Mutation of a Conserved Threonine in the Third Transmembrane Helix of \(\alpha\)- and \(\beta\)-Connexins Creates a Dominant-negative Closed Gap Junction Channel*

Received for publication, June 15, 2005, and in revised form, December 15, 2005 Published, JBC Papers in Press, December 28, 2005, DOI 10.1074/jbc.M506533200

Derek L. Beahm†, 1, Atsunori Oshima‡1, 2, Guido M. Gaietta§, Galen M. Hand¶, Amy E. Smock∫, Shoshanna N. Zucker∫, Masoud M. Toloue∫, 3, Anjana Chandrasekhar†, 3, Bruce J. Nicholson‡3, and Gina E. Sosinsky‡ 3, 4

From the †Department of Biological Sciences, State University of New York, Buffalo, New York 14260 and ‡National Center for Microscopy and Imaging Research, Department of Neurosciences, University of California, San Diego, La Jolla, California 92039-0608

Single site mutations in connexins have provided insights about the influence specific amino acids have on gap junction synthesis, assembly, trafficking, and functionality. We have discovered a single point mutation that eliminates functionality without interfering with gap junction formation. The mutation occurs at a threonine residue located near the cytoplasmic end of the third transmembrane helix. This threonine is strictly conserved among members of the \(\alpha\) - and \(\beta\)-connexin subgroups but not the \(\gamma\)-subgroup. In HeLa cells, connexin43 and connexin26 mutants are synthesized, traffic to the plasma membrane, and make gap junctions with the same overall appearance as wild type. We have isolated connexin26T135A gap junctions both from HeLa cells and baculovirus-infected insect SF9 cells. By using cryoelectron microscopy and correlation averaging, difference images revealed a small but significant size change within the pore region and a slight rearrangement of the subunits between mutant and wild-type connexins expressed in SF9 cells. Purified, detergent-solubilized mutant connexins contain both hexameric and partially disassembled structures, although wild-type connexins are almost all hexameric, suggesting that the three-dimensional mutant connexin is unstable. Mammalian cells expressing gap junction plaques composed of either connexin43T154A or connexin26T135A showed an absence of dye coupling. When expressed in Xenopus oocytes, these mutants, as well as a cysteine substitution mutant of connexin50 (connexin50T157C), failed to produce electrical coupling in homotypic and heteromeric pairings with wild type in a dominant-negative effect. This mutant may be useful as a tool for knocking down or knocking out connexin function in vitro or in vivo.

Intercellular communication is a fundamental feature of all multicellular organisms. Gap junctions are one means by which cells communicate with each other and arise as tissue cells grow and abut each other. The morphologically distinctive cell-cell junctional areas allow the exchange of ions, nutrients, and small metabolites between neighboring cells. Gap junction structures are found throughout vertebrates and invertebrates, although the primary sequences of constituent proteins are different from each other even though electron micrographs and physiological assays indicate similar quaternary structure and functionality. Gap junctions are composed of two oligomeric channel structures called connexons, with each cell supplying one connexon that docks with the other at their extracellular surfaces. The connexin family of proteins has a very conserved protein folding topology with highly conserved transmembrane and extracellular primary sequences, but conserved sequences are found in GenBankTM from various species ranging the evolutionary scale from fish to human.

The correct synthesis and assembly of gap junctions are critically important for development (2–4), signaling (5, 6), and homeostasis (7). Recently, the genetic characterizations of connexin diseases have led to studies focused on understanding how single amino acid changes in connexins result in macroscopic symptoms seen in patients (reviewed in Refs. 8 and 9) and whether these mutations arise from defects in synthesis, trafficking, docking, or channel function. Mutations in connexin genes have been demonstrated to be the cause of several diseases such as X-linked Charcot-Marie-Tooth (CMTX) syndrome (defects in Cx32 causing a peripheral neuropathy (10, 11), non-syndromic hereditary deafness (Cx26 (12)), erythrokeratoderma variabilis (Cx31, a skin disorder (13)), cataracts (Cx46 and Cx50 (14, 15)), oculodentodigital dysplasia, a pleiotropic syndrome displaying craniofacial and limb dysmorphisms, spastic paraplegia, and neurodegeneration (Cx43 (16)); and a few cases of viscerointestinal heterotaxia manifested as cardiac developmental disorders (17)). In each case, no systematic pattern has emerged as to the cause and effect of these mutations; however, these naturally occurring mutations are useful in trying to build three-dimensional models based on bioinformatics considerations (18).

Here we present the functional and structural characterization of a mutation of a threonine residue situated in the third transmembrane (TM3) helix. Except as part of a larger mutagenesis study (19), the position and amino acid make up at this site have not been investigated. The

* This work was supported by National Institutes of Health Grants GM065937 (to G. E. S.), GM072861 (to G. E. S.), GM048773 (to B. J. N.), and CA048049 (to B. J. N.) and National Science Foundation Grant MCB-0131425 (to G. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Both authors contributed equally to this work.

‡ Present address: Dept. of Biophysics, Kyoto University, Kyoto 606-8502, Japan.

§ Present address: Dept. of Biochemistry, University of Texas Health Sciences Center, San Antonio, TX 78229-3900.

To whom correspondence should be addressed: University of California, San Diego, 1070 Basic Science Bldg. MC 0608, 9500 Gilman Dr., La Jolla, CA 92039-0608. Tel.: 858-534-0128; Fax: 858-534-7497; E-mail: giosinsky@ucsd.edu.

‡1 Atsunori Oshima,‡2 Guido M. Gaietta,‡3 Anjana Chandrasekhar,‡3 Bruce J. Nicholson,‡3, and Gina E. Sosinsky‡ 3

5 The abbreviations used are: Cx, connexin; wt, wild type; TC, tetracysteine; GFP, green fluorescent protein; SCAM, substituted cysteine accessibility method; CMTX, X-linked Charcot-Marie-Tooth; TM, transmembrane; MP, MPCCPGCGGS; ER, endoplasmic reticulum; EDT, 1,2-ethanedithiol.
presence of this mutation does not impede the formation of channels, which in conventional EM displays the same morphology as wild-type (wt) channels. However, as we show in dye transfer and electrophysiological assays, the channels carrying this mutation are nonfunctional and may mimic a "closed pore" state. This residue is strictly conserved among the α- and β-connexins. Because the majority of the naturally occurring disease-related mutations are site-specific ones that result in mis-folding or mis-trafficking of connexins, this mutation is interesting because the connexin carrying this relatively minor substitution is correctly synthesized, trafficked, inserted into the plasma membrane, and capable of docking with a partner connexon, yet it is completely non-functional. We show electron microscopic data indicating small differences in the mutant from wild-type structure. We have mutated this residue at the equivalent positions in three connexin isoforms, and we found that function is eliminated in all three cases in intercellular channels in a dominant-negative fashion. We also show that a loss of function occurs in Cx50 hemichannels in homomeric and heteromeric combinations of wt and mutant connexins. Hence, in addition to being interesting from a structure/function relationship point of view, this mutation is potentially very useful as a tool for knocking out connexin functionality without having to delete connexin genes or for examining potential heteromeric interactions between different connexins.

MATERIALS AND METHODS

Sequence Analysis of Connexins—Connexin sequences were obtained from the National Center for Biotechnology Information (NCBI) protein sequence search web site and collated into a file format readable by the ClustalW set of sequence alignment programs (European Bioinformatics Institute). These aligned sequences were used as input for dendrograms using the program TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) and also used to generate the TM3 sequences shown in Table 1.

