgenic mouse expressing epidermal targeted mutant Cx26 (D66H) and showed that it mimicked Vohwinkel syndrome. However, given the number of mutations that are responsible for deafness (50,51) and the lack of an epidermal phenotype in the majority of these cases suggests that Cx26 is not crucial for normal epidermal differentiation. The most likely explanation for apparent normal development and differentiation of the skin in the absence of functional Cx26 is that other Cxs co-expressed by epidermal keratinocytes compensate for the loss of Cx26. This raises the important question as to why some specific mutations in Cx26 do indeed manifest in the form of both deafness and skin disease.
Clinically, individuals affected by the D66H mutation exhibited progressive sensorineural deafness as well as horny and warty papules and other lesions including pseudo-ainhum consistent with mutilating palmoplantar keratoderma (18). The G59A mutation causes a less severe palmoplantar hyperkeratosis and sensorineural deafness (22). Preliminary studies by Di et al., 2001 showed that D66H was retained within an unidentified intracellular compartment when expressed in HeLa cells (17) and intracellular retained D66H was also observed in suprabasal keratinocytes in D66H transgenic mice (53). During the preparation of this manuscript, Marziano et al. (2003) reported that D66H and G59A mutations were both retained in a perinuclear localization and furthermore D66H exerted a dominant negative effect on Cx26 but not Cx30 while G59A had a dominant negative effect on Cx30 but surprisingly not on wild-type Cx26 (54). Our results differ from these in that, while D66H was retained intracellularly, a population of G59A was able to be transported to the cell surface and form a number of Triton-X-100 resistant gap junctions. In addition, as may be expected, both D66H and G59A exerted dominant negative effects on wild-type Cx26. Since we examined our mutants in three different cell lines including HeLa cells as used by Marziano et al (2003), we cannot necessarily attribute the fact that G59A was transported to the cell surface in our studies to cell type differences (54). However, it is possible that a population of mutant Cx26 escapes intracellular retention when expressed in high quantities. Nevertheless, in both studies, G59A did not form functional gap junctions and had Cx-specific trans-dominant effects.

In our studies we examined the cytoplasmic retention of D66H and found it was specifically retained within the TGN and, consequently, did not form functional gap junction channels. Interestingly, co-expression with wild-type Cx26, Cx30 and Cx32, but not Cx43, led to the transport rescue of the D66H mutant and its assembly into gap junction plaques at the cell
Since heteromeric channels composed of Cx32 and Cx26 have been well documented \cite{55,56} it seems probable that the delivery of D66H mutants to the cell surface is facilitated by heteromeric oligomerization with wild-type Cxs. Another possibility is that the D66H mutation in Cx26 targets the molecule for degradation and co-expression with wild-type Cx26 rescues this premature degradation. This seems unlikely, however, since incubation of cells expressing D66H-GFP with lysosome and proteasome inhibitors failed to rescue D66H-GFP transport to the cell surface. Interestingly, we were able to estimate that D66H-GFP had a significantly longer half-life relative to Cx26-GFP and G59A-GFP. One possible explanation is that because D66H-GFP is trapped within the Golgi apparatus, a compartment not associated with any sort of protein degradation machinery, D66H-GFP is not readily accessible to either lysosomal or proteasomal degradation.

D66 is found within a highly conserved region within the first extracellular loop of Cx26 and this portion of the Cx is thought to be involved in voltage gating \cite{57,58}, connexon-connexon interactions \cite{59} as well as the oligomerization of Cxs into connexons \cite{60}. The functional importance of this domain within the first extracellular loop of Cx26 was supported further by the finding that the cysteine at position 64 and the arginine at position 75 are critical for the formation of a functional gap junction channel \cite{61}.

Given the retention of D66H within the TGN and its subsequent transport to the cell surface when co-expressed with select wild-type Cxs suggests that the D66H mutant may fail to properly fold or oligomerize when expressed alone. One hypothesis is that a negatively charged amino acid at position 66 is crucial and a change to a basic amino acid at this site ionically alters the conformation of this domain enough to disrupt Cx folding or subunit interactions. Our experiments in which three more mutations at D66 were analyzed suggest this may be the case.
Both D66H and D66K were retained within the Golgi apparatus while D66A and D66E formed apparent gap junctions at the cell surface. Another possibility is that a change from a negative to a positive amino acid at position 66 alters interactions with molecular chaperones or quality control mechanisms within the Golgi apparatus and subsequently, D66H and D66K are abnormally retained within that organelle. Perhaps more intriguing is that even though structures reminiscent of gap junctions were formed by D66A-GFP and D66E-GFP, permeability to fluorescent dye was greatly reduced suggesting that this residue is not only important for transport and assembly of Cx26 but also plays a role in connexon docking and the eventual function of the gap junction channel. Surprisingly, the change from an aspartic acid to a glutamic acid, resulting in only the addition of a small CH2 group to the residue side chain, had a large effect on the function of the channel, suggesting this is a critical and important Cx motif.

