Different consequences of cataract-associated mutations at adjacent positions in the first extracellular boundary of connexin50

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Tong JJ, Minogue PJ, Guo W, Chen TL, Beyer EC, Berthoud VM, Ebihara L. Different consequences of cataract-associated mutations at adjacent positions in the first extracellular boundary of connexin50. Am J Physiol Cell Physiol 300: C1055–C1064, 2011. First published January 12, 2011; doi:10.1152/ajpcell.00384.2010.—Gap junction channels, which are made of connexins, are critical for intercellular communication, a function that may be disrupted in a variety of diseases. We studied the consequences of two cataract-associated mutations at adjacent positions at the first extracellular boundary in human connexin50 (Cx50), W45S and G46V. Both of these mutants formed gap junctional plaques when they were expressed in HeLa cells, suggesting that they trafficked to the plasma membrane properly. However, their functional properties differed. Dual two-microelectrode voltage-clamp studies showed that W45S did not form functional intercellular channels in paired Xenopus oocytes or hemichannel currents in single oocytes. When W45S was coexpressed with wild-type Cx50, the mutant acted as a dominant negative inhibitor of wild-type function. In contrast, G46V formed both functional gap junctional channels and hemichannels. G46V exhibited greatly enhanced currents compared with wild-type Cx50 in the presence of physiological calcium concentrations. This increase in hemichannel activity persisted when G46V was coexpressed with wild-type lens connexins, consistent with a dominant gain of hemichannel function for G46V. These data suggest that although these two mutations are in adjacent amino acids, they have very different effects on connexin function and cause disease by different mechanisms: W45S inhibits gap junctional channel function; G46V reduces cell viability by forming open hemichannels.

MATERIALS AND METHODS

Generation of Cx50 constructs. Wild-type Cx50 and Cx50G46V were previously subcloned into pSP64TII (for RNA transcription and Xenopus oocyte expression) and pcDNA 3.1/Hygro(+) (for expression in transfected mammalian cells) (2, 10, 21). DNA encoding W45S was obtained by polymerase chain reaction using oligonucleotides encoding the nucleotide substitution, Phusion DNA polymerase (New England BioLabs, Ipswich, MA), and plasmid templates containing wild-type Cx50 in pSP64TII and pcDNA 3.1/Hygro(+). Wild-type Cx50-GFP and G46V-GFP were obtained by appending the Emerald variant (Invitrogen Life Technologies, Carlsbad, CA) of green fluorescent protein (GFP) to the COOH-terminus of wild-type Cx50 and Cx50G46V. Generation of Cx50 constructs. Wild-type Cx50 and Cx50G46V were previously subcloned into pSP64TII (for RNA transcription and Xenopus oocyte expression) and pcDNA 3.1/Hygro(+) (for expression in transfected mammalian cells) (2, 10, 21). DNA encoding W45S was obtained by polymerase chain reaction using oligonucleotides encoding the nucleotide substitution, Phusion DNA polymerase (New England BioLabs, Ipswich, MA), and plasmid templates containing wild-type Cx50 in pSP64TII and pcDNA 3.1/Hygro(+). Wild-type Cx50-GFP and G46V-GFP were obtained by appending the Emerald variant (Invitrogen Life Technologies, Carlsbad, CA) of green fluorescent protein (GFP) to the COOH-terminus of wild-type Cx50 and Cx50G46V. The coding regions of all amplified constructs were fully sequenced at the Cancer Research Center DNA Sequencing Facility of the University of Chicago (Chicago, IL) to confirm the absence of additional unwanted mutations.

Immunofluorescence. Parental HeLa cells were transiently transfected with wild-type Cx50 or W45S in pcDNA3.1/Hygro(+) (Invitrogen Life Technologies), and HeLa cells stably transfected to inducibly express G46V were induced by treatment with 1 μM ponasterone A. Forty-eight hours later, cells were rinsed with phosphate-buffered saline (PBS), fixed for 15 min in 4% paraformaldehyde at room temperature, and permeabilized with 1% Triton X-100 in PBS. Immunofluorescence was carried out using affinity-purified rabbit polyclonal anti-Cx50 antibodies and Cy2-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA) as previously described (4).
Preparations enriched in plasma membranes from Xenopus oocytes were made according to White et al. (38) and Gupta et al. (13). Briefly, four control Xenopus oocytes or oocytes injected with cRNAs encoding wild-type Cx50 (Cx50), W45S, or G46V were homogenized in 5 mM Tris·HCl, 1 mM EDTA, 1 mM EGTA, and 2 mM PMSF, pH 8.0. The homogenates were centrifuged at 3,000 g for 5 min at 4°C. The supernatants were collected and centrifuged at 100,000 g for 1 h at 4°C. The supernatants were discarded and the pellets were resuspended in Laemmli sample buffer. Immunoblotting was performed using affinity-purified rabbit polyclonal anti-Cx50 antibodies (3) and horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (Jackson ImmunoResearch). Detection of secondary antibody binding was performed by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) as previously described (4).