Generation of Connexin Constructs6 and Site-directed Mutagenesis—Rat Cx43 and Cx26 cDNAs were subcloned into the prokaryotic vector Bluescript (Stratagene, La Jolla, CA). Rat Cx43T154A and rat Cx26T135A were generated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The mutation T154A was introduced with the primers 5'-AAAAAGCTTGCCACCATGGTG-3' and 5'-AAAAAGCTTGATCCAACTCCAGGTG-3'. The resulting wt and mutated connexin cDNAs were subcloned at HindIII and XhoI restriction sites into the eukaryotic expression vector pcDNA3.1 (Invitrogen) for transient expression or into the retroviral vector pCLNCX of the RetroMax system (Imgenex, La Jolla, CA) for transduction and stable expression. Wt and mutated connexins were tagged at the C terminus by appending the peptide AEAAAREACCREC (Cx43-TC and Cx26-TC), the peptide ESSGSMPCPPGCCGCS (Cx43-MP and Cx26-MP), the peptide ESSGFSNCCPGCC-MEPGGR (Cx43-4C and Cx26-4C), and the fusion tag GFP-4C. The tags TC, MP, and 4C (plus linkers) were introduced into the target cDNA by PCR.

Transient transfections in HeLa cells were performed using FuGENE (Roche Diagnostics). Transfections were performed with recombinant virus generated in HEK293 cells. pCLNCX Cx43-GFP-4C was co-transfected with the Imagenex packaging vector pCL-Ampho. HeLa cell lines stably expressing the wt or mutant connexins were isolated after a 2-week selection in 100 μg/ml hygromycin in growth medium (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 1% penicillin/streptomycin).

Dye Injection Studies—Dye injection was performed by injecting Alexa 568 (Molecular Probes) into HeLa cells that were transiently transfected with connexins containing the GFP-TC tag. Images were recorded in epifluorescence mode on a BX50W1 Olympus light microscope using a fluorescein isothiocyanate filter set before injection to image the Cx-GFP-TC expression and after injection using a rhodamine filter set to image the Alexa 568. Image acquisition was performed with a Nikon SLR digital camera. Only cells showing fluorescent gap junctions were injected. The images shown in Fig. 6 were obtained by superimposing before and after pictures.

ReAsH Labeling of TC Constructs, Light Microscopy, and Photooxidation for Electron Microscopy—Cells expressing protein with tetracysteine tags were labeled with FlAsH-EDT2 or ReAsH-EDT2 similarly to what we described previously (20). Briefly, FlAsH-EDT2 or ReAsH-EDT2 was used at final concentrations of 0.5 and 1.25 μM, respectively, in the presence of EDT (12.5 μM). The labeling was performed for 1 h at 37 °C in 1 × Hanks’ balanced salt solution (Invitrogen). Free and non-specifically bound ligands were removed by washing with EDT/Hanks’ balanced salt solution (250 μM EDT for TC-tagged connexins, 300 μM EDT for MP-tagged connexins, 600 μM EDT for 4C-tagged connexins). After washing, cells were either fixed in 4% paraformaldehyde (light microscopy imaging) or imaged live (using a Bio-Rad MRC-1024 confocal microscope) in complete growth media (phenol red-free Dulbecco’s medium supplemented with 10% fetal calf serum). GFP and ReSh were easily discriminated from one another using standard fluorescein and rhodamine settings, respectively.

Cx43-GFP-TC-expressing cells were photooxidized as described previously (24) except that the excitation was achieved using 490 nm light. At this wavelength, direct excitation of ReSh (and thus photooxidation of diaminobenzidine by non-GFP-bound ReSh) was minimal. Prior to photoconversion, cells were fixed with 2% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 20 min, rinsed in buffer, and treated for 30 min in KCN (20 mM), amnotriazole (5 mM), glycine (50 mM), and H2O2 (0.01%) in cacodylate buffer to reduce nonspecific background. Diaminobenzidine (1 mg/ml) in oxygenated sodium cacodylate (0.1 M) was added to the culture dish, and the cells were irradiated with 585 nm light from a xenon lamp for 10–15 min until a brownish reaction product appeared in place of the red fluorescence. After photoconversion, cells were washed in buffer and post-fixed in 1% osmium tetroxide for 30 min. Cells were rinsed in double distilled water and incubated in 2% uranyl acetate overnight at 4 °C and rinsed again in double distilled water dehydrated in ethanol, embedded in Durcupan ACM resin, and polymerized at 60 °C for 48 h. Sections or 7–8 nm thickness were cut with a Leica Ultracut UTC microtome (Leica, Bannockburn, IL).

Baculovirus Expression of Cx26 T135A Mutant with Insect Cells—Cx26T135A-His6 cDNA was subcloned into the vector Bac-N-Blue (Invitrogen) at EcoRI and HindIII sites and was used to generate recombinant baculovirus. A plaque assay was performed for purifying recombinant viruses. Insect S9 cells were cultured in the S9001-SEM media supplemented with 2% fetal bovine serum and 0.1% antibiotic/antimycotic (Invitrogen) at 27 °C. Cells were infected at a density of 1.5 × 106 cells/ml for expression and then recovered 65 h after infection.

Gap Junction and Hemichannel Purification—Gap junctions were purified from HeLa cells grown to confluency by following a procedure published previously (25) or isolated from baculovirus-infected S9 cells according to published methods (26). The baculovirus-expressed con-
nexins contained a His$_6$ tag for affinity purification (26). Solubilized connexons were prepared from isolated Sf9 membranes by incubation in 2% dodecyl maltoside in HEPES buffer (10 mM HEPES, 200 mM NaCl), and the protein was purified using the C-terminal His$_6$ tag and nickel-nitrilotriacetic acid-agarose. Samples were negatively stained with 2% uranyl acetate for EM observation.

Native gels were carried out according to Schagger (27) and visualized with enhanced chemiluminescence Western blotting procedures. Electrophoresis was performed using a 4–20% gradient gel (NuPAGE Novex and Invitrogen).

Electron Microscopy and Image Processing Procedures—Conventional EM images were recorded either on a JEOL 1200 EX operating at 80 kV or a JEOL 2000 at 200 kV. Cryoelectron microscopy (cryo-EM) was performed using a JEOL 2000 at 200 kV equipped with a Tietz Video and Image Processing System low dose tomography package and Tietz Video and Image Processing System TemCam-F224 2k × 2k CCD camera. Images were acquired at 40,000 magnification with a 3.76 Å/pixel magnification and had an effective pixel size of 3.76 Å/pixel on the specimen. For correlation averaging, sub-areas were chosen based on a correlational search using a reference image central gap junction channel surrounded by six neighbors obtained by local Fourier filtering. The program QINDEX in the EMAN package of three-dimensional reconstruction single particle programs (28) performs both the local averaging and correlation search. Images were first high pass filtered to eliminate or dampen any correlations with inconsistent background values. A threshold of 160–300 of the highest correlation was chosen based on a correlational search using a reference image.

Electrophysiology (Oocytes)—Voltage clamp experiments began 18–48 h after cRNA injections. Voltage clamp recordings were obtained using a two-electrode voltage clamp technique for single oocytes or a dual two-electrode voltage clamp technique for oocyte pairs. GeneClamp 500B voltage clamps (Axon Instruments, Inc., Foster City, CA) were used in one or two oocyte configurations using a 1 L head stage for voltage recording and a 10 × MG head stage for passing current. The bath potential was actively clamped to 0 mV using a 100 × VG head stage. Voltage recording and current passing electrodes were pulled from borosilicate glass on a horizontal puller (Flaming-Brown P-87, Sutter Instruments, Novato, CA). Electrodes had resistances between 0.5 and 3 megohms using an internal pipette solution of 150 mM KCl, 10 mM EGTA, 10 mM HEPES, pH 7.2. The amplifiers were interfaced to a personal computer through a Digidata 1320 A/D converter, and data were acquired and analyzed using pClamp 8 software (Axon Instruments, Inc.). Currents were filtered at 50–200 Hz and acquired directly from the hard drive.