Unlike the D66H mutant, a population of the G59A mutant was transported to the cell surface and assembled into apparent gap junctions which were resistant to extraction by Triton-X-100. There was, however, a notable intracellular retention of G59A-GFP when compared to wild-type Cx26-GFP which may suggest this mutant also has compromised transport properties as supported by Marziano and colleagues (54). Heathcote et al. (2000) hypothesized that since G59 is found within the highly conserved amino acid motif 57QPGC60 which is suggested to form a reverse turn (62), the replacement of glycine with the more bulky alanine would disrupt this structure and hence disrupt connexon-connexon interactions (22). Although a population of G59A did reach the plasma membrane, the resulting channels were clearly defective.

Both D66H and G59A were able to exert a dominant-negative effect on wild-type Cx26 channel function. Therefore, even though co-expression with Cx26 lead to D66H transport to the cell surface, gap junction channel function was impaired. These results would suggest that mixed
oligomers formed from varying ratios of wild-type and D66H mutants are defective. This effect likely explains why deafness is dominantly inherited. Moreover, the fact that the D66H mutation completely ablated channel function while the G59A mutant retained a minimal amount of channel activity could partially explain why the D66H mutation exhibits a more severe clinical phenotype (22). Intriguingly, G59A, but not D66H, exerted a trans-dominant effect on Cx32 function. This may reflect the participation of select and specific amino acids within the first extracellular loop in regulating compatible Cx interactions.

Consistent with *Xenopus* oocyte studies (23), D66H was found to exert a small trans-dominant negative effect on Cx43 channel function even in the absence of any detectable changes in the subcellular distribution of either Cx43 or the D66H mutant. Additionally, G59A was also able to exert a significant trans-dominant effect on Cx43 function. Since wild-type Cx43 and Cx26 do not typically form heteromeric channels, these trans-dominant negative effects are difficult to explain. As mentioned earlier, it is possible that changing the amino acids within the first extracellular domain alters the compatibility for oligomerization partners. Alternatively, the bolus of D66H mutant retained within the TGN may indirectly affect the transport of wild-type Cx43 to the cell surface resulting in reduced dye transfer. Another possibility is that the interdigitation of non-functional G59A channels amongst functional Cx43 channels may reduce the overall cell-cell coupling capacity of adjacent cells. Consequently, while direct heteromeric interactions between mutant and wild-type connexins may seem to be the most obvious explanation for the dominant and trans-dominant effects we can not completely eliminate indirect effects linked to impaired Cx transport and gap junction assembly.

In summary, we have explored the localization and function of two disease-causing Cx26 mutations, D66H and G59A. Both of these Cx26 mutants exerted dominant-negative as well as
trans-dominant negative effects on co-expressed wild-type Cxs. Our results suggest the ability of these mutants to exhibit both deafness and differential severities of skin disease could be based on the ability of the mutants to trans-dominantly interact with other Cx members. Finally, we have clearly shown that residue 66 plays a vital role in not only Cx26 transport but also in the formation of a functional gap junction channel.

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Figure Legends

Figure 1: Cx26 mutants, G59A-GFP and D66H-GFP, exhibit distinct subcellular localizations. HBL-100 cells expressing Cx26-GFP, G59A-GFP, or D66H-GFP (A, D, G) were fixed and immunolabelled for the constituent resident protein of the Golgi apparatus, giantin (B, E, H). Similar to Cx26-GFP, G59A-GFP was localized to the cell surface in typical appearing gap junction plaques (A, D, arrows) as well as to perinuclear locations (A, D, double arrows) in proximity to giantin (B, C, E, F double arrows). D66H-GFP, however, was localized to distinct juxtanuclear locations (G, double arrows) that completely co-localize with giantin (Fig. 2 G-I, double arrows). Bar = 10 µm.
Figure 2: Cx26-GFP and G59A-GFP formed gap junction-like structures at the cell surface which were resistant to Triton-X-100 extraction. HBL-100 cells expressing Cx26-GFP (A, B), G59A-GFP (C, D) or D66H-GFP (E, F) were incubated in regular medium (A, C, E) or medium containing 1% Triton-X-100 for 15 minutes (B, D, F). Triton X-100 extracted the majority of the D66H mutant (F) while leaving the architecture of the cells intact (F, insert) while many Cx26-GFP (B) and G59A-GFP (D) gap junctions remained. Bar = 10 µm.

