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Ⅳ．研究成果の刊行物・別刷り

A Connexin40 Mutation Associated With a Malignant Variant of Progressive Familial Heart Block Type I

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Background—Progressive familial heart block type I (PFHBI) is a hereditary arrhythmia characterized by progressive conduction disturbances in the His-Purkinje system. PFHBI has been linked to genes such as *SCN5A* that influence cardiac excitability but not to genes that influence cell-to-cell communication. Our goal was to explore whether nucleotide substitutions in genes coding for connexin proteins would associate with clinical cases of PFHBI and if so, to establish a genotype-cell phenotype correlation for that mutation.

Methods and Results—We screened 156 probands with PFHBI. In addition to 12 sodium channel mutations, we found a germ line *GJA5* (connexin40 [Cx40]) mutation (Q58L) in 1 family. Heterologous expression of Cx40-Q58L in connexin-deficient neuroblastoma cells resulted in marked reduction of junctional conductance (Cx40-wild type [WT], 22.2±1.7 nS, n=14; Cx40-Q58L, 0.56±0.34 nS, n=14; $P<0.001$) and diffuse localization of immunoreactive proteins in the vicinity of the plasma membrane without formation of gap junctions. Heteromeric cotransfection of Cx40-WT and Cx40-Q58L resulted in homogenous distribution of proteins in the plasma membrane rather than in membrane plaques in ≈50% of cells; well-defined gap junctions were observed in other cells. Junctional conductance values correlated with the distribution of gap junction plaques.

Conclusions—Mutation Cx40-Q58L impairs gap junction formation at cell-cell interfaces. This is the first demonstration of a germ line mutation in a connexin gene that associates with inherited ventricular arrhythmias and emphasizes the importance of Cx40 in normal propagation in the specialized conduction system. **(*Circ Arrhythm Electrophysiol.* 2012; 5:163-172.)**

Key Words: heart block ■ genes ■ ion channels ■ death sudden ■ gap junctions

Cardiac myocyte excitability in atria, His-Purkinje system, and ventricles is largely determined by the properties of voltage-gated sodium channels. Once activated, excitatory currents rapidly propagate to neighboring cells through low-resistance intercellular channels called gap junctions, which facilitate the synchronous contraction of the heart.[1,2] Loss of expression and function of cardiac gap junctions and sodium currents can severely impair action potential propagation, which sets the stage for life-threatening arrhythmias.[1,2] Although multiple mutations in genes coding for components of the voltage-gated sodium channel complex have been previously described in relation to arrhythmias and sudden death in young persons[3] and connexin40 (Cx40) mutations have been implicated in atrial fibrillation,[4,5] no study has identified an

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association between germ line mutations in gap junction proteins and inherited ventricular arrhythmias in humans.

In this study, we investigated a group of patients with progressive familial heart block type I (PFHBI) (Online Mendelian Inheritance in Man 113900), also known as progressive cardiac conduction defect or Lenègre-Lev disease,[6,7] is a dominant inherited disorder of the His-Purkinje system. Affected individuals show electrocardiographic evidence of bundle branch disease (ie, right bundle branch block, left anterior or posterior hemiblock, complete heart block) with broad QRS complexes. The disease can progress from a normal ECG to right bundle branch block and from the latter to complete heart block. Affected individuals often present with family history of syncope, pacemaker implantation, and sudden death.[8] Although structural abnormalities have been invoked as a cause of the disease,[6,7] a number of patients present with normal cardiac structure and contractile function. Linkage analysis in a large South African PFHBI kindred[9] and a Lebanese kindred[10] mapped a causal locus on chromosome 19q13.3, and further work identified mutations in genes encoding for the transient receptor potential nonselective cation channel, subfamily M, member 4 (*TRPM4*) gene[11] at this locus. Haploinsufficiency of *SCN5A* and aging have been implicated in PFHBI,[8] and age-dependent manifestations of the disease have been recapitulated in mice.[12]

Here, we sought to expand on the association between PFHBI and mutations in genes relevant to action potential propagation; in particular, we assessed the possible association between nucleotide substitutions in connexin-coding genes and PFHBI. We evaluated 156 probands of diverse ethnic backgrounds from Asia, Europe, and North America given a clinical diagnosis of PFHBI. In addition to the sodium channel mutations previously reported,[13–15] we identified a germ line missense mutation in *GJA5* in a family with severe, early onset disease. This gene codes for the gap junction protein connexin40 (Cx40), which predominantly expresses in the atria and His-Purkinje system.[16] Heterologous expression experiments revealed that this novel mutation (Cx40-Q58L) significantly impaired the ability of Cx40 to form gap junction channels. Confocal microscopy showed that the Cx40-Q58L mutant but not the wild type (WT) failed to form plaques at sites of cell-cell apposition. Coexpression experiments indicated that the Cx40-WT protein provided only partial rescue of the Cx40-Q58L cellular phenotype. To our knowledge, this is the first description of a germ line mutation in a connexin gene associated with inherited ventricular arrhythmias. The results open the possibility of *GJA5* as a candidate gene for screening in patients with PFHB1, yet in the absence of further evidence, screening may be limited to the research environment rather than included as a part of the routine diagnostic examination.[17] The data also emphasize the importance of Cx40 in the maintenance of normal propagation in the specialized conduction system of the human heart.

