Novel Mechanism Associated With an Inherited Cardiac Arrhythmia
Defective Protein Trafficking by the Mutant HERG (G601S) Potassium Channel

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Background—The congenital long-QT syndrome (LQTS) is an inherited disorder characterized by a prolonged cardiac action potential and a QT interval that leads to arrhythmia. Mutations in the human ether-a-go-go–related gene (HERG), which encodes the rapidly activating component of the delayed rectifier current (I\textsubscript{Kr}), cause chromosome 7–linked LQTS (LQT2). Studies of mutant HERG channels in heterologous systems indicate that the mechanisms mediating LQT2 are varied and include mutant subunits that form channels with altered kinetic properties or nonfunctional mutant subunits. We recently reported a novel missense mutation of HERG (G601S) in an LQTS family that we have characterized in the present work.

Methods and Results—To elucidate the electrophysiological properties of the G601S mutant channels, we expressed these channels in mammalian cells and \textit{Xenopus} oocytes. The G601S mutant produced less current than wild-type channels but exhibited no change in kinetic properties or dominant-negative suppression when coexpressed with wild-type subunits. To examine the cellular trafficking of mutant HERG channel subunits, enhanced green fluorescent protein tagging and Western blot analyses were performed. These showed deficient protein trafficking of the G601S mutant to the plasma membrane.

Conclusions—Our results from both the \textit{Xenopus} oocyte and HEK293 cell expression systems and green fluorescent protein tagging and Western blot analyses support the conclusion that the G601S mutant is a hypomorphic mutation, resulting in a reduced current amplitude. Thus, it represents a novel mechanism underlying LQT2. (\textit{Circulation}. 1999;99:2290-2294.)

Key Words: genes ■ arrhythmia ■ long-QT syndrome

Long-QT syndrome (LQTS) is characterized by prolongation of the QT interval and is an inheritable, autosomal-dominant susceptibility to cardiac arrhythmias. LQTS was originally identified clinically as a lengthened QT interval on the ECG that can lead to torsade de pointes, syncope, and sudden death.\textsuperscript{1,2} Recent findings have led to advances in the understanding of the genetic basis of LQTS. Four ion channel genes are now associated with LQTS, and mutations have been discovered within these genes.\textsuperscript{3–8} One of these genes, \textit{HERG}, encodes a component of the cardiac rapidly activating delayed rectifier current, I\textsubscript{Kr}, which helps terminate the cardiac action potential.\textsuperscript{9–12} A reduction in I\textsubscript{Kr} causes delayed repolarization\textsuperscript{13} and increased risk of LQTS.

Existing mechanisms by which mutations in \textit{HERG} are thought to cause LQTS are diverse. They include a gene dosage effect in which the mutant subunits are not expressed and therefore do not affect wild-type subunits on coexpression or a dominant-negative effect in which the mutant subunit suppresses wild-type function on coassembly. Some mutations alter channel kinetics when expressed alone or with wild-type subunits, and this can be coupled with dominant-negative suppression.\textsuperscript{14,15} We recently reported that a glycine-to-serine mutation at position 601 in \textit{HERG} (G601S) is present in an LQTS family.\textsuperscript{16} This mutation is located in the extracellular loop between the S5 domain and the pore domain (Figure 1A). In the present work, we show that the
G601S mutant expresses less current than wild-type channels but exhibits no dominant-negative suppression of wild-type subunits and has no apparent alteration in gating kinetics. A large percentage of the G601S mutant channels do not traffic to the plasma membrane. Thus, this mutant represents a novel mechanism of altering currents in vivo, reduced expression levels.

**Methods**

**Generation of the G601S Expression Construct**

The G601S mutant construct was generated by reverse transcriptase–polymer chain reaction from an LQTS patient carrying the mutation. The polymerase chain reaction product was subcloned into an HERG/pcDNA3 expression construct. The insert region, including the cloning sites, was sequenced directly. Also, HERG sequence was inserted downstream of the coding region of an enhanced green fluorescent protein (GFP), cloned in a pEGFP-C2 vector (CLONTECH). The first ATG was 57 bp downstream of the final GFP codon. The insert region, including the cloning sites, was sequenced directly.

