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ORIGINAL ARTICLE

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Clinical and electrophysiological characterization of a novel mutation R863X in HERG C-terminus associated with long QT syndrome

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Abstract We have found a novel nonsense mutation in the C-terminus of *HERG* in a four-generation Chinese family with long QT syndrome and investigated the molecular mechanism of this mutation in vitro. Six family members, including the proband, were clinically affected. Syncope and ventricular tachycardia of torsades de pointes were triggered by startling or emotional stress, and β-adrenergic blockade treatment was ineffective. Haplotype analysis showed that only LQT_2 markers cosegregated with the disease, and sequence analysis revealed a substitution of T with C at nucleotide position 2770 of the *HERG* gene (U04270), which creates a stop codon at amino acid position 863 (R863X) of the HERG protein, leading to a deletion of 296 amino acids. Whole cell patch clamp studies showed that the R863X HERG could not induce time-dependent current. Coexpression of R863X with wild-type HERG showed reduced current densities and accelerated voltage-dependent inactivation of HERG channels. Subcellular localization of R863X-EGFP revealed that the mutant did not traffic to the cell surface. These data suggest that R863X failed to form functional HERG channels, contributing to a prolongation of the QT interval and long QT syndrome with a dominant phenotype. These findings provide new insights into the structure-function relationships of the HERG C-terminus.

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Keywords Arrhythmias · Long QT syndrome · K-channel · *HERG*

Introduction

Familial long QT syndrome (LQTS) is an inherited cardiac disorder characterized by delayed cardiac repolarization, ventricular arrhythmia, syncope, and sudden death [1]. Although there is a variable spectrum of cardiac electrical abnormalities and penetrance, the majority of LQTS pedigrees show dominant transmission.

Six LQTS-associated genes have been hitherto identified [2, 3]. The human *ether-a-go-go* related gene (*HERG*) is responsible for one form of long QT syndrome (LQT_2) and encodes the pore-forming subunit of the rapidly activating, delayed rectifier current I_{kr}, which plays an important role in the repolarization of the cardiac action potential [4, 5]. Although the role of other factors contributing to I_{kr} current, such as *HERG* splice variants and interacting β-subunits, is still under investigation [6, 7, 8, 9], mutations in *HERG* are one of the most common causes for congenital LQTS [10].

So far, more than 100 mutations in the *HERG* gene have been identified in LQT_2 patients [10]. Various mechanisms underlying the dysfunction of the HERG channel in these mutants have been suggested, including abnormal channel processing [11, 12, 13], generation of nonfunctional channels [14, 15] and altered channel gating [16, 17, 18, 19].

From the primary HERG structure point of view, there are several sites where mutations have occurred that could result in HERG channel dysfunction. For example, mutations in the N-terminus usually accelerate HERG channel deactivation [19], while mutations in the pore region affect HERG channel inactivation or ion selectivity [15, 17, 18]. More recently, mutations in LQT_2 have been mapped to the C-terminus which contains regions essential for I_{kr} function [20], but the mechanisms underlying HERG channel dysfunction in association with C-terminal mutations have not been fully characterized yet.

In this study, a novel mutation, R863X, of *HERG* was identified in a Chinese LQTS family, leading to a deletion of 296 amino acids in the C-terminus of HERG protein. The functional consequences of this mutation were characterized in vitro.

Materials and methods

Subjects

This study was conducted in a four-generation Chinese family with LQTS (Fig. 1A). Informed consent was obtained from the family members in accordance with the study protocols approved by the Review Board of Center for Molecular Cardiology, Chinese Academic of Medical Sciences. Detailed medical histories, physical examinations and 12-lead electrocardiography (ECG) and a 24-h Holter recording were obtained from 26 familial members. Diagnosis of LQTS was made on the basis of symptoms and corrected QT interval (QTc) on ECG according to the criteria of Schwartz et al. [21].

One hundred normal controls without heart or other systemic diseases were recruited based on medical history, physical examination, echocardiography, ECG and serum biochemical analysis. Peripheral blood was collected from all participants.

