The Dominant Negative LQT2 Mutation A561V Reduces Wild-type HERG Expression*

(Received for publication, December 3, 1999, and in revised form, January 19, 2000)

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HERG K⁺ channel mutations are responsible for one form of dominantly inherited long QT syndrome (LQT). Some LQT mutations exert a dominant negative effect on wild-type current expression. To investigate mechanisms of dominant-negative behavior, we co-expressed wild-type HERG with the A561V mutant in mammalian cells. Transfection with various cDNA ratios produced HERG K⁺ current densities that approached a predicted binomial distribution where mutant and wild-type subunits co-assemble in a tetramer with nearly complete dominance. Using C terminus myc-tagged wild-type HERG we specifically followed the mutant's effect on full-length wild-type HERG protein expression. Co-expression with A561V reduced the abundance of full-length wild-type HERG protein comparable to the current reduction. Reduction of wild-type protein was due to decreased synthesis and increased turnover. Conditions facilitating protein folding (growth at 30 °C, or in 10% glycerol) resulted in partial rescue from the dominant effect, as did the 26 S proteosome inhibitor ALLN. Thus, for A561V, dominant negative effects result from assembly of wild-type subunits with mutant very early in production leading to rapid recognition of mutant channels and targeting for proteolysis. These results establish protein misfolding, cellular proofreading, and bystander involvement as contributing mechanisms for dominant effects in LQT2.

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cDNA (1 µg). Varying amounts of A561V HERG cDNA were added for a final mutant HERG molar ratios of 0, 0.2, 0.33, 0.5, 0.6, and 0.8 of total HERG cDNA. Cells were studied with voltage clamp at 20–36 h after transfection.

Electrophysiology—Cells were plated and grown on sterile glass coverslips within 35-mm tissue culture dishes. The coverslips were taken directly from the cell culture incubator and placed in an acrylic/polystyrene perfusion chamber (Warner Instruments) for immediate electrophysiological measurements. Extracellular solution was 150 mM NaCl, 1.8 mM CaCl2, 4 mM KCl, 1 mM MgCl2, 5 mM glucose, and 10 mM HEPES buffer, pH 7.4, at room temperature. Intracellular pipette solution was 128 mM KCl, 4 mM MgCl2·6H2O, 2 mM MgSO4·7H2O, 5 mM NaCl, 25 mM HEPES buffer, pH 7.2, at room temperature. Osmolarity was adjusted to maintain the internal solution 20–30 mOsm less than the external solution. The whole cell patch clamp configuration was used to study HERG channels (17). Patch pipettes were pulled and polished to obtain a tip resistance of 2–3 megaohm in the above solutions. An Axopatch-1D patch clamp amplifier (Axon Instruments) was used for voltage clamp measurements and voltage protocols were controlled via PC using pClamp6 acquisition and analysis software. To elicit HERG K+ currents depolarizing voltage pulses were applied to various levels from a holding potential of ~75 mV for 3.5–4.0 s followed by partial repolarization to ~40 mV and then to ~120 mV briefly to measure outward and inward tail currents. Current densities were calculated as current (nA) divided by cell capacitance (pF). The values shown for transfection efficiency, current densities, were normalized to the control group daily based on average current density of cells transfected with 100% wild type.

Immunoblot Analysis—Cells were harvested for analysis 36–48 h after transient transfection by detaching cells with RPMI supplemented with 10 mM EDTA at 37 °C. Resuspended cells were subsequently pelleted by centrifugation at 300 × g for 5 min at 4 °C and washed with ice-cold phosphate-buffered saline. All subsequent steps were performed at 4 °C. For whole cell lysate analysis, cell pellets were resuspended in 500 µl of lysis buffer and pelleted by centrifugation at 13,200 rpm for 5 min at 4 °C. Membranes were dissolved in 50 µl of lysis buffer and analyzed as above.

