Hum Genet (2004) 115: 191–199
DOI 10.1007/s00439-004-1142-6

ORIGINAL INVESTIGATION

Gülistan Meşe · Eric Londin · Rickie Mui · Peter R. Brink · Thomas W. White

Altered gating properties of functional Cx26 mutants associated with recessive non-syndromic hearing loss

Received: 10 March 2004 / Accepted: 21 April 2004 / Published online: 7 July 2004

Abstract Connexins (Cx) form gap junctions that allow the exchange of small metabolites and ions. In the inner ear, Cx26 is the major gap junction protein and mutations in the Cx26-encoding gene, *GJB2*, are the most frequent cause of autosomal recessive non-syndromic hearing loss (DFNB1). We have functionally analyzed five Cx26 mutations associated with DFNB1, comprising the following single amino-acid substitutions: T8M, R143W, V153I, N206S and L214P. Coupling of cells expressing wild-type or mutant Cx26 was measured in the paired *Xenopus* oocyte assay. We found that the R143W, V153I and L214P mutations were unable to form functional channels. In contrast, the T8M and N206S mutants did electrically couple cells, though their voltage gating properties were different from wild-type Cx26 channels. The electrical coupling of oocytes expressing the T8M and N206S mutants suggest that these channels may retain high permeability to potassium ions. Therefore, deafness associated with Cx26 mutations may not only depend on reduced potassium re-circulation in the inner ear. Instead, abnormalities in the exchange of other metabolites through the cochlear gap junction network may also produce deafness.

Introduction

Gap junction channels facilitate the communication of neighboring cells by allowing the exchange of ions, small metabolites and secondary messengers (D'Andrea et al. 2002; Marziano et al. 2003; White and Bruzzone 1996; White and Paul 1999). This communication coordinates a variety of cellular events, including cell synchronization, cell growth, and cell differentiation (Oshima et al. 2003; Richard et al. 1998). A family of genes encodes the connexin (Cx) proteins, which form gap junction channels. These proteins share a similar topology, having a cytoplasmic amino-terminus, four transmembrane domains, a cytoplasmic carboxy-terminus, two extracellular domains and a cytoplasmic loop (Lefebvre and Van De Water 2000; Marziano et al. 2003; Rabionet et al. 2002). Six connexins oligomerize to form hemichannels, called connexons, which span the plasma membrane. Interaction at the extracellular space enables the completion of the intercellular gap junction channels that link the cytoplasm of adjoining cells (Bruzzone et al. 2003; Evans and Martin 2002; Marziano et al. 2003; White 2000).

Gap junctional communication has been implicated in several hereditary human diseases, like cataracts, X-linked Charcot-Marie Tooth disease, skin disorders and sensorineural hearing loss (Kelsell et al. 1997; Rabionet et al. 2002; White and Paul 1999). Linkage of connexin gene mutations to these diverse pathologies improves understanding of the in vivo functions of connexins. Expression patterns of several connexins have been described in the inner ear, where they play important roles in several processes of auditory transduction, such as recycling of K^+ ions in the endolymph and exchange of metabolites between supporting cells (Bruzzone et al. 2003; Kelley et al. 2000; Kikuchi et al. 1995, 2000; Marziano et al. 2003; Richard et al. 1998). Cx26, Cx30 and Cx31 are all expressed in the cochlear supporting cells, and mutations in these connexins have been associated with both syndromic and non-syndromic forms of genetic deafness (Bruzzone et al. 2003; Marziano et al. 2003; Petit et al. 2001; Rabionet et al. 2002) (http://www.crg.es/deafness/).

Sensorineural deafness affects one child in 1,000 at birth and 35–40% of these cases have a genetic basis (Kenna et al. 2001; Lefebvre and Van De Water 2000; Rabionet et al. 2000). Approximately 80% of genetic

G. Meşe · E. Londin · T. W. White
Graduate Program in Genetics, State University of New York,
BST 5-147,
Stony Brook, NY 11794-8661, USA

R. Mui · P. R. Brink · T. W. White (✉)
Department of Physiology and Biophysics, State University of New York,
BST 5-147,
Stony Brook, NY 11794-8661, USA
e-mail: thomas.white@sunysb.edu
Tel.: +1-631-4449683
Fax: +1-631-4443432

deafness is of the non-syndromic form, presenting with impaired hearing and no other clinical features (Estivill et al. 1998; Kenna et al. 2001; Kenneson et al. 2002). Mutations in the *GJB2* locus were found to be associated with pathogenesis of most autosomal recessive non-syndromic hearing loss (DFNB1), autosomal dominant non-syndromic hearing loss (DFNA3) and sporadic congenital deafness (Estivill et al. 1998; Kelsell et al. 1997; Kenneson et al. 2002; Rabionet et al. 2000). The mutations involved in deafness include nonsense or insertion/deletion, which results in the premature termination of protein translation, and missense mutations that alter a single amino acid. The most frequently found mutation in the Cx26 gene, *GJB2*, involved in 70–85% of Cx26-related deafness is 35delG (Zelante et al. 1997). Deletion of this one base results in a frameshift, introducing a premature stop codon and resulting in termination of protein translation. In addition to 35delG, over 50 missense mutations have been identified throughout *GJB2* (http://www.crg.es/deafness/) and some of them have been analyzed for functional properties. For example, Bruzzone et al. (2003) have analyzed the F83L polymorphism and V84L mutation, and they have shown that this mutant and polymorphism were able to form functional channels and that voltage-gating properties of F83L, V84L and wild-type Cx26 channels shared similar features. In another study by D'Andrea et al. (2002), six more Cx26 mutations have been analyzed, and G12V, S19T, M34T and R184P were not able to form active channels in transfected HeLa cells, while L90P and R127H showed very low levels of Lucifer Yellow transfer. Thus, not all deafness-causing mutations resulted in loss of activity and the functional mutations may provide insights into the specific function of the Cx26 gene in hearing.