Methods

Genetic Screening of PFHB1

Genomic screening by polymerase chain reaction and DNA sequencing was performed for *GJA5* (Cx40), *GJA1* (Cx43), *GJC1* (Cx45), *KCNQ1*, *KCNH2*, *SCN5A*, *KCNE1*, *KCNE2*, *KCNJ2*, *SCN1B*, *SCN4B*, *HCN4*. Primer information is provided in the online-only Data Supplement. All participating probands and family members gave written informed consent in accordance with standards (Declaration of Helsinki) and local ethics committees.

Plasmid Construction

A 1.1-kb Cx40-DNA fragment was subcloned into bicystronic plasmids pIRES2-EGFP and pIRES2-DsRED2. An EGFP or FLAG epitope was added at Cx40 C terminal to generate EGFP- or FLAG-tagged Cx40. Site-directed mutagenesis (Q58L) was performed with QuikChange. Primer information and additional details are provided in the online-only Data Supplement.

Cell Culture and Transfection

Constructs were introduced into connexin-deficient HeLa cells or mouse neuroblastoma (N2A) cells using Lipofectamine as per manufacturer's protocol.

Electrophysiology

Gap junction currents were recorded from transiently transfected N2A cell pairs using whole-cell double-patch clamp techniques as previously described.[18,19] Further details are provided in the online-only Data Supplement.

Immunocytochemistry and Western Blotting

HeLa cells, transfected with pEGFPN1-Cx40-WT, pCMV-FLAG-Cx40-Q58L, or both, were stained with anti-FLAG M2 antibody and Alexa546-labeled secondary antibody. EGFP and Alexa546 fluorescence images were recorded by confocal microscopy. For western blotting, N2A cells were transiently transfected with 3 μg of Cx40 plasmids. Two days after transfection, cells were lysed, and proteins were extracted and separated by conventional methods. Further details are provided in the online-only Data Supplement.

Statistical Analysis

Results are presented as mean±SEM. Mann-Whitney rank sum tests with Bonferroni post hoc correction were used in comparisons for which normality or equal variance assumptions were invalid. In other instances, differences between groups were assessed by 1-way ANOVA followed by Bonferroni post hoc correction. Statistical significance was assumed for $P<0.05$.

Results

Genetic Screening of PFHBI Probands

We genetically screened 156 probands given a clinical diagnosis of PFHBI. We identified 4 novel and 5 previously reported mutations in *SCN5A*,[13,15] 3 mutations in *SCN1B*,[14] and a novel germ line heterozygous missense mutation in exon 2 of the Cx40 gene *GJA5* (online-only Data Supplement Table I). Mutations were not found in connexin genes *GJA1* (Cx43) or *GJC1* (Cx45) or in the other genes screened (*KCNQ1, KCNH2, KCNE1, KCNE2, KCNJ2, HCN4,* or *SCN4B*). Of the novel *SCN5A* mutations, 1 caused a modification of the amplitude and voltage gating kinetics of the sodium current in heterologously expressing cells (online-only Data Supplement Figure I); 3 other mutant constructs failed to express functional channels, suggesting that patients carrying the mutation were functionally haploinsufficient for Nav1.5 (online-only Data Supplement Figure I). The *GJA5* mutation (c.173A>T) caused an amino acid substitution (glutamine [Q] replaced by leucine [L]) at position 58 in Cx40 (Cx40-Q58L) (Figure 1A and 1B). The mutation was absent in 400 alleles from unaffected control subjects and in the other 155 PFHBI probands. Screening of the entire gene