To assay hemichannel currents, individual oocytes were subjected to a two-pulse voltage protocol before and after exchanging the bath media from ND96 containing 2 mM CaCl$_2$ to ND96 with no added calcium. The voltage protocol consisted of a 500-ms −10-mV prepulse followed by a 5-s voltage step ranging from −120 to 60 mV in 10–20-mV increments from a holding potential of −20 mV. Whole-cell hemichannel conductance was determined as the current elicited by the pre-pulse divided by 10 mV. To assay gap junctional currents, both oocytes in a pair were clamped to −20 mV, and one oocyte was subjected to a similar voltage pulse protocol as above but using a series of voltage steps ranging from −120 to 80 mV to generate transjunctional potentials of −100 to 100 mV. In gap junction experiments, the pre-pulse current recorded in the nondonor oocyte was divided by the transjunctional voltage of 10 mV to obtain a measurement of the junctional conductance.

Fluorescent Imaging (Oocytes)—For fluorescent measurement of dye transfer, oocytes were prepared as for electrophysiological recording. After recording the initial junctional conductance, the donor oocyte was injected with 41.4 nl of 10 mM hydrazide sodium salt of Alexa 488 (Molecular Probes). At 1, 3, and 6 h after injection, the oocyte pair was imaged with a Zeiss Axiovert S100 fluorescence microscope using a CCD camera (model RTE/CCD-1300-Y, Princeton Instruments, Trenton,
N.J.). D450→490 and LP520 (Chroma Technology) excitation and emission filter sets were used for Alexa 488. To quantify the diffusion of dye, uniformly sized boxes were centered over both donor (D) and acceptor (A) oocytes of a pair, and their pixel intensity ratio (acceptor:donor) was measured using Metamorph 6.2R6 software (Universal Imaging Corp., West Chester, PA). For visualization, the software also assigned color based on fluorescent intensity over a range of 0–4095 pixels, with the blue end of the spectrum being the lower and red the higher intensities. 0 was assigned to black and 4095 to white.

Expression of Cx43T154A in Keratinocytes and Dye Transfer Assays for Functionality—Cx43wt and Cx43T154A cDNAs were subcloned into the pBMNIGFP vector (a kind gift of Gary Nolan) and transfected into the packaging cell line PA317 (34) using FuGENE 6 (Roche Diagnostics). Recombinant virus was harvested and used to transduce primary keratinocytes isolated from human foreskin according to a protocol described previously (35). The keratinocytes were microinjected using an Eppendorf Femtojet injector equipped with an Eppendorf Injectman manipulator using an Olympus 1X70 microscope and sterile femtotips (Brinkmann Instruments). The cells were maintained in Dulbecco’s modified Eagle’s medium + 20 mM HEPES and injected with 10 mM Alexa 350 dye (Molecular Probes, Eugene, OR). Cells were photographed with the appropriate filters for GFP (488 emission) and Alexa 350 (UV emission).

RESULTS

Site-specific mutants are commonly used as a tool for exploring structure/function relationships. In particular, there has been great interest in finding connexin mutants that form gap junction structures but fail to establish open channels, because such mutants would allow us to distinguish functions of connexins associated with the protein or the structures per se, and those associated with their function in intercellular communication. It would also provide us a way of stably isolating closed pores. Strictly or absolutely conserved amino acids often indicate critical positions that can eliminate functionality if mutated or perturbed. Here we became interested in a threonine amino acid located at the cytoplasmic end of TM3. This mutation was fortuitously discovered in some Cx43 sequences we had generated, and we chose to study it further because of the conservation within the connexin family, its loss of function when mutated, and its three-dimensional location within the connexin polypeptide.

Sequence Analysis of the TM3 Helix and Conservation of the Threonine Position

We examined the sequence of the third transmembrane helix and found a threonine that was strictly conserved among the α- and β-connexins but not the γ-connexins. The diagram in Fig. 1A shows the approximate location of this amino acid on the connexin topology diagram. This amino acid sits on the N-terminal side of a strictly conserved tyrosine that is found in 100% of the connexin sequences and was mapped in Cx32 as the most cytoplasmic pore-lining residue (19). The amino acid on the other side varies among the connexins (arginine, asparagine, glycine, and tryptophan being the most common substitutions). Next, we searched the NCBI database for all connexin sequences and aligned the sequences using the ClustalW set of programs. The *bar graph* of the aligned sequences shown in Fig. 1B shows that this threonine occurs in ~71% of the connexins. Deviations from having Thr in this position only occurred among highly divergent species and isoforms. For example, nonconservation of this amino acid occurred in all Cx45 (Ile) and Cx36 (Phe) homologues. Using ClustalW and TREEVIEW-PPC, we plotted the connexin sequences from mouse to see the familial relationships (Fig. 1C). When viewed as a radial plot, connexins with deviations from Thr at this position cluster in a manner that is separate from the α- and β-subgroups.

Expression and Trafficking of the Threonine Mutant in HeLa Cells

When the Cx26T135A mutant construct is transiently or stably expressed in HeLa cells, “normal” gap junction patterns are seen in confocal micrographs (Fig. 2A). We also generated a Cx43T154A-GFP-4C construct and transiently expressed it in HeLa cells to elucidate whether the protein trafficked in a native manner. This particular construct was generated to perform correlated light and electron microscopy; however, the confocal microscopy reinforces the result seen with Cx26T135A that this Cx43 mutant forms normal appearing gap junctions. Fig. 2B shows immunofluorescence from the GFP and from ReAsH labeling demonstrating not only the large gap junction plaque but also intracellular labeling indicative of trafficking vesicles and lyso-
somess. When GFP is excited, it transfers energy to the ReAsH molecule in a fluorescence resonance energy transfer–dependent manner (22, 23).

The confocal image shown in Fig. 2B is of an area prior to photoconversion. The green signal is from the GFP, and the red is ReAsH labeling. Note that in the large gap junction the GFP and ReAsH signal overlap. These large gap junctions are typical of tagged Cx43 (20, 36, 37).

The ability to perform photoconversion on these tetracysteine-tagged connexins allows us to examine trafficking pathways at higher resolution provided by electron microscopy. The excited ReAsH label then triggers the photo-oxidation of diaminobenzidine, which precipitates in situ to create an osmiophilic electron-dense stain (20). Fig. 2C demonstrates that EM staining occurs at the gap junction as well as in lysosomal compartments. This area is different from the one shown in Fig. 2B but was part of the same set of experiments. Another micrograph at higher magnification is shown in Fig. 2D. This micrograph was selected because it clearly showed the gap junction as well as trafficking intermediates. This image contains the edge of a gap junction plaque between the two cells with indications of a hemichannel plaque next to it. However, it should be pointed out that Cx43-GFP-4C transient expression in HeLa cells produces gap junctions significantly larger than in endogenously expressing Cx43 cell lines. In this image, a large hemichannel plaque is found at the side of the gap junction, consistent with the idea that connexons are added to the outer edges of the plaque (20, 40). These large gap junctions are typical of those obtained with transiently expressed tagged connexins (36). The expectation is that in endogenously expressing Cx43 native cell lines and tissues where the plaque size is small, there are small “hemijunctions” below the detection level for EM or light microscopy that add to the edges of the plaque. Hemichannel plaques have been imaged previously with atomic force microscopy in isolated preparations (38) and using freeze fracture and thin section electron microscopy in Xenopus oocytes expressing exogenous Cx50 (39). Selectively stained trafficking vesicles are also indicated in this image. A section of a gap junction at higher magnification is shown in the inset in Fig. 2D and demonstrates the classical pentalaminar appearance of a gap junction. Therefore, from our light microscopy and EM studies, it would appear that the Cx26T135A and Cx43T154A traffic in a mode similar to wt.

