Connexin mutation that causes dominant congenital cataracts inhibits gap junctions, but not hemichannels, in a dominant negative manner


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Summary
The connexin (Cx) 50, E48K, mutation is associated with a human dominant congenital cataract; however, the underlying molecular mechanism has not been characterized. The glutamate (E) residue at position 48 is highly conserved across animal species and types of connexins. When expressed in paired Xenopus oocytes, human (h) and chicken (ch) Cx50 E48K mutants showed no electrical coupling. In addition, this mutation acts in a dominant negative manner when paired hetero-typically or hetero-merically with wild-type Cx50, but has no such effect on Cx46, the other lens fiber connexin. A similar loss-of-function and dominant negative effect was observed using dye transfer assays in the same system. By using two different dye transfer methods, with two different tracer dyes, we found chCx50 E48K expressed in chicken lens embryonic fibroblast cells by retroviral infection similarly failed to induce dye coupling, and prevented wild-type chCx50 from forming functional gap junctions. In contrast to its effect on gap junctions, the E48K mutation has no effect on hemichannel activity when assayed using electrical conductance in oocytes, and mechanically induced dye uptake in cells. Cx50 is functionally involved in cell differentiation and lens development, and the E48K mutant promotes primary lens cell differentiation indistinguishable from wild-type chCx50, despite its lack of junctional channel function. Together the data show that mutations affecting gap junctions but not hemichannel function of Cx50 can lead to dominant congenital cataracts in humans. This clearly supports the model of intercellular coupling of fiber cells creating a microcirculation of nutrients and metabolites required for lens transparency.

Key words: Cataract, Connexin, Gap junction, Hemichannel, Lens

Introduction
Gap junctions, formed by connexin (Cx) molecules, are transmembrane channels allowing the exchange of small molecules (Mr≤1000 Da), such as metabolites, ions and second messengers between contacting cells. This type of cell-cell communication is crucial in maintaining normal cell and tissue functions (Goodenough et al., 1996). Gap junctions are formed by a family of proteins named connexins, with approximately 20 different members in humans (Willecke et al., 2002). Mutations of connexin genes are linked to multiple human diseases, including defective myelination diseases such as Charcot-Marie-Tooth disease (Cx32) (Ressot and Bruzzone, 2000), Pelizaeus-Merzbacher-like disease (Cx47) (Orthmann-Murphy et al., 2007), congenital sensorineural hearing loss (Cx26, Cx30, Cx30.3) (Petit et al., 2001) and oculodentodigital dysplasia (ODDD) (Cx43) (Paznekas et al., 2003).

The eye lens, suspended from the ciliary body, retains a stem cell population that continues to proliferate and differentiate throughout an organism’s life (Berry et al., 1999; Mathias et al., 1997). Fibers at the lens center are coupled with cells at the lens surface through a highly developed gap junction-mediated intercellular communication network. This extensive network is vital, because it facilitates ion and metabolite exchange throughout the avascular lens, maintaining osmotic and metabolic homeostasis and ultimately, lens transparency (Goodenough, 1992). Three connexins have been identified in the mammalian lens, Cx43, Cx46 and Cx50, of which Cx43 is specific to the lens epithelium, whereas Cx46 and Cx50 predominantly co-localize in lens fibers and form heteromeric connexons (Jiang and Goodenough, 1996). The physiological importance of lens gap junctions has been realized in the last decade through the identification of connexin mutations linked to lens congenital cataracts in humans and the lens phenotypes displayed in connexin-deficient mouse models. Multiple mutations of Cx46 and Cx50 have been identified that are directly linked to human autosomal congenital cataracts (reviewed by Gerido and White, 2004). Among these mutations, the Cx50 missense mutant E48K in the first extracellular loop domain was first mapped from linkage analysis of a three generation family of Pakistani origin (Berry et al., 1999). The functional properties of Cx50 E48K that may contribute to lens cataracts have not been characterized.

In gene knockout models, mice lacking either fiber-specific connexin, Cx46 or Cx50, develop lens cataracts (Gong et al., 1997; Rong et al., 2002; White et al., 1998). However, only Cx50-deficient mice develop smaller eyes, defined as microphthalmia (Rong et al., 2002; White et al., 1998) and exhibit a delayed lens-fiber maturation (Rong et al., 2002). We previously showed that only chicken (ch) Cx50 stimulates epithelial-fiber-cell differentiation and expression of major differentiation markers. The other two types of lens connexins do not (Gu et al., 2003). This stimulatory effect appears to be independent of intercellular coupling (Banks et al., 2007). These studies provide compelling evidence that Cx50 is functionally involved in lens development and fiber differentiation.
When expressed in oocytes (Beahm and Hall, 2002; Srinivas et al., 2005; Srinivas et al., 2006) and HeLa cells (Valiunas and Weingart, 2000), Cx50 was not only found to form gap junctions, but also hemichannels, the unapposed halves of gap junctions that form large pores to the extracellular environment. Morphological studies also suggest the possible existence of hemichannels in lens fibers in situ (Zampighi, 2003). Cx46 hemichannels are mechanosensitive and are postulated to assist lens accommodation by providing a path for volume flow as the lens changes shape (Bao et al., 2005).