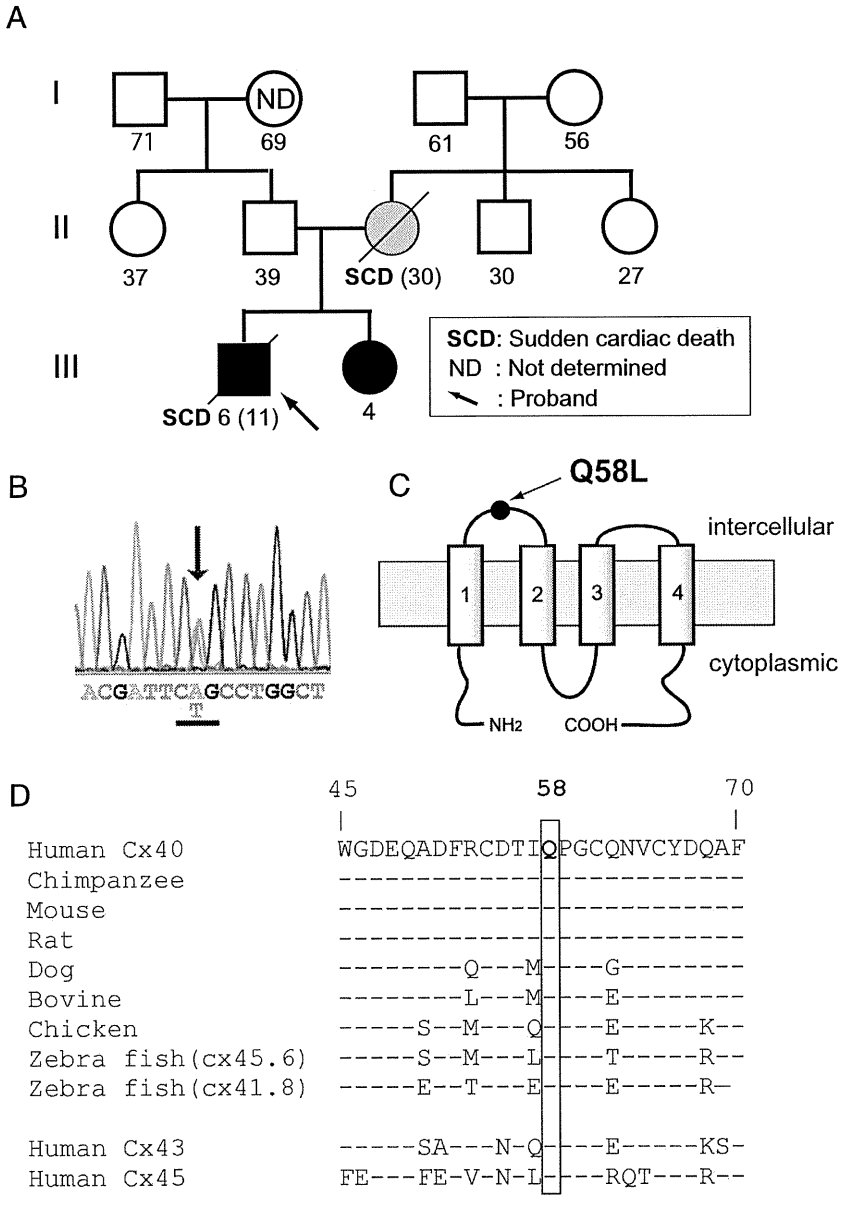

Figure 1. *GJA5* mutation identified in a family given the clinical diagnosis of progressive familial heart block type I. **A**, Family pedigree. Genetically affected and unaffected individuals are shown with closed and open symbols, respectively. The hatched circle indicates the proband's mother not genotyped; clinical data suggest that she was a de novo mutation carrier. Number below each symbol indicates the age at registration or age of SCD (parenthesis). **B**, Sequence electropherogram of exon 2 *GJA5* of proband. Arrow indicates heterozygous missense mutation of leucine (CTG) for glutamine-58 (CAG). **C**, Cx40 predicted membrane topology indicating position Q58 in first extracellular loop. **D**, Sequence alignment of human Cx40 and its homologues (residues 45–70). Notice the conservation in human Cx43 and Cx45. Dashes indicate residues identical with the top sequence. Cx indicates connexin.

panel (including *SCN5A* and *SCN1B*) revealed no other sequence modification in the DNA of this proband. Topological analysis placed amino acid 58 of Cx40 within the first extracellular loop (Figure 1C). The presence of glutamine in this position is highly conserved among *GJA5* orthologs, and 2 other cardiac connexins, Cx43 and Cx45 (Figure 1D). The clinical and genotypic characteristics of proband and tested family members are described next.

Clinical Phenotypes and Genotype of the PFHBI Pedigree With the *GJA5* Mutation

The proband, an 11-year-old boy at time of death, was first referred for evaluation when he was age 6 years because of ECG abnormalities. Although asymptomatic at that time, his ECG showed advanced atrioventricular block, complete left bundle branch block, and left axis deviation (Figure 2A). Echocardiography and cardiac scintigraphy did not reveal signs of structural heart disease. He experienced an episode of syncope at age 9; implantation of a permanent pacemaker was recommended by the physician but not authorized by the legal guardian. The proband died suddenly 2 years later during exercise (running), and the family declined postmortem examination. The proband's younger sister shares the Cx40-Q58L mutation. She is asymptomatic, with a QRS duration at the upper limit of normal, left axis deviation that has been progressive (online-only Data Supplement Table II), and QRS notch. These findings are consistent with impaired intraventricular conduction (Figure 2B). The mother died suddenly at age 30 after delivering the second child. An ECG on record, obtained when she was age 16, was similar to that of the proband (compare Figure 2C with 2A). In addition, a ventricular tachycardia was recorded during the recovery phase of an exercise stress test (online-only Data Supplement Figure II). DNA from the mother was not available for