In the present study, we have characterized five missense mutations [T8M, R143W, V153I, N206S and L214P (Brobby et al. 1998; Hamelmann et al. 2001; Kenna et al. 2001; Marlin et al. 2001)] associated with nonsyndromic hearing loss. We tested their ability to form functional channels in the paired *Xenopus* oocyte expression system. We found that three of these Cx26 mutants were unable to form functional channels, while the other two formed partially functional channels with altered voltage-gating properties. Thus, not all deafness-associated Cx26 mutations produce a loss of ionic coupling and, although not directly examined in the present study, the exchange of larger metabolites through Cx26 gap junction channels may also be required to prevent deafness.

Materials and methods

Molecular cloning

Wild-type human Cx26 was subcloned into the *Xba*I-*Bam*HI sites of the pCS2+ expression vector for functional expression studies in *Xenopus* oocytes. The following mutations were introduced using standard oligonucletide directed mutagenesis: L214P, N206S, R143W, T8M and V153I, and then subcloned into the pCS2+ vector. Mutated constructs were sequenced on both strands.

In vitro transcription, oocyte microinjection and pairing

All constructs were linearized using *Not*I, and the Message Machine kit (Ambion) was used to transcribe RNAs. Adult *Xenopus* females were anesthetized and ovarian lobes were surgically removed. To collect stage V–VI oocytes, lobes were defollicullated in a solution containing 50 mg/ml collagenase B and 50 mg/ml hyaluronidase in modified Barth's medium (MB) without Ca^{++}. Cells were subsequently cultured in MB medium at room temperature. For physiological analysis, cells were first injected with 10 ng of an antisense *Xenopus* Cx38 oligonucleotide to eliminate the conductance of endogenous intercellular channels. After a 24-h incubation, antisense oligonucleotide-injected oocytes were re-injected with specified Cx26 RNAs (5 ng/cell) or H_2O as a negative control. Subsequently, vitelline envelopes were removed in a hypertonic solution, and the oocytes were manually paired with the vegetal poles apposed in MB medium containing Petri dishes.

Dual-cell voltage clamp

Following an overnight incubation, gap junction coupling between oocyte pairs was measured using dual-cell voltage-clamp techniques (Bruzzone et al. 2003; Spray et al. 1981). Current and voltage electrodes (1.2 mm diameter, omega dot; Glass Company of America, Millville, N.J., USA) were pulled to a resistance of 1–2 MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with a solution containing 3 M KCl, 10 mM EGTA and 10 mM HEPES, pH 7.4. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instruments, Foster City, Calif., USA) controlled by a PC-compatible computer through a Digidata 1320A interface (Axon Instruments). The pCLAMP 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms. Current outputs were filtered at 50 Hz and the sampling interval was 10 min. For simple measurements of junctional conductance, both cells of a pair were initially clamped at −40 mV to ensure zero transjunctional potential and alternating pulses of ±10–20 mV were imposed to one cell. Current delivered to the cell clamped at −40 mV during the voltage pulse was equal in magnitude to the junctional current, and was divided by the voltage to yield the conductance.

To determine voltage-gating properties, transjunctional potentials (V_j) of opposite polarity were generated by hyperpolarizing or depolarizing one cell in 20 mV steps (over a range of ±120 mV), while clamping the second cell at −40 mV. Currents were measured at the end of the voltage pulse, at which time they approached steady state (I_{jss}) for the majority of tested voltages, and the macroscopic conductance (G_{jss}) was calculated by dividing I_{jss} by V_j. G_{jss} was then normalized to the values determined at ±20 mV, and plotted against V_j. Data describing the relationship of G_{jss} as a function of V_j were analyzed using Origin 6.0 (Microcal Software, Northampton, Mass., USA) and fit to a Boltzmann relation of the form: $G_{jss}=(G_{jmax}-G_{jmin})/1+\exp[A(V_j-V_0)]+G_{jmin}$, where G_{jss} is the steady-state junctional conductance, G_{jmax} (normalized to unity) is the maximum conductance, G_{jmin} is the residual conductance at large values of V_j, and V_0 is the transjunctional voltage at which $G_{jss}=(G_{jmax}-G_{jmin})/2$. The constant A ($=nq/kT$) represents the voltage sensitivity in terms of gating charge as the equivalent number (n) of electron charges (q) moving through the membrane, k is the Boltzmann constant, and T is the absolute temperature.

Preparation of oocyte samples for Western blot analysis

Oocytes were collected and lysed in 1 ml of lysis buffer containing 5 mM Tris pH 8.0, 5 mM EDTA and protease inhibitors (White et al.

1992). The oocytes were sheared using a 1-ml syringe and a series of needles of diminishing caliber (20, 22, 26 Ga). After homogenization extracts were centrifuged at 1,000 *g* at 4°C for 5 min in a microcentrifuge. The supernatant was transferred to a new tube and centrifuged at 100,000 *g* (45,000 rpm) at 4°C for 30 min in a TLC45 ultracentrifuge. Membrane pellets were dissolved in SDS sample buffer and samples were separated on 15% SDS gels and then transferred to nitrocellulose membranes. Blots were blocked with 3% BSA in PBS with 0.02% NaN_3 for 1 h and then probed with polyclonal antibody anti-rat Cx26 (Zymed) at a 1:1,000 dilution. Band intensities were quantified using Kodak 1D Image Analysis software (Eastman Kodak, Rochester, N.Y., USA). Values from three independent experiments were normalized to the mean value of band intensity of the wild-type Cx26 sample. Statistical analyses between wild-type Cx26, T8M and N206S levels were performed using the one-way ANOVA test with a significance level of 0.05.

Results

Formation of functional gap junction channels by T8M and N206S

We have used the *Xenopus* oocyte expression system to analyze the ability of Cx26 mutations associated with DFNB1 to form functional gap junction channels. We tested five missense mutations, T8M, R143W, V153I, N206S, and L214P as depicted in Fig. 1. Oocytes were pretreated with an antisense oligonucleotide against *Xenopus* Cx38 to reduce the background conductance provided by endogenous gap junctions in this system (Barrio et al. 1991; Bruzzone et al. 1993). After mutant or wild-type Cx26 cRNA injection, oocytes were paired and intercellular currents were quantified and analyzed. Water injected antisense oligonucleotide treated cells were used as negative controls.