Here we characterize the functional properties of Cx50 E48K, a mutation linked to a human congenital cataract. We show that the Cx50 E48K mutant does not form functional gap junctions but exerts a dominant negative effect when coexpressed or paired with wild-type Cx50, but not with Cx46. Moreover, this mutant is expressed on the cell surface and forms functional hemichannels similar to wild-type Cx50. Furthermore, chCx50 E48K promotes differentiation in a manner comparable to wild-type chCx50. These results suggest that the Cx50 E48K residue at the first extracellular loop domain is involved in gap junction, but not hemichannel, function. Impaired lens cell coupling due to the dominant negative function of E48K is likely to be the underlying mechanism for cataract development.

Results

Cx50 E48K is a loss-of-gap-junction function, dominant negative mutant

The E48K mutation is located in the first extracellular loop domain of Cx50, one of the two docking domains responsible for formation of functional gap junction channels (Fig. 1A). The first extracellular domain, as well as E48, is highly conserved across animal species, including human, rodent, ovine, bovine and chicken (Fig. 1B). E48 is also highly conserved between various types of connexin molecules (Fig. 1C). We first investigated whether the E48K mutation has any discernable effect on gap junction coupling in paired Xenopus oocytes (Fig. 2), a well-established assay system for gap junction formation (Barrio et al., 1991). Oocytes with endogenous junctional conductance that was eliminated through injection of antisense oligonucleotides (see Materials and Methods) were injected with either wild-type chCx50, chCx46 or the mutant chCx50 E48K cRNA. Another set of oocytes was injected with both wild-type chCx50 and chCx50 E48K cRNA or wild-type chCx46 and chCx50 E48K in 1:1 ratios. The total amount of cRNA injected was held constant for all oocytes examined. Paired oocytes were then analyzed for junctional conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance. Oocyte pairs expressing wild-type chCx50 or chCx46 in homotypic or heterotypic combinations exhibited robust electrical conductance.

This test also incidentally confirmed that the mutant Cx50 was expressed in these cells. When oocytes coexpressing both wild-type and mutant chCx50 were paired with those expressing chCx50 wild type, the junctional conductance was decreased by 80%, demonstrating that the E48K mutant inhibits wild-type chCx50 function in a dominant negative manner when expressed in the same cell (as would occur in heterozygotic patients). Consistent with the ability of the chCx50 mutant to pair heterotypically with wild-type chCx46, this dominant negative effect was not observed when chCx50 E48K was coexpressed with Cx46, the other lens fiber connexin, even when the mutant was present in both oocytes. This is a function of the heterotypic pairing interface, as oocytes heteromerically coexpressing chCx46 and chCx50 E48K did show reduced coupling when paired with oocytes expressing wild-type chCx50.

Quantitative dye transfer was also analyzed in paired oocytes using a previously developed method (Cao et al., 1998) (Fig. 2B). Analogous to the electrical conductance, dye transfer was evident between oocytes expressing wild-type Cx50 (chCx50wt:chCx50wt), but not between oocytes expressing the E48K mutant (chCx50E48K:chCx50E48K). The dominant negative function of E48K mutation was also evident from the absence of dye coupling between chCx50wt:chCx50wt and chCx50E48K:chCx50E48K pairs. The electrical conductance of the oocyte pairs used in dye coupling (indicated above each bar, Fig. 2B, right panel) is comparable to the values associated with the data shown in Fig. 2A except in the case of chCx50wt:chCx50wt and chCx50E48K:chCx50E48K, where the highest conducting pairs (7, 8 and 18 μS) were chosen for dye coupling experiments. Typically, dye coupling is not detected in oocyte pairs with conductances <5 μS, even in the case of wild-type connexins. In this case we biased the sample for the few high conductance pairs to determine if there were any changes in channel properties.

Fig. 1. E48K mutation of Cx50 is associated with human congenital cataracts. (A) A membrane topological diagram showing the location of E48K at the first extracellular domain of the chCx50 protein. (B,C) Sequence comparisons showing that E48 is a highly conserved amino acid residue of Cx50 across various animal species (B) and also between various human connexin isotypes (C).
Nevertheless, the average dye transfer was no higher than between cells expressing homomeric mutant pairs, and much lower than would have been expected based on average conductance of the pairs compared with wtCx50 pairings [we have observed dye coupling in oocytes coupled with wild-type connexins at the level of ~10 μS (E.B., M.M.T. and Q. Shi et al., unpublished)]. Together, the conductance and dye transfer measurements indicate that Cx50 E48K cannot form functional gap junctions and has a dominant negative effect on coexpressed Cx50, where the residual channels appear to have a lower permeability for dyes than wild-type channels.