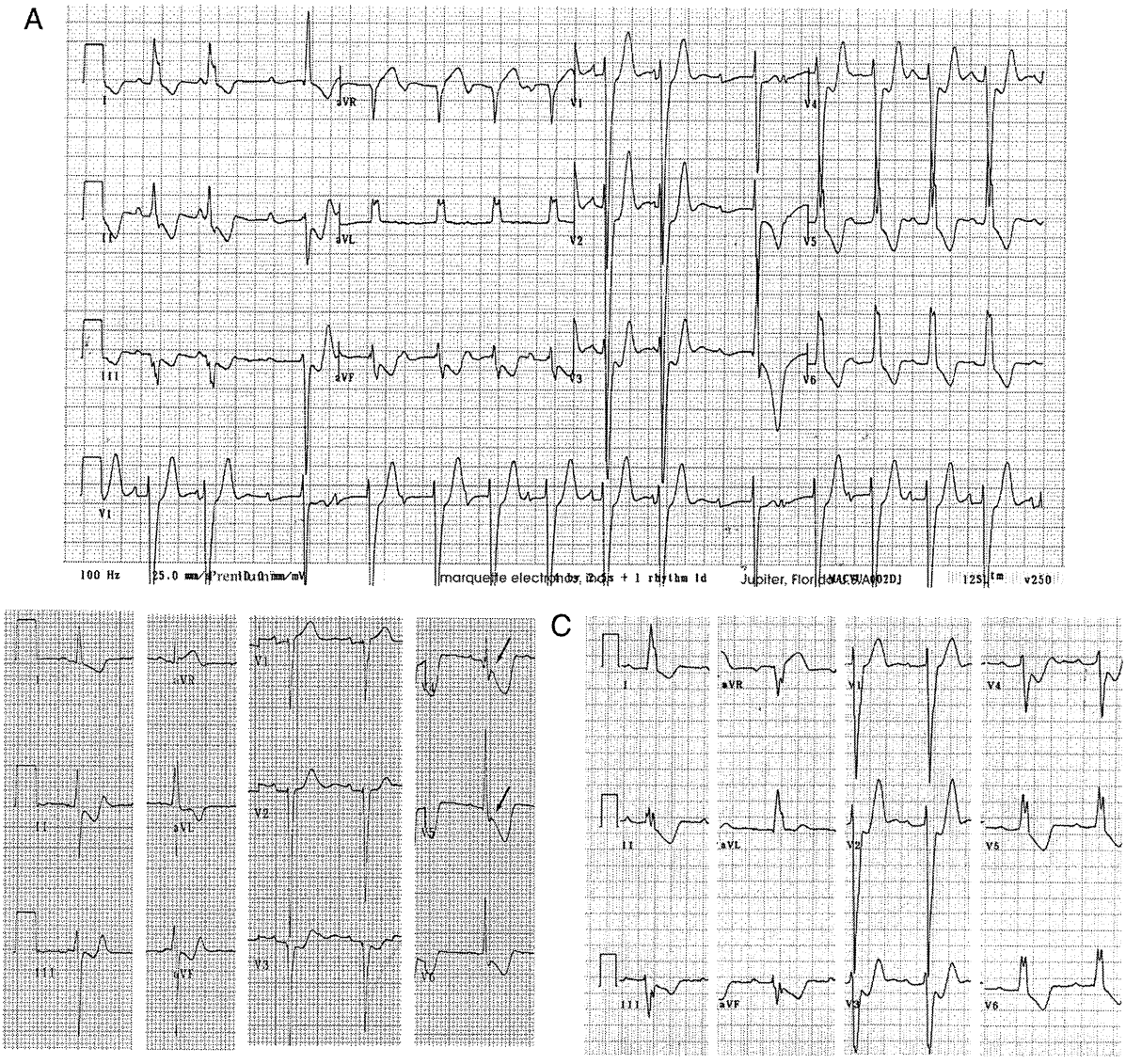

Figure 2. ECGs of proband and affected family members. **A**, ECG of proband at age 6 years, showing advanced atrioventricular block, complete left bundle branch block, and left axis deviation. Patient died suddenly 5 years later. **B**, ECG of proband's sister at age 6 years, showing QRS duration at the upper limit of normal, left axis deviation that has been progressive, and QRS notch in leads V4 and V5 (arrows) consistent with impaired intraventricular conduction. **C**, ECG of proband's mother at age 16 years, showing complete left bundle branch block and left axis deviation. She died suddenly at age 30.

examination. Other family members, including the proband's father, showed normal ECGs. DNA analysis of proband's father and maternal grandparents revealed absence of the Cx40-Q58L mutation. On the basis of clinical data and genotypic features of the proband and sister, it is most likely that the Cx40-Q58L mutation appeared de novo in the proband's mother. The data also indicate an early onset of PFHBI in this family compared with the natural history of the disease in most other cases.[8] As an initial step to assess the functional implications of the Cx40-Q58L mutation, modified constructs were transiently expressed in an exogenous system and evaluated for localization and function.

Electrophysiological Properties of Mutant Cx40-Q58L Channels

Connexin-deficient N2A cells were transiently transfected with cDNA for Cx40-WT or Cx40-Q58L; electrophysiological properties of homologous Cx40 channels were analyzed by conventional dual whole-cell patch clamp. Figure 3A shows representative junctional current traces elicited by a transjunctional voltage gradient of −60 mV. Average junctional conductance (Gj) decreased from 22.2±1.7 nS in cells expressing Cx40-WT (n=14) to 0.56±0.34 nS in cells expressing the Cx40-Q58L mutant (n=14; $P<0.001$). The probability of functional coupling, calculated by dividing the number of electrically coupled pairs by the number of pairs tested, was 100% and 57.1% for Cx40-WT and Cx40-Q58L, respectively.

