Molecular mechanism underlying a Cx50-linked congenital cataract

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Molecular mechanism underlying a Cx50-linked congenital cataract. Am. J. Physiol. 276 (Cell Physiol. 45): C1443–C1446, 1999.—Mutations in gap junctional channels have been linked to certain forms of inherited congenital cataract (D. Mackay, A. Ionides, V. Berry, A. Moore, S. Bhattacharya, and A. Shiels. Am. J. Hum. Genet. 60: 1474–1478, 1997; A. Shiels, D. Mackay, A. Ionides, V. Berry, A. Moore, and S. Bhattacharya. Am. J. Hum. Genet. 62: 526–532, 1998). We used the Xenopus oocyte pair system to investigate the functional properties of a missense mutation in the human connexin 50 gene (P88S) associated with zonular pulverulent cataract. The associated phenotype for the mutation is transmitted in an autosomal dominant fashion. Xenopus oocytes injected with wild-type connexin 50 cRNA developed gap junctional conductances of ~5 µS 4–7 h after pairing. In contrast, the P88S mutant connexin failed to form functional gap junctional channels when paired homotypically. Moreover, the P88S mutant functioned in a dominant negative manner as an inhibitor of human connexin 50 gap junctional channels when co-injected with wild-type connexin 50 cRNA. Cells injected with 1:5 and 1:11 ratios of P88S mutant to wild-type cRNA exhibited gap junctional coupling of ~8% and 39% of wild-type coupling, respectively. Based on these findings, we conclude that only one P88S mutant subunit is necessary per gap junctional channel to abolish channel function.

INHERITED CONGENITAL CATARACT is a heterogeneous lens disorder that is often transmitted as an autosomal dominant Mendelian trait. Autosomal dominant cataract has been mapped to at least 10 separate loci (8). One type of congenital cataract is the zonular pulverulent cataract that, in one case, has been linked to a proline-to-serine mutation at position 88 in human connexin 50 (Cx50) (17). Cx50 is a member of the connexin family of gap junctional proteins and is expressed primarily in the lens, together with Cx46, where it forms intercellular channels between adjacent fiber cells (6). These intercellular channels consist of 12 connexons, arranged in two hexameric connexons or hemichannels, which are located in the plasma membranes of adjacent cells. The topology of connexins in the plasma membrane predicts that the P88S mutation lies within the second transmembrane domain (Fig. 1A). This proline is conserved throughout the connexin family and is thought to be involved in the voltage gating of connexins. Suchyna et al. (21) demonstrated that mutations of this proline in Cx26 prevented the formation of homotypic (same connexons) gap junctional channels and caused a reversal of voltage-gating polarity when paired heterotypically (different connexons) with wild-type Cx26. Here we have used the paired Xenopus oocyte system to characterize the voltage-dependent gating properties of human Cx50 gap junctional channels and to examine the functional consequences of a mutation in Cx50 associated with congenital cataract.

METHODS

Wild-type and mutant Cx50 alleles were PCR amplified from an affected individual of the Ev. family (15, 16). The two primers correspond to codons 1–7 and 428-Stop of the human Cx50 gene with EcoRI linkers. The PCR conditions were as described previously (17). We sequenced the entire coding region to determine whether the PCR products encoded the mutant or wild-type allele and to verify that PCR amplification did not introduce any random errors. The PCR products were then subcloned into the RNA expression vector SP64TII (3). The plasmids were linearized with Sal I, and capped RNAs were synthesized using the mMessage mMachine SP6 in vitro transcription kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The amount of RNA was quantitated by measuring the absorbance at 260 nm.

