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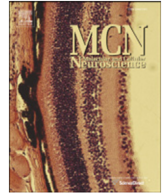

Dominant Cx26 mutants associated with hearing loss have dominant-negative effects on wild type Cx26

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ABSTRACT

Mutations in *GJB2*, the gene encoding the human gap junction protein connexin26 (Cx26), cause either non-syndromic hearing loss or syndromes affecting both hearing and skin. We have investigated whether dominant Cx26 mutants can interact physically with wild type Cx26. HeLa cells stably expressing wild type Cx26 were transiently transfected to co-express nine individual dominant Cx26 mutants; six associated with non-syndromic hearing loss (W44C, W44S, R143Q, D179N, R184Q, and C202F) and three associated with hearing loss and palmoplantar keratoderma (G59A, R75Q, and R75W). All mutants co-localized and co-immunoprecipitated with wild type Cx26, indicating that they interact physically, likely by forming admixed heteromeric/heterotypic channels. Furthermore, all nine mutants inhibited the transfer of calcein in cells stably expressing Cx26, demonstrating that they each have dominant effects on wild type Cx26. Taken together, these results show that dominant-negative effects of these Cx26 mutants likely contribute to the pathogenesis of hearing loss.

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Introduction

Gap junctions (GJs) are intercellular channels that allow the direct passage of ions and small molecules (typically <1000 Da) between adjacent cells, and are thought to have diverse functions, including the propagation of electrical signals, metabolic cooperation, spatial buffering of ions, growth control, and cellular differentiation (Bruzzone et al., 1996). A complete channel is formed when one hemichannel (or connexon) docks with a compatible hemichannel on an apposed cell membrane; each hemichannel is comprised of six compatible connexin molecules—a large family of highly conserved proteins, named according to their predicted molecular mass (Willecke et al., 2002). Individual hemichannels can be composed of one (homomeric) or more than one (heteromeric) type of connexins. Similarly, channels can be composed of hemichannels comprised of the same (homotypic) or different (heterotypic) connexins (Kumar and Gilula, 1996; White and Bruzzone, 1996). The configuration and molecular composition of channels affect the biophysical properties such as permeability and gating (Harris, 2001).

Mutations in *GJB2*, *GJB6*, and *GJB3*, the genes that encode the human gap junction proteins connexin26 (Cx26), Cx30, and Cx31, respectively, cause hearing loss (Estivill et al., 1998; Grifa et al., 1999; Kelsell et al., 1997; Xia et al., 1998). Recessive mutations of *GJB2* are the most common cause of hereditary non-syndromic hearing loss, accounting for up to 50% of such patients, with over 90 recessive mutations reported (http://davinci.crg.es/deafness/). At least 30 dominant mutations in *GJB2* have also been reported to cause hearing loss, either in isolation (non-syndromic) or as part of a syndrome with various skin disorders, including palmoplantar keratoderma (PPK). Recessive *GJB2* mutations likely cause simple loss of function, whereas dominant *GJB2* mutations likely cause gain of function, including dominant-negative effects on wild type (WT) Cx26 and/or Cx30 (Marziano et al., 2003; Yum et al., 2010).

Cx26 is widely expressed throughout non-sensory epithelial and connective tissue cells, and is largely co-expressed with Cx30 in the inner ear (Ahmad et al., 2003; Forge et al., 2002; Jagger and Forge, 2006; Kikuchi et al., 1995; Lautermann et al., 1998; Sun et al., 2005). We reported that 9 dominant Cx26 mutants co-immunoprecipitated with Cx30, indicating that they formed admixed heteromeric/heterotypic channels and 8 of them had trans-dominant effects on Cx30 (Yum et al., 2010). Cx26 mutants are proposed to exert their dominant-negative effect on WT Cx26 by forming heteromeric/heterotypic channels as well (Marziano et al., 2003; Oshima et al., 2003; Piazza et al., 2005), but this has not been directly demonstrated. In this study, we extended the analysis of these nine dominant Cx26 mutants (Suppl. Fig. 1)—six (W44C, W44S, R143Q, D179N, R184Q and C202F) that cause non-syndromic hearing loss (NSHL) and three (G59A, R75Q and R75W) that cause syndromic hearing loss (SHL) associated with PPK, and demonstrated that all nine co-localized and co-immunoprecipitated with WT Cx26, indicating that these individual mutants co-assembled with WT Cx26 into heteromeric/

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heterotypic channels, and they either partially or completely inhibited dye transfer of WT Cx26. Dominant-negative effects of these Cx26 mutants likely contribute to the pathogenesis of hearing loss.