Figure 3B depicts representative single-channel recordings elicited by a transjunctional voltage of −60 mV in cell pairs expressing Cx40-WT or Cx40-Q58L. Unitary events for WT channels displayed current transitions corresponding to 2 conducting states (O_1 and O_2) of 43.3 and 119.5 pS, respectively. Figure 3C shows the event histograms for both cell

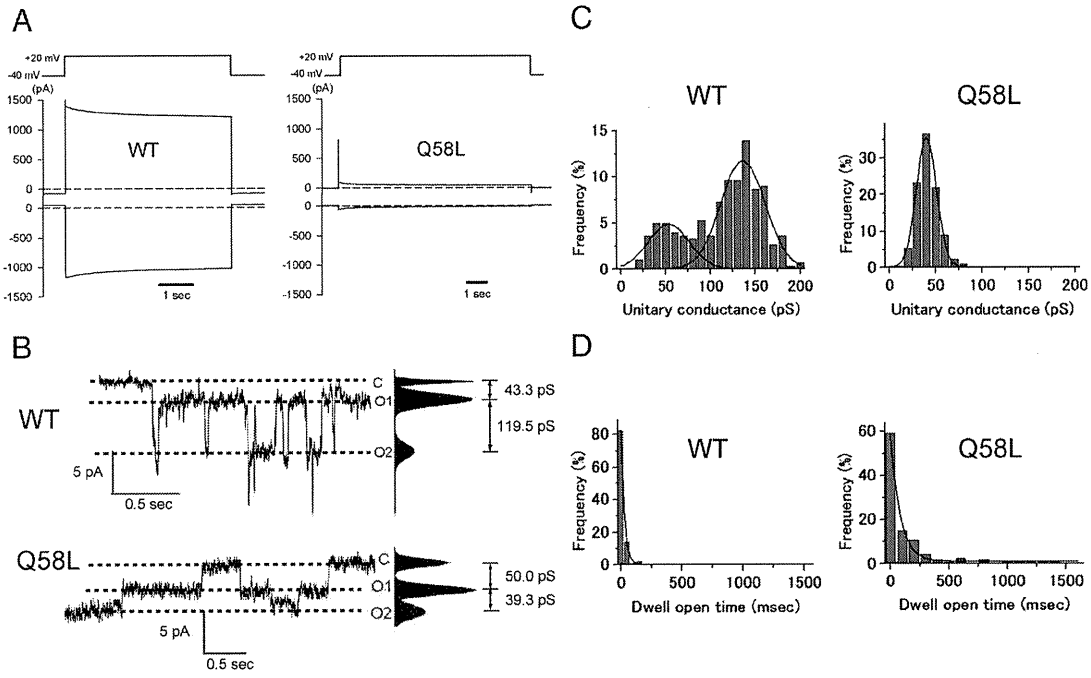

Figure 3. Whole-cell and single-channel properties of connexin40 (Cx40)-WT and Cx40–Q58L channels. **A**, Voltage pulse (top) and junctional current (bottom) from a homomeric WT cell pair (junctional conductance, 12.9 nS) and a Q58L cell pair (junctional conductance, 1.2 nS). **B**, Unitary currents recorded from homomeric Cx40-WT and Cx40–Q58L channels. O_1 and O_2 refer to 2 conducting (open) unitary levels of current. **C**, All-event histograms pooled from WT (n=3) and Q58L (n=3) cells with homologous channels. For WT, Gaussian peaks centered at 136.2±2.3 and 53.1±5.3 pS. For Q58L, best fit by a single Gaussian distribution centered at 40.2±0.3 pS (n=3). **D**, Frequency of events in relation to dwell open time. Binned data were fit by single exponentials (τ_{open} WT, 27.9±0.5 ms, 4 cells, 186 events; τ_{open} Q58L, 92.0±7.8 ms, 3 cells, 163 events). WT indicates wild type.

types (Cx40-WT, 3 cell pairs and 303 events; Cx40-Q58L, 3 cell pairs and 416 events). The histogram for the Cx40-WT channels was best described by 2 Gaussian distributions centered at 136.2±2.3 and 53.1±5.3 pS. In contrast, the histogram for Cx40-Q58L channels was best described by a single Gaussian function centered at 40.2±0.3 pS. Moreover, the length of time that a channel dwelled in the open state (dwell open time) was substantially longer for the Cx40-Q58L channels (92.0±7.8 ms, 3 cell pairs, 163 events) than for Cx40-WT channels (27.9±0.5 ms, 4 cell pairs, and 186 events) (Figure 3D). Of note, the Q58L mutation had a strong dominant effect on formation of heterotypic functional gap junctions. Cells were transfected with either pIRES2-EGFP-Cx40-WT or pIRES2-DsRED2-Cx40-Q58L, and heterotypic pairs were identified by fluorescence microscopy (an EGFP-expressing cell paired with a DsRED2-expressing cell). We recorded from 8 cell pairs and detected unitary current events in only 2 pairs. A total of 57 events were recorded, and average macroscopic junctional conductance was 0.04±0.03 nS. Collectively, the data demonstrated that the Q58L mutation significantly affects the biophysical properties of Cx40 channels and the overall ability of Cx40 gap junctions to form a low-resistance pathway between cells.