Expression of connexins in Xenopus oocytes. Connexin cRNAs were synthesized using the mMessage mMachine in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The amount of cRNA was quantitated by measuring the absorbance at 260 nm.

Adult female Xenopus laevis frogs were anesthetized with tricaine, and a partial ovariectomy was performed in accordance with protocols approved by the Rosalind Franklin University Animal Care and Use Committee. The oocytes were manually defolliculated after treating them with collagenase IA (Worthington Biochemical, Lakewood, NJ). Stage V and VI oocytes were selected and pressure injected using a Nanoject variable microinjection apparatus (model no. 3-000-203, Drummond Scientific, Broomall, PA) with 36.8 nl of 0.5–600 ng/μl of connexin cRNA and 5 ng/36.8 nl of oligonucleotides antisense to mRNA for Xenopus Cx38 as previously described (9). The oocytes were incubated overnight at 18°C in L-15 medium (Invitrogen Life Technologies) containing 2 mM CaCl₂ and 2 mM CoCl₂ before electrophysiological experiments were performed.

Electrophysiological measurements. For measurement of gap junctional coupling, connexin cRNA-injected oocytes were devitellinized and paired as previously described (8). Double two-microelectrode voltage-clamp experiments were performed using Geneclamp 500 and an Axoclamp 2A voltage-clamp amplifier (Axon Instruments, Union City, CA). The microelectrodes were filled with 3 M KCl and had a resistance of 10–15 MΩ.

Fig. 1. Wild-type -and mutant connexin50 (Cx50) form gap junction plaques. Photomicrographs show the distribution of Cx50 immunoreactivity in HeLa cells expressing wild-type Cx50 (WT Cx50), W45S, or G46V. Scale bar, 31 μm.

Fig. 2. G46V and W45S differ in their abilities to form intercellular channels. A–C: bar graphs showing the junctional conductances (means ± SE) in pairs of Xenopus oocytes expressing wild-type Cx50, G46V, W45S, or Cx46, or combinations of these connexins as determined by double whole cell voltage clamp. All oocytes were injected with antisense oligonucleotides (AS) to block endogenous Cx38 junctional currents. The number of pairs is indicated in parentheses. **P < 0.001 compared with wild-type Cx50-injected oocyte pairs; ***P < 0.001 compared with Cx46-injected oocyte pairs.
resistance between 0.1 and 0.6 MΩ. To prevent electrode leakage, the tips of the electrodes were backfilled with 1% agar in 3 M KCl. For simple measurements of gap junctional coupling, both cells of a pair were held initially at -40 mV, and voltage-clamp steps were applied to one cell while the second cell was held at -40 mV. Under these conditions, the change in current measured in the second cell would be equal to the junctional current (Ij), but of opposite polarity. The junctional conductance (gj) was calculated as (gj = Ij/Vj), where Vj (transjunctional voltage) = Vcell 2 - Vcell 1.

For measurements of hemichannel currents in single oocytes, a series of voltage-clamp steps were applied between -70 and +30 mV in increments of 10 mV from a holding potential of -80 mV. The standard external bath solution contained (in mM) 88 NaCl, 1 KCl, 2.4 NaHCO3, 1 MgCl2, and 15 HEPES, pH 7.6 to which different concentrations of calcium were added.

Pulse generation and data acquisition were performed using a PC computer equipped with PCLAMP6 software and a TL-1 acquisition system (Axon Instruments). Currents were filtered at 20–50 Hz and digitized using PCLAMP6 software and a Digidata 1200 (Axon Instruments). All experiments were performed at room temperature (20–22°C).