Pulse-Chase and Metabolic Labeling—16 h after transfection, cells were subjected to short (5 min) or long (30 min) 35S labeling pulses. Cells were starved of cysteine and methionine in a serum-free Dulbecco’s modified Eagle’s medium with high glucose, without l-glutamine, l-methionine, or l-cysteine for 1 h at 37 °C. Cells were suspended in above media supplemented with 10 mM EDTA and pelleted at 1,200 rpm for 5 min. Labeling pulses were carried out by resuspending cells in above media containing 230 µCi/ml of [35S]cysteine and [35S]methionine (ICN, Trans-label) and incubating for 5 min (short) or 30 min (long) at 37 °C. Pulses were terminated by replacing labeling medium with chase medium (10 mM each of unlabeled cysteine and methionine, 10% fetal calf serum RPMI), and incubating at 37 °C for various times. At the end of the chase interval, cells were collected by centrifugation at 1,200 rpm for 5 min at 4 °C, homogenized with 250 µl of lysis buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.4% deoxycholic acid, 1% Nonidet P-40). Lysates were cleared by centrifugation at 13,200 rpm for 5 min at 4 °C. Immunoprecipitation from the cleared lysates was performed by pre-clearing with 30 µl of protein G-agarose (Pierce, UltraLink) for 30 min at 4 °C, followed by incubation with monoclonal 9E10 anti-myc asces for 2 h at 4 °C. Protein-antibody complexes were precipitated with protein G-agarose for 1 h at 4 °C and centrifuged at 13,200 rpm for 3 min. For quantified immunoprecipitation serial incubations with additional antibody and protein G-agarose were performed. When normalizing the total amount of HERG protein a limiting amount of antibody (10 µl) was used. After washing the pellet 3 times with 500 µl of immunoprecipitation buffer precipitated complexes were eluted with 40 µl of 2× SDS-PAGE sample buffer for 5 min at 50 °C, and separated on 7.5% SDS-PAGE. The gel was stained with Coomassie Blue overnight, destained (5% MeOH, 10% acetic acid) for 30 min, and soaked in 0.5 µm sodium salicylate 30% MeOH for 2 h. The gel was then vacuum-dried and subjected to fluorography. Each gel figure is a representative of at least three experiments with similar results.

RESULTS

Electrophysiological Interactions of Wild-type and A561V HERG—To evaluate the functional effects of the A561V allele on wild-type HERG in a mammalian expression system, both cDNAs were co-transfected into CHO cells and analyzed by whole cell current measurement. Whole-type HERG was kept constant (5 µg of plasmid) while varying the amount of A561V. Examples of patch clamp recording from transfected cells are shown in Figure 1a. Dominant negative HERG mutations (13,200). When the data were normalized to unity (Fig. 1b), curves for a mutant that freely and randomly associates with wild-type A561V also have HERG K+ currents, albeit of smaller amplitude. The mutant-dependent reduction of current is graphically demonstrated in Fig. 1b, where current-voltage and voltage-dependent activation curves show a graded reduction in current at all voltages as the fraction of A561V increases. The mean current reduction measured during repolarization tails when the wild-type and A561V mutant were expressed in equimolar amounts was 85 ± 5% (n = 33). When the data were normalized to unity (Fig. 1b, insets) a 10–15 mV hyperpolarizing shift in voltage-dependent activation was seen in cells expressing wild-type and A561V HERG (V1/2 HERG = 0.7 ± 0.3 mV, V1/2 HERG:A561V = −10.9 ± 0.26 mV). The summary current density data of different ratios of wild-type and mutant co-transfections is replotted in Fig. 1c. A curve for a mutant that freely and randomly associates with wild-type subunits in a tetramer and is completely dominant is superimposed over the data points for comparison. The ideal predicted curve plotted was derived from the binomial function:

\[
I = f_w Z w + f_m Z m + f_w f_m Z w m + \ldots + f_w^n Z w^n + f_m^n Z m^n \quad \text{(Eq. 1)}
\]

where I is the whole cell current, n is the subunit stoichiometry, f_w is the fraction of wild-type subunits, f_m is the fraction of mutant subunits, and Z_w is the fraction of wild-type current passed by channels with x mutant subunits. If n is four and the mutant is completely dominant over wild type then Z_w = 0 for
Assuming completely random association of mutant and wild-type subunits the equation reduces to:

\[ I = I_{wt} \times (f_{wt})^4. \]

The experimental data, however, was best fit to a model where \( \frac{1}{6} \) the amount of current remained if one subunit was mutant and negligible current arose from tetramers with more than one mutant subunit (\( Z_1 = 0.166, Z_2 = 0.0267, \) and \( Z_3 = 0.0046 \)). Taken together, these findings suggest that the small amount of residual current in cells expressing both wild-type and mutant subunits is produced by a combination of wild-type homotetramers and mutant/wild-type heterotetramers.