Xenopus oocytes were prepared and tested as described previously, except that the oocytes were incubated for 2 days after injection of cRNA and studied 4–7 h after pairing. To measure the junctional conductance, cell pairs were studied using the dual two-microelectrode technique described by Spray et al. (19). Families of junctional currents were generated by applying transjunctional voltage-clamp steps to ±70 mV from a holding potential of −40 mV. Changes in junctional conductance during the experiment were normalized by applying a 5-mV prepulse 1 s before the initiation of the test pulse. Only cell pairs with resting membrane potentials

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In vitro synthesized cRNAs for wild-type and mutant human Cx50 were injected into Xenopus oocytes, either singly or in combination. The expression of wild-type and mutant human Cx50 in cRNA-injected oocytes was verified by Western blot analysis of cell membrane-enriched preparations using antisera to Cx50 (Fig. 1B) (12). A bovine lens homogenate was used as the positive control. Cells injected with either wild-type or mutant Cx50 exhibited a predominant band with an electrophoretic mobility of ~70 kDa. No immunoreactive bands were detected in oocytes injected with antisense oligonucleotide to endogenous Cx38 only.

To characterize the voltage-dependent characteristics of human Cx50 gap junctional channels, Xenopus oocytes were injected and paired as described previously. Transjunctional coupling was assayed using the dual two-microelectrode voltage-clamp technique (19). Endogenous coupling was inhibited by injecting the cells with Cx38 antisense oligonucleotide (1). Wild-type human Cx50 efficiently made gap junctional channels (Table 1), and characteristic junctional current traces are shown in Fig. 2A. The junctional current decayed to new steady-state levels on application of depolarizing or hyperpolarizing transjunctional voltage-clamp steps to potentials greater than ±20 mV. The normalized initial and steady-state conductance-voltage relationships are shown in Fig. 2B. The solid lines are the best fit of the steady-state data to a Boltzmann equation with \( A = 0.08, V_o = 34 \text{ mV}, G_{\text{max}} = 1.1 \), and \( G_{\text{min}} = 0.22 \) for positive transjunctional voltages and \( A = -0.08, V_o = -32 \text{ mV}, G_{\text{max}} = 1.1 \), and \( G_{\text{min}} = 0.24 \) for negative \( V_i \) values, where \( A \) is the steepness factor, \( V_o \) and \( V_i \) are midpoint and junctional voltage, respectively, and \( G_{\text{max}} \) and \( G_{\text{min}} \) are maximum and minimum conductance, respectively. These findings indicate that human Cx50 is less voltage sensitive than mouse Cx50 (22).

We tested the ability of the P88S mutation to induce gap junctional coupling, either alone or in combination with wild-type Cx50. The results of these experiments are summarized in Table 1. Expression of the P88S mutant failed to induce coupling in homomeric oocyte pairs (same connexins in both cells), and the mutant cRNA also did not cause the formation of hemichannels that could interact with wild-type Cx50 in a heterotypic manner (different connexins in each member of the cell pair).

The effect of mixing was examined by coinjecting oocytes with different ratios of mutant to wild-type CxRNA while keeping the total amount of injected cRNA constant. When equal amounts of mutant and wild-

<table>
<thead>
<tr>
<th>Oocyte Injection</th>
<th>Gap Junctional Coupling Mean conductance, µS</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>No RNA</td>
<td>0.16 ± 0.10</td>
<td>12</td>
</tr>
<tr>
<td>WT Cx50</td>
<td>5.31 ± 0.44</td>
<td>12</td>
</tr>
<tr>
<td>P88S</td>
<td>0.27 ± 0.15*</td>
<td>8</td>
</tr>
<tr>
<td>P88S + WT (1:1)</td>
<td>0.18 ± 0.14*</td>
<td>10</td>
</tr>
<tr>
<td>P88S + WT (1:5)</td>
<td>0.22 ± 0.13*</td>
<td>8</td>
</tr>
<tr>
<td>P88S + WT (1:11)</td>
<td>0.43 ± 0.16*</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \) is no. of cell pairs. Wild-type and mutant human connexin 50 (Cx50) gap junctional coupling in heteromeric and heterotypic configurations. Each cell was injected with a total of 1.8 ng of CxRNA. * \( P < 0.05 \) compared with wild-type Cx50 by Student's t-test.
subunits

\[ F_i = \frac{n!}{i!(n-i)!} f_{\text{mut}}^i f_{\text{wt}}^{n-i} \]

where \( y \) is the fractional expected current, \( n \) is the number of subunits in the channel, \( a \) is the maximum number of mutant subunits permissible in a functional channel, \( f_{\text{mut}} \) is the mutant mole fraction, and \( f_{\text{wt}} \) is the wild-type mole fraction. This equation is valid for cases where the translation efficiencies of the cRNAs are comparable and the connexins aggregate randomly in the cell membrane. For the case where only one mutant subunit is required to abolish channel function, \( a = 0 \), and Eq. 2 reduces to

\[ y = (1 - f_{\text{mut}})^2 \quad (3) \]