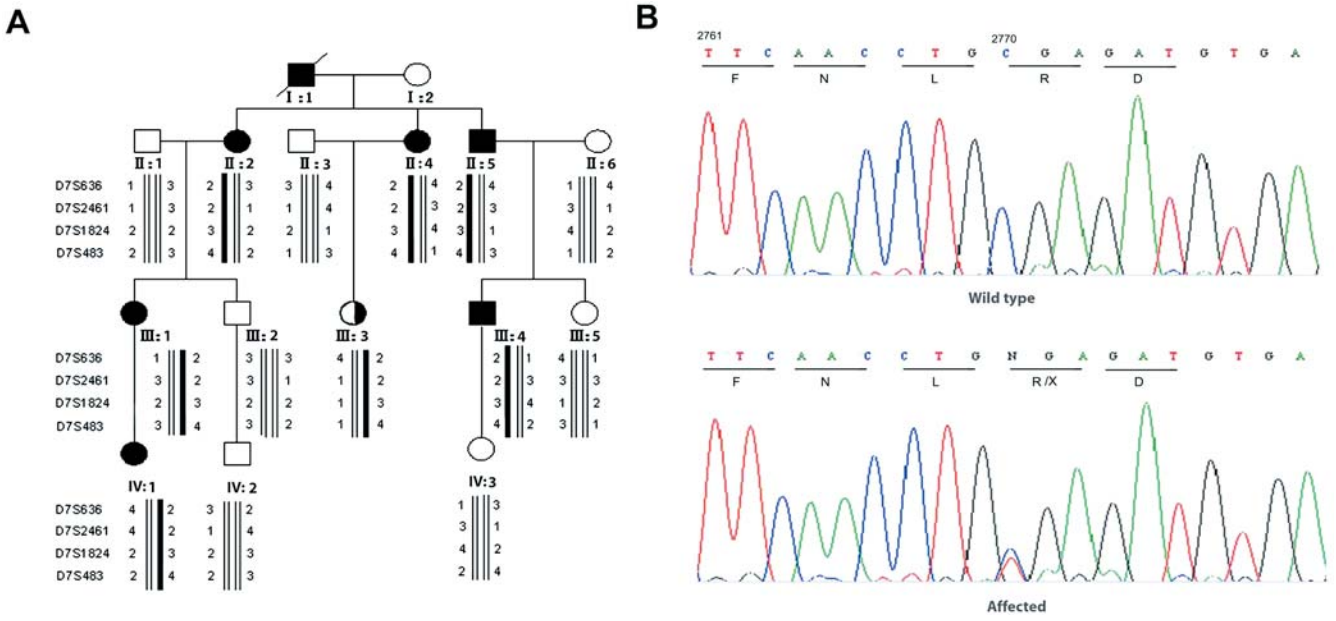

Fig. 1 **A** Pedigree of the LQTS family and their genotypes obtained with *HERG* microsatellite markers. *Squares* indicate men, *circles* women, *solid symbols* affected individuals, *slashed symbols* deceased individuals, and *half-solid symbols* individuals with an uncertain phenotypic status. **B** Sequence identification of a C to T transition of one *HERG* allele in an affected individual (both alleles shown), causing replacement of arginine 863 by a stop codon (R863X)

Haplotype analysis

Genomic DNA was extracted from blood samples by standard procedures. Polymorphic microsatellite markers covering *HERG*, *KCNQ1*, *SCN5A*, *KCNE1* and *KCNE2* loci were amplified by polymerase chain reaction (PCR) as described (http://www.gdb.org), including *D3S1298* and *D3S1767* for *SCN5A*; *D7S483*, *D7S636*, *D7S1824* and *D7S2461* for *HERG*; *D11S1323*, *D11S2362* and D11S1318 for *KCNQ1*; and *D21S2049* and *D21S1435* for *KCNE1* and *KCNE2*. These sequences were based on the data in the genome databases. After PCR, aliquots of 2 μl reaction mixture were denatured and separated on 7% or 10% polyacrylamide gels including 7 M urea. The gels were then silver-stained and dried. Two independent investigators with no knowledge of each individual's status scored the alleles. Genotypes were grouped into the most likely paternal and maternal haplotypes. Haplotype analysis was performed to confirm the cosegregation of the haplotypes with the disease.