**Immunoblot Analysis Mutant Effects on Wild-type HERG Protein**—To investigate whether A561V HERG mutant expression affects wild-type HERG protein accumulation we developed a co-expression system for use in mammalian cell lines. In this system (Fig. 2a) mammalian cells are co-transfected with equimolar amounts of myc-tagged wild-type HERG (HERG-myc) and either untagged wild-type or untagged mutant HERG. The biochemical and electrophysiological behavior of HERG-myc was indistinguishable from HERG (Fig. 2b) and has been previously reported (16). Because wild-type HERG is epitope-tagged at its C terminus with c-myc, the c-myc antibody detects only the full-length HERG-myc rather than the co-transfected mutant protein. Thus any changes in HERG-myc will come from interactions with co-transfected untagged cDNAs. In substituting untagged mutant for untagged wild-type HERG cDNAs we control for plasmid competition for transcriptional machinery. HERG channels are presumed to function as tetrameric complexes as do other voltage-gated \( K^+ \) channels (18). Assuming that HERG subunits do not exist as monomers once fully formed, measurements of full-length wild-type HERG-myc in co-transfection experiments will reflect assembled channel tetramer interactions. When CHO cells were co-transfected with A561V HERG and wild-type HERG-myc, we observed a marked reduction in HERG-myc expression levels in the plasma membrane fraction (Fig. 2c). When transfec-
tions were carried out with equimolar amounts of wild-type HERG-myc and the A561V mutant, the abundance of wild-type protein was reduced 92 \( \pm \) 5%, a value in agreement with our electrophysiology studies. The observed effect of A561V HERG on wild-type HERG-myc was not cell-line specific, since full-length HERG-myc expression levels were comparably reduced when identical studies were carried out in CHO, baby hamster kidney, and HEK 293 cells. When membrane fractions from co-transfected cells were examined, neither full-length HERG-myc nor proteolytic fragments were detected in the cytosolic/microsomal fraction (Fig. 2d).

**A561V Effects on HERG Protein Synthesis and Degradation Studied by Metabolic Labeling**—The A561V-dependent reduction of wild-type HERG-myc protein could be due either to decreased synthesis or increased degradation. To determine the mechanism(s) by which A561V influenced the accumulation of wild-type HERG protein, we next performed pulse-chase experiments. Synthesis and stability of wild-type HERG-myc was determined by anti-myc immunoprecipitation of \( ^{35}S \)-la-
beled protein. Previous experiments had shown that we could quantitatively precipitate all HERG-myc by serial antibody incubations or by using an excess of a-myc antibody and protein G (16). Compared with cells expressing only wild-type HERG, less labeled full-length HERG-myc was recovered from cell co-transfected with the A561V mutant after a 20-min pulse (Fig. 3a).

To better analyze the relative stability of labeled full-length HERG-myc protein during the chase, immunoprecipitations were repeated using non-quantitative amounts of a-myc antibody (Fig. 3b). This ensured that the amount of total HERG-myc in each lane was a fixed and equal amount. 35S-Labeled wild-type HERG-myc co-transfected with the A561V mutant after a 20-min pulse (Fig. 3a).

Mechanisms of Dominant Negative Effects of HERG-myc Synthesis by A561V—The above findings indicate that A561V co-expression exerts its effect on wild-type HERG through early interactions resulting in both decreased biogenesis of full-length protein and increased turnover. To examine whether misfolding of the mutant protein is responsible for the observed effects of on wild-type HERG expression, we modified the cell culture conditions to enhance protein folding. Glycerol is a known chemical chaperone that rapidly enters cells and facilitates folding of proteins (19, 20). Decreasing the culture temperatures is another method that has been shown to augment the correct folding of proteins, particularly some proteins in which mutations cause misfolding (21, 22). Fig. 4a shows partial rescue of HERG-myc from dominant negative effect of A561V HERG when cells were grown in medium supplemented with 10% glycerol. Similarly, when co-transfected cells were cultured at 30 °C the accumulation of wild-type HERG was greater than that observed under standard culture conditions. The actions of 10% glycerol and reduced growth temperature were not additive, suggesting that they affect the same process. The partial rescue of HERG protein expression by reduced
If misfolding is responsible for the dominant negative effect of A561V on HERG-myc, misfolded tetrameric assembly may be recognized by cellular proofreading mechanisms and targeted toward early degradation. The 26 S proteosome complex is known to participate in a variety of early protein editing and degradation pathways (23, 24). To investigate whether proteasomal activity might contribute to the enhanced degradation of wild-type HERG, we studied the effects of ALLN, a potent, cell-permeable, low molecular weight, peptide inhibitor of the 26 S proteosome (25, 26). Fig. 4c shows that ALLN inhibition of the proteosome complex partially rescued the reduction of HERG-myc caused by co-expression with A561V. Dimethyl sulfoxide itself, at the concentrations used in this study (<1% \( v/v \)), had no effect on HERG stability.