For single-channel measurements, the oocyte vitelline membrane was removed and the channels were studied with the cell-attached patch-clamp technique. All measurements were performed at room temperature. Pipettes were pulled using a Flaming/Brown micropipette puller (model P-87; Sutter Instruments, Novato, CA). The patch pipettes had resistances of 2–5 MΩ when filled with standard internal solution containing (in mM) 140 KCl, 1 EGTA, 0.5 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.6. The bath chamber contained the same solution as the pipettes. Single-channel currents were recorded in the cell-attached patch configuration using an Axopatch 200B amplifier (Axon Instruments). Signals were filtered at 1 kHz and digitized with a Digidata 1322A analog/digital converter (Axon Instruments) at 10 kHz using pClamp 9.2 (Axon Instruments). Single-channel current-voltage (I-V) curves were determined using 200-msec voltage-clamp ramps between -100 mV and 100 mV. The I-V curves of the main open state were constructed by subtracting a segmented average trace of the baseline current from a single current trace or a segmented average trace of the current when the channel was in the main open state.

Confocal microscopy. Oocytes expressing GFP-tagged connexins were placed on collagen-coated glass-bottom culture dishes (MatTek, Ashland, MA), and the vegetal hemispheres were observed using a confocal laser scanning system (VisiTech Infinity 3, Sunderland, UK) based on an Olympus IX71 inverted microscope with a ×20 objective. GFP was excited at 488 nm by an argon laser, and fluorescent emission was collected at wavelengths above 500 nm with a long pass barrier filter. Acquisition conditions were kept the same for all samples. The average intensity of connexins localized to the oocyte plasma membrane was quantified using a region of interest (ROI) defining a portion of the ring-like signal at the boundary of the oocytes, and subtracting the fluorescence intensity measured using a similar ROI in the extracellular space.

Table 1. Hemichannel currents induced by wild-type and mutant Cx50

<table>
<thead>
<tr>
<th>cRNA Injected</th>
<th>cRNA Amount, ng</th>
<th>Hemichannel Current, nA</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>136.50 ± 6.64</td>
<td>4</td>
</tr>
<tr>
<td>WT Cx50</td>
<td>400</td>
<td>390.88 ± 45.90</td>
<td>17</td>
</tr>
<tr>
<td>W45S</td>
<td>400</td>
<td>81.57 ± 11.21</td>
<td>17</td>
</tr>
<tr>
<td>G46V</td>
<td>0.5</td>
<td>998.10 ± 270.21*</td>
<td>12</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of oocytes. Hemichannel currents were measured at +30 mV in single Xenopus oocytes. *P < 0.001 compared with wild-type connexin50 (WT Cx50) cRNA-injected cells by Student’s t-test.

Fig. 3. Levels of wild-type Cx50, G46V, and W45S are similar in cRNA-injected oocytes. Immunoblot shows the levels of wild-type and mutant Cx50 in samples enriched in plasma membranes prepared from control Xenopus oocytes or oocytes injected with cRNAs encoding wild-type Cx50, G46V, or W45S. All oocytes were injected with antisense Cx38 oligonucleotides.

Oocyte viability experiments. Oocyte viability was assessed by injecting oocytes with 6.9 ng of Cx46, Cx50, or G46V cRNA and incubating them in modified Barth’s solution (MBS) containing 1 mM Ca2+ for 1, 24, 48, and 72 h at 18°C. In the case of the oocytes coinjected with a 1:1 mixture of Cx46 and G46V cRNA, the total amount of cRNA injected into each oocyte was 6.9 ng. For the oocytes coinjected with a 1:1 mixture of Cx50 and G46V cRNA, the total amount of cRNA injected into each oocyte was 13.8 ng. Cells were scored (dead or alive) by evaluating their appearances for evidence of

Table 4. Average fluorescence intensity in the cell membrane of oocytes injected with wild-type Cx50-GFP or G46V-GFP cRNA. B: histogram comparing the average fluorescence intensity in the cell membrane of oocytes injected with wild-type Cx50-GFP or G46V-GFP cRNA. The boxes indicate the regions of interest used to quantify fluorescence intensity. The number of cells is indicated in parentheses. AU, arbitrary units.
cellular blebbing, irregularities in the border between animal and vegetal pole, and discoloration of the animal pole.

RESULTS

The capacity of W45S and G46V to form gap junctional plaques was assessed by immunofluorescence microscopy of HeLa cells transfected with wild-type Cx50, W45S, or G46V. Similar to wild-type Cx50, W45S and G46V localized at appositional membranes, where they formed gap junctional plaques, and in the perinuclear region, probably the Golgi compartment (Fig. 1).