Mutation screening

The DNA fragments encoding the *HERG* gene were amplified using the primer sets as described [22] on a GeneAmp PCR System 9700 Thermal Cycler (Perkin Elmer, Foster City, Calif.) in the presence of 1 U *Taq* DNA polymerase, 0.2 mM deoxyribonucleotides, 1.5 mM $MgCl_2$, 100 ng forward and reverse primers and 0.2 μg genomic DNA. PCR fragments were subsequently screened with single strand conformation polymorphism assays (SSCP) according to standard procedures at 4°C and the gels were silver-stained. If abnormal SSCP patterns were found, the same fragments (all family members and the 100 unrelated normal controls) were reamplified and subjected to dye-terminator sequencing using an ABI 377 automatic sequencer. The primer sequences used to detect the *HERG* gene in exon 10 were 5′ AGTTCTCCGACCACTTCT 3′ and 5′ TCAATGTCACAC AGCAAA 3′.

Construction of wild-type *HERG* (WT) and R863X *HERG*

WT *HERG* cDNA cloned into the pSP64 vector was a kind gift from Dr. Michael Sanguinetti (University of Utah) and was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, Calif.) through the *Hin*dIII and *Bam*HI sites. The pEGFP-*HERG* plasmid was a kind gift from Dr. Thomas V. McDonald (Albert Einstein College of Medicine, N.Y.)

R863X-*HERG* was constructed by PCR based-mutagenesis. The sequence was amplified using the forward primer 5′-CCCTGTC-CTCTC CATGGCCT-3′ and the reverse primer 5′-TGGATC-CACAGGTTGAAGGTG-3′ with *Pfx* DNA polymerase (Gibco, Rockville, Md.). The resultant PCR products were verified by sequencing and subcloned into pcDNA3.1 vector through the *Hin*dIII and *Bam*HI sites and the pEGFP-C_2 vector through the *Eco*RI and *Bam*HI sites.

Cell culture and transfection

CHO cells were maintained in MEM-media supplemented with 10% fetal calf serum (Gibco-BRL, Rockville, Md.) and penicillin/streptomycin at 37°C under 5% CO_2. Gene transfer was performed using 1–5 μg of Qiagen Midiprep purified plasmid DNA (QIAGEN,Germany). The cells were transfected using LipofectAMINE according to the manufacturer's instructions (Gibco-BRL). The pcDNA3/GFP plasmid encoding green fluorescent protein was cotransfected with the test plasmid and the target cells were identified by fluorescence microscopy. Patch clamp recordings were conducted 24–48 h after transfection.

Electrophysiologic experiments and data analysis

Whole cell patch clamp recordings were performed using an EPC-9 amplifier (HEKA Elektronik, Lambrecht). The pulse software 8.0 (HEKA Elektronik) was used for the generation of voltage pulse protocols and data acquisition. All experiments were conducted at room temperature. The extracellular solution contained 150 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES and 10 mM sucrose, pH 7.4. The pipette solution consisted of 120 mM KCl, 2 mM $MgSO_4$, 5 mM EGTA, 0.5 mM CaCl2, 10 mM HEPES, pH 7.2. Series resistance was actively compensated and was below 5 Mω. The holding potential was −80 mV in all experiments.

The voltage protocols are presented in the figure legends. The current densities were calculated by dividing the maximal peak current by the capacities of the cells tested. Voltage activation data were plotted as peak tail current amplitudes against the test potential values and were fitted to a Boltzmann function, $I/I_{max}=1/(1+\exp[(V_{1/2}-V)/k])$, where I is the measured tail current, V is the applied membrane voltage, $V_{1/2}$ is the voltage at half-maximal activation, and k is the slope factor. Activation, deactivation, and inactivation kinetics were fitted with a single or double exponential function. Data analysis and drawings were performed by using IGOR software (WaveMetrics, Lake Oswego, Ore.) and kaleidagraph 3.5 software (Synergy, Reading, Pa.). All data were expressed as means±SEM. Statistical differences were assessed by Student's t-test, $P<0.05$ was considered significant.

Confocal imaging

CHO cells were cultivated as described in the previous section and transiently transfected using LipofectAMINE with pEGFP-HERG or pEGFP-R863X. After 24–48 h, the transfected cells were replated on chambers with glass coverslips and allowed to attach for at least 12 h prior to imaging. The confocal images were obtained using a Leica TCS NT laser scanning microscope (Leica, Bensheim). Image analysis was performed by using Imagepro and Photoshop 7.0 software.