DISCUSSION

We have investigated the mechanism of dominant negative inhibition of \( I_{Kr} \) by the HERG A561V LQT2 mutant. We found that the A561V mutant polypeptide interacts with wild-type HERG subunits and that this interaction causes a reduction in the total amount of HERG channel protein. This reduction in protein expression is comparable to the suppression of current caused by co-expression with the mutant. The degree to which the A561V mutant depresses current from wild-type HERG closely approximates what would be predicted for randomly associating subunits forming tetramers wherein any amount of mutant protein completely abolishes channel function. Our results further show that the mutant-dependent reduction in current is paralleled by a corresponding diminution of wild-type HERG abundance. The means by which the mutant depresses normal HERG expression include both decreased biogenesis of full-length channel protein and accelerated degradation of newly synthesized protein. Furthermore, we show that maneuvers known to enhance correct protein folding partially reversed the mutant effect over normal HERG protein. This suggests that misfolded mutant subunits within tetramers that include normal subunits are recognized by cellular editing/proofreading systems, leading to aborted synthesis and early degradation of those tetramers that are completed. That ALLN also partially rescues the mutant effect on wild-type HERG indicates that the 26 S proteosome is involved to some extent in this quality control system.

Several of the LQT2 mutations have been shown to be functionally dominant when co-expressed in Xenopus oocytes (11), however, the mechanism(s) involved have not been directly demonstrated. Zhou and co-workers (13, 14) have shown that homotetramers of some LQT2 mutants are abnormally proc-
essed when expressed in mammalian cells and result in instability of the channel protein and retention within intracellular compartments. Furthermore, they demonstrated that expression of a current-producing, non-dominant mutant, N470D, could be increased by small molecule chaperones and lowered culture temperature, implicating misfolding as a mechanism of channel defect (15). Such a mechanism has been well documented for the autosomal recessive genetic defect in cystic fibrosis where a single amino acid deletion results in temperature-sensitive misfolding, and abnormal processing of the cystic fibrosis transmembrane conductance regulator Cl channel (22, 27–32).

In this report we show that co-expression of A561V reduces the abundance of wild-type HERG protein to produce the dominant effect. By epitope tagging the wild-type cDNA we were able to specifically assay for expression of the normal HERG protein independently from the mutant protein. Our experiments did not detect retained HERG-myc protein in cellular fractions containing endoplasmic reticulum or Golgi, as had been reported for homotetrameric LQT2 mutants. This may reflect either a difference in processing of A561V heterotetramers or the fact that the C-terminal myc tag will allow detection of only full-length protein. If proteolysis has begun with cleavage from the C terminus then no protein would be detected.

A mutant-dependent reduction in the abundance of wild-type HERG-myc assayed by immunoblot could be the result of increased turnover or decreased synthesis. Our results in metabolically labeled cells indicate that both processes contribute to the mutant's effect on full-length HERG. That we observed reduced labeling of full-length HERG-myc at even the earliest time points indicates that newly formed or forming mutant and wild-type subunits are interacting. This result is consistent with the findings that the N terminus of HERG (33) and other voltage-gated K⁺ channels (34) contain association domains that can assemble into tetramers rapidly, even in the absence of C-terminal sequences. Taken together, these data would support our model of early, co-translational association of nascent HERG polypeptides where the inclusion of mutant subunits results in a misfolding tetrameric complex (Fig. 4d).

Recognition of this aberrant assembly by the cellular proof-reading system would target the complex toward rapid proteolysis.

Misfolding of proteins is a well recognized cause of genetic disease, however, elucidation of cellular mechanisms involved in recognition and processing abnormal proteins is ongoing. Precedence for a co-translational folding and editing scenario has recently been demonstrated in eukaryotic cells (35) that would support early assembly of multimeric protein complexes.

**Fig. 4. Misfolding of assembling channels as a mechanism for A561V effects.** a, conditions that enhance protein folding partially reverse the effects of A561V on full-length HERG-myc. Control condition is incubation at 37 °C without glycerol. HERG = HERG-myc + wild type; A561V = HERG-myc + A561V HERG. b, growth of cells co-transfected with 1:1 ratio of wild-type and A561V HERG at 30 °C partially reverses to functional suppression of current. c, role of proteosome inhibitor on dominant effect of A561V HERG on HERG-myc. Inhibition of 26 S proteosome complex results in partial reversal of A561V effect on wild-type HERG-myc. d, model for co-translational quality control of assembling channel tetramers. Association of the N termini would occur prior to full-length translation. Incorporation of mutant chain causes misfolding of complex that is recognized by processing systems resulting in targeting toward rapid proteolysis.
Specific, co-translational proteolytic processing of NF-xB inhibitor has also been documented, where the proteosome was shown to cleave nascent polypeptides as they formed (36). Wigley and co-workers (37) have shown that concentration of the proteosome, chaperones, and ubiquitin at the centrosome, near endoplasmic reticulum and Golgi, provide a means to localize protein processing machinery in close proximity to foci of protein translation. Furthermore, they showed that the ΔF508 mutant cystic fibrosis transmembrane conductance regulator caused an even greater localized accumulation of these processing proteins at the site where new cystic fibrosis transmembrane conductance regulator was being synthesized. Thus, evidence is accumulating to support a model of simultaneous translation, folding, and complex assembly of proteins where mutant alleles could be recognized and edited.