As shown in Fig. 1B, the amino acid residue E48 is conserved across species, so we expected the inhibitory effect of this mutant on gap junctions to be generalizable to the human situation. This proved to be true, as similar to chCx50, oocyte pairs expressing wild-type human (h) Cx50 have robust electrical coupling but low or no coupling when expressed in heterotypic pairings, or heteromERICALLY, with hCx50 E48K, demonstrating a similar dominant negative effect for coexpressed Cx50, where the residual channels appear to have a lower permeability for dyes than wild-type channels.

The impact of the E48K mutation on gap junctions was also investigated in cells. Wild-type Cx50 and the E48K mutant proteins could be expressed efficiently in chicken lens embryonic fibroblast (CEF) cells by cell infection with recombinant retroviruses containing the respective DNA constructs. This approach permits the expression of exogenous connexins in virtually all cells at physiological levels (compared to lens tissue in situ) without overexpression (Jiang and Goodenough, 1998b). Western blots show that expression levels of virally introduced chCx50 E48K were similar to wild-type chCx50 when compared to β-actin controls (Fig. 4C).

Functional gap junction expression was assessed by scrape loading dye transfer or cell parachuting with two different dyes [Lucifer Yellow (LY) and calcein], as previously established in our laboratory (Banks et al., 2007). LY transfer between wild-type chCx50 cells was more than twofold greater than between uninfected and mutant-expressing cells (Fig. 4A), which showed no significant difference. The extent of rhodamine (RD) spread was approximately the same for all three cultures (approximately 25 μm). To complement the scrape loading studies, a non-invasive parachuting dye transfer method was used. Fluorescence microscopy with merged images shows how the dye transfer emanated from pre-loaded dye transfer area. There was no significant difference between chCx50 E48K and control (Fig. 4B, left panel). Wild-type chCx50-expressing cells showed more than twofold difference when compared with chCx50 E48K and uninfected (control) cells, as measured by calcein dye transfer method was used. Fluorescence microscopy with merged images shows how the dye transfer emanated from pre-loaded dye transfer area. There was no significant difference between chCx50 E48K and control (Fig. 4B, right panel). Dil served as a pre-loaded cell membrane marker that could not be transferred. There is low level of coupling for the control, implying the possibility of certain endogenous connexin(s). However, we did not detect the expression of Cx43, Cx50, Cx46, Cx45, Cx32 and Cx26, although we cannot exclude the existence of other connexins (Banks et al., 2007).

To compare the dominant negative effects of the chCx50 E48K mutant in CEF cells, wild-type chCx50 or chCx46 were coexpressed
extracellular Ca\textsuperscript{2+} (data not shown), but, hemichannels of chCx50 or its mutant (Fig. 6E).

In conclusion, Cx50 E48K mutation has no discernible effect on stimulation of lens cell differentiation

Cx50 has been shown to be important in lens development (Rong et al., 2002; White et al., 1998). We have also demonstrated the role of Cx50 in stimulating epithelial-fiber differentiation in primary lens cultures (Gu et al., 2003) and this stimulatory effect appears to be independent of gap junction function (Banks et al., 2007). Given that the E48K mutations appear to selectively ablate gap junction function, while leaving hemichannel function intact, we examined whether the E48K mutation exerts any effects on lens differentiation using primary chick lens cultures infected by retrovirus containing the chCx50 E48K coding region (Fig. 8).

Western blots showed an approximate 1.5-fold increase in total chCx50 amount when either exogenous chCx50 (Fig. 8A, lane 2), or chCx50 E48K (lane 3) were expressed (lane 1). Consistent with our previous study, some phosphorylated chCx50 was also detected (lower migrating band) (Jiang et al., 1994; Jiang and Goodenough, 1998a; Yin et al., 2000). Confocal microscopy shows that the E48K mutant co-distributes with endogenous chCx50 in lentoid structures (Fig. 8B).

The formation of lentoid structures was visualized by phase microscopy and DAPI fluorescence, and the maturation of lens cell differentiation was assessed by the level of lens fiber marker, MIP(AQP0). Expression of chCx50 E48K induced an almost twofold increase in MIP(AQP0) over control (Fig. 9A), which mimics the rise in MIP(AQP0) with overexpression of wild-type chCx50. Immunolabeling of adherent primary lens cells with MIP(AQP0) antibodies showed the formation of mature, differentiated fiber cells, whereas counterstaining with phalloidin clearly showed the interfaces of differentiating fiber cells indicated by filamentous actin (F-actin) (Fig. 9B). Quantification of MIP(AQP0)-positive stained area confirmed a nearly twofold increase in MIP(AQP0) expression in infected cultures (Fig. 9B, lower panel). Consistent with this elevation in the differentiation marker of AQP0, we also observed an approximate twofold increase in numbers of lentoids induced by chCx50 E48K, similar to the level induced by wild-type chCx50 (Fig. 9C). Moreover, expression of chCx50 E48K increased the protein levels of two other lens differentiation marker proteins, filensin and CP49 (Fig. 9D) (Blankenship et al., 2001). Taken together, our results suggest that, even though the E48K mutant prevents the formation of functional gap junction channels, it forms functional hemichannels and stimulates epithelial-fiber differentiation indistinguishable from wild-type Cx50.