Results

Clinical presentation

In this family, six members were clinically diagnosed as LQTS. The proband was a 57-year-old woman, who experienced her first syncope with seizure at age 27, with ECG documented torsade de pointes ventricular tachycardia (Tdp). The ECG revealed a prolonged QTc interval (580 ms at rest and 480 ms after exercise) and a notched T wave or a T-U-wave complex in several leads. The Tdp attacks were easily and repeatedly provoked by startling or emotional stress. The proband was treated with atenolol or propranolol but remained symptomatic.

Molecular genetics

Microsatellite markers for *KCNQ1*, *SCN5A*, *KCNE1* and *KCNE2* were used to identify the affected individuals by haplotype analysis.The most likely haplotypes obtained with these *HERG* microsatellite markers indicated that the haplotype 2-2-3-4 cosegregated with the disease, suggesting that the disease gene in the family is linked to this locus Fig. 1A. The family was then screened for

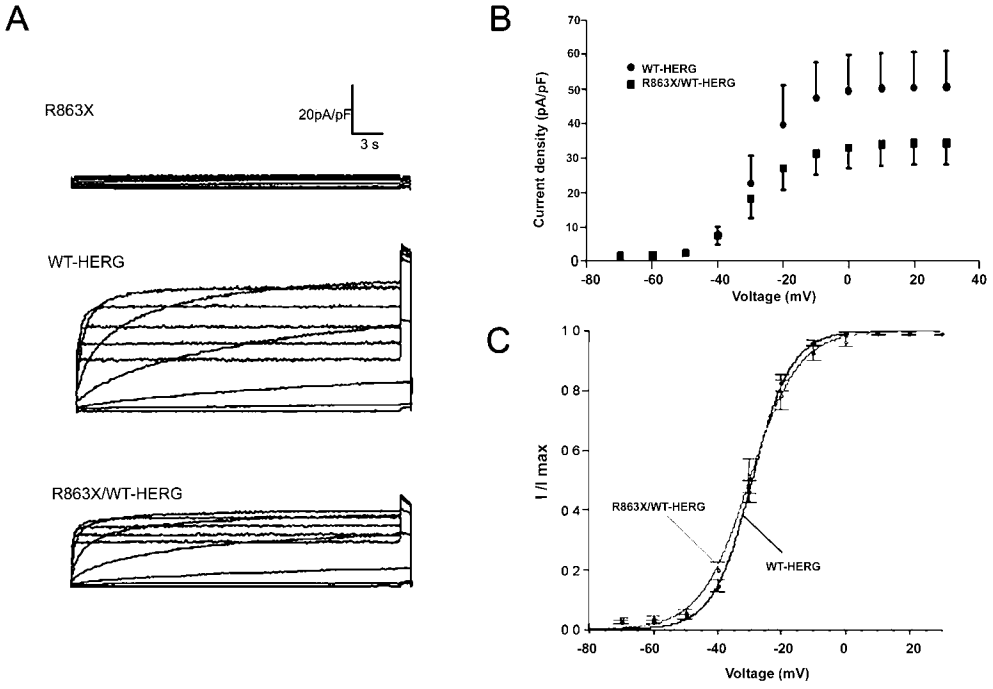

Fig. 2A–C Steady state activation kinetics of WT and R863X/WT. **A** Typical current traces in CHO cells transfected with the constructs indicated. The currents were elicited with 30 sec pulses to potentials ranging from −70 to 30 mV and the tail currents were measured on return of the voltage to −40 mV. **B** The relationship of tail current density and test pulses. **C** *I-V* relationships of the tail currents in CHO cells transfected with WT and R863X/WT. Amplitudes of tail currents were normalized and plotted as a function of the test potential and were fitted to a Boltzmann function

mutations by PCR-SSCP, with intronic PCR primers covering the coding regions of the *HERG* gene. The resultant PCR products generated from *HERG* exon 10 had an aberrant pattern in the patient, and were not observed in DNA samples from 100 control subjects. DNA sequencing revealed a heterozygous C to T transversion at nucleotide position 2770 of the *HERG* gene (GenBank accession U04270), causing the substitution of a conserved arginine residue at position 863 for a stop codon (R863X) with a deletion of 296 amino acids in the HERG C-terminus (Fig. 1B). All clinically affected family members (diagnosed by ECG) were found to be heterozygous for R863X. The proband's niece (III-3) had a normal QTc, but carried this mutation. The mutation was not identified in other unaffected family members nor in 100 normal controls.