As expected, wild-type Cx26 formed functional channels with junctional conductance that was 100-fold higher than background levels recorded in antisense-oligonucleotide-injected control pairs (Fig. 2a). In contrast, the junctional conductances of the R143W, V153I and L214P mutants were equal to or less than background levels ($P>0.05$, Student's unpaired *t*-test), establishing the inabilities of these mutants to form functional intercellular channels (Fig. 2a). Oocyte pairs injected with the two remaining mutants, T8M and N206S, developed junctional coupling above background levels, N206S had a mean conductance five-fold higher than background, while T8M conductance was twofold higher than negative controls ($P<0.05$, compared with antisense oligonucleotide injected controls). However, both functional mutants had macroscopic conductance significantly lower than wild-type Cx26 ($P<0.05$, compared with Cx26). A scatter plot of all the data revealed that the incidence of coupling above background was 57% for N206S, and 31% for T8M, both of which were lower than the 100% coupling incidence of wild-type Cx26 (Fig. 2b). Thus the T8M and N206S mutations retained the ability to form functional gap junction channels, but with a reduction in both the magnitude and incidence of coupling compared with wild-type Cx26.

Synthesis of mutant connexins in *Xenopus* oocytes

The reduction of intercellular coupling for T8M and N206S may have resulted from reduced levels of protein synthesis or stability for these mutated Cx26 isoforms. To analyze translation of the injected RNAs, oocytes that had received equal quantities of wild-type Cx26, T8M and N206S cRNAs were collected after dual-cell voltage clamp and analyzed by Western blot using a Cx26-specific antibody. Immunoblot analysis of *Xenopus* oocytes injected with the specified RNAs showed that both mutated constructs were expressed in oocytes, at similar levels to wild-type Cx26 (Fig. 3a). This observation was verified by plotting the mean densitometry values of replicate blots ($n=3$) which failed to reveal any significant differences (one-way ANOVA, $P>0.05$) between the expression levels

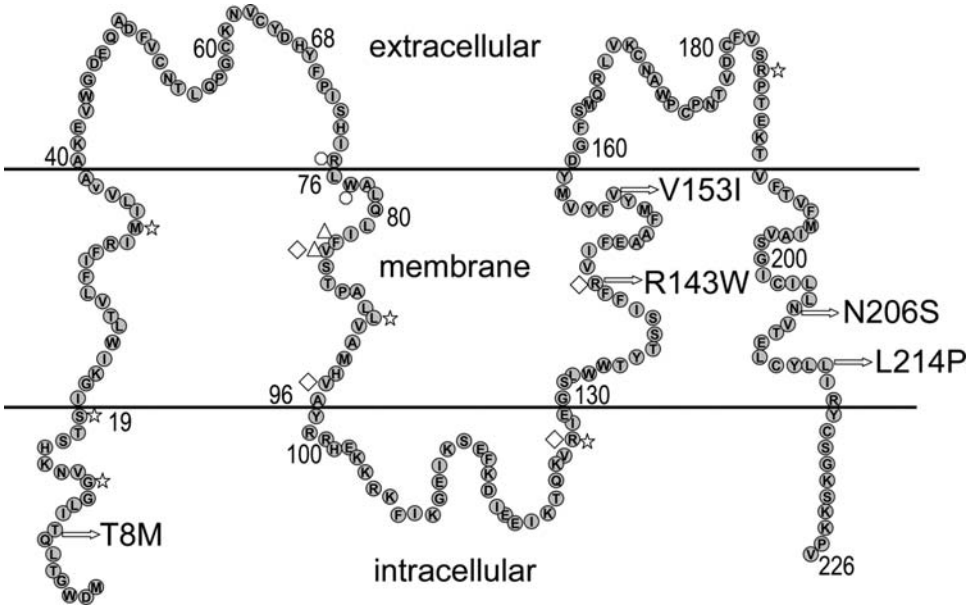

Fig. 1 Schematic representation of Cx26 relative to the plasma membrane showing the mutations analyzed in this paper. The *arrows* indicate the five missense mutations analyzed in the present study, T8M, R143W, V153I, N206S and L214P. For comparison, the *triangles* show mutations previously investigated by (Bruzzone et al. 2003), the *circles* specify the mutations evaluated by (Richard et al. 1998), the *stars* designate mutations tested by (D'Andrea et al. 2002) and *diamonds* are the mutations analyzed by (Wang et al. 2003)