**Discussion**

The E48K mutation is directly linked to a human congenital dominant cataract; however, the role of this mutant in cataract formation has not been evaluated. In this study, we demonstrate that the Cx50 E48K mutant fails to form functional gap junctional channels, but can form functional hemichannels and promote lens epithelial differentiation. This mutation shows similar surface expression and hemichannel formation as the wild type but, by contrast, entirely compromised gap junction function. Moreover, this mutant functions in a dominant negative manner to block gap...
junctions formed by wild-type Cx50, but has no effect on Cx46, whether in heteromeric or heterotypic pairings.

We examined the Cx50 E48K mutant protein in paired oocytes using junctional conductance and dye transfer, and also measured the dye transfer in CEF cells using various methods and tracer dyes. The dye transfer results in paired oocytes and cells correlate well with our electrophysiology data. Our results show that the Cx50 E48K mutant cannot form functional gap junctions. Our evidence implies that E48 in the first extracellular loop domain of Cx50 is likely to be a crucial amino acid residue for docking of this connexin required in the formation of gap junction channels. The change from a negatively charged glutamate (E) to a positively charged lysine (K) would alter the local charge of the docking interface by +24 in purely mutant channels (two charges per subunit, six subunits per hemichannel, two hemichannels per gap junction channel), but significantly less than 12 in heterotypic/heteromeric pairings, where channel function is still markedly suppressed. In the case of Cx50, this destabilizes formation of gap junction channels, even when only 50% of the subunits of one hemichannel are mutant. However, this effect is highly connexin-specific, as this site has no discernable effect on docking with Cx46, despite the conserved nature of the site. This dramatically illustrates that the docking of connexins is not, despite conservation of the extracellular loop sequences, a universally conserved mechanism, but that it can be quite specific to each connexin pairing. Hence, charge changes at other positions in E1 have been found to affect properties such as selectivity, conductance and rectification (Trexler et al., 2000), but not gap junction formation, in other connexins. In fact, the D66H mutation in Cx26 causes different problems again, associated with transport to the cell surface (Thomas et al., 2004). Thus, mutations in the E1 domain seem to be both site- and connexin-specific.

The E48K mutant acts in a dominant negative manner to prevent formation of gap junctions by Cx50. The dominant negative function of this mutation is well correlated with the dominant inheritance of congenital cataracts developed in the affected family members (Berry et al., 1999). This mutant is capable of properly expressing on the
cell surface, which excludes the possibility of a dominant negative effect on Cx50 intracellular transport. A possible explanation could be that mixed connexons formed by wild-type and mutant Cx50 cannot interact or dock properly with partner connexons. The paired oocyte assay demonstrates that even if one cell in the pair coexpresses both wild-type and mutant Cx50, it is sufficient to decrease the coupling with oocytes expressing wild-type Cx50 alone. The dominant negative behavior is consistent with a requirement for most of the connexins in a hemichannel to interact with the apposed hemichannel to form a functional gap junction channel. Our analysis of chCx50 wt:chCx50E48K pairs revealed that a subset had notably higher conductances (7.18 μS) than the mean (3 μS) (Fig. 2B), presumably representing the subpopulation of oocytes in which the wild-type subunits were expressed more efficiently than mutant subunits. However, when these were analyzed for transfer of Alexa 488 dye, this subpopulation showed no transfer above that seen in mutant-mutant, or wtCx50:oligo pairings. Given that oocytes displaying similar conductances to wild-type connexins have been seen to show detectable dye transfer (M.M.T., unpublished observation), this suggested that hemichannels with a minority of mutant subunits are capable of forming gap junctional channels with wild-type Cx50. However, our results suggest that heteromeric channels, with a minority of mutant subunits are modified in that they can still pass small ions, but not the larger dyes that move freely through wild-type channels. Similar dominant negative effects were previously reported for the P88S mutation of Cx50. This mutation does not form active gap junctions but does act as a dominant negative, needing perhaps only one subunit in a wild-type gap junction channel to reduce junctional conductance (Pal et al., 1999). In contrast to E48K, the P88S mutant tends to affect the targeting of Cx50 to the cell surface, associated with accumulation at yet-to-be identified structures inside the transfected cells (Berthoud et al., 2003). Another Cx50 mutation, D47A, the site adjacent to E48, was also reported to induce cataract formation in mice (Steele et al., 1998). D47A was described as a loss-of-function mutation without dominant inhibition (Xu and Ebihara, 1999). Our previous results demonstrate that the D47A mutant does not form active gap junctions (Banks et al., 2007). However, we also observed a partial dominant negative function, which may correlate with semidominant cataract phenotypes induced by this mutation, as the heterozygote has a less severe obstruction than the homozygote in the nuclear opacity 2 (op2) mouse (Steele et al., 1998). The overall charge alteration in D47A is less, which may explain its reduced dominant negative function compared with the E48K mutation. In addition, should the structure of this region be predominantly beta-sheet, proposed by Foote et al. (Foote et al., 1998), these residues could face in opposite directions in the structure.