**Connexon Structure by Negative Staining and Cryo-EM**

To study the structural appearance of gap junction plaques between mutant and wt, we isolated Cx26T135A gap junctions from both HeLa cells (Fig. 3, A and B) and from baculovirus-infected Sf9 cells (Fig. 3C). We chose to perform our structural studies with Cx26 because of our previous successes in the isolation and analysis of gap junctions composed of this isoform (25, 41). Gap junctions isolated from both HeLa cells and Sf9 cells showed the classic doughnut appearance with plaques ranging from larger and less ordered (Fig. 3, A and C) to occasional plaques that were relatively small but had better lattice order. Under these low resolution and high irradiation conditions, the channels appear similar to wild-type Cx26 (Fig. 3D).

We applied cryo-EM and image analysis methods to isolated Sf9 gap junction membranes to evaluate structural changes in the averaged projection maps between mutant and wt connexons. Because of the inherent lattice disorder in these samples, standard Fourier averaging could not be used effectively, and instead a correlation average approach was used (42). Lattice filtered subregions of images for the wt (Fig. 4A) and Cx26T135 mutant (Fig. 4B) show small differences where the ice density within the pore in the wt is more diffuse than in the mutant as indicated by its black appearance. We used correlation averaging of frozen-hydrated wt (Fig. 4C) and mutant gap junctions (Fig. 4D) and computed a difference map from the subtraction of wt minus mutant (Fig. 4E). The wt average contains about ~400 unit cells, although the mutant image was computed from ~600 unit cells. The average and difference maps have been rotationally filtered to enhance the 6-fold symmetry of the
FIGURE 3. Isolated gap junctions of Cx26T135A and wt gap junctions show very similar structures. A and B, conventional EM of a negatively stained Cx26T135A gap junction from HeLa cells shows wt appearance at this resolution and under high irradiation conditions (scale bar for A and B the same). C, negatively stained Cx26T135A-His6 gap junction obtained from expressing the mutant connexin in Sf9 cells with baculovirus expression system. D, negatively stained EM image of a Cx26wt-His6 gap junction is shown here for comparison. Under these imaging conditions, the mutant and wild-type connexin channels are indistinguishable.
Analysis of a Closed Pore Connexin Mutant

Connexon. The difference map (Fig. 4E) has been displayed on a step gray scale where the largest differences are either black (negative differences) or white (positive differences). The zero difference level would appear as a medium gray. We find that the pore size is larger in the wild-type average than in the mutant, and there is some rearrangement of features within the lobes the connexons. There is an ~17% reduction in the pore size from wt to mutant (~27 to ~22.5 Å), although it is not possible in these projection images to distinguish whether this reduction occurs at the cytoplasmic surface or at the extracellular surface, or if a larger constriction could occur at a local point within the pore. Single site mutations often result in instability of the hexamer when released from the membrane (26) and indicate a potential role in maintaining intra-molecular or inter-molecular contacts. To test the stability of the connexon, Cx26T135A-His6 and wt-His6 connexons were detergent-solubilized in 2% dodecyl maltoside and purified with a nickel-nitrilotriacetic acid resin. We found that Cx26T135A-His6 connexons became unstable as judged by their appearance using conventional, negative-stained EM (Fig. 5A). Although wt-His6 maintained a uniform “doughnut” appearance (Fig. 5B, circles), images of Cx26T135A-His6 contained aggregates of smaller substructures (arrows) as well as some round shaped particles (circles). Native gel PAGE analysis was performed with these purified samples (Fig. 5C). The Cx26T135A-His6 mutant sample (Fig. 5C, left lane) has strong bands at the dimer, trimer, tetramer, and hexamer positions, whereas the wt-His6 (Fig. 5C, right lane) shows a strong band at the hexamer molecular weight (~156 daltons). The relative amount of each species in the mutant was variable in three preparations examined by native gel analysis (data not shown); however, in the mutant lane shown in this figure the ratio of wt:tetramer:trimer: dimer is 1:~0.8:~0.65:~0.35. Because both wt and mutant connexons have a His6 tag, the difference in appearance of the oligomers can only be explained by the difference between the connexin isotypes. These results suggest that mutant protein is in a slightly altered conformation from the wt because it is still retained as hexamers but also dissociates to smaller oligomers. A highly disruptive mutation would cause disruption into monomers and aggregates or fail to assemble in vivo.

Functional Analysis of Constructs in Oocytes

The Tetracysteine Tag Does Not Interfere with Normal Channel Function of Connexins—We had found that using tetracysteine-tagged versions of Cx43 and Cx26 resulted in a nonfunctional channel based on dye-coupling assays. To determine whether the C-terminal tetracysteine domain interfered with channel function, we employed a more sensitive electrical assay to examine tagged versions of wt Cx26 and Cx43.

TC-tagged connexins were expressed in the paired Xenopus oocyte assay system and assessed for changes in electrophysiological behavior from wt. An example of the functional currents elicited in oocyte pairs expressing either Cx26wt or Cx26-TC is presented in Fig. 6A. The average normalized initial and steady-state conductance-voltage relation-
ships obtained from three different pairs indicates that tagged and Cx26wt gap junctions behave very similarly (Fig. 6B). These experiments were performed in different batches of oocytes, where junctional currents were elicited by imposing transjunctional potentials of −120 to +120 mV in 20-mV increments for Cx26wt and Cx26-TC oocyte pairs and −130 to +130 mV in 20-mV increments for the Cx26wt pairs. Fig. 6C show examples of Cx43wt and Cx43-TC gap junctional currents elicited by imposing transjunctional potentials of −130 to +130 mV in 20-mV increments. The average normalized initial and steady-state conductance-voltage relationships obtained from three pairs each are presented in Fig. 6D. As had been the case for Cx26, the Cx43T154A channels behaved nearly identical to wt channels.

Initial conductance-voltage values in Fig. 8, B and D, were obtained by normalizing the initial conductances resolved between 20 and 30 ms at each Vj to the initial conductance at ±10 mV, although the “steady-state” conductance values were estimated by normalizing the conductances at the end of the voltage step to the conductance at ±10 mV. Clearly, the voltage dependence of both Cx26 and Cx43 were weak and sufficiently slow that junctional currents did not reach steady state by the end of the voltage pulse, leading to systematic overestimates of...
steady-state conductances for both wt and mutant. In addition, the oocyte pairs typically developed conductances exceeding 10 μS (traces not shown), so that a quantitative macroscopic assessment of the channel properties is influenced by access resistance in the oocytes, leading to an underestimate of the voltage dependence of the channels (43, 44). However, this affects all channel types similarly, and a comparison of pairs of wt and mutant with similar conductance levels (e.g. Fig. 8 for Cx43) reveals almost identical responses of the mutant and wt channels (31, 45). In addition, a comparison of all recordings, even with a broad range of current levels, shows no significant difference between wt and mutant profiles (see Cx26 (Fig. 6B) or Cx43 (Fig. 6D)).

Functionality and Dominant-negative Effects of Cx26T135A—We next tested the functionality of the Cx26T135A mutant and the possible heteromeric effects it may have when co-expressed with Cx26wt in the paired Xenopus oocyte expression system. Data from different batches of oocytes were combined by normalizing the junctional conductances of each oocyte pair from a batch of oocytes to the average junctional conductance measured in the wt-wt pairs for that batch (Fig. 7A).