The ability of W45S and G46V to form functional gap junctional channels was tested by double two-electrode voltage clamp in pairs of *Xenopus* oocytes injected with the corresponding cRNAs. We have previously shown that pairs of oocytes expressing G46V exhibit similar levels of gap junctional conductance to oocytes expressing wild-type Cx50 (21). We confirmed these results in the current study (Fig. 2A).

While homotypic oocyte pairs expressing G46V or wild-type Cx50 were well coupled, oocytes injected with cRNA for W45S failed to induce the formation of functional gap junctional channels when paired homotypically (Fig. 2B). Furthermore, heterotypic pairing of oocytes expressing wild-type Cx50 with oocytes expressing W45S did not induce significant coupling (Fig. 2B).

To determine whether coexpression of W45S affected the junctional conductance produced by wild-type lens connexins, we studied pairs of oocytes coinjected with equal amounts of W45S and wild-type Cx50 or wild-type Cx46 cRNA. Coexpression of W45S decreased the junctional conductance induced by wild-type Cx50 and wild-type Cx46 by 61% and 66%, respectively (Fig. 2, B and C). These results suggest that the W45S mutant inhibits both wild-type Cx50 and wild-type Cx46 in a dominant negative manner.

![Fig. 5. Wild-type and mutant Cx50 hemichannels have similar single-channel properties. A and B: single-channel current traces for wild-type Cx50 (A) and G46V (B) in the cell-attached mode. Voltage-clamp steps of ±40 mV were applied from a holding potential of 0 mV. C and D: open channel current-voltage (I-V) curves for wild-type Cx50 (C) and G46V (D) obtained by application of a 200-ms voltage ramp between −100 mV and +100 mV to cell-attached patches containing only one active channel. Voltage-clamp ramp data were corrected for leakage currents as described in MATERIALS AND METHODS.](http://ajpcell.physiology.org/)
mutant cRNA exhibited a predominant band with an electrophoretic mobility of ~60 kDa (Fig. 3). No immunoreactive bands were detected in antisense-injected control oocytes (Fig. 3). The mean intensities of the bands corresponding to wild-type Cx50, G46V, and W45S were consistently similar, indicating that levels of wild-type and mutant Cx50 were comparable.

We next examined the possibility that the dramatic differences in the magnitudes of wild-type Cx50 and G46V hemichannel currents might reflect dissimilarities in their abundances in the oocyte plasma membranes by comparing the fluorescence intensities in oocytes injected with GFP-tagged versions of these proteins. The fluorescence intensity in the cell membrane of oocytes injected with G46V-GFP cRNA appeared similar to that of oocytes injected with wild-type Cx50-GFP cRNA (Fig. 4A). Figure 4B summarizes the results of quantification of these experiments; there was no significant difference in surface fluorescence intensity between the two tagged proteins. Consistent with the behavior of the untagged connexins, the G46V-GFP cRNA-injected oocytes developed much larger hemichannel currents than wild-type Cx50-GFP cRNA-injected oocytes (data not shown).

To explore the possibility that the increased hemichannel currents produced by the G46V mutation were due to alterations of Cx50 single-channel properties, we studied these properties in oocytes using the cell-attached patch-clamp technique. Both wild-type Cx50 and G46V channels were predominantly in the open state at 0 mV and closed to a long-lasting substate after application of a voltage-clamp step to +40 mV. When the potential was changed to −40 mV, the channels reopened and remained in the fully open state for the duration of the pulse as illustrated in Fig. 5. Wild-type and mutant Cx50 hemichannels also showed similar open channel I-V curves. The slope conductances at −40 mV were 652 ± 53 pS and 670 ± 10.5 pS for wild-type Cx50 (n = 2) and G46V (n = 2), respectively.

We also considered the possibility that the G to V missense mutation at position 46 might alter the regulation of Cx50 channels. Replacement of extracellular potassium with sodium while maintaining extracellular calcium constant resulted in a large reduction in the magnitude of both wild-type Cx50- and G46V-induced currents (Fig. 6, A and B); thus, these currents appeared to be similarly regulated by external monovalent cations. Like wild-type Cx50, the G46V-induced currents were inhibited by raising the extracellular calcium concentration ([Ca2+]o) (Fig. 6, C–F). Both wild-type Cx50 and G46V were blocked to a similar extent in 1 mM [Ca2+]o. Thus, the G46V mutant did not appear to exhibit a significantly altered sensitivity to extracellular calcium.