The Cx50 E48K hemichannels formed on the cell surface behave similarly to wild-type Cx50. The data generated from both electrical measurement in oocytes and dye uptake by mechanical stimulation in cells is consistent with wild-type hemichannel function of this mutant. In addition to connexins, pannexins can also form hemichannels (Scemes et al., 2007). In this study, we cannot exclude the possibility that a pannexin-forming hemichannel is upregulated with the expression of Cx50. However, functional hemichannels alone are not sufficient to maintain lens transparency, whether formed by connexins or pannexins. The electrical conductance observed in Xenopus oocytes expressing connexins has gating characteristics of hemichannels formed by Cx50 or Cx46. Moreover, the data from dye uptake studies in the cell and conductance measurement in oocytes are similar, which implies the major involvement of connexin hemichannels.

A previous cataract-causing mutant, N63S of Cx46, was reported to have reduced ability to form functional hemichannels when expressed in Xenopus oocytes (Ebihara et al., 2003; Pal et al., 2000). This mutation results in hemichannels with increased sensitivity to magnesium compared with wild-type Cx46 (Ebihara et al., 2003). By contrast, the N63Q mutant of Cx46 failed to produce functional hemichannels. Additionally, a frameshift mutant S380Qp of Cx46 fails to form hemichannels in Xenopus oocytes (Pal et al., 2000). However, in all the above studies, the type of impairment (gap junction or hemichannel) that contributes to cataract formation cannot be distinguished. Our results demonstrate that deficiency of gap junctions can result in cataractogenesis, whereas the presence of functional hemichannels is not sufficient for maintaining lens transparency. While the calcium sensitivity and dye permeability of the hemichannels seem unchanged by the mutation, we could not exclude other properties that may be altered, such as sensitivity to endogenous modulators or permeability to cytoplasmic molecules. These changes could contribute to the development of cataracts. The E48K residue is highly conserved in Cx50 across animal
species, suggesting that this connexin residue plays a general role in docking and formation of functional homotypic Cx50 gap junction channels.

Several lines of evidence have suggested a close functional relationship between Cx46 and Cx50. A knockin Cx46 on the locus of Cx50 rescued the severe cataract by the G22R mutation of Cx50 (Xia et al., 2006b). Although the Cx50(G22R) mutant failed to mediate gap junctional communication, coexpression with Cx46 partially restored junctional conductance, which supports the hypothesis that the G22R mutant interacts with wild-type Cx46 to form functional channels and rescue the lens defect. The lens defect caused by the S50P mutation of Cx50 was corrected in Cx50S50P/Cx46+/+ mice, which lack wild-type Cx50 (Xia et al., 2006a). The mixed expression of Cx50 S50P mutant with either wild-type Cx50 or Cx46 resulted in functional heteromeric gap junctional channels and hemichannels (DeRosa et al., 2007). These studies illustrate the close functional relationship between Cx46 and Cx50 in the lens. In contrast to these previous observations, the Cx50E48K mutation only functions in a dominant negative manner with wild-type Cx50, but not Cx46, when coexpressed in the same cell (heteromERICally), or opposed cells (heterotypICally). This establishes E48 as a unique residue responsible in selective docking of Cx50 hemichannels.

The Cx50E48K mutant protein has comparable capability of promoting lens cell differentiation as wild-type Cx50. This is in agreement with our previous findings showing that mutants that fail to form functional gap junctions stimulate lens cell differentiation in a similar manner to wild type (Banks et al., 2007). This further strengthens the hypothesis that surface expression of Cx50, and its C-terminal domain, is the signaling platform for differentiation in the absence of gap junctional coupling. In terms of the etiology of lens cataracts, the current study is also illuminating. Models to explain cataract formation have been proposed that invoke a role for gap junctions in creating a ‘microcirculation pathway’ for nutrients and waste through the avascular lens (Donaldson et al., 2001; Mathias et al., 1997). However, other models have proposed a role on lens fiber cells and flux of material across the membrane. There has been no definitive evidence to distinguish these models to date. However, the demonstration here that a cataract-causing mutation of Cx50 fails to form gap junctions, but still forms hemichannels that are indistinguishable in terms of gating and permeability to wild-type channels, strongly supports the argument that hemichannel function is not sufficient to prevent cataract formation. Thus, our results would strongly support a role for gap junction intercellular communication in maintaining lens transparency. Finally, due to the highly conserved feature of this amino acid residue across various species and connexin types, the Cx50E48K mutation can be used as a novel, potent tool in generating animal models with the specific blockage of intrinsic functions of one class of gap junctions, but not hemichannels or gap junctions predominantly involving the other lens fiber connexin, Cx46.