Oocytes injected with either 10 or 20 ng of Cx26T135A cRNA developed no detectable gap junctional coupling beyond that observed in oligonucleotide-injected control oocyte pairs over the course of 2 days, compared with similar levels of wt Cx26 cRNA that produced 40 – 62 μS of conductance. The lack of junctional coupling suggests that the mutation rendered the connexin nonfunctional but does not indicate whether the protein was synthesized or correctly processed and trafficked to the plasma membrane, although this seemed likely from the microscopy results shown in Fig. 2. One way of assessing this was to determine whether Cx26T135A had a dominant-negative effect on wt junctional conductance. Oocytes were injected with either wt (20 ng), mutant (20 ng), or an equal mix of mutant and wt cRNA (20 ng each) and were subsequently paired with either the same type of oocyte or an oocyte injected with only wt cRNA. All oocytes also received 5 ng of an antisense oligonucleotide to endogenous Cx38 to suppress contamination of results by endogenous coupling. Because oocytes received the same amount of wt cRNA, if the mutant were unable to interact with wt connexin, then the junctional conductance levels between oocytes injected with a combination of wt and mutant cRNA should be comparable with the conductance levels between oocytes injected with only wt cRNA. We had shown previously in oocytes that at these levels of RNA, there were minimal effects because of competition for translational machinery (46), although this was also confirmed in these experiments (see below). As shown in Fig. 7A, the mutant Cx26T135A clearly inhib-
Analysis of a Closed Pore Connexin Mutant

Combining data from experiments in four different batches of oocytes, Cx26T135A never induced coupling above background in the system (Fig. 7A). Furthermore, co-injection of the mutant with Cx26wt reduced conductance of the Cx26wt-Cx26wt pairs to 15.4 ± 2.3% when paired with Cx26wt cells and to near background levels when two co-injected cells were paired (6.2 ± 1.8% of Cx26wt-Cx26wt pairs versus 4.6 ± 1.1% and 5.1 ± 1.1% of wt for Cx26T135A-Cx26wt pairs and pairs receiving only antisense oligonucleotide, respectively).

Theoretically, the binomial theorem can predict the subunit composition of hemichannels formed in oocytes injected with different ratios of mutant and wt cRNA. The binomial theorem predicts that equal amounts of mutant and wt RNA will generate mainly heteromeric hemichannels (>95%), assuming that the two cRNAs are translated with equal efficiency and assuming that the connexin polypeptides are homogeneously mixed in the ER and can assemble into a hemichannel with equal probability. For example, if there are equal numbers of mutant and wt subunits, the probability of having a homomeric wt hemichannel would be (1/2)² or 1.5% (i.e. the probability of any one subunit being wt raised to the power of the number of subunits). Similar probabilities can be calculated for any heteromeric mix by multiplying this probability by the number of possible combinations of the particular kind of heteromeric channel.

Because of the complexity of accurately quantifying the levels of plasma membrane resident Cx26 and Cx26T135 within the same cell, we did not attempt to determine the minimum number of mutant subunits required to render a hemichannel nonfunctional by manipulating subunit stoichiometry using various ratios of wt and T135A cRNA. However, if the conditions of the binomial theorem were met, the absence of detectable junctional coupling between wt oocytes and oocytes co-expressing wt and mutant in the third round of experiments suggests that a single mutant subunit may be capable of rendering the channel nonfunctional (i.e. based on the example above, only 1.5% of all channels would be functional, homomeric wt if the mutant and wt proteins were equally expressed).

The lower level of dominant-negative effects at the higher RNA concentration used in the first two experiments described above might suggest a higher stoichiometry of mutant channels is needed to ablate function of wt Cx26. However, the high levels of conductance in wt pairs is likely to lead to a significant underestimate, i.e. ~40% (47) of the actual junctional conductance, once access resistance is taken into account. The inhibition by co-injection of mutant would then be closer to the 95% predicted by the Poisson distribution, assuming equal affinity of the two types of subunits.

The Dominant-negative Effect of the Mutation in Cx43 Gap Junctions—To determine whether the specific mutation at the equivalent position of Cx26T135 affected other types of connexins, experiments were performed using oocytes injected with Cx43wt (4 ng), Cx43T154A (4 ng), or a combination of Cx43wt (4 ng) + Cx43T154A (4 ng). In these experiments, oocytes were pre-injected with 5 ng of Cx38 antisense oligonucleotide 2 days prior to injecting the connexin cRNAs to reduce the potential interactions between Cx43 and Cx38. The results of various pairing configurations are shown in Fig. 7B and resemble the Cx26 experiments with the Cx43T154A mutation showing a clear dominant-negative effect on the formation of functional Cx43 gap junction channels, reducing the conductance from 12.4 ± 2.6 μS for Cx43wt (n = 7) to 2.4 ± 0.9 μS for Cx43wt-(Cx43wt + Cx43T154A) pairs (n = 9) and to background levels 0.3 ± 0.2 μS (n = 4) when mutant and wt were mixed in both oocytes. The ~80% reduction seen in the heterotypic pairing is

FIGURE 8. Dominant-negative effect of mutation on Cx50 hemichannels. A representative whole-cell currents from oocytes, injected with the cRNAs indicated on the left before and after changing the bath solution from ND96 with 2 mM CaCl₂ (left column) to ND96 with 0 added calcium (right column). Oocytes were held at −20 mV and subjected to a series of voltage steps ranging from −120 to +60 mV (30-mV increments shown above). The scale bar represents 5 μA (vertical) and 2 s (horizontal). The hemichannel currents characteristic of Cx50wt are not seen in the mutant and only minimally detected if wt and mutant are co-expressed in the same oocyte. B, histograms of the average whole-cell conductance after removing calcium from the medium show that Cx50T157C does not produce functional hemichannels alone and suppresses the ability of wt to make hemichannels. The control consists of injection of the same solution as in the other conditions (Cx38 mRNA only) but lacks any exogenous Cx50 connexin mRNA.

ite the formation of functional gap junction channels when co-expressed with Cx50wt, reducing the conductance in two experiments from 40.1 ± 7.5 μS (n = 11) and 61.8 ± 17.0 μS (n = 9) for wt Cx50 to 8.7 ± 2.0 μS (n = 11) and 11.2 ± 1.6 μS (n = 15), respectively when Cx50wt and Cx26T153A were co-expressed in only one of the pairs. The properties of the gap junctional currents (kinetics and voltage dependence) measured in the Cx50wt-(Cx50wt + Cx26T153A) pairs were similar to those of Cx50wt-Cx26wt pairs (data not shown). When mutant and wt Cx50 were co-expressed in both cells, the conductance was reduced even further so as not to be significantly different to pairs injected only with antisense oligonucleotide to the endogenous connexin.

In a third batch of oocytes, the cRNA levels injected into oocytes were reduced by 50% to ensure that the inhibition caused by co-injecting Cx26T135A was not a result of exceeding the translational capacity of the oocytes. In this experiment, the average junctional conductances were 19.6 ± 5.0 μS (n = 7) for the Cx26wt-Cx26wt pairs and 0.5 ± 0.2 μS (n = 8) for the Cx26wt-(Cx26wt + Cx26T135A) pairs. This reduction of ~97% was even greater than that seen in the first two experiments (~78 and 82%, respectively).

MARCH 24, 2006•VOLUME 281•NUMBER 12
JOURNAL OF BIOLOGICAL CHEMISTRY 8003
somewhat less than that seen with Cx26 or predicted by the Poisson distribution, as discussed above. This could also reflect a less efficient suppression in the case of Cx43 or differential translation of the mutant and wt Cx43 constructs.