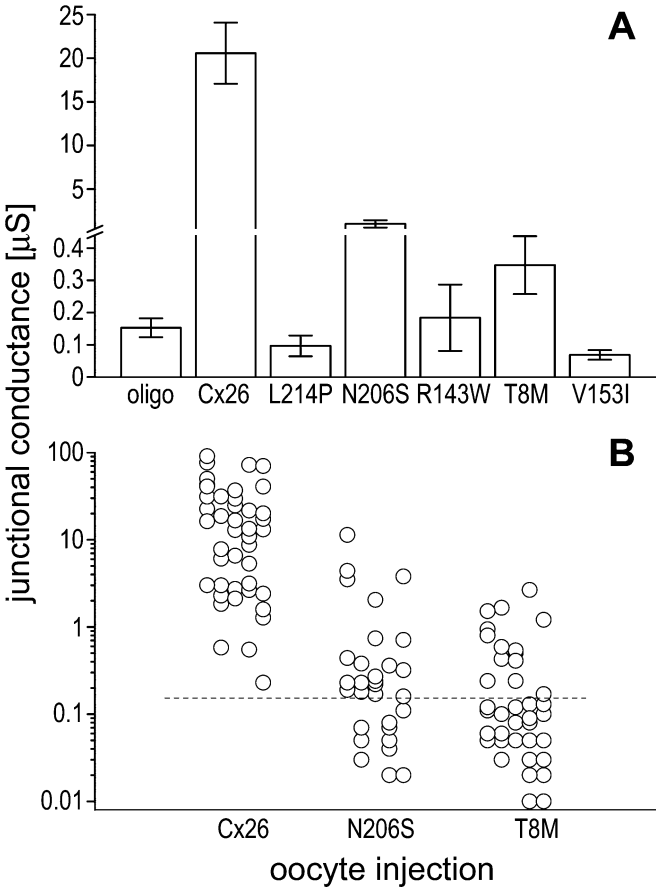

Fig. 2a, b Analysis of the ability of Cx26 mutants associated with DFNB1 to form functional gap junction channels. **a** The junctional conductance of wild-type Cx26 (n=43), T8M (n=38), R143W (n=10), V153I (n=15), N206S (n=30) and L214P (n=12) mutants were compared with that of antisense oligonucleotide injected control oocytes (n=43). R143W-, V153I- and L214P-injected cell pairs produced junctional conductance equal to or less than the background levels measured in antisense oligonucleotide treated control pairs. Wild-type Cx26 produced junctional conductance 100-fold higher than background, while T8M and N206S showed lower junctional conductance levels than wild-type channels, but still twofold and five-fold higher than background level, respectively. **b** *Scatter plot* showing the incidence of coupling for wild-type Cx26, T8M and N206S channels. The *dashed line* indicates the mean level of background conductance. For wild type, the incidence of coupling was 100% and the magnitude of coupling was higher than either mutant. In contrast, the incidence of coupling higher than background was 31% for T8M and 57% for N206S

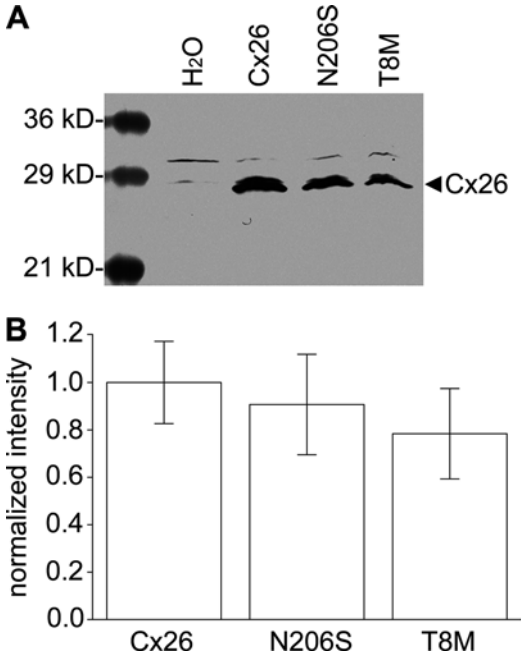

Fig. 3a, b Immunoblot analysis of *Xenopus* oocyte extracts indicates that wild-type and mutant connexins are equally synthesized. **a** Equal numbers of oocytes from each cRNA-injected group were pooled and membrane fractions were prepared. Following resuspension in sample buffer, 20% of the preparation was loaded in each lane for each condition. The Western blot was probed with an affinity-purified rabbit anti-Cx26 antibody. Despite resulting in both a lower magnitude and reduced incidence of coupling, the T8M and N206S mutants had similar expression levels to wild-type Cx26 protein. **b** Quantitation of wild-type Cx26, N206S and T8M expression. Relative synthesis of wild-type and mutant connexins were not statically different (P>0.05). Values are the mean±SE of three independent experiments

of wild-type Cx26, or the T8M and N206S mutations (Fig. 3b). These results suggest that the reductions in the incidence and magnitude of coupling were not simply due to reduced protein translation or stability with the two functional mutant constructs.

Voltage-gating properties of T8M and N206S channels

We next analyzed the gating properties of the two functional mutants, N206S and T8M. To achieve this goal, we examined the response of wild-type, N206S and T8M to a range of hyperpolarizing and depolarizing transjunctional voltages. Figure 4 shows the junctional currents (I_j) and the relationship between steady-state junctional conductance (G_j) and transjunctional voltage (V_j) for wild-type Cx26 and the two mutants. As previously reported (Barrio et al. 1991; Bruzzone et al. 2003), at potentials greater than ±80 mV, junctional currents of wild-type Cx26 pairs decayed slowly and asymmetrically over the course of the voltage step (Fig. 4a). In contrast, the T8M and N206S channels illustrated altered gating characteristics from wild-type Cx26. For N206S, the decay in I_j was similar to Cx26, but appeared qualitatively slower than wild-type (Fig. 4c). Similar to wild-type Cx26, N206S pairs also showed asymmetry in their gating. The I_j of T8M pairs had very distinctive characteristics. For positive V_j values, the junctional currents initially increased over the duration of the voltage step, showing a slight decay only at the highest values of V_j. For negative values of V_j, the junctional current also increased over the time of the voltage step (Fig. 4e).

To quantify differences in the magnitude of the voltage gating, conductance values at the end of the imposed pulse were normalized, plotted against V_j and were fit to a Boltzmann equation (Table 1). These data revealed that for wild-type Cx26 the transjunctional voltage required to