Fig. 6. chCx50 E48K has no effect on hemichannel function in Xenopus oocytes. Post-injection of cRNAs of wild type (chCx50wt) (A) or mutant (chCx50E48K) (B) into Xenopus oocytes, the transmembrane hemichannel conductance was measured in the presence of 0.1 mM (left panels) or 4 mM Ca$^{2+}$ (right panels). (C) Average hemichannel conductance of oocytes injected with antisense oligonucleotide (Oligo), wild-type (chCx50wt) or mutant (chCx50E48K) in the presence of either 0.1 mM or 4 mM Ca$^{2+}$ showed that both wild-type and mutant constructs induced Ca$^{2+}$-sensitive membrane currents significantly different to oligo-only controls. The numbers of oocytes tested are indicated at the top of the bar. (D) Average hemichannel conductance of oocytes injected with antisense oligonucleotide (Oligo), wild-type chCx46 (chCx46wt), chCx50 (chCx50wt) or mutant (chCx50E48K) and other combinations in the presence of either 0.1 mM or 4 mM Ca$^{2+}$ showed that both wild-type Cx46 and Cx50 and mutant constructs induced Ca$^{2+}$-sensitive membrane currents significantly different from oligo-only controls. The numbers of oocytes tested are indicated at the top of the bar. (E) Average hemichannel conductance of wild-type and mutant hCx50 revealed similar results to that with the chick connexin. The numbers of oocytes tested are indicated at the top of the bar. **P<0.005.
Dominant negative mutant of lens connexin in gap junctions

Materials and Methods

Materials

Fertilized, unincubated white leghorn chicken eggs were obtained from Ideal Poultry (Cameron, TX) and incubated for 11 days in a humidified 37°C incubator. Anti-chCx50 (also called Cx45.6) antibody was generated and affinity-purified as previously reported (Jiang et al., 1994). Anti-chick MIP(AQP0) monoclonal antibody was a generous gift from Erica Tenbroek and Ross Johnson from the University of Minnesota (St Paul, MN); anti-filensin and CP49 antibodies were generous gifts from Paul Fitzgerald from the University of California (Davis, CA); human Cx50 construct in SP64T vector was a generous gift from Lisa Ebihara from Rosalind Franklin University of Medicine and Science. Anti-MIP(AQP0) polyclonal antibody was purchased from Alpha Diagnostics (San Antonio, TX); mMEssage mMACHINE for in vitro transcription from Ambion (Austin, TX); rhodamine-conjugated goat anti-mouse IgG and bicinchoninic acid (BCA) microprotein assay kit from Pierce Chemical (Rockford, IL); fluorescein-conjugated goat anti-rabbit IgG from ICN (Costa Mesa, CA); paraformaldehyde (16% stock solution) from Electron Microscopy Science (Fort Washington, PA); fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT); QuikChange Site-directed Mutagenesis Kit from Stratagene (La Jolla, CA); Vectashield fluorescence mounting medium from Vector Laboratories (Burlingame, CA); chemiluminescence kit (ECL) from Amersham Pharmacia Biotech (Piscataway, NJ); Alexa 488, Lucifer yellow (LY), rhodamine dextran, calcein acetoxymethyl ester (AM), 4′,6-diamidino-2-phenylindole (DAPI) and dialkylcarbocyanine dye (DiI) from Molecular Probes (Eugene, OR). All other chemicals were obtained from either Sigma (St Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Site-directed mutagenesis, and preparation of human and chicken Cx50 and Cx50 E48K cRNA and oocyte for microinjection

With human or chicken Cx50 in the SP64T vector as templates, E48K mutants were generated with the QuikChange Site-directed Mutagenesis Kit according to the manufacturer’s instructions with the following pairs of primers: hCx50(E48K) (Sense: GTTCGTGTGGGGGATAAGCAATCCGACTTCG. Antisense: CGAAGTCGGATTGTTATCCCTCCACACAGAA); chCx50(E48K) (Sense: GAACTAAGTTGATGGGGAGATAAACAGTCAGACTTTGTGTGC. Antisense: GCACACTAGTCTGACTGTTTATCTCCCCATACTAGTTC). RCAS(A)-hCx50(E48K) was constructed by changing GAG encoding Glu to AAG encoding Lys; chCx50(E48K) by changing codon GAA encoding Glu to AAA encoding Lys. The constructs were confirmed by sequencing, linearized with NcoI for human constructs and EcoRI for chicken constructs, and transcribed in vitro using the mMessage mMachine kit (Ambion Inc., Austin TX) in accordance with the manufacturer’s instructions. RNA concentrations were estimated by non-denaturing gel electrophoresis with ethidium bromide staining. Diluted working stock solutions were prepared with RNase-free water and stored at –80°C.

Fig. 7. The opening of chCx50 and chCx46-forming hemichannels induced by mechanical stimulation was not affected by E48K mutation. CEF cells were infected with RCAS(A) (Vehicle), wild-type Cx50 (chCx50wt), mutant chCx50(E48K) (chCx50E48K), different combination or non-infected control (Ctrl), and cultured at low cell density without (chCx50E48K) were fixed, labeled with DAPI (blue) or anti-FLAG (green) or anti-chCx50 (red) antibody. The primary antibodies were detected by fluorescein-conjugated anti-mouse IgG for anti-FLAG antibody and rhodamine-conjugated anti-rabbit IgG for anti-chCx50 antibody. The corresponding merged images (Merged) are shown on the right. Bar, 10 μm.