![Fig. 6](http://ajpcell.physiology.org/)

A: membrane currents recorded in oocytes expressing wild-type Cx50 (A) and G46V (B) during repetitive voltage-clamp steps to 50 mV from a holding potential of −40 mV. The oocytes were initially bathed in 89 mM KCl containing zero added extracellular Ca2+ concentration ([Ca2+]o; black trace). Equimolar replacement of K+ with Na+ in the continued presence of zero added [Ca2+]o caused a large reduction in the amplitude of the hemichannel current (red trace). Equimolar replacement of K+ with Na+ in the continued presence of zero added [Ca2+]o caused a large reduction in the amplitude of the hemichannel current (red trace).

B: bar graph summarizing the concentration-response data for Ca2+ inhibition of wild-type Cx50 hemichannel currents in external K+. The current amplitudes at each Ca2+ concentration were normalized to the responses obtained in 0 mM [Ca2+]o. Data are presented as means ± SE (n = 7). E: example of membrane currents recorded from a G46V-expressing oocyte at different [Ca2+]o. The oocyte was sequentially exposed to 89 mM KCl containing Ca2+ concentrations of 0, 0.2, 1, and 5 mM. F: bar graph summarizing the concentration-response data for Ca2+ inhibition of G46V hemichannel currents in external K+. The current amplitudes at each Ca2+ concentration were normalized to the responses obtained in 0 mM [Ca2+]o. Data are means ± SE (n = 6). To reduce the size of the G46V hemichannel currents to levels comparable to those observed in wild-type Cx50-expressing oocytes, we injected oocytes with ~800-fold lower amounts of G46V than wild-type Cx50 cRNA.
To gain further insight regarding the impact of the G46V mutation in the lens, we examined the effect of coexpressing G46V with other lens connexins on oocyte survival. The results obtained from several oocyte batches are presented in Fig. 7, A and B. Nearly all control oocytes and oocytes expressing wild-type Cx50 remained healthy when incubated in MBS containing 1 mM [Ca\(^{2+}\)]\(_{o}\) and 1 mM [Mg\(^{2+}\)]\(_{o}\), for at least 72 h, the longest time point studied (Fig. 7A). Oocytes expressing Cx46 exhibited 100% survival 24 h postinjection, but signs of cellular degeneration were observed at longer times after Cx46 cRNA injection; oocyte viability decreased to approximately 81% and 69% after 48 and 72 h postinjection, respectively (Fig. 7B). Oocytes expressing G46V deteriorated even more rapidly than Cx46-injected oocytes, although the exact time course of deterioration differed among different batches of oocytes (Fig. 7, A and B). In all experiments, >80% of G46V-expressing oocytes showed degenerative changes by 48 h after cRNA injection (viability was <20%). Furthermore, oocytes co-injected with G46V cRNA and wild-type Cx50 or Cx46 cRNA deteriorated to approximately the same extent as oocytes injected with G46V cRNA alone 48–72 h after cRNA injection (Fig. 7, A and B).

To determine whether the differences in oocyte survival could be attributed to differences in the magnitude or voltage gating properties of the hemichannel currents produced by G46V and wild-type connexins, the biophysical properties of macroscopic hemichannel currents were investigated in single oocytes expressing Cx46, wild-type Cx50, or G46V (in NaCl solutions containing 0.7 mM [Ca\(^{2+}\)]\(_{o}\) and 1 mM [Mg\(^{2+}\)]\(_{o}\)). Both G46V and Cx46 cRNA-injected oocytes developed time-dependent currents that activated on depolarization and had a reversal potential near −10 mV (Fig. 8, A and B). No hemichannel currents greater than those detected in antisense-injected oocytes were observed in oocytes injected with wild-type Cx50 alone (Fig. 8, C and D). G46V hemichannel currents could be distinguished from the Cx46 hemichannel currents by their faster time course of activation and slower time course of deactivation. In addition, the G46V hemichannels activated at more negative voltages than Cx46. The voltage dependence of activation of Cx46 and G46V hemichannels was quantified by plotting the amplitude of the initial tail current at −80 mV as a function of test potential. Cx46 hemichannels had a threshold for activation of approximately −10 mV while G46V hemichannels had a threshold for activation of approximately −80 mV. Thus, while G46V hemichannels exhibited a substantial open probability at hyperpolarized potentials, Cx46 hemichannels were mostly closed at potentials negative to −10 mV (Fig. 8, D and E).