**Transfection and Voltage Clamp of HEK293 Cells**

For expression in mammalian cells, we used the human embryonic kidney 293 cells (HEK293, ATCC No. CRL-1573). The cells were cultured to 60% to 70% confluence in a 35-mm tissue culture plate and were transfected with the lipofectamine method (Gibco-BRL) with 2.5 µg of the following: wild-type HERG/pcDNA3, G601S HERG/pcDNA3, the GFP-tagged wild-type HERG (GFP-WT), G601S mutant HERG (GFP-G601S), and pEGFP-C2. Electrical recording was performed 48 hours after initiation of transfection. The whole-cell voltage-clamp method used was the same as that described previously.17–19 Experiments were performed at room temperature. The membrane capacitance was 17.4 ± 3.1 pF (n = 11 cells) in wild-type HERG and 16.9 ± 2.1 pF (n = 8 cells, values represent mean ± SEM) in the G601S mutant; these values were not significantly different among these 2 groups (2-way Student’s t test, P > 0.05). The normal Tyrode’s solution contained the following (in mmol/L): NaCl 136.9, KCl 5.4, CaCl2 1.8, NaH2PO4 0.33, glucose 5, and HEPES 5 (pH adjusted to 7.4 with NaOH). The standard pipette solution contained (in mmol/L) KOH 110, KCl 20, EGTA 10, MgCl2 2, K2-ATP 5, K2-creatine phosphate 5, and HEPES 5 (pH 7.4 with aspartic acid, ~60 mmol/L).

**Voltage Clamp of Xenopus Oocytes**

RNA was prepared from 1 µg of template DNA in the pGH19 expression vector20 by use of T7 RNA polymerase from the message machine kit (Ambion). DNase I was then used to remove the template, and RNA was precipitated with 7.5 mol/L LiCl. The concentration of the purified RNA was then determined spectrophotometrically. The RNA was diluted with sterile water to yield ~2.5 ng RNA per injection at a volume of 36.8 nL. Cells injected with 1.25 ng RNA were injected with 18.4 nL of the same batch of RNA. Oocytes were stored at 18°C in ND-96 solution21 supplemented with 1 mmol/L gentamycin and 1 mg/mL BSA.

Figure 1. G601S mutant expressed in HEK293 cells. A, G601S mutation lies in extracellular region between S5 and pore domain. B through E, Currents from wild-type HERG-transfected cells. F through I, Currents from G601S-transfected cells. Tail current was recorded at –40 mV. I-V relationship obtained at end of depolarizing pulse revealed characteristic inward rectification (D and H), and I-V relationship for peak tail current amplitude showed voltage dependence of activation (E and I). J, Confocal-imaged GFP tagging of G601S and wild-type HERG channels in HEK293 cells. Left, Merged images of Nomarski with GFP fluorescence; right, GFP fluorescence images. GFP-WT signal was present intracellularly in endoplasmic reticulum and perinuclear space and in plasma membrane (fluorescent circular image). Unstained region within cell is nucleus. For GFP-G601S, signal was present intracellularly but with reduced plasma membrane signal. K, Western blot showing HERG-WT and G601S mutant proteins.
Properties of Mutant HERG (G601S)

Two-electrode voltage clamp recordings were performed at room temperature 1 to 4 days after injection as previously described. The external bath solution contained (in mmol/L) KCl 5, NaCl 95, CaCl$_2$ 0.3, MgCl$_2$ 1.0, and HEPES 5, pH 7.4.

Laser Confocal Microscopy
Cells were cultured for 36 to 48 hours after transfection and photographed at 660x magnification under a microscope (LSM510, Zeiss). Confocal analysis was performed with an argon-krypton laser. Cells were observed after being treated with 0.1% trypsin (which causes the cells to become round) and resuspended in PBS.

Western Blot Analysis
Western blot analysis was performed as previously described. The membrane proteins were subjected to SDS-PAGE. They were then electrophoretically transferred onto nitrocellulose membranes. The nitrocellulose membranes were incubated with the HERG antifusion protein antibody (1:20 000 dilution) at room temperature overnight, and the antibody was detected with an ECL detection kit. The HERG antibody and its specificity have been previously described.