Effects of the Mutation on Cx50 Hemichannels and Gap Junction Channels—We also examined a third connexin, Cx50. This construct was originally generated for substituted cysteine accessibility method (SCAM) analyses of Cx50. We had generated a mutation of the equivalent site in Cx50 (Thr-157), albeit to Cys rather than the Ala we had introduced in the other two connexins. When expressed in paired *Xenopus* oocytes, this mutant failed to form functional gap junction channels.

7 D. L. Beahm, M. M. Toloue, and B. J. Nicholson, unpublished observations.
TABLE 1

Naturally occurring single amino acid site mutations in the region spanning TM3 among the nine connexins that have been shown to be the cause of human hereditary connexon-opathies

This table shows those mutations found in the 3rd transmembrane helix (shown in larger text and underlined) as compared with the conserved threonine residue that we have shown to eliminate functionality (as shown by the shaded boxed T). This table summarizes information in the Human Gene Mutation Data Base at the Institute of Medical Genetics in Cardiff (archive.uea.ac.uk/umcm/hgmd0.html) and from Refs. 70 and 71. Beginning and ending amino acids numbers for TM3 are shown at the right- and left-hand sides, respectively, in the middle column. The disease phenotypes are as follows: 1, deafness (undefined); 2, non-syndromic autosomal dominant deafness; 3, non-syndromic autosomal dominant deafness; 4, keratitis-ichthyosis-deafness syndrome; 5, deafness and palmoplantar keratoderma; 6, Vohwinkel syndrome; 7, hidrotic ectodermal dysplasia; 8, erythrokeratodermia variabilis; 9, Charcot-Marie-Tooth syndrome; 10, susceptibility to atherosclerosis; 11, association with atrial standstill; 12, cardiac malformations; 13, oculodentodigital dysplasia; 14, Hallerman-Streiff syndrome/ODDD; 15, congenital cataracts; and 16, zonular nuclear pulverulent cataracts.

| Disease Phenotype | Nucleotide Change | Amino Acid Change | Mutation Site |
|-------------------|-------------------|-------------------|---------------|
| Deafness (undefined) | G > A | E > K | TM3 |
| Non-syndromic Autosomal Dominant Deafness | G > A | E > K | TM3 |
| Non-syndromic Autosomal Dominant Deafness | G > A | E > K | TM3 |
| Keratitis-Ichthyosis-Deafness Syndrome | G > A | E > K | TM3 |
| Deafness and Palmoplantar Keratoderma | G > A | E > K | TM3 |
| Vohwinkel Syndrome | G > A | E > K | TM3 |
| Hidrotic Ectodermal Dysplasia | G > A | E > K | TM3 |
| Erythrokeratodermia Variabilis | G > A | E > K | TM3 |
| Charcot-Marie-Tooth Syndrome | G > A | E > K | TM3 |
| Susceptibility to Atherosclerosis | G > A | E > K | TM3 |
| Association with Atrial Standstill | G > A | E > K | TM3 |
| Cardiac Malformations | G > A | E > K | TM3 |
| Oculodentodigital Dysplasia | G > A | E > K | TM3 |
| Hallerman-Streiff Syndrome/ODDD | G > A | E > K | TM3 |
| Congenital Cataracts | G > A | E > K | TM3 |
| Zonular Nuclear Pulverulent Cataracts | G > A | E > K | TM3 |

As had been the case in the other connexins, it also suppressed the development of gap junction currents when co-expressed with wt (Fig. 7C), reducing the currents between wt pairs from 32 ± 12 μS (n = 13) to 4.8 ± 1.6 μS in Cx50wt-(Cx50wt + Cx50T157C) pairs (n = 10) and 3.8 ± 1.4 μS in (Cx50wt + Cx50T157C)-(Cx50wt + Cx50T157C) pairs (n = 7). As in the other connexin experiments, background was 0.1–0.6 μS. This ~85% reduction was comparable with that seen with Cx43 and with Cx26 prior to correction for access resistance.

Using Cx50 also allowed for the possibility of assessing the effects of the mutation at this site on the function of hemichannels. Expression of
can be seen in the acceptor cell is similar to that seen in the antisense oligonucleotide-injected controls (Fig. 9E) and appears to be an optical artifact of the system. Expression of the mutant protein was confirmed by demonstrating its dominant-negative effect on the wt connexin, as seen by the lack of dye coupling in the Cx26 + Cx26T135A co-injected pairs (Fig. 9F). Note that the dye intensity in these images is expressed in pseudocolor, according to the scale shown in Fig. 9G.

These observations were quantified by placing boxes of equivalent area within the donor and acceptor cells and measuring pixel intensity using Metamorph software. The resulting ratio of intensity in the acceptor cell to donor box was then measured at 1-, 3- (data not shown), and 6-h time points (Fig. 8). The latter is shown in Fig. 9H. Cx43 and Cx26 passed dye efficiently. Although the average conductances of the pairs tested were the same for each connexin (37.6 and 37 μS, respectively), there were more Cx43 channels connecting the cells, as this connexin has a lower single channel conductance than Cx26 (90 and 130 pS, respectively). Cx43 channels also have a higher permeability for this dye than Cx26 channels (~2-fold) (40). Together, this would explain the higher acceptor:donor ratio that is observed for Cx43. Most importantly, all pairs expressing the mutant form of either protein, including those where it was co-injected with wt (in the case of Cx26), showed no transfer of dye above that seen in the antisense oligonucleotide-injected negative controls. Hence, dye injection studies reflect the identical pattern to that seen in the electrophysiological analyses of these channels, demonstrating that these mutants are equally impermeable to ions or larger molecules.

Functional Analysis of Constructs in Mammalian Cells

Dye Transfer Assays in Mammalian Cells—In addition to oocytes, we also tested the functionality of these mutants through dye transfer between mammalian cells. Initially, we employed dye coupling of HeLa transfectants that had been shown to have no detectable expression of endogenous connexins. Alexa 564, a dye that should pass through the channel pore (47) and whose emission does not significantly overlap with GFP, was employed in these experiments. Gap junctions were monitored using a construct that was a chimera of GFP with a 4C tetracycline peptide appended to the C terminus of the GFP. These constructs were used in combination with fluorescence photodetection for obtaining the EM images in Fig. 2 and allow us to monitor the protein and gap junction expression (green color) and to unambiguously select only cell pairs containing gap junctions. Typical images of mutant versus wt dye transfer experiments are shown in Fig. 10. Images are presented for Cx26T135A mutant (Fig. 10A) and wt (Fig. 10B) and Cx43T154A mutant (Fig. 10C) and wt (Fig. 10D). In both isoforms, Alexa 564 (red color) was transferred in the wt but not in the mutant, indicating a loss of function in the mutant.

The dominant-negative effects of the Cx43 mutant was also tested using primary human keratinocytes. These cells were transfected with either the pBMNIGFP vector alone or with Cx43wt or T154A mutant inserted into this vector. Transfected cells were microinjected with Alexa 350 dye, which has also been shown to pass through Cx43 channels (40), and photographed after 15 s (Fig. 11). The results show that the expression of Cx43wt enhances dye transfer, although the expression of Cx43T154A significantly reduces the dye transfer. However, when the time course was allowed to progress beyond 15 s, dye transfer was detected even with the mutant. This might be explained if the mutant were expressed at lower levels than the endogenous protein, leading to incomplete “knockdown” of endogenous Cx43. However, the Alexa 350 dye is transported by all of the connexins surveyed to date (47), and there are a total of seven connexins expressed in human skin (48). Although Cx43T154A may have dominant-negative effects on connexins other than Cx43, it seems likely that it may not affect all of the endogenous connexins. In future experiments, use of a combination of larger and smaller Alexa dyes (e.g. Alexa 350 and Alexa 564) may help to discriminate between different isoform channels in cells expressing multiple connexins.