Fig. 8. Exogenous chCx50 E48K expression is comparable to wild-type chCx50. (A) Lens primary cultures were infected with retroviruses containing RCAS(A) vector (Vehicle), cDNAs of wild-type chCx50 (chCx50wt) (lane 2) and mutant Cx50 (chCx50E48K) (lane 3). After 8 days of infection, crude membranes were prepared and analyzed by western blots probed with anti-chCx50 antibody. Stripped membrane replicas were re-probed with monoclonal antibody against β-actin. The intensity of the bands on western blots was quantified by densitometric measurement (right panel). Data are presented as mean ± s.e.m. (n=3). chCx50wt and chCx50E48K versus Vehicle, ***P<0.001. (B) At 8 days after infection, lens primary culture expressing exogenous wild-type (chCx50wt) and mutant (chCx50E48K) Cx50 were fixed, labeled with DAPI (blue) or anti-FLAG (green) or anti-chCx50 (red) antibody. The primary antibodies were detected by fluorescein-conjugated anti-mouse IgG for anti-FLAG antibody and rhodamine-conjugated anti-rabbit IgG for anti-chCx50 antibody. The corresponding merged images (Merged) are shown on the right. Bar, 10 μm.
Oocytes were taken from ovarian lobe tissue, surgically removed from adult female *Xenopus laevis* after anesthetization in 0.2% tricaine chilled to 4-6°C. The tissue was then separated into smaller segments with about 6-12 oocytes and incubated at 22°C for 1 hour in OR2 buffer (83 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, pH 7.4 with NaOH) containing 1.5 mg/ml collagenase. After washing with OR2, stage V and VI oocytes were separated and manually defolliculated as necessary. They were then incubated at 17°C for 24 hours in half-strength L-15 media (Sigma), supplemented with 0.05 mg/ml gentamicin. Stage V/VI oocytes were injected with a 23-46 nl solution of subject connexin cRNA along with 3-5 ng of Cx38 antisense RNA for endogenous Cx38 suppression (Barrio et al., 1991). Subject oocytes were then incubated for 1-2 days before analysis as single or paired entities.

**Fig. 9.** chCx50 E48K mutant has similar capability to promote lens cell differentiation to wild-type chCx50. Lens primary cultures were infected with retroviruses containing RCAS(A) (Vehicle), RCAS(A)-chCx50 (chCx50wt) and RCAS(A)-chCx50(E48K) (chCx50E48K) for 8 days. (A) Crude membrane preparations from respective lens cell cultures: chCx50wt (lane 1), chCx50E48K (lane 2) and Vehicle (lane 3) were immunoblotted with anti-MIP(AQP0). The stripped membrane replicas were re-probed for β-actin. The intensity of the bands from western blot analysis was quantified by densitometry (lower panel). chCx50wt and chCx50E48K versus Vehicle, data presented as mean±s.e.m. (n=3). (B) Lens primary cells were immunolabeled with monoclonal MIP(AQP0) antibody and counterstained with Alex 488 phalloidin and DAPI. The primary antibody was detected by fluorescein-conjugated anti-mouse IgG, and the images were captured by fluorescence microscope. The intensity of the MIP(AQP0)-stained area was quantified (UTHSCSA ImageTool Software) and presented as a percentage in the x-axis (lower panel). chCx50wt and chCx50E48K versus Vehicle, data presented as mean±s.e.m. (n=3). (C) Lens cell differentiation was assessed by counting and quantifying numbers of lentoids. chCx50wt and chCx50E48K versus Vehicle, data presented as mean±s.e.m. (n=3). (D) Lysates from cells infected with retroviruses RCAS(A) (Vehicle) (lane 1), RCAS(A)-chCx50 (chCx50wt) (lane 2), and RCAS(A)-Cx45.6(E48K) (lane 3) were immunoblotted with anti-filensin, CP49 or β-actin antibodies. The CP49 protein bands from three separate western blot analyses were quantified by densitometry (right panel). chCx50wt and chCx50E48K versus Vehicle, data presented as mean±s.e.m. (n=3). Bar, 10 μm. * and **, P<0.05.
Electrophysiological measurement

Oocyte morphology was determined by voltage clamp experiments 18-48 hours after cRNA injections. Single oocyte voltage clamp recordings were taken using two electrode techniques while oocyte pairs were analyzed with dual electrode voltage clamps. In both single and paired oocyte arrangements GenoClamp 500B voltage clamps (Axon Instruments, Inc., Foster City CA) were used. A 1 X MG head stage was used for voltage recording and a 10 X MG head stage was used for passing current. Bath temperature was routinely cycled to 0 mM using an internal pipette solution (140 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 5 mM glucose and 25 mM Tris at pH 7.4), and digested with 0.1% trypsin in TD buffer at 37°C, and then broken apart by pipetting up and down in M199 media (plus 10% FBS and 1% penicillin/streptomycin). Cells were collected and reseeded in M199 media. Living cells were then counted and seeded at 3 x 10^4 cells per well of 12-well culture plates. The next day after the primary culture was started, retroviruses containing wild-type and mutant cDNA of chx50 diluted in M199 were added to primary lens cells for overexpression of connexins. The cultures were incubated at 37°C with 5% CO2 and were returned to regular media the next day. A total of 5 days cells proliferated on the culture plates, but not fiber cells. After 3-4 days lens epithelial cells became confluent and began to differentiate and form fiber-like 'lentoid' structures.