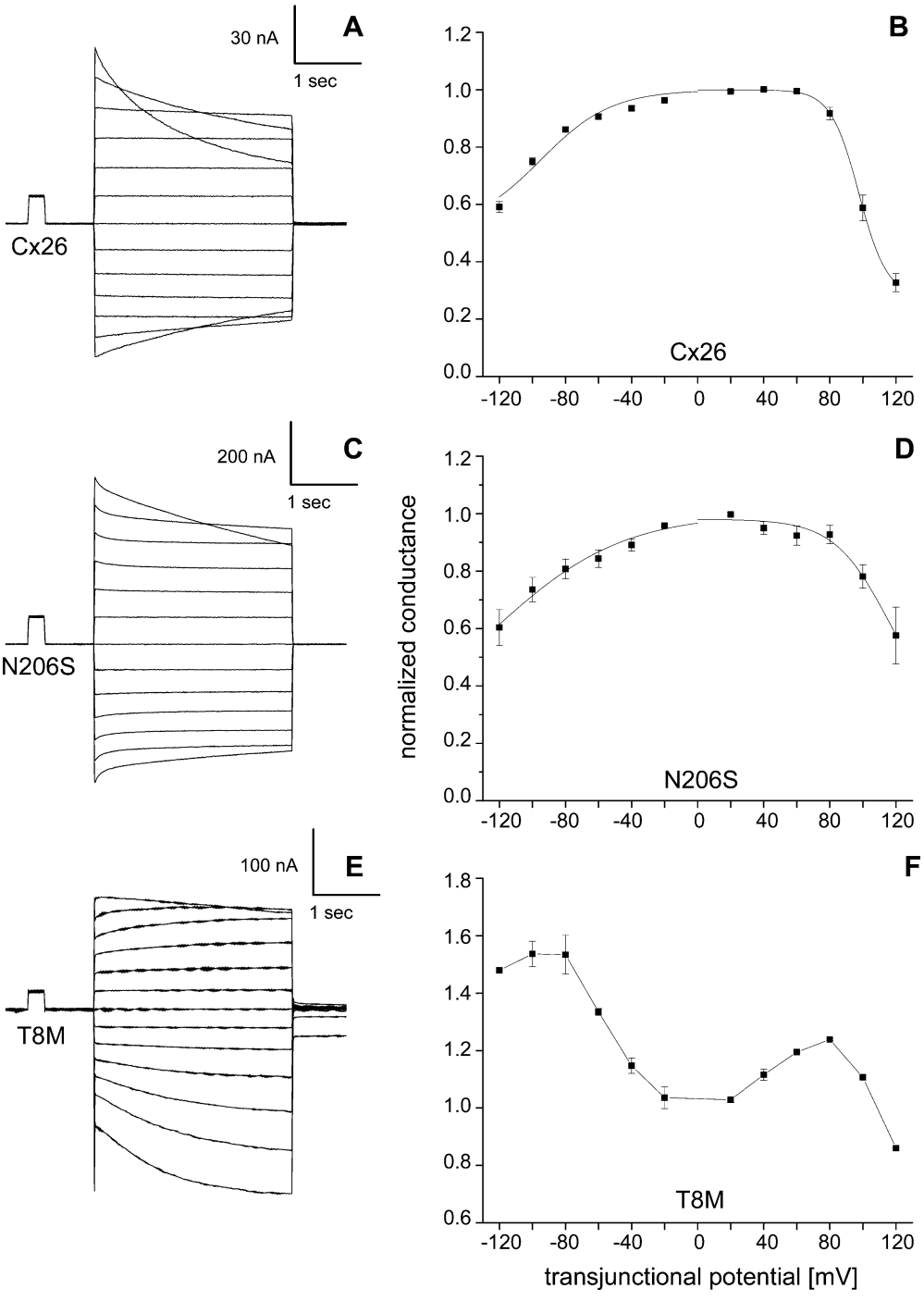

Fig. 4a–f Gating properties of wild-type Cx26, T8M and N206S channels. The change in junctional currents (I_j) induced by transjunctional voltage (V_j) was plotted as a function of time for mutant and wild-type channels. **a** For Cx26 channels, I_js decreased slowly for V_js ≥±80 mV. **b** The decay in I_js for N206S over time was slower than wild-type. **c** In contrast, T8M has showed increased I_js for V_j≤+120 mV with the exception of V_j=+120 mV, where I_j slightly decreased. For all negative V_js, the I_j increased over the course of the voltage step. **d–f** The relationship of V_j to normalized steady-state conductance (G_{jss}). The smooth line shows the best fits to the Boltzmann equation and the parameters are given in Table 1. N206S channels showed a reduced sensitivity to voltage compared with wild-type channels. T8M channels, in contrast, had characteristics markedly different from wild-type channels that could not be fit by the Boltzmann equation

yield a conductance midway (V_0) between maximum (G_{jmax}) and minimum conductance (G_{jmin}) had a value of 98 mV for positive V_js, and a value of −95 mV for negative V_js. Conversely, the V_0s for N206S were slightly larger, 116 mV for positive values and −123 mV for negative values, consistent with a reduced sensitivity to voltage for this mutant channel. As the T8M data were not possible to fit with the Boltzmann equation, we could not quantify its voltage dependence.

To quantify differences in the kinetics of the voltage gating, I_js were plotted against time and fit to a single exponential decay equation. The mean time constant (τ) for the rate of decline in I_j over time for wild-type Cx26 at a V_j of +120 mV was 0.99±0.07 s (mean±SE, n=11, Fig. 5a). In contrast, N206S channels closed significantly slower at V_j of +120 mV, the average time constant for channel closure was 1.99±0.26 s (n=7, P<0.05, Fig. 5b). As the T8M currents were not possible to fit with the exponential decay equation, we couldn't quantify the kinetics of its voltage dependent closure. In any case, these data clearly show that the magnitude and kinetics of the voltage-gating properties of T8M and N206S both differ quantitatively from those of wild-type Cx26 channels.

Table 1 Comparison of the Boltzmann parameters for Cx26 and N206S gap junctional channels in the *Xenopus* oocyte expression system. Junctional conductance (G_j) developed between pairs of *Xenopus* oocytes was measured by dual voltage clamp in response to increasing transjunctional potentials (V_j) of opposite polarity and normalized to the conductance measured at a V_j of ±20 mV (G_{jmax}, set as unity), as described in the Materials and methods section. Data were fit to a Boltzmann equation of the form given in the text. G_{jmin} is the minimum conductance value as estimated from the Boltzmann fit, and V_0 is the voltage at which half-maximal decrease of G_j is measured. The cooperativity constant (A), reflecting the voltage sensitivity of the channel, reflects the equivalent number of electron charges moving through the transjunctional field. The plus and minus signs for V_j refer to the polarity of the transjunctional potential

	V_j	V_0	G_{jmin}	A
Cx26	+	98	0.27	0.12
Cx26	−	−95	0.51	0.05
N206S	+	116	0.27	0.06
N206S	−	−123	0.20	0.03

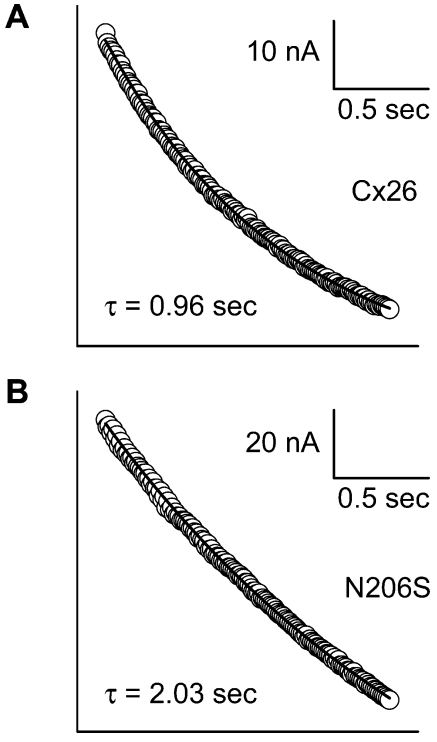

Fig. 5a, b Comparative kinetic analysis of voltage-dependent channel closure for wild-type Cx26 (**a**) and N206S (**b**) channels. Representative current decays after the imposition of a V_j of +120 mV could be well fit by a monoexponential relation with the time constants (τ) indicated. The mean time channel closure constant (τ) for N206S was significantly higher than wild-type Cx26 channels (mean values are given in the text)