For the case where two and three mutant subunits are required to abolish channel function, the resulting equations are

\[ y = (1 - f_{\text{mut}})^2 + 12 f_{\text{mut}} (1 - f_{\text{mut}})^1 \]

\[ y = (1 - f_{\text{mut}})^2 + 12 f_{\text{mut}} (1 - f_{\text{mut}})^1 + 66 f_{\text{mut}}^2 (1 - f_{\text{mut}})^0 \]

In the derivation of this equation, we assumed the arrangement of the mutant subunits within the gap junctional channel to be insignificant. If placement of the mutant connexins is important, then Eqs. 4 and 5 will underestimate the true amount of coupling. Figure

![Fig. 2. A: representative family of junctional current traces recorded from a cell pair injected with wild-type Cx50 cRNA. Cells were initially voltage clamped at -40 mV. Junctional current traces were recorded in response to transjunctional voltage-clamp steps between +70 and -70 mV in 10-mV increments. Changes in junctional conductance (G_j) during the experiment were normalized by applying a 5-mV prepulse 1 s before initiation of test pulse. For this pair, gap junctional conductance was measured to be 5.02 µS. B: normalized initial and steady-state conductance-voltage relationship for Cx50 gap junctional channels. Solid lines are best fit of steady-state data to a Boltzmann equation with \( V_o = 5\text{mV}, V_50 = 5\text{mV}, 5\text{mV}_{\text{max}} = 1.1, \text{and} 5\text{mV}_{\text{min}} = 0.22 \) for positive transjunctional voltages and \( V_o = -32\text{mV}, V_50 = 1.1, \text{and} 5\text{mV}_{\text{min}} = 0.24 \) for negative \( V_j \) values; \( n = 2 \) cell pairs.](image)

![Fig. 3. Predicted and measured inhibition of gap junctional coupling by P88S. Theoretical inhibition of current through a dodecameric channel by 1, 2, or 3 subunits, respectively, was modeled by the following functions: \( y = (1 - x)^2 \), \( y = (1 - x)^2 + 12x(1 - x)^1 \), and \( y = (1 - x)^2 + 12x(1 - x)^1 + 66x^2(1 - x)^0 \), where \( x \) represents the mutant mole fraction. Theoretical and measured inhibition of current was normalized to coupling measured in oocytes injected with wild-type Cx50. Data are plotted as mean conductance ± SE (µS; \( n = 9 \) for each data point).](image)
3 compares the inhibition of coupling due to the P88S mutation with the predicted reduction if one or multiple subunits were necessary to abolish channel function. The measured inhibition of coupling follows Eq. 3 and indicates that only one mutant subunit is necessary to abolish channel function.

**DISCUSSION**

Gap junctions are thought to play an important role not only in lens homeostasis but in virtually every organ system of the body. Mutations in connexin genes have been associated with X-linked Charcot-Marie-Tooth disease (9, 14), hereditary nonsyndromic deafness (10, 11), and congenital heart defect (2, 18). With regard to the lens, recent studies have shown that disruption of the genes for Cx46 or Cx50 by homologous recombination leads to cataractogenesis in mice (5, 25). In addition, a missense mutation in the Cx50 gene has been associated with the mouse No2 cataract (20). In the present study, we investigated the molecular mechanisms involved in cataractogenesis associated with a mutation of the human Cx50 gene. Our results indicate that the P88S mutant interacts with wild-type connexins and can abolish channel function by virtue of a single mutant subunit per gap junctional channel. This type of behavior can explain the autosomal dominant pattern of inheritance observed in this family. Recently, White et al. (24) reported similar findings regarding a Cx26 mutant in a family with profound deafness inherited as a dominant trait. Thus dominant negative behavior of connexins may be a general phenomenon to explain autosomal dominantly inherited diseases due to connexin mutations.

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