The magnitudes and voltage dependent gating of hemichannel currents were also studied in single oocytes expressing G46V in combination with wild-type Cx50. Coinjection of G46V with wild-type Cx50 cRNA at a ratio of 1:1 induced hemichannel currents whose voltage dependence of activation resembled those of G46V (Fig. 9). The magnitudes of the heteromeric hemichannel currents were slightly smaller than those of the homomeric G46V hemichannel currents.

The effect of mixing of wild-type or mutant Cx50 with Cx46 on hemichannel function was examined by coinjecting oocytes with different ratios of wild-type or mutant Cx50 cRNA to Cx46 cRNA while keeping the amount of injected Cx46 cRNA constant (Fig. 10). The main effect of coexpressing wild-type Cx50 with Cx46 was to reduce the magnitude of the hemichannel currents. This effect was more pronounced at higher ratios of wild-type Cx50:wild-type Cx46 (Fig. 10, C and D). Coexpression of wild-type Cx50 with Cx46 at ratios of 1:5 and 5:1 also caused a slowing of the time course of both activation and deactivation of hemichannel currents but did not shift the threshold for Cx46 hemichannel activation. In contrast, when G46V cRNA was co-injected with Cx46 cRNA at a ratio of 1:5 (to obtain an average of one mutant subunit per hemichannel), there was a small shift in the threshold for hemichannel activation to more negative potentials, an acceleration in the time course of hemichannel current activation and a slowing in the time course of hemichannel current deactivation, but the magnitude of the currents did not change significantly. Increasing the ratio of G46V to Cx46 cRNA caused a progressively larger negative shift in the threshold of activation and increased the magnitude of the tail currents. These findings demonstrate that although both wild-type Cx50 and G46V can interact with Cx46 to form heteromeric hemichannels, the identity of the amino acid residue at position 46 can have differential effects on hemichannel gating.
DISCUSSION

In this study, we investigated the functional and cellular properties of two congenital cataract-associated mutations that alter amino acids located at the TM1/E1 boundary of Cx50, W45S, and G46V. Both of these mutants formed gap junctional plaques when they were expressed in transfected HeLa cells, suggesting that they were able to oligomerize and traffic to the plasma membrane properly. However, the functional behavior of the two mutants differed.

We expressed W45S and G46V in Xenopus oocytes, because this is an excellent system to study the channel properties of proteins that oligomerize and traffic to the plasma membrane properly. However, the functional behavior of the two mutants differed.

We expressed W45S and G46V in Xenopus oocytes, because this is an excellent system to study the channel properties of proteins that oligomerize and traffic to the plasma membrane properly like these mutants. Usually, the functional properties of connexin channels in Xenopus oocytes closely parallel their properties in mammalian cells. In the Xenopus oocyte expression system, levels of the expressed connexin can be manipulated by injecting different amounts of cRNA and the consequences of mixing of wild-type and mutant connexins can be evaluated by coinjecting different ratios of their cRNAs. Potential disadvantages of this system may include altered connexin behavior due to the lower temperature in which oocytes are maintained and possible differences in posttranslational modifications.

W45S did not form functional intercellular channels or hemichannels. Moreover, it acted as a dominant negative inhibitor of wild-type Cx50 and Cx46 gap junctional channels. The tryptophan at position 45 is highly conserved within the connexin family. Indeed, 14 of the 21 human connexins contain a tryptophan at this residue; the only substitutions are other aromatic residues, Y and F (even in other species). The 3.5 Å resolution crystal structure of Cx26 shows that the tryptophan at the corresponding position is packed within the hydrophobic environment of the four helix bundle and is involved in interprotomer interactions that stabilize hexamer structures.

Fig. 8. Membrane currents from hemichannels composed of Cx46 and G46V exhibit distinct voltage-dependent properties. A–C: representative families of current traces recorded from single oocytes injected with similar amounts of cRNA for Cx46 (A), G46V (B), or wild-type Cx50 (C) in NaCl solution containing 0.7 mM [Ca²⁺]o and 1 mM [Mg²⁺]o. The oocytes were held at −80 mV between pulse sequences. Voltage-clamp steps were applied to voltages between −80 mV and 30 mV in increments of 10 mV. The data were not corrected for leakage current. Dashed line represents zero current. D: quasi-steady-state I–V relationships for Cx46 (a, n = 3), G46V (c, n = 4), and wild-type Cx50 (○, n = 5) determined by measuring the current at the end of the test pulse and plotting it as a function of pulse potential. hCx50, human Cx50; rCx46, rat Cx46. E: iso-chronal conductance-voltage relationships for Cx46 (a, n = 3) and G46V (c, n = 4). Initial amplitude of tail currents were measured at −80 mV after 20-s pulses to different potentials and then normalized to the amplitude of the tail current after a voltage-clamp pulse to +30 mV.