Immunofluorescence and confocal laser microscopy

For immunolabeling of connexins expressed in the primary lens culture, a glass coverslip was placed into each well of 12-well plate before cell seeding. After approximately 6-8 days of culture, lens epithelial cells were substantially differentiated into lens fiber cells, associated with the formation of 'lentoid' structures. Cells were fixed using 2% PFA for 30 minutes, and then incubated with blocking solution [2% bovine serum albumin (BSA), 2% fish skin gelatin, 0.25% Triton-X-100 and 1% BSA in Hank’s balanced salt solution (HBSS)] for another 30 minutes. Alexa Fluor 488 conjugated secondary antibody (1:100 dilution) was used to counterstain F-actin for 1 hour before staining with DAPI. The specimens were analyzed using a confocal laser scanning microscope (Fluoview; Olympus Optical, Tokyo, Japan). Acquisition conditions were kept constant for each sample. FITC fluorescence was excited at 488 nm by an argon laser with corresponding excitation wavelengths from 455-468 nm using a barrier filter. Rhodamine was excited at 543 nm with a HeNe-G laser with a corresponding emission wavelength of 560 nm using a long pass barrier filter. DAPI fluorescence was excited at 405 nm with corresponding excitation wavelengths from 430-460 nm using a barrier filter. For visual MIP(AQP0) quantification we used a method previously developed in our laboratory. Primary cultures grown on plastic and glass coverslips in 12-well culture dishes were used for MIP(AQP0) quantification. MIP(AQP0) images from each culture dish were used to determine the MIP(AQP0) staining area versus the whole image area (Gu et al., 2003). The threshold was adjusted to clearly distinguish lentoid boundaries for each image used for lentoid measurement. Fluorescent images were then converted to a binary black/white scale, according to which black represented the fluorescence and white represented the total black/white field and reported as calcein-positive area. Five representative images for each condition tested were used to assess calcein dye transfer per measurement.

SDS gel electrophoresis, fluorography and western blot

Cultured cells were collected in lysis buffer (5 mM Tris pH 8.0 and 5 mM EDTA/E GTA) and then ruptured by pipetting through a 26/0 gauge needle. Cell lysates were then centrifuged for 3 minutes at 2000 g to remove cell debris. The supernatant was then centrifuged at 100,000 g for 30 minutes (SW 60 Ti rotor, Beckman). The pellet was then resuspended in lysis buffer and boiled in 0.6% SDS for 3 minutes. Lysates were analyzed on 12% SDS-PAGE. Western blots were performed by probing with anti-MIP(AQP0) (1:300 dilution), anti-filensin (1:1000 dilution), anti-CP49 (1:1000 dilution), affinity-purified anti-chx50 (1:5000 dilution), and anti-f-actin antisa (1:5000 dilution). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000 dilution) or anti-mouse IgG (1:5000 dilution) using chemiluminescence reagent kit (ECL). The intensity of the bands on western blot was quantified by densitometry.

Primary lens cell culture

Lens primary cultures were prepared by a modified method as described previously (Menko et al., 1984). Lenses from 10- to 11-day-old chick embryos were dissected, washed with TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, 5 mM glucose and 25 mM Tris at pH 7.4), and digested with 0.1% trypsin in TD buffer at 37°C, and then broken apart by pipetting up and down in M199 media (plus 10% FBS and 1% penicillin/streptomycin). Cells were collected and reseeded in M199 media. Living cells were then counted and seeded at 3 x 10^4 cells per well of 12-well culture plates. The next day after the primary culture was started, retroviruses containing wild-type and mutant cDNA of chx50 diluted in M199 were added to primary lens cultures for overexpression of connexins. The cultures were incubated at 37°C with 5% CO2 and were returned to regular media the next day. In the beginning of culturing, only monolayer lens epithelial cells proliferated on the culture plates, but not fiber cells. After 3-4 days lens epithelial cells became confluent and began to differentiate and form fiber-like ‘lentoid’ structures.
Dye uptake assay
CEP cells were grown to ensure that the majority of the cells were not physically in contact. LY was used as a tracer for hemichannel activity and RD (M, 10 kDa) was used as a negative control. LY/RD solution was directly ‘dropped’ to cells maintained in Ca2+-free MEM by pipette at a fixed distance. Cells were washed with medium containing 1.8 mM Ca2+ and then fixed with 1% paraformaldehyde. Similar fields were observed under the fluorescence microscope. Dye uptake was presented as a percentage of fluorescent cells.

Statistical analysis
Data were analyzed by one-way ANOVA and Newman-Keuls multiple comparison test along with a biostatistics program (Prism). Data are presented as the mean ± s.e.m. of at least three measurements. Asterisks represent the degree of significance compared to control (*, P<0.05; **, P<0.005; ***, P<0.001).

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