DISCUSSION

The specific mechanisms for connexin channel gating have yet to be identified despite examinations of gating in response to calcium (49) or electrophysiological dissection of different voltage gates (for a review see Ref. 50). The absence of a widely accepted structural model for gating is partially due to the fact that although closed and open states have been characterized via functional methods such as electrophysiology and dye transfer, the isolation or preparation of distinct and homogenous open and closed states for structural analysis has been problematic. Atomic force microscopy investigations have shown opening and closing of the channel pore (41, 51); however, because these images are surface views, they cannot provide an insight into internal protein changes. Perturbation analysis studies from potassium channels have shown that single site amino acid substitutions can be useful in defining inter-helical associations (52). One way to achieve this goal is the isolation of mutant connexons or channels whereby the mutation causes the hexamer or dodecamer to be locked into a permanent open or closed state. Here we have shown by using correlative structural and functional studies that a dominant-negative nonfunctional mutant is closed, and we observed small structural differences in projection maps between the mutant connexon and wt. Given that this is a conservative amino acid substitution, it is important to understand what effect the mutation has on neighboring amino acids, the structure of TM3, and its three-dimensional spatial inter-helical and intra-helical interactions.

Comparative Sequence Analysis of the TM3 at the Threonine or Equivalent Position—Conservation of residues can provide insights into the importance of certain amino acid positions. A search of the connexin sequences in GenBank™ revealed that this threonine is conserved among a subset of connexins that group into α- and β-subfamilies. However, the tyrosine that is next to the threonine (e.g. Tyr-136 in Cx26 and Tyr-155 in Cx43), one amino acid down into the transmembrane region, is absolutely conserved throughout the connexin family. In addition, an analysis of hereditary mutations leading to connexin diseases reveals that the 8 connexins linked to diseases are all α and β, therefore, all contain this Thr. Table 1 contains the TM3 sequences of naturally occurring mutations found in connexin diseases with mutations highlighted in a large boldface and underlined font. There are no published mutations within the TM3 sequences of Cx37-, Cx46-, and Cx50-caused diseases, although this is most likely due to the small number (1–10) of mutations for each of these connexin diseases that have been documented as compared with Cx26 and Cx32, where over 100 and 250 distinct mutations, respectively, have been identified and published (Human Gene Mutation Data base at the Institute of Medical Genetics in Cardiff; archive.uwcm.ac.uk/uwcm/mg/hgmd0.html). The mutation of Cx26Y136 into a termination codon as been shown to be one cause of nonsyndromic hereditary deafness (53), whereas a mutation of Cx32Y135, the neighbor of Thr-134, to a Cys creates manifestations of CMTX (54). In addition, mutation of Trp-133, the other Thr-134 neighboring amino acid in Cx32, to an Arg-55, Cys-56, or stop codon (57) also resulted in patients displaying CMTX. It is interesting to note that in CMTX, although mutations span the entire sequence of

---

*S. N. Zucker, A. Chandrasekhar, and B. J. Nicholson, unpublished data.*
TM3 with the four upstream residues and one downstream amino acid from this Thr, this threonine residue has yet to be identified as a disease mutation or in Cx26-caused diseases. There are three reasons why this might be so: 1) cases have not yet been identified; 2) mutations at this position cause no change in the expression and functionality of the connexin, or 3) this mutation would be embryonic lethal. Given the functional data presented in this paper showing an almost complete knock-out of function in a dominant-negative fashion, we believe that the third hypothesis, lethality of this mutation in vivo, is the most likely explanation (58). Although most mutations would only ablate the function of the connexin mutated, these mutants would be expected to cause widespread effects on co-expressed connexins, eliminating any “redundancy” protections in tissues. The biological consequences of such a mutation are likely to be more severe. This being said, it is still likely that mutations in connexins with limited expression (e.g. Cx46 and Cx50 in the eye lens) would not be expected to be lethal.

The Third Transmembrane Helix Tertiary Structure—Although such sequence comparisons are useful, these bioinformatics approaches are not enough to explain observed differences in functionality and trafficking between isoforms even in such highly conserved regions as TM3. Lagere et al. (59) reported that replacement of the residues at 152 and 153 of Cx43 with those occurring in Cx32 (L152W and R153W, respectively) resulted in formation of gap junctions, but the L152W had markedly reduced dye transfer (59). In addition, the neighboring residue to L153 of Cx43 with those occurring in Cx32 (L152W and R153W, respectively) agreed between isoforms even in such highly conserved regions as TM3. Not enough to explain observed differences in functionality and trafficking of the connexin mutated, these mutants would be expected to cause widespread effects on co-expressed connexins, eliminating any “redundancy” protections in tissues. The biological consequences of such a mutation are likely to be more severe. This being said, it is still likely that mutations in connexins with limited expression (e.g. Cx46 and Cx50 in the eye lens) would not be expected to be lethal.

A new model of the arrangement of the α-carbon atoms of the transmembrane α-helices has been proposed by Fleishman et al. (18). This model was based on a three-dimensional map, 5.7 Å in plane and 19.8 Å perpendicular to the membrane plane, which was obtained by data analysis refinement of the original 7 Å data of Unger et al. (63). The four transmembrane segments in the oleamide-treated Cx43 truncation mutant structure were traced as canonical α-helices, and the α-carbons were assigned to specific amino acid residues in the protein sequence. The criteria for these assignments were evolutionary conservation of residues, hydrophobicity of the amino acid residues, biochemical and phylogenetic data from mutagenesis studies, and analysis of naturally occurring disease mutations in Cx32. Because Cx32 mutations in CMTX are the most highly characterized, with over 250 published mutations that extend over the entire length of the sequence, the amino acid sequence of Cx32 was used for assigning the α-carbons to the canonical α-helices. In this model, it is assumed that highly conserved domains will share a common architecture between isoforms that highly conserved amino acid residues will preferentially pack at helix-helix interfaces and perturbations of the helix-helix packing will manifest as CMTX mutations. Using this computational approach, Fleishman et al. (18) suggest that the major pore-lining helix is TM3, and the minor pore-lining helix is TM1. The TM2 and TM4 helices face the lipid environment with TM2 closely apposed to TM1 and TM4 closely apposed to TM3 in this model. It is important to point out that this is not a consensus model, because the errors in assignment of the helix orientations are within 40°, and the helical register could vary by ~1 turn of a helix because of the decreased vertical resolution in the EM map. More importantly, mutagenesis studies often differ greatly with this interpretation (19, 67, 68) with portions of TM1, TM2, and/or TM3 all being identified as contributing to the pore lining. However, this model provides a first approximation for its spatial location and insights into why it plays a critical functional role.

Within the Fleishman et al. (18) model, the Thr-134 position of Cx32 is in a region of the TM3 helix that is buried on all sides by other helices at the cytoplasmic end of the bilayer. This is consistent with the deductions from the SCAM studies (19) described above where Thr-134 might be expected to interact with adjacent helices. The cytoplasmic end of TM3 is the region of the connexin structure that shows the tightest packing of helices in the electron crystallographic model (61). Thus, Thr-134 could form a contact with the TM4 helix by possibly interacting with the Glu-208 residue and therefore may be important for determining inter-helix contacts. Whether the Thr OH group forms hydrogen bonds with amino acids nearby in three-dimensional space, but in a different part of the sequence, remains to be determined. Certainly, a test of this theory is whether replacements of the Thr with a Val (same size but no OH group) or a Ser (maintains the OH group but is smaller) would maintain function of the channels. This would indicate that hydrogen bonding at this position plays a role in inter-helical packing or whether it is the specific size of the Thr that is important in maintaining the helix integrity or both are a factor. Notably, cysteine substitutions for this threonine in both Cx32 and Cx50 fail to preserve function, despite retaining some H-bonding potential in the introduced SH group, albeit reduced compared with the endogenous OH group.
However, the cysteine does not have the β-branched structure of Thr and is likely to present the SH group in a distinct orientation.