Results

All nine dominant Cx26 mutants co-localized with WT Cx26 in the gap junction plaques

We have recently shown that nine dominant Cx26 dominant mutants (W44C, W44S, G59A, R75Q, R75W, R143Q, D179N, R184Q and C202F) form gap junction plaques at apposed cell borders, similar in appearance to those formed by WT Cx26 (Yum et al., 2010). To determine whether these dominant mutants can interact with WT Cx26, we added various tags (EGFP or DsRed, as well as V5, myc, or FLAG epitopes) to the C-terminus of WT Cx26. Immunostaining transiently transfected HeLa cells showed that WT Cx26 with a V5 tag (Cx26V5) had the most similar expression pattern to that of (untagged) WT Cx26 (Suppl. Fig. 2), and that the V5 tag "blocked" the staining of a rabbit antiserum against the C-terminus of Cx26 (RbaCx26-C) but not that of a rabbit antiserum against the cytoplasmic loop of Cx26 (RbαCx26-L), as shown in Fig. 1A. This serendipitous finding allowed us to localize Cx26V5 (with MαV5) and untagged Cx26 (with RbαCx26-C) separately in HeLa cells expressing both of them (Fig. 1A). Using this approach, we found that each of the 9 dominant Cx26 mutants formed gap junction plaques at apposed cell borders, as in cells transfected to express the individual mutants alone at the same time (Fig. 1C), and were largely co-localized with Cx26V5 in gap junction plaques at apposed cell borders (Fig. 1B). We have performed the transfection at least 5 times for each mutant with similar results. Thus, none of the nine Cx26 mutants affected the trafficking and localization of WT Cx26.

Cx26V5 and Cx26 mutants co-immunoprecipitate

The co-localization of Cx26V5 and Cx26 mutants in co-transfected cells suggested that they formed heteromeric hemichannels. To evaluate directly this possibility, we immunoprecipitated Cx26V5 with a mouse monoclonal antibody against V5 and blotted the immunoprecipitates with the rabbit antiserum against the C-terminus of Cx26. As shown in Fig. 2, WT Cx26 as well as all 9 different mutants co-immunoprecipitated with Cx26V5. In contrast, Cx43 did not co-immunoprecipitate with Cx26V5 (Fig. 2A, right panel), in keeping with the evidence that these two connexins did not interact in mammalian cells (Gemel et al., 2004; Yum et al., 2007). To validate our approach, we demonstrated directly that two different V5 antibodies co-immunoprecipitate Cx26V5 and WT Cx26 (untagged) when they were co-expressed in the cells, but did not immunoprecipitate WT Cx26 when it was expressed alone, and that the V5 epitope tag largely blocked the binding of the rabbit antiserum against the C-terminus of Cx26 (Suppl. Fig. 3). We have performed these experiments three times with similar results, and conclude that the dominant Cx26 mutants likely interact physically with Cx26WT, probably by forming heteromers.

Functional analysis of cells expressing Cx26V5 and/or Cx26 mutants

To investigate whether these dominant Cx26 mutants affect the function of WT Cx26, we compared the fluorescence recovery after photobleaching (FRAP), a quantitative analysis of dye transfer over time, in HeLa cells stably expressing Cx26 (Yum et al., 2007) that were transiently transfected with a pIRES2-DsRed bicistronic vector containing WT *GJB2* or one of the nine *GJB2* mutations. After transfection, confluent monolayers of cells were incubated in calcein AM, which is cleaved within cells, yielding calcein, a small (623 Da; −4 charge) fluorescent molecule. The number of neighboring cells needed to surround individual cells completely in the culture ranged from 4 to 7 depending on the size and the shape of the cells; fewer cells if they were larger or if they were relatively elongated. Therefore, we selected individual DsRed-positive cells that were in close contact with at least four other DsRed-positive cells for photobleaching. The images were acquired immediately before and after bleaching, and every 10 s thereafter for 400 s; examples are shown in Fig. 3A. The fluorescence was measured as mean pixel density in the bleached cell in every image. We normalized the data for each cell, assigning the fluorescent signal present in each cell immediately prior to and immediately after photobleaching as 100% and 0%, respectively, so that the data from different cells could be pooled. As shown in Fig. 3B, all 9 mutants significantly suppressed calcein transfer of WT Cx26 ($p<0.0001$), although the D179N mutant had less severe inhibition comparing to other mutants ($p<0.0001$).