Discussion

Functional analysis of Cx26 mutations linked to deafness may improve the understanding of the role played by intercellular communication in cochlear homeostasis. Our hypothesis is that deafness caused by mutations in Cx26 is not due to a simple loss of ionic junctional communication, but rather by changes in junctional permeability between cochlear cells induced by selective loss of one out of the three connexin isoforms normally available. Thus, one can predict that a subset of the missense Cx26 mutations causing deafness may retain ionic coupling, but show altered permeability to larger solutes. Indeed, such mutations could provide valuable tools for probing the type of molecules cochlear cells need to share to preserve organ homeostasis and prevent hearing loss. Toward this goal, we have analyzed five Cx26 missense mutations associated with DFNB1 to identify disease-causing mutants that retained functional activity. We found that while the R143W, V153I and L214P mutants completely lost their abilities to form functional gap junction channels, the T8M and N206S mutants retained the ability to provide intercellular communication, although at lower conductance levels, and with gating properties that differed from wild-type Cx26 channels.

Many deafness associated missense Cx26 mutations studied thus far resulted in a complete loss of ability to form functional channels (Martin et al. 1999; White et al. 1998). These studies indicated that the failure of many mutants to form functional channels depended on improper targeting to the plasma membrane, reduced protein stability, failure to oligomerize into hemichannels, or impaired docking into complete channels (Thomas et al. 2004; Thonnissen et al. 2002; White 2000). Three of the five mutants we tested failed to induce intercellular coupling in paired *Xenopus* oocytes. Our biochemical analysis showed that all of the mutants were expressed at similar levels to wild-type Cx26 (Fig. 3, and data not shown). Thus the inability to form functional channels was not likely a result of reduced protein expression. Further experimentation will be required to determine at which level of assembly these mutations fail; possibilities include altered trafficking, reduced unitary conductance, or reduced open probability of the channels. Further investigation of these same parameters may also help to explain why the T8M and N206S mutations exhibited a reduced magnitude of coupling despite showing similar levels of synthesis to wild-type Cx26.

Our data demonstrated that the V153I mutation was unable to form functional channels. However, this mutation has been alternatively reported as either a polymorphism or a recessive pathogenic allele. Marlin et al. (2001) identified the V153I mutation in normal-hearing parents in association with 35delG mutation on the other allele, so that they considered the V153I change as a polymorphism. In addition, Bayazit et al. (2003) claimed V153I as a polymorphism rather than a pathogenic change. In contrast, Wu et al. (2002) identified this mutation in deaf subjects in a compound heterozygous state with the T8M missense mutation and proposed it to be a pathogenic mutation. Moreover, Rickard et al. (2001) identified a deaf woman homozygous for V153I, although linkage analysis has not yet established pathogenicity. Our study may support the view that V153I can be a pathogenic change, as V153I mutants could not form functional channels, producing junctional conductance lower than background

levels. R143W and L214P have been shown to be pathogenic, as two studies have identified deaf subjects carrying homozygous R143W missense mutations and the R143W mutation as compound heretozygous with L214P (Brobby et al. 1998; Hamelmann et al. 2001). This is consistent with the results in this study as these mutants had junctional conductance less than or equal to background levels.

A few Cx26 mutations have previously been reported to retain some level of functional properties (Bruzzone et al. 2003; D'Andrea et al. 2002; Wang et al. 2003). In this study, the T8M and N206S mutations displayed some level of functional activity, having junctional conductance higher than background levels. Several studies indicated that these mutants are pathogenic (Kenna et al. 2001; Marlin et al. 2001; Wu et al. 2002) and are inherited in an autosomal recessive manner (Kenna et al. 2001). As described above, T8M was found in compound heterozygotes with the V153I missense mutation in deaf subjects (Kenna et al. 2001; Marlin et al. 2001). Patients carrying the N206S mutation were either heterozygous for this change or in compound heterozygotes state with 35delG or V37I (Kenna et al. 2001; Marlin et al. 2001; Wu et al. 2002). In these studies, patients carrying the N206S mutation were observed to exhibit mild hearing loss, causing the authors to speculate that this mutation may not severely compromise the gap junctional communication system in the inner ear (Kenna et al. 2001). This is consistent with our results, as the N206S mutants show similar voltage-gating properties to wild-type channels.

In the inner ear, there are two main gap junctional systems: the non-sensory epithelial cell system including cochlear supporting cells and root cells, and the connective tissue cell system in the cochlear lateral wall comprised of fibrocytes in the spiral ligament, and cells in the stria vascularis (Forge et al. 2002; Kikuchi et al. 2000). These gap junction systems form a network that functionally couples these cells in the inner ear and is thought to provide the re-circulation of K^+ ions back to the endolymph after activation of auditory transduction (Forge et al. 2002; Kemperman et al. 2002; Kikuchi et al. 2000; Wilcox et al. 2000). Cx26 expression corresponded to the distribution of gap junctions throughout the inner ear (Kikuchi et al. 1994; Kikuchi et al. 2000). In addition, Cx30 and Cx31 were also expressed in the inner ear, and Cx30 co-localizes with Cx26 (Marziano et al. 2003; Rabionet et al. 2000). Mutations in these connexin genes were also found to be involved in genetic deafness (Bruzzone et al. 2003; Marziano et al. 2003; Petit et al. 2001; Rabionet et al. 2002).