Results
Expression of HERG (G601S) Mutant Channels in HEK293 Cells
Figure 1 compares the basic properties of HERG-WT and G601S mutant channels expressed in HEK293 cells. Figure 1B and 1C show HERG-WT currents elicited by depolarizing pulses between −40 and 0 mV (Figure 1B) and 10 and 50 mV (Figure 1C) from a holding potential of −80 mV. The outward current was activated in response to test potentials to ＞−50 mV and then progressively decreased with test potentials of ＞0 mV (Figure 1D). The amplitude of the tail current at −40 mV saturated at ≈10 mV (Figure 1C). The current-voltage (I-V) relationships revealed that the peak outward current decreased at potentials to 10 mV, reflecting the inward rectifying property caused by the fast inactivation of HERG current (Figure 1D). The voltage dependence of channel activation was obtained by measuring the relative amplitude of the tail current as a function of test potentials. The voltage of half-maximal activation (V$_{0.5}$) was －18.80±0.18 mV, and the slope factor was 8.46±0.10 mV per e-fold change in conductance in HERG-WT (Figure 1E). The current density was quantified by measurement of the tail currents at −40 mV after a 3-second depolarizing test pulse to 20 mV. Currents were normalized for plasma membrane surface area by cell capacitance, giving a current density of 63.1±5.5 pA/pF (n=11 cells) in HERG-WT. The corresponding properties of G601S mutant current in HEK293 cells are shown in Figure 1F through 1H. G601S also showed characteristic inwardly rectifying outward current similar to HERG-WT, but the current density of 9.8±0.7 pA/pF (n=8 cells) in G601S mutant is only 15.5% of the HERG-WT current. The voltage dependence of channel activation was similar to that of HERG-WT, with an V$_{0.5}$ of －17.90±0.11 mV and a slope factor of 8.38±0.15 mV per e-fold change in conductance in G601S mutant. These results indicated that the voltage dependence and kinetics of the G601S mutant were similar to those of HERG-WT channels, except that its current density was substantially smaller and inactivation at the most positive voltages was slightly greater.

GFP Tagging and Western Blot Analyses
To examine the cellular trafficking of mutant HERG channel subunits, GFP was tagged to the N-terminus of HERG subunits and was expressed in HEK293 cells. As shown in Figure 1J, a strong confocal fluorescence signal associated with GFP-WT was present in the endoplasmic reticulum, perinuclear space, and plasma membrane. In contrast, a weak confocal fluorescence signal with GFP-WT was seen in the plasma membrane, whereas a strong confocal signal was present in the endoplasmic reticulum and perinuclear space. The GFP-tagged G601S mutant HERG channels produced current on patch clamping of these cells (data not shown). In addition, the fluorescence signal associated with pEGFP-C2 was seen in the cytoplasm (data not shown).

We also investigated the Western blot analysis of the expression of HERG-WT and G601S mutant HERG channel protein in transiently transfected HEK293 cells (Figure 1K). The HERG-WT shows 2 bands of HERG protein, a weaker upper broad band with a molecular mass of ≈155 kDa and a stronger lower band with a molecular mass of ≈135 kDa. For G601S mutant HERG protein, only a lower band (≈135 kDa) was present.