Discussion

To simulate the *in vivo* situation in patients with dominant *GJB2* mutations, where mutant and WT Cx26 subunits are co-expressed due to the heterozygous state of the mutations, we investigated the interaction between nine individual dominant Cx26 mutants and WT Cx26 in a model system. The lack of binding of antibodies against the C-terminus of Cx26 to Cx26V5 enabled us to distinguish WT Cx26 (Cx26V5) from the untagged Cx26 mutants. In this way, we showed that nine dominant Cx26 mutants interact directly with WT Cx26—all mutants were co-localized and co-immunoprecipitated with Cx26V5. Furthermore, we used a novel adaptation of a FRAP assay to demonstrate directly that all nine mutants diminished the function of WT Cx26. Our results, taken together, provide the first comprehensive demonstration that dominant Cx26 mutants interact physically with, and have dominant effects upon, WT Cx26, likely by forming heteromeric/heterotypic channels.

All nine dominant Cx26 mutants interact physically with WT Cx26

Up to 30 different dominant mutations in *GJB2* have been reported; 10 are associated with non-syndromic hearing loss (NSHL), and the others cause syndromic hearing loss (SHL) with various skin diseases. In this study, we focused our investigation on mutants associated with NSHL or SHL associated with the milder form of skin disease PKK. We previously reported that these nine dominant Cx26 mutants formed gap junction plaques in transfected cells when expressed alone, or when co-expressed with Cx30, with which they were co-localized (Yum et al., 2010). We extended the analysis here by showing that these nine mutants were also co-localized with Cx26V5, forming normal-appearing gap junction plaques. Our findings are consistent with previous reports that W44S, G59A, G59A-EGFP, W75Q-EYFP, or R75W-GFP formed gap junction plaques at the cell borders in cells co-expressing WT Cx26 (Marziano et al., 2003; Oshima et al., 2003; Piazza et al., 2005), but some of these reports did

Fig. 1. Dominant Cx26 mutants co-localize with WT Cx26 at gap junction plaques. These are confocal images of transiently transfected HeLa cells that express WT Cx26 with a C-terminal V5 epitope tag (Cx26V5) alone (A), or co-express Cx26V5 and WT Cx26 (Cx26WT) or the indicated Cx26 mutants (B), or the individual Cx26 mutants alone as indicated (C). (A) These cells were co-labeled with a mouse antibody against V5 (MaV5) and a rabbit antiserum against the C-terminus (RbaCx26-C, left panel) or the cytoplasmic loop (RbaCx26-L, right panel) of Cx26. Note that the V5 tag did not alter the trafficking of Cx26V5 to gap junction plaques at apposed cell borders as visualized with MaV5 or RbaCx26-L (right panel), whereas the immunoreactivity to the RbaCx26-C was minimal and never seen at the gap junction plaques (left panel), indicating that the V5 tag prevented the binding of the RbaCx26-C antiserum. (B) These cells were co-labeled with RbaCx26-C (to visualize the untagged WT or mutant Cx26) and MaV5 (to visualize Cx26V5). Similar to Cx26WT, all of these Cx26 mutants were co-localized with Cx26V5, including at gap junction plaques. Scale bar: 10 μm. (C) These are confocal images of HeLa cells transiently transfected to express WT Cx26 (Cx26WT) or the indicated Cx26 mutants. The cells were labeled with a rabbit antiserum against the C-terminus of Cx26. Similar to the WT Cx26, all of these mutants formed gap junction plaques at apposed cell borders. Scale bar: 10 μm.

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