Figure 3: A significant population of D66H-GFP was found in the trans Golgi network (TGN). HBL-100 cells expressing D66H-GFP were either untreated (A, B, E, F) or treated with BFA for 30 min (C, D, G, H). Cells were immunolabelled for giantin (B, D) or BODIPY-TR ceramide (F, H). BFA induced the redistribution of giantin (D), while a distinct population of D66H-GFP adjacent to the nucleus remained BFA-insensitive (C, arrow). In contrast, after 30 minutes of BFA treatment, a significant population of D66H-GFP co-localized with BFA-insensitive BODIPY-TR ceramide indicative of the TGN (G, H, arrows). Bar = 10 µm.

Figure 4: Neither G59A-GFP or D66H-GFP form functional gap junction channels as assessed by permeability to Lucifer yellow. GJIC-deficient HeLa cells expressing wtCx26-GFP (A-C), G59A-GFP (D-F) or D66H-GFP (G-I) were microinjected with Lucifer yellow. While HeLa cells expressing Cx26-GFP readily allowed dye to spread to several neighbouring cells, cells expressing G59A-GFP or D66H-GFP did not pass dye to neighbouring cells. HeLa and HBL-100 cells expressing wild-type or mutants are graphically summarized in (J) and data is expressed as mean +/- SEM. Bar = 20 µm.
Figure 5: Connexin-specific rescue of D66H-GFP transport to the cell surface and assembly. In HBL-100 cells co-expressing Cx26 and D66H-GFP, gap junction plaques composed of both Cx26 and D66H-GFP were commonly observed (A-C, arrows). Co-expression of Cx32 and D66H-GFP in HeLa cells also led to the appearance of gap junction plaques containing both Cx32 and D66H-GFP (D-F). When D66H-GFP was co-expressed with Cx43 in HBL-100 cells, however, D66H-GFP remained within the Golgi apparatus (G-I, arrow denotes a Cx43 gap junction). In HeLa cells that were co-transfected with equal amounts of cDNA encoding Cx30 and D66H-GFP, gap junctions composed of both types of Cxs were observed (J-L, arrows). A cell expressing only Cx30 is denoted by the double arrow (J-L). As controls, gap junctions readily assembled in HBL-100 cells expressing only Cx26 (M, arrows) or Cx43 (O, arrows) as well as in HeLa cells that expressed only Cx32 (N, arrows). Bar = 10 µm.

Figure 6: Inhibition of lysosomal and proteasomal degradation fails to rescue D66H-GFP transport to the cell surface. HBL-100 cells expressing Cx26-GFP or D66H-GFP were grown in the absence (A, B) or presence of leupeptin (C, D) or lactacystin (E, F) to inhibit lysosomal or proteasomal degradation, respectively. In the presence of leupeptin, an increase in intracellular Cx26 was observed in cells expressing either Cx26-GFP (C) or D66H-GFP (D). Lactacystin-treatment did not significantly alter the distribution of either Cx26-GFP or D66H-GFP (E, F). Bar = 10 µm.

Figure 7: D66H-GFP exhibited a prolong half-life compared to Cx26-GFP and G59A-GFP. HBL-100 cells expressing Cx26-GFP, G59A-GFP or D66H-GFP were treated with
cycloheximide (CHX) for up to 5 h. Normalized Western blots using anti-GFP and anti-vimentin antibodies (A) revealed that D66H had a significantly prolonged half-life compared to wild type Cx26 or the G59A mutant (B). Variance reported as +/-SEM using 3 independent experiments.

**Figure 8: Cx26 mutants, G59A and D66H exhibit dominant and trans-dominant negative effects on co-expressed connexins.** HBL-100 cells co-expressing Cx26 and either G59A-GFP or D66H-GFP exhibited an >80% decrease in dye transfer suggesting that both G59A and D66H exert a dominant negative effect on wild-type Cx26 function (A). HeLa cells co-expressing Cx32 and G59A-GFP exhibited a 50% decrease in dye permeability while the D66H-GFP mutant had no effect on dye transfer (B). Interestingly, NRK cells expressing endogenous Cx43 and G59A-GFP or D66H-GFP also showed a 50% and 30% decrease in dye transfer, respectively (C). Results are expressed as mean +/- SEM. * p<0.05 and *** p<0.001.