Electrophysiological Properties and Gap Junction Plaque Formation in Cells Coexpressing WT and Q58L Proteins

In the clinical cases identified, the Q58L mutation was detected in only 1 carrier allele. Therefore, we assessed the function of gap junctions in cells coexpressing WT and mutant proteins. N2A cells were cotransfected with cDNA for both GFP-tagged Cx40-WT and Cx40-Q58L (0.5 μg of pEGFPN1-Cx40-WT combined with 0.5 μg of pEGFPN1-Cx40-Q58L). Results were compared with those obtained when only 1 of the constructs (1 μg) was transfected. Cells expressing both constructs (WT/Q58L) showed intermediate conductance (15.4±3.7 nS, n=16) between WT (28.8±3.6 nS, n=16, $P<0.001$) and Q58L (0.28±0.11 nS, n=14, $P<0.001$) (Figure 4A). These values were comparable to those obtained using the bicistronic pIRES2-EGFP constructs (WT, 22.2±1.7 nS, n=14; WT/Q58L, 13.0±2.4 nS, n=17; Q58L, 0.56±0.34 nS, n=14). The coexpression results were consistent with those obtained using pIRES plasmids that tagged the cells both green and red, if cotransfected (online-only Data Supplement Figure I). The probability of finding functional coupling in cotransfected cells was 76.5%, which was intermediately between WT (100%) and Q58L (57.1%).

The characteristics of gap junction plaques observed in cells coexpressing WT and Q58L varied significantly between pairs (Figure 4B). Nearly one half of transfected (fluorescence-positive) cells exhibited clear and discrete gap junction plaques (arrow a), whereas the rest of fluorescent-positive cells showed a diffuse expression pattern and absence of well-defined plaques (arrow b). Fluorescence-positive and gap junction plaque-positive cells were counted in 10 different views for each group, and efficacy of gap junction plaque formation was statistically analyzed (Figure 4C) by calculating the ratio of cells with gap junction plaques