Our results indicated that some of the Cx26 mutants were still able to form functional channels since the junctional conductance of T8M and N206S was higher than that of water-injected oocytes. Given that T8M and N206S channels were electrically coupled, and that potassium is the dominant permeant ion of cytoplasm, it is likely that these mutant channels retain high permeability to K^+ ions, as has been demonstrated for all gap junction channels (Valiunas et al. 2002). Thus, deafness associated with these missense mutations in the Cx26-encoding gene may not solely depend on the loss of K^+ coupling and K^+ re-circulation. Instead, these mutations may interfere with the coupling of larger biochemical solutes in the inner ear. Cohen-Salmon et al. (2002) have shown that targeted deletion of Cx26 in the inner ear resulted in degeneration of cells within the cochlea, including inner and outer hair cells and in distortion of the organ of Corti. However, Cx30 distribution and expression within the inner ear was normal in these mice. Furthermore, Cx26 and Cx30 have similar functional properties and Kudo et al. (2003) suggested that connexins especially Cx30 in the cochlea might compensate the K^+ cycling since K^+ can also efficiently pass through these junctional channels (Valiunas et al. 1999). Therefore, hearing loss resulting from functional Cx26 mutations may be due to failure in the passage of other small metabolites or secondary messengers rather than K^+.

Many studies have shown that gap junctional channels formed by different connexins have distinctive permeability for larger solutes like cyclic nucleotides and inositol phosphates (Manthey et al. 2001; Valiunas et al. 2002). In addition, Manthey et al. (2001) documented permeation differences between Cx26 and Cx30, showing that they display differences in the diffusion of dyes of the same size and charge as the larger metabolites. Therefore, loss of Cx26 in the cochlea may alter the exchange of certain metabolites or secondary messengers between cells in the inner ear. Further study of partially functional channels formed by V84L (Bruzzone et al. 2003), N206S and T8M mutants (present work) may improve the characterization of molecules exchanged within the gap junctional systems in the inner ear that fail to pass through the mutant gap junctions resulting in deafness. One potential candidate molecule is implied by recent studies of mice with a conditional inactivation of Cx26 in the inner ear, suggesting that Cx26 may play an essential role in preventing the accumulation of glutamate that leads to apoptotic cell death (Cohen-Salmon et al. 2002). Glutamate is normally taken up by the supporting sensory epithelium and the inner hair cells, and could be spatially buffered through the Cx26 gap junctional syncitium as has been proposed for K^+ ions. Demonstration of a reduction in glutamate permeability in the functional Cx26 mutants awaits further experimentation.

Acknowledgements Supported by NIH grants DC06652 (TWW) and GM55263 (PRB).

References

Barrio LC, Suchyna T, Bargiello T, Xu LX, Roginski RS, Bennett MV, Nicholson BJ (1991) Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by applied voltage. Proc Natl Acad Sci USA 88:8410–8414

Bayazit YA, Cable BB, Cataloluk O, Kara C, Chamberlin P, Smith RJ, Kanlikama M, Ozer E, Cakmak EA, Mumbuc S, Arslan A (2003) *GJB2* gene mutations causing familial hereditary deafness in Turkey. Int J Pediatr Otorhinolaryngol 67:1331–1335

Brobby GW, Muller-Myhsok B, Horstmann RD (1998) Connexin 26 R143 W mutation associated with recessive nonsyndromic sensorineural deafness in Africa. N Engl J Med 338:548–550

Bruzzone R, Haefliger JA, Gimlich RL, Paul DL (1993) Connexin40, a component of gap-junctions in vascular endothelium, is restricted in its ability to interact with other connexins. Mol Biol Cell 4:7–20

Bruzzone R, Veronesi V, Gomes D, Bicego M, Duval N, Marlin S, Petit C, D'Andrea P, White TW (2003) Loss-of-function and residual channel activity of connexin26 mutations associated with non-syndromic deafness. FEBS Lett 533:79–88

Cohen-Salmon M, Ott T, Michel V, Hardelin JP, Perfettini I, Eybalin M, Wu T, Marcus DC, Wangemann P, Willecke K, Petit C (2002) Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. Curr Biol 12:1106–1111

D'Andrea P, Veronesi V, Bicego M, Melchionda S, Zelante L, Di Iorio E, Bruzzone R, Gasparini P (2002) Hearing loss: frequency and functional studies of the most common connexin26 alleles. Biochem Biophys Res Commun 296:685–691

Estivill X, Fortina P, Surrey S, Rabionet R, Melchionda S, D'Agruma L, Mansfield E, Rappaport E, Govea N, Mila M, Zelante L, Gasparini P (1998) Connexin-26 mutations in sporadic and inherited sensorineural deafness. Lancet 351:394–398

Evans WH, Martin PE (2002) Gap junctions: structure and function (Review). Mol Membr Biol 19:121–136

Forge A, Becker D, Casalotti S, Edwards J, Marziano N, Nickel R (2002) Connexins and gap junctions in the inner ear. Audiol Neurootol 7:141–145

Hamelmann C, Amedofu GK, Albrecht K, Muntau B, Gelhaus A, Brobby GW, Horstmann RD (2001) Pattern of connexin 26 (GJB2) mutations causing sensorineural hearing impairment in Ghana. Hum Mutat 18:84–85

Kelley PM, Cohn E, Kimberling WJ (2000) Connexin 26: required for normal auditory function. Brain Res Brain Res Rev 32:184–188

Kelsell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, Parry G, Mueller RF, Leigh IM (1997) Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature 387:80–83

Kemperman MH, Hoefsloot LH, Cremers CW (2002) Hearing loss and connexin 26. J R Soc Med 95:171–177

Kenna MA, Wu BL, Cotanche DA, Korf BR, Rehm HL (2001) Connexin 26 studies in patients with sensorineural hearing loss. Arch Otolaryngol Head Neck Surg 127:1037–1042

Kenneson A, Van Naarden BK, Boyle C (2002) GJB2 (connexin 26) variants and nonsyndromic sensorineural hearing loss: a HuGE review. Genet Med.4:258–274

Kikuchi T, Adams JC, Paul DL, Kimura RS (1994) Gap junction systems in the rat vestibular labyrinth: immunohistochemical and ultrastructural analysis. Acta Otolaryngol 114:520–528