Fig. 9. G46V has a dominant behavior when mixed with wild-type Cx50. A: representative family of current traces recorded from single oocytes coexpressed with Cx50 cRNA for G46V and wild-type Cx50 in NaCl solution containing 0.7 mM [Ca²⁺]o and 1 mM [Mg²⁺]o. Voltage-clamp steps were applied to voltages between −80 mV and +30 mV in 10-mV increments from a holding potential of −80 mV. No leakage current correction was applied. Dashed line represents zero current. B: tail I–V relationships for G46V (●, n = 5), Cx50 (○, n = 1), or G46V + Cx50 (1:1, ⊙, n = 4). Initial amplitudes of tail currents were measured at −80 mV after 20-s pulses to different potentials and plotted as a function of pulse potential. The total amount of cRNA injected was 0.147 ng/oocyte, except in the case of Cx50 + G46V where it was 0.294 ng/oocyte.
Thus, it is not surprising that introduction of a polar residue, as in the W45S mutation, would result in loss of channel function. Our results may be generalizable to other connexins. Replacement of the corresponding residue with S or C in Cx26, with S in Cx46, and with L in Cx32 is associated with deafness, cataracts, and X-linked Charcot-Marie-Tooth disease, respectively (5, 6, 17). Consistent with our findings that W45S acted as a dominant negative inhibitor of coexpressed wild-type Cx50, the deafness and cataracts due to the substitutions in Cx26 and Cx46 are inherited as dominant traits. The functional consequences of substitution at this position have only been studied for Cx46 and Cx26; replacement with cysteines resulted in loss of channel function in both cases (16, 19). Similar to our findings for W45S, the cysteine-substituted Cx26 formed gap junctional plaques (19).

In contrast, the results presented here and in our previous study (21) showed that the G46V mutant is functional. Indeed, it shows increased hemichannel activity compared with wild-type Cx50 at normal membrane potentials. This enhanced hemichannel activity would tend to depolarize the cells and increase entry/exit of metabolites and ions, thus explaining the observed decrease in cell viability. The increase in hemichannel activity of G46V did not appear to be the result of altered sensitivity to factors previously shown to regulate Cx50 [i.e., external calcium (3), monovalent cations (30), [pH]o (3)], nor could it be explained by increased total connexin levels or amounts of connexin at the plasma membrane. However, previous studies using cysteine substitutions have shown that G46 lies in a region of the pore that narrows during hemichannel closure by external calcium and hyperpolarization, suggesting that G46 is involved in Cx50 hemichannel gating (27).

Fig. 10. Mixing of mutant Cx50 but not wild-type Cx50 with Cx46 results in increased hemichannel opening at negative potentials. A–D: membrane currents recorded in response to voltage-clamp steps from −80 mV to +30 mV in 10-mV increments from a holding potential of −80 mV. Equal amounts of Cx46 cRNA were injected into each oocyte. Oocytes were bathed in NaCl solution containing 0.7 mM Ca2+ and 1 mM Mg2+. E: tail I-V relationships for Cx46 (●, n = 3), Cx46 + G46V (5:1, ○, n = 3), and Cx46 + G46V (1:2, △, n = 3). Initial amplitudes of tail currents were measured at −80 mV after 20-s pulses to different potentials and plotted as a function of pulse potential. F: tail I-V relationships for Cx46 (●, n = 3), Cx46 + Cx50 (5:1, ○, n = 3), and Cx46 + Cx50 (1:5, △, n = 3). Initial amplitudes of tail currents were measured at −80 mV after 20-s pulses to different potentials and plotted as a function of pulse potential.
Our data are also consistent with a dominant gain of hemi-
channel function for G46V, which rapidly killed Xenopus
oocytes even when coexpressed with wild-type lens connexins.
Thus, the cellular toxicity due to the mutant allele is a domi-
nant trait that is not ameliorated by the presence of either
wild-type Cx50 or Cx46. Interestingly, heteromeric hemichan-
gels formed by G46V and Cx46 had clearly different voltage
gating properties compared with heteromeric channels formed
by wild-type Cx50 and Cx46. Coexpression of G46V with
wild-type Cx46 had a deleterious effect on oocyte survival and
 correlated with an increase in hemichannel activity at negative
potentials. In contrast, coexpression of wild-type Cx50 with
wild-type Cx46 appeared to have a protective effect (data not
shown) and was associated with a diminution of hemichannel
activity. These results indicate that the interactions of wild-
type Cx50 or G46V with wild-type Cx46 result in formation of
hemichannels with different behaviors.