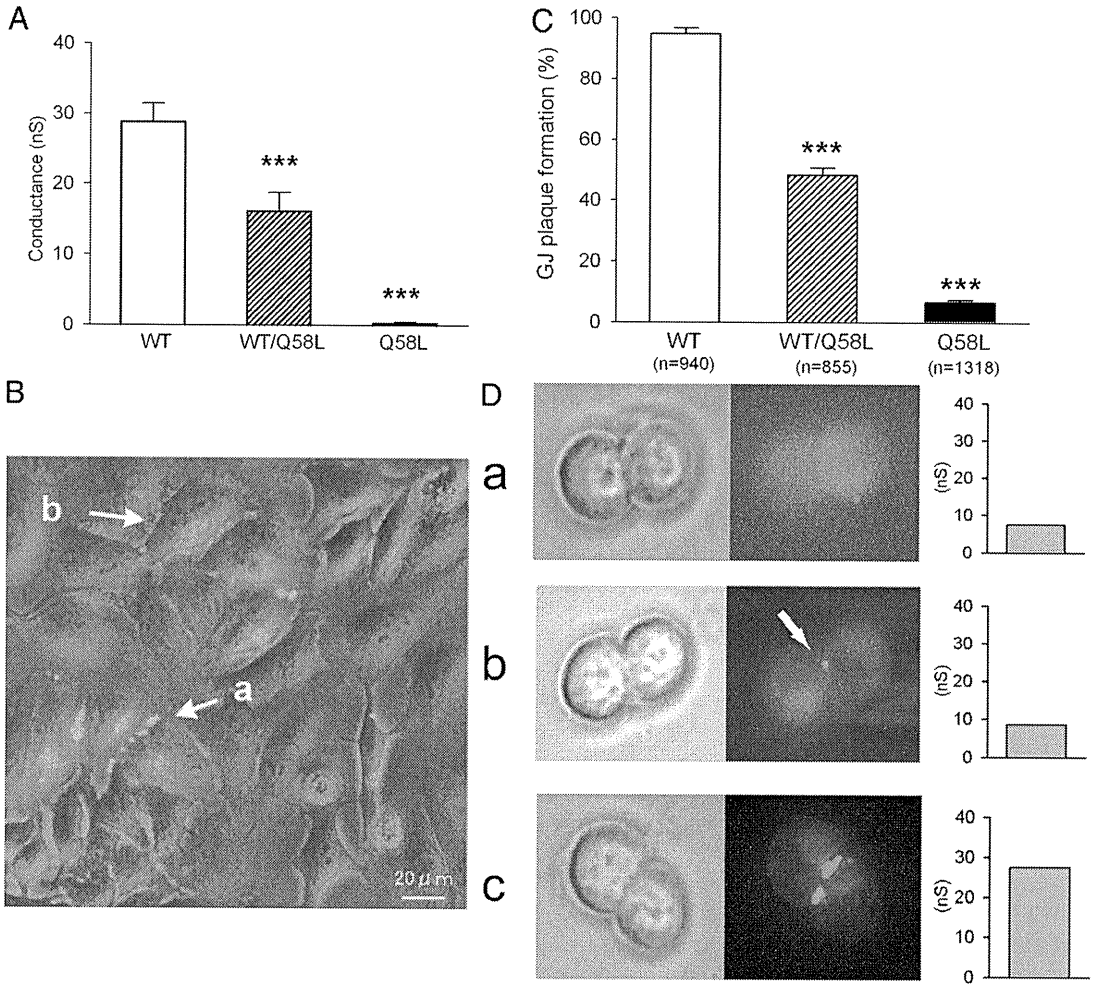

Figure 4. Macroscopic conductance and gap junction plaque morphology in cells coexpressing connexin40 (Cx40)-WT and Cx40-Q58L. **A**, Junctional conductance of cells transfected with plasmid pEGFPN1-Cx40-WT (1 μg), pEGFPN1-Cx40-Q58L (1 μg), or cotransfected with WT and Q58L (WT/Q58L, pEGFPN1-Cx40-WT 0.5μg+pEGFPN1-Cx40-Q58L 0.5 μg). **B**, Phase contrast/fluorescence overlay image of neuroblastoma cells transfected with WT/Q58L constructs. Arrow *a* points to gap junction plaque; arrow *b* points to an example of cells transfected but devoid of gap junction plaque. **C**, Efficacy of gap junction plaque formation was measured as the ratio between the number of gap junction plaque-positive cells and the number of fluorescent-positive cells (WT, n=940; WT/Q58L, n=855; Q58L, n=1318). **D**, Representative images of phase contrast (left), EGFP fluorescence (middle), and junctional conductance (right) from neuroblastoma cells cotransfected with pEGFPN1-Cx40-WT (0.25 μg) and pEGFPN1-Cx40-Q58L (0.25 μg). Three different examples illustrate the relation between plaque morphology and recorded junctional conductance. WT indicates wild type. ***$P<0.001$ compared with WT.

to the number of fluorescence-positive cells. In the Cx40-WT group, almost all fluorescent-positive cells exhibited clear gap junction plaques (94.9±1.9%, n=940), whereas there was a more-diffuse and homogenous pattern with only occasional plaque formation in the Cx40-Q58L group (6.6±0.7%, n=1318, $P<0.001$ compared with WT). In contrast, results varied widely in cells cotransfected with WT/Q58L; nearly one half of fluorescence-positive cells exhibited gap junction plaques similar to those observed in cells transfected with the WT construct (48.2±2.4%, n=855, $P<0.001$), whereas the rest showed a diffuse expression pattern similar to that of Cx40-Q58L. To establish a better correlation between plaque formation and junctional conductance, both variables were measured concurrently in the same cell pair for 39 N2A cell pairs where GFP-tagged plasmids of Cx40-WT and Cx40-Q58L were cotransfected. As shown in Figure 4D, about one half of GFP-positive cell pairs showed a very small Gj (<5 nS) and very few or negligible gap junction plaques (a). In the other half of cell pairs, small, dot-like junctional plaques correlated with intermediate Gj values (b), and there were clear, extensive gap junction plaques associated with Gj values >25 nS (c). Overall, we found significant heterogeneity in the extent of electric coupling, although the measurements of Gj correlated with the localization of proteins in transfected cells. These results indicate that the Q58L mutation significantly impairs the ability of cells to form gap junction plaques, although the effect is not purely dominant when both WT and mutant proteins are coexpressed.

Subcellular Distribution of WT and Q58L Cx40 in Transiently Transfected Cells

To further analyze the subcellular distribution of Cx40-WT and Cx40-Q58L proteins, the C terminal of Cx40-WT was