Kikuchi T, Kimura RS, Paul DL, Adams JC (1995) Gap junctions in the rat cochlea: immunohistochemical and ultrastructural analysis. Anat Embryol (Berl) 191:101–118

Kikuchi T, Adams JC, Miyabe Y, So E, Kobayashi T (2000) Potassium ion recycling pathway via gap junction systems in the mammalian cochlea and its interruption in hereditary nonsyndromic deafness. Med Electron Microsc 33:51–56

Kudo T, Kure S, Ikeda K, Xia AP, Katori Y, Suzuki M, Kojima K, Ichinohe A, Suzuki Y, Aoki Y, Kobayashi T, Matsubara Y (2003) Transgenic expression of a dominant-negative connexin26 causes degeneration of the organ of Corti and nonsyndromic deafness. Hum Mol Genet 12:995–1004

Lefebvre PP, Van De Water TR (2000) Connexins, hearing and deafness: clinical aspects of mutations in the connexin 26 gene. Brain Res Brain Res Rev 32:159–162

Manthey D, Banach K, Desplantez T, Lee CG, Kozak CA, Traub O, Weingart R, Willecke K (2001) Intracellular domains of mouse connexin26 and −30 affect diffusional and electrical properties of gap junction channels. J Membr Biol 181:137–148

Marlin S, Garabedian EN, Roger G, Moatti L, Matha N, Lewin P, Petit C, Denoyelle F (2001) Connexin 26 gene mutations in congenitally deaf children: pitfalls for genetic counseling. Arch Otolaryngol Head Neck Surg 127:927–933

Martin PE, Coleman SL, Casalotti SO, Forge A, Evans WH (1999) Properties of connexin26 gap junctional proteins derived from mutations associated with non-syndromal heriditary deafness. Hum Mol Genet 8:2369–2376

Marziano NK, Casalotti SO, Portelli AE, Becker DL, Forge A (2003) Mutations in the gene for connexin 26 (*GJB2*) that cause hearing loss have a dominant negative effect on connexin 30. Hum Mol Genet 12:805–812

Oshima A, Doi T, Mitsuoka K, Maeda S, Fujiyoshi Y (2003) Roles of Met-34, Cys-64, and Arg-75 in the assembly of human connexin 26. Implication for key amino acid residues for channel formation and function. J Biol Chem 278:1807–1816

Petit C, Levilliers J, Hardelin JP (2001) Molecular genetics of hearing loss. Annu Rev Genet 35:589–646

Rabionet R, Gasparini P, Estivill X (2000) Molecular genetics of hearing impairment due to mutations in gap junction genes encoding beta connexins. Hum Mutat 16:190–202

Rabionet R, Lopez-Bigas N, Arbones ML, Estivill X (2002) Connexin mutations in hearing loss, dermatological and neurological disorders. Trends Mol Med 8:205–212

Richard G, White TW, Smith LE, Bailey RA, Compton JG, Paul DL, Bale SJ (1998) Functional defects of Cx26 resulting from a heterozygous missense mutation in a family with dominant deaf-mutism and palmoplantar keratoderma. Hum Genet 103:393–399

Rickard S, Kelsell DP, Sirimana T, Rajput K, MacArdle B, Bitner-Glindzicz M (2001) Recurrent mutations in the deafness gene *GJB2* (connexin 26) in British Asian families. J Med Genet 38:530–533

Spray DC, Harris AL, Bennett MV (1981) Equilibrium properties of a voltage-dependent junctional conductance. J Gen Physiol 77:77–93

Thomas T, Telford D, Laird DW (2004) Functional domain mapping and selective trans-dominant effects exhibited by Cx26 disease-causing mutations. J Biol Chem 279:19157–19168

Thonnissen E, Rabionet R, Arbones L, Estivill X, Willecke K, Ott T (2002) Human connexin26 (GJB2) deafness mutations affect the function of gap junction channels at different levels of protein expression. Hum Genet 111:190–197

Valiunas V, Manthey D, Vogel R, Willecke K, Weingart R (1999) Biophysical properties of mouse connexin30 gap junction channels studied in transfected human HeLa cells. J Physiol 519:631–644

Valiunas V, Beyer EC, Brink PR (2002) Cardiac gap junction channels show quantitative differences in selectivity. Circ Res 91:104–111

Wang HL, Chang WT, Li AH, Yeh TH, Wu CY, Chen MS, Huang PC (2003) Functional analysis of connexin-26 mutants associated with hereditary recessive deafness. J Neurochem 84:735–742

White TW (2000) Functional analysis of human Cx26 mutations associated with deafness. Brain Res Rev 32:181–183

White TW, Bruzzone R (1996) Multiple connexin proteins in single intercellular channels: connexin compatibility and functional consequences. J Bioenerg Biomembr 28:339–350

White TW, Paul DL (1999) Genetic diseases and gene knockouts reveal diverse connexin functions. Annu Rev Physiol 61:283–310

White TW, Bruzzone R, Goodenough DA, Paul DL (1992) Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein Mp70. Mol Biol Cell 3:711–720

White TW, Deans MR, Kelsell DP, Paul DL (1998) Connexin mutations in deafness. Nature 394:630–631

Wilcox SA, Saunders K, Osborn AH, Arnold A, Wunderlich J, Kelly T, Collins V, Wilcox LJ, McKinlay Gardner RJ, Kamarinos M, Cone-Wesson B, Williamson R, Dahl HH (2000) High frequency hearing loss correlated with mutations in the *GJB2* gene. Hum Genet 106:399–405

Wu BL, Lindeman N, Lip V, Adams A, Amato RS, Cox G, Irons M, Kenna M, Korf B, Raisen J, Platt O (2002) Effectiveness of sequencing connexin 26 (GJB2) in cases of familial or sporadic childhood deafness referred for molecular diagnostic testing. Genet Med 4:279–288

Zelante L, Gasparini P, Estivill X, Melchionda S, D'Agruma L, Govea N, Mila M, Monica MD, Lutfi J, Shohat M, Mansfield E, Delgrosso K, Rappaport E, Surrey S, Fortina P (1997) Connexin26 mutations associated with the most common form of non-syndromic neurosensory autosomal recessive deafness (DFNB1) in Mediterraneans. Hum Mol Genet 6:1605–1609