Expression of Mutant HERG (G601S) and Coexpression With HERG-WT in Xenopus Oocytes
We used the Xenopus oocyte expression system to confirm our results in the HEK293 cells and to test for a dominant-negative phenotype. Our results with Xenopus oocytes are consistent with those in cultured cells, with the G601S mutant producing less current than HERG-WT channels. Injection of identical amounts of RNA resulted in G601S mutant currents that were ≈39% smaller than wild-type currents (Figure 2A, 2B, and 2F; 2-way Student’s t test, P＜0.001). The smaller currents from G601S channels are not obviously due to alteration of channel gating mechanisms, because the steady-state voltage dependence of activation (Figure 2C) and inactivation (Figure 2D and 2E) are identical to those of wild-type HERG channels. The V$_{0.5}$ was －30.15±0.15 mV with a slope factor of 10.34±0.13 (millivolts per e-fold change in conductance) for wild type (mean±SEM; n=10 cells) compared with a V$_{0.5}$ of －29.30±0.21 mV and slope factor of 9.80±0.19 for the mutant (n=7 cells). Differences in V$_{0.5}$ between the oocytes and the HEK293 cells are attributable to differences in external Ca$^{2+}$ used in the 2 preparations. The V$_{0.5}$ of the steady-state inactivation curve was －76.17±0.99 mV with a slope factor of 19.96±0.84 for wild-type channels (n=9 cells) compared with a V$_{0.5}$ of －78.89±0.81 mV and a slope factor of 20.09±0.69 for the G601S mutant channels (n=7 cells). The midpoints and slope factors of the mutant channels are not significantly different from the corresponding parameters of the wild-type channels (2-way Student’s t test, P＞0.05).

We tested for a dominant-negative suppression of HERG expression by the G601S mutant using a quantitative coinjection approach in oocytes. Care was taken to ensure that the resulting current expressed was roughly proportional to the amount of RNA injected at the 2 concentrations. Doubling the amount of RNA injected from 1.25 to 2.5 ng resulted in...
Figure 2. Coexpression of HERG-WT and G601S mutant channels in oocytes. Families of currents evoked from oocytes were injected with 2.5 ng wild-type RNA (A) and G601S RNA (B). C, Steady-state conductance-voltage (g/gmax versus V) relation for HERG-WT and G601S mutant channels was determined from analysis of tail currents. D, Inset. Tail currents were fit with y = A1 e^(-V-V0.5)/k + A2 e^(-V-V0.5)/k and extrapolated to onset of repolarizing pulse to correct for fast recovery from inactivation as monoexponential rising phase in tails. Extrapolated values were plotted versus voltage and fit with single-power Boltzmann function y = 1/[1 + e^(-0.5(V-V0.5)/k)]. D and E, Steady-state inactivation relation was obtained for HERG-WT and G601S mutant by use of 3-pulse voltage protocol (D, inset). Channels were fully activated by 1-second pulse to 40 mV, followed by 30-ms conditioning pulses to range of voltages from 70 to −150 mV, during which channels equilibrated between open and inactive state. Number of channels in open state was reported by subsequent pulses to 40 mV.2 Resulting instantaneous current (D, arrow) was plotted versus voltage (after correction for closing at hyperpolarized potentials) and fit with single-power Boltzmann function y = 1/[1 + e^(-0.5(V-V0.5)/k)]. All data in Figure 2 is from same batch of oocytes because different batches of cells expressed different amounts of current. All were recorded 48 hours after injection.

Discussion

The G601S mutant channel phenotype is a reduction in current. Evidence in support of this conclusion includes the reduced current amplitude of G601S compared with wild-type channels in 2 heterologous expression systems, persistence of normal gating mechanisms in mutant channels, and lack of a dominant-negative interaction of mutant with wild-type subunits. Instead, the G601S mutant phenotype may be the result of fewer channels in the plasma membrane.

To date, the phenotypes of several chromosome 7–linked LQTS (LQT2) mutations have been characterized by expression of the mutant HERG channels in heterologous systems,14,15,22,24 and more studies of other mutants are expected. Electrophysiological studies have shown that some mutations cause loss of or altered channel function. Some mutations also cause dominant-negative suppression of HERG-WT channels or may shift the voltage dependence of channel inactivation.14,24 Recently, HERG-WT and LQT2 protein processing has been studied through biochemical approaches.12,22 These studies showed that HERG-WT generates 2 protein bands on Western blot: the upper band represents the mature protein that forms functional channels in the surface membrane, whereas the lower band represents immature protein in the endoplasmic reticulum.22 Furthermore, some LQT2-associated HERG mutations generated different patterns on Western blot: 2 mutations (Y611H and V822 M) caused defects in the biosynthesis of HERG channels with the protein retained in the endoplasmic reticulum; 2 other mutations (I593R and G628S) were processed similarly to HERG-WT protein, but the mutations did not produce functional channels; and 1 mutation (T474I) was processed similarly to the wild-type protein and expressed HERG current with altered gating properties. These findings suggested that the loss of HERG channel function in LQT2 mutations is caused by multiple mechanisms, including abnormal channel processing, generation of nonfunctional channels, and altered channel gating, as well as by dominant-negative suppression of HERG-WT current.