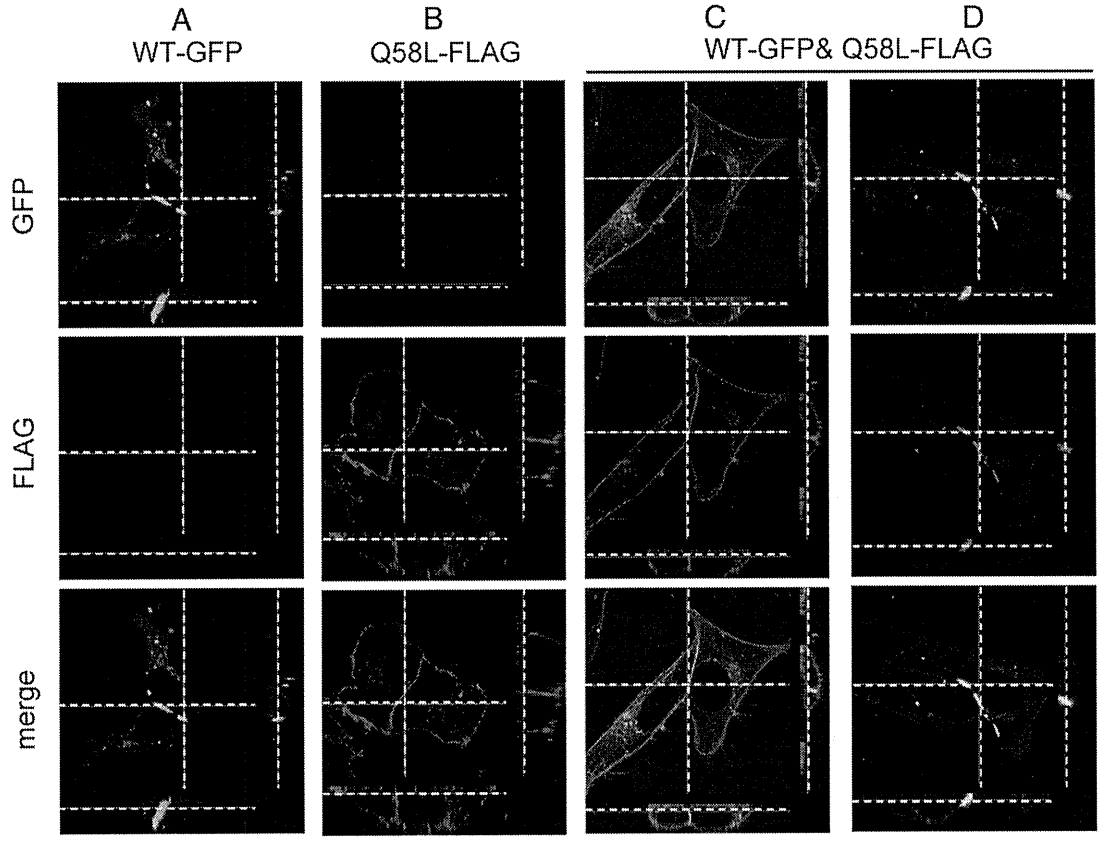

Figure 5. Subcellular distribution of connexin40 (Cx40)-WT and Cx40-Q58L in transiently transfected cells. HeLa cells were transiently transfected with pEGFPN1-Cx40-WT (3.0 μg) (**A**), pCMV-FLAG-Cx40-Q58L (3.0 μg) (**B**), or pEGFPN1-Cx40-WT (1.5 μg) plus pCMV-FLAG-Cx40-Q58L (1.5 μg) (**C**); immunostained for the respective tag protein; and visualized by confocal laser scanning microscopy. Notice that gap junction plaques (**A**) are absent in Q58L transfectants (**B**) and present in some (**D**) but not all (**C**) cotransfected cells. Bar=20 μm. WT indicates wild type.

tagged with GFP, whereas the C terminal of Cx40-Q58L was FLAG tagged. After transfection of N2A cells with the tagged constructs, the distribution of each protein was examined by confocal microscopy. As shown in Figure 5, green color indicates the position of GFP-tagged molecules, whereas red indicates the position of FLAG-tagged molecules. In cells transfected only with GFP-tagged Cx40-WT, fluorescence was consistently detected at sites of cell-cell apposition, following the pattern previously described for GFP-labeled gap junction plaques (Figure 5A). A similar distribution was found when cells were transfected with FLAG-tagged Cx40-WT (not shown). In contrast, most FLAG-tagged Cx40-Q58L signals were evenly distributed around the cell in the vicinity of the plasma membrane (Figure 5B). Biotinylation experiments showed that the Q58L mutation did not prevent the Cx40 protein from inserting into the membrane and presenting a domain-reachable form in the extracellular space (online-only Data Supplement Figure II). Micoscopy experiments in cells coexpressing GFP-tagged Cx40-WT and FLAG-tagged Cx40-Q58L proteins yielded results intermediate to those obtained when only 1 construct was expressed. Nearly one half of cell pairs showed that both proteins distributed homogenously at or near the cell membrane, without the formation of well-defined gap junction plaques (Figure 5C). These images resembled those obtained when only Cx40-Q58L proteins were expressed (Figure 5B, FLAG). In contrast, other cell pairs showed clustering of fluorescent signals within closely confined areas that appeared to be gap junction plaques (Figure 5D).

The experiments described herein led us to speculate that the distribution and function of heteromeric connexons is determined by their mutant subunit content, whereby formation (or not) of plaques and channels are determined, at least in part, by the abundance of expression of one protein over the other. As an initial step to probe this hypothesis, we took advantage of the characteristics of the bicistronic plasmid pIRES, in which the expression rate of the upstream gene is several-fold greater than that of the downstream gene,[20] and explored the functional properties of heteromeric connexons. Cx40-WT and GFP-tagged Cx40-Q58L were subcloned into the pIRES vector, either alone or in combination, in the specific orientations shown in Figure 6A. Protein expression levels of Cx40-WT and Cx40-Q58L were determined by immunochemistry. In contrast to the data obtained when Cx40-WT and GFP-tagged Cx40-Q58L plasmids were cotransfected at a 1:1 ratio (lane 6), expression of heteromeric pIRES plasmids WT-IRES-Q58L-EGFP (lane 3) and Q58L-EGFP-IRES-WT (lane 4) resulted in uneven protein expression levels of WT (40 kDa) and Q58L-EGFP (67 kDa), depending on their orientation in the pIRES vector. Based on