Lowering of cell culture growing temperature or addition of chemical chaperones such as glycerol have been shown to partially reverse misfolding of mutant proteins cystic fibrosis transmembrane conductance regulator ΔF508 (20) and the N470D HERG mutation (15). With lower temperature or with 10% glycerol we not only observed a partial reversal of the mutant’s effect but there was also an increase in expression of wild-type HERG in the absence of the mutant. This may reflect either a degree of inherent misfolding and processing of even the normal protein, or inability of processing systems to match the forced expression rates. When both lowered culture temperature and glycerol were combined there was no additive effect on HERG expression, suggesting that both maneuvers affect the same process. ALLN is a potent, cell-permeable peptide inhibitor of the 26 S proteosome proteolytic activity. Use of ALLN also partially reversed the mutant-dependent suppression of normal HERG expression supporting the role of proteosomal processing. Although ALLN can also inhibit lysosomal proteases and calpains it seems unlikely that the early disappearance of normal HERG during mutant co-expression would involve lysosomal participation (23). That ALLN only partially rescues HERG expression may indicate that other processing or proteolytic systems are involved.

Although the A561V mutant potently suppresses current from co-expressed wild-type HERG in a dominant fashion, our data deviate to a small degree from the ideal curve for a completely dominant effect. When the molar ratio of mutant: wild-type HERG was between 0.2 and 0.5 we observed a slightly greater current density than expected (Fig. 1c). Such a deviation could result from a non-random assembly of mutant and normal subunits skewing the distribution toward tetramers excluding mutants. Another possibility is that the mutant exerts an incomplete dominance over the wild type resulting in some fraction of heterotetramers being functionally expressed. If we assume that one mutant subunit in a tetramer reduces the current to 1/2 of normal then the binomial distribution function is changed with Z = (0.167)^n x and the resulting function more closely describes the data. This model would result in a small fraction of current being passed by channels possessing mutant subunits. That we observed a current density slightly greater than expected and that the residual current exhibited a hyperpolarized shift in voltage activation kinetics (Fig. 1b) can be interpreted as some fraction of mutant containing heterotetrameric channels present in the membrane. A high proportion of dysfunctional mutant heterotetramers in the plasma membrane is one potential mechanism of dominant suppression as seen in Barter’s syndrome (38), however, our results suggest that this is a minor mechanism for HERG A561V.

That the degree of dominant suppression by A561V in the present study was significantly greater than previously reported (11) may reflect the growth of mammalian cells at 37 °C in contrast to Xenopus oocytes where expression typically takes place at 18–22 °C. Thus, at the higher temperatures there is more rapid and fault-prone folding of newly translated proteins. This is supported by the findings of Zhou et al. (15) where homotetramers of N470D HERG were more labile at 37 °C. In preliminary experiments (data not shown) we have observed similar dominant suppression of wild-type HERG by three other LQT2 mutants (N470D, ΔI500-F508, and ΔI261) not previously observed to be dominant in Xenopus expression. Thus, our present findings on the HERG mutant A561V may indicate a general mechanism of dominant effects by other alleles.

In summary, our data provide the first insight into the dominant mechanisms of mutant protein actions in LQTS. Unanswered questions remain, however, such as specific identification of chaperones or processing proteins that may interact with newly forming HERG channels and whether cellular systems other than the proteosome are involved. Although we have shown that four different LQT2 mutants reduce the abundance of co-expressed normal HERG, it remains to be determined if each mutant acts in the same manner as A561V. Likewise, there are an increasing number of LQT mutations being described whose mechanisms of pathogenesis are yet to be investigated. Last, the use of small molecule chemical chaperones as potential therapeutic modalities appears to be a promising area to explore.

Acknowledgment—We thank Kami Kim for helpful discussion.

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J. Biol. Chem. 2000, 275:11241-11248.
doi: 10.1074/jbc.275.15.11241

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