We can explain the effects of the G46V mutation on Cx50
behavior using the following simple kinetic scheme:

\[
\begin{align*}
\text{ultraslow} & \quad \text{slow} & \quad \text{fast} \\
A & \quad B & \quad C & \quad O & \quad \text{WT Cx50} \\
[\text{Ca}] & \\
A & \quad B & \quad C & \quad O & \quad \text{G46V}
\end{align*}
\]

where \(A\), \(B\), and \(C\) are closed states and \(O\) is an open state.
According to this model, nearly all of wild-type Cx50 channels
reside in \(A\), where \(A\) is a very long lived closed state, even in
the presence of low \([\text{Ca}^2+]_o\). The main effect of the G46V
mutation is to destabilize \(A\) either by slowing entry into \(A\) or by
increasing exit from \(A\). This will increase the number of
channels in \(B\) that are available for opening following applica-
tion of a depolarizing step or reduction of calcium. The
behavior of the heteromeric channels can also be explained
using this simple kinetic scheme by assuming that their behav-
ior is intermediate between that of the wild-type Cx50 (or
Cx46) channels and the G46V mutant channels and depends on
the ratio of mutant to wild-type subunits. Channels containing
five wild-type and one mutant subunit would be expected to
behave more closely resembles that of wild-type
Cx50 (or Cx46). In contrast, channels containing five mutant
subunits and one wild-type subunit would be expected to
behave like G46V.

Comparison of the functional behavior of the G46V muta-
tion to the behavior of a disease-associated form of Cx26 with
a missense mutation at the corresponding position (Cx26G45E)
shows a number of similarities as well as some differences (11,
29, 31). Like G46V, Cx26G45E formed both gap junctional
channels and hemichannels. Furthermore, the single-channel
conductance, voltage-gating properties, and calcium sensitivity
of the Cx26G45E hemichannels were similar to wild type.
There are conflicting data about the effect of the G45E muta-
tion on the size of the macroscopic Cx26 hemichannel current.
While Gerido et al. (11) reported significantly larger currents
for Cx26G45E compared with wild-type Cx26, Sánchez et al.
(29) did not. Sánchez et al. (29) also reported that Cx26G45E
channels exhibited altered permeability to calcium. Our results
more closely resemble those of Gerido et al. (11). We were
unable to test whether G46V hemichannels showed altered
permeability to calcium because of the extremely small size of
wild-type Cx50 currents in the presence of physiological calc-
ium concentrations.

Many of the cataracts that develop in individuals with
mutations in the GJA8 gene have been described as nuclear,
sometimes with a pulverulent appearance. The cataracts in
members of the family with the W45S mutation have a fanlike
opacity that tends to hide the deeper structure of the opacity
(36). In contrast, the individual carrying the G46V mutation
had a total cataract (21). The different effects of W45S and
G46V on channel function may contribute to the difference in
cataract phenotypes observed in people carrying the mutations.
Since W45S acts as a dominant negative inhibitor of coex-
pressed wild-type Cx50 or Cx46, it would be expected to
decrease gap junctional coupling between lens fiber cells. In
contrast, it is likely that increased hemichannel function is the
primary cause of cataracts in people carrying the G46V muta-
tion. Sustained, high hemichannel activity would be expected
to cause alterations in ionic homeostasis, loss of plasma mem-
brane potential, and depletion of vital metabolites such as ATP
and glutathione, leading to cell death. However, the relations-
ships between Cx50 mutant genotype and cataract phenotype
must be labeled as speculative. Indeed, the same Cx50 mutant,
Cx50P88Q, has been associated with cataracts of different
phenotypes in families of different ethnic backgrounds (2, 35).

In summary, our data suggest that mutations at adjacent
residues at the TM1/E1 boundary lead to differential effects on
hemichannel and gap junction channel function, and that these
mutations may differentially affect the function of wild-type
connexins.

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