**Figure 9: The charge and side chain of amino acid 66 is critical for proper transport and assembly of Cx26.** HeLa cells were transiently transfected with cDNA encoding D66A-GFP (A), D66E-GFP (B) or D66K-GFP (C). Gap junction plaques were formed by both D66A-GFP and D66E-GFP (A, B, arrows). D66K-GFP, like D66H-GFP, was not transported to the cell surface (C, double arrows). Bar = 10 µm.

**Figure 10. Gap junctions composed of D66A-GFP or D66E-GFP were not functional.** HeLa cell pairs that were transiently transfected with D66A-GFP or D66E-GFP were
microinjected with Lucifer yellow. Neither D66A-GFP (A, B) nor D66E-GFP (C, D) exhibited dye transfer to the adjacent cell. Bar = 20 µm.

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Figure 1:

A. Cx26-GFP
B. Anti-Giantin
C. Overlay

D. G59A-GFP
E. Anti-Giantin
F. Overlay

G. D66H-GFP
H. Anti-Giantin
I. Overlay

Legend:
- Arrows indicate areas of interest.
Figure 2

Control

A Cx26-GFP

B Triton-X-100

C G59A-GFP

D

E D66H-GFP

F
Figure 3:

**D66H-GFP**

A. Control

B. 30 min BFA

**Anti-Giantin**

C. Control

D. 30 min BFA

**D66H-GFP**

E. Control

F. 30 min BFA

**BODIPY**

G. Control

H. 30 min BFA
Figure 4:

|                  | Phase contrast | Pre-injection | Post-injection |
|------------------|----------------|---------------|----------------|
| **A** *wtCx26-GFP* | ![Image]       | ![Image]      | ![Image]       |
| **D** *G59A-GFP*  | ![Image]       | ![Image]      | ![Image]       |
| **G** *D66H-GFP*  | ![Image]       | ![Image]      | ![Image]       |

**J**

|                      | **HBL-100** | **HeLa** |
|----------------------|-------------|----------|
| *wt*                 | ![Column]   | ![Column]|
| *Cx26-GFP*           | ![Column]   | ![Column]|
| *G59A-GFP*           | ![Column]   | ![Column]|
| *D66H-GFP*           | ![Column]   | ![Column]|

![Bar graph with data points and error bars]
Figure 5:

D66H-GFP

Overlay

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

Anti-Cx26

Anti-Cx32

Anti-Cx43

Anti-Cx32

Anti-Cx43

Anti-Cx30

Anti-Cx26

Anti-Cx32

Anti-Cx43
Figure 6:

A Cx26-GFP Control
B D66H-GFP
C Leupeptin
D
E Lactacystin
F
Figure 7

A.

Cx26-GFP

G59A-GFP

D66H-GFP

Vimentin

B.

| CHX treatment (h) | Cx26-GFP | G59A-GFP | D66H-GFP |
|------------------|----------|----------|----------|
| 0                | 100      | 100      | 100      |
| 3                | 70       | 50       | 80       |
| 5                | 40       | 30       | 60       |

*p<0.05
Figure 8

A

Number of neighbouring cells receiving dye

Cx26
Cx26/Cx26-GFP
Cx26/G59A-GFP
Cx26/D66H-GFP

B

Number of neighbouring cells receiving dye

Cx32
Cx32/Cx26-GFP
Cx32/G59A-GFP
Cx32/D66H-GFP

C

Number of neighbouring cells receiving dye

Cx43
Cx43/Cx26-GFP
Cx43/G59A-GFP
Cx43/D66H-GFP

*** ***
***
*

Mean number of neighbouring cells receiving dye...
Figure 9
**Figure 10**

| Cx26 mutant expressed in HeLa cells | Number of cell pairs microinjected | Number of cell pairs exhibiting dye transfer | Percentage of cell pairs exhibiting dye transfer |
|-----------------------------------|-----------------------------------|---------------------------------------------|-----------------------------------------------|
| D66A-GFP                          | 20                                | 1                                           | 5%                                            |
| D66E-GFP                          | 20                                | 3                                           | 15%                                           |

![Image](http://www.jbc.org/Downloaded from)
Functional domain mapping and selective trans-dominant effects exhibited by Cx26 disease-causing mutations

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