In the present work, the G601S mutation represents another mechanism for LQT2. As shown in Figures 1B through I and 2, this mutation produces current that is similar to HERG-WT current but with reduced amplitude. The GFP-tagging and Western blot data in Figure 1J and 1K show that G601S mutant protein is generated as the immature protein within the endoplasmic reticulum but that very little is processed to the mature form of the protein. Hence, for the G601S mutant, very little GFP-tagged protein is visible in the plasma membrane, and the amount of mature protein in the transiently transfected cells is insufficient to be detected on the Western blot. Thus, the G601S mutation is the first example of a trafficking-deficient, normal-gating mutant HERG channel. Similar findings have been observed with hypomorphic mutations with altered protein trafficking in CFTR channels.25

The extent of G601S mutant expression compared with that of HERG-WT is markedly different in the 2 heterologous expression systems. In HEK293 cells, the mutant expression level was only ∼15% relative to HERG-WT, whereas in Xenopus oocytes, the mutant expressed ∼61% of the current

a 78% increase from 2503 ± 161 (n=10 cells) to 4457 ± 215 (n=16 cells) nA for wild-type currents and a 75% increase from 1581 ± 133 (n=10 cells) to 2771 ± 214 (n=10 cells) nA for G601S currents. If the G601S mutant exhibits dominant-negative suppression, the currents produced by coinjection of oocytes, the mutant expressed that of HERG-WT is markedly different in the 2 heterologous expression systems,14,15,22,24 and more studies of other mutants are expected. Electrophysiological studies have shown that some mutations cause loss of or altered channel function. Some mutations also cause dominant-negative suppression of HERG-WT channels or may shift the voltage dependence of channel inactivation.14,24 Recently, HERG-WT and LQT2 protein processing has been studied through biochemical approaches.12,22 These studies showed that HERG-WT generates 2 protein bands on Western blot: the upper band represents the mature protein that forms functional channels in the surface membrane, whereas the lower band represents immature protein in the endoplasmic reticulum.22 Furthermore, some LQT2-associated HERG mutations generated different patterns on Western blot: 2 mutations (Y611H and V822 M) caused defects in the biosynthesis of HERG channels with the protein retained in the endoplasmic reticulum; 2 other mutations (I593R and G628S) were processed similarly to HERG-WT protein, but the mutations did not produce functional channels; and 1 mutation (T474I) was processed similarly to the wild-type protein and expressed HERG current with altered gating properties. These findings suggested that the loss of HERG channel function in LQT2 mutations is caused by multiple mechanisms, including abnormal channel processing, generation of nonfunctional channels, and altered channel gating, as well as by dominant-negative suppression of HERG-WT current.

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seen with HERG-WT. The reason for this difference in current amplitudes is unknown, but it may be attributable to temperature-dependent differences in levels of protein expression in HEK293 cells, which are incubated at 37°C, compared with the oocytes, which are incubated at 18°C. Mutant CFTR ion channel proteins show a temperature-dependent increase in protein expression as incubation temperatures are lowered from 37°C to 23°C. Whether a similar dependence of G601S mutant expression on temperature accounts for the differences exhibited between the heterologous systems will require further experimentation.

The reduction in current observed on expression of LQT2-associated mutations in HERG suggests that a reduction in native \( I_{\text{K}} \) causes susceptibility to cardiac arrhythmia. The reduction in expression of the G601S mutant, which is in the extracellular loop between S5 and the pore domain, is a less severe phenotype than those previously described for other mutations located in other domains, which have either gene dosage or dominant-negative effects. It will be interesting to determine whether the degree of HERG channel dysfunction correlates with the severity of the disease state.

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References

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