Although the Fleishman model might suggest an interaction of Thr-134 with Glu-208 in TM4 as described above, the mapping of Tyr-135 as being within the pore by Skerrett et al. (19) would place Thr-134 on the opposite face to TM4 based on the models presented in both Refs. 19 and 18. This would suggest an interaction with either TM1 or TM2, depending on the model. This is consistent with the demonstration that Arg-142 (8 residues away and hence on the opposite side of the helix) interacts with TM4, based on the R142C mutant forming a spontaneous disulfide with Cys-201 in TM4 that locks the channel in the closed state (19). Comparison of the models deduced by Fleishman et al. (18) and Skerrett et al. (19) is further complicated by the fact that the SCAM analysis was based on accessibility in open channels, whereas the Fleishman et al. (18) model was constructed from an oleamidie-treated sample, where the channel has been speculated to be in a closed or partially closed state. Hence, should TM3 undergo rotation during gating, Thr-134 could interact with different helices in the open and closed states.

Consequences of Destabilizing Structure—Our EM data indicate that substitution at the Thr position causes only minor alterations in the structure as seen in our projection images but causes profound changes on the physiology of the mutant connexin channel. In particular, in all of our electrophysiological and dye transfer assays, the pore is essentially closed. Whether this reflects a true closed state or an artificial one is a subject for further investigation. Nonetheless, to our knowledge, this is the first time that a direct structure/function relationship has been shown for a mutant connexon at the resolutions that EM provides. Specifically, we see a smaller pore size consistent with the idea that the pore is closed in this mutant and a rearrangement of the subunit structure that would indicate a shifting of subunit substructure between wt and mutant, perhaps reflecting a destabilizing effect of this substitution on this structure. The reduction in measured pore diameter is ~17%, although in projection images, it is difficult to assign that reduction to specific areas of the structure. The expectation is that the relative decrease in the diameter at the extracellular surface (also called the extracellular “loop” gate (69)) would be greater than the cytoplasmic gate based on atomic force microscopy measurements of the extracellular surface pore diameter before and after calcium-induced closure (41).

Our EM data argue for the role of Thr in maintaining either inter-helical or intra-helical stability that is consistent with both models discussed above, because detergent solubilization of the channels results in breakdown of the hexamer. The normal appearance of mutant gap junction plaques signifies that this mutant could form the complex within the membrane. Because the substitution is a relatively modest one, when held together in the membrane, the lipid environment helps to stabilize the connexin channel. As the complex is solubilized and released from the membrane, one see a series of structures in the EM depending on the model. This is consistent with the demonstration that the substitution causes a modest destabilization in the structure. If the modification had caused a dramatic change in structure, there should have been only been aggregates in the detergent and no hexameric forms. This contrasts to studies by Oshima et al. (26), which showed that Cx26M34T or Cx26M34A expressed and purified from baculovirus-infected S9 insect cells remained intact when solubilized in dodecyl maltoside, but have greatly reduced dye transfer permeability arguing that the structural change affected the pore but left inter-subunit contacts intact. Other residues have also been implicated in the stability of subunit interactions, including Arg-75 in E1 and Arg-153 and Glu-173 (58, 59) in TM3 of Cx43.

Potential Use of This Mutant for Knocking Out or Down Connexin Functionality—Beyond the interest in this mutant for the role it plays in gap junction structure, we propose that this mutant is also useful as a tool for the gap junction community. As shown in Fig. 11, transfection of keratinocytes with Cx43T154A significantly reduced initial Alexa 350 dye transfer, presumably by making heteromeric combinations with Cx43wt but also with any other connexins that are compatible for heteromeric combinations with Cx43. Because keratinocytes contain multiple connexins, the eventual dye transfer is probably due to transfer through other connexin channels found in smaller amounts than Cx43. We believe it is easier to transfect cells with this construct to knock down function rather than either generating knock-out animals or using small interfering RNA interference, which would be specific to a single connexin isotype. Our studies indicate a broad dominant-negative phenotype among both α-class (Cx43 and 50) and β-class (Cx26 and possibly Cx32) connexins.

Conclusions—Mutation of the Thr residue at the cytoplasmic end of TM3 in connexins of the α- and β-subgroups does not interfere with synthesis, trafficking, and docking, yet it leads to the formation of non-functional channels with dramatically reduced dye and ion transfer properties, a smaller pore size, and structural instability of the connexin oligomer.

The Thr mutation in this location also has a dominant-negative effect, as we show in homotypic, heterotypic, and heteromeric combinations of Cx43, Cx26, and Cx50. This mutation may be useful as a tool for knocking down or knocking out connexin functionality in endogenously expressing connexin cell lines.

Acknowledgments—We thank Dr. Steve Ludtke and Robert Ashmore for their image processing programs, development of the software for this study, and aid in implementing them. We thank Brent Martin for first identification of this mutant and pointing this out to us and Dr. Jean Jiang for advice with the native gels, Dr. Stelios Andreadis for provision of the human keratinocytes and help with infection studies, and Dr. Martha Skerrett for help with the electrophysiology. We also thank Dr. D. Miller for the gift of the PA317 packaging cells. The EMAN development is supported by National Institutes of Health Grant R01GM59580. Some of the work included here was conducted at the National Center for Microscopy and Imaging Research at San Diego, which is supported by National Institutes of Health Grant RR04050 awarded to Dr. Mark Ellisman.

REFERENCES

1. Willecke, K., Eiberger, J., Dejen, J., Eckardt, D., Romualdi, A., Guldenagel, M., Deutsch, U., and Sohl, G. (2002) Biol. Chem. 383, 725–737
2. Reuime, A. G., de Souza, P. A., Kulkarni, S., Langille, G. L., Zhu, D., Davies, T. C., Junega, S. C., Kidder, G. M., and Rossant, J. (1995) Science 267, 1831–1834
3. Nelles, E., Butzler, C., Jung, D., Temme, A., Gabriel, H. D., Dahl, U., Traub, O., Stumpel, F., Jungermann, K., Zielesk, J., Toyka, K. V., Dermetzizel, R., and Willecke, K. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9565–9570
4. Gourdie, R. G., and Lo, C. W. (2000) in Gap Junctions: Molecular Basis of Cell Communication in Health and Disease (Peracchhi, C., ed) Vol. 49, pp. 581–602, Academic Press, San Diego
5. Goldberg, G. S., Lamp, D. P., and Nicholson, B. J. (1999) Nat. Cell Biol. 1, 457–459
6. Beltramelllo, M., Piazza, V., Bukauskas, F. F., Pozzan, T., and Mammano, F. (2005) Nat. Cell Biol. 7, 63–69
7. Gilula, N. R., Reeves, O. R., and Steinbach, A. (1972) Nature 235, 262–265
8. Bennett, M. V. L., and Abrams, C. K. (2000) in Gap Junctions: Molecular Basis of Cell Communication in Health and Disease (Peracchhi, C., ed) Vol. 49, pp. 423–459, Academic Press, San Diego
9. Krotovskvkk, V., and Yamaski, H. (2000) Mutat. Res. 462, 197–207
10. Patel, P. I., and Lupski, J. R. (1994) Trends Genet. 10, 128–133
11. Scherer, S. S., Bone, L. J., Deschênes, S. M., Abel, A., Balice-Gordon, R. J., and Fischbeck, K. H. (1999) Novartis Found. Symp. 219, 175–187