R863X *HERG* alone could not form functional channels

To investigate the functional consequence of this mutation, we measured the whole cell K^+ currents from CHO cells transiently transfected with either WT or R863X *HERG* plasmids. Typical whole cell current traces are seen in Fig. 2A. The mutation could not produce voltage-gated K^+ current when expressed alone. This indicates that R863X *HERG* alone could not form functional channels.

Coexpression of WT with R863X *HERG* reduced the current densities of HERG channels

To assess the interaction between WT and the mutant proteins, we coexpressed equal amounts (1.5 μg) of WT and R863X *HERG* in CHO cells and characterized the currents, making quantitative analysis feasible. Typical whole cell current traces are seen in Fig. 2A. The tail currents were measured at −40 mV after a depolarizing pulse from −70 mV to +30 mV and the peak tail current density was 52.47±6.73 pA/pF (n=25) for WT, and 35.44±3.16 pA/pF (n=24) for R863X/WT (P<0.01) (Fig. 2B). Coexpression of WT and R863X *HERG* reduced HERG current densities about 30%. The apparent degree of HERG current reduction, however, indicated that co-expression of WT with R863X *HERG* did not exert a dominant negative effect on WT *HERG* expression. In addition, the maximal tail current amplitudes were obtained at the peak of the third pulse in the inactivation protocol, when almost all of the channels were active (Fig. 3). The results also demonstrated that the current densities of R863X/WT *HERG* were lower than that of WT (WT, 357±78 pA/pF, n=8; R863X/WT 256±82 pA/pF, n=8, P<0.05).

Coexpression of WT with R863X *HERG* also modified the gating property of HERG channels

Figure 2C represents the normalized *I-V* relationships for amplitudes of tail currents for WT and R863X/WT. The voltage at which the current was half-activated ($V_{1/2}$) for R863X/WT was not much different from that for WT, but the slope factor k for R863X/WT (7.29±1.1) was

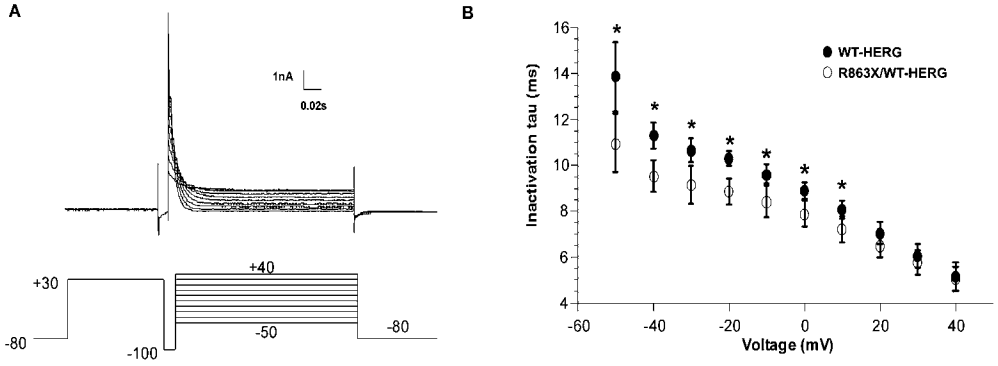

Fig. 3A, B Time courses of inactivation of expressed currents in CHO cells transfected with WT or R863X/WT. The *inset* illustrates the voltage protocol. **A** Representative current recordings were those in CHO cells transfected with WT or R863X/WT. Inactivation time constants (τ) were measured by fitting inactivating currents during test pulses at each potential with a single-exponential function. **B** τ values representing inactivation time constants for expressed currents in CHO cells transfected with WT and R863X/WT were plotted as a function of test potential (*VT*). **P*<0.05 for time constant between R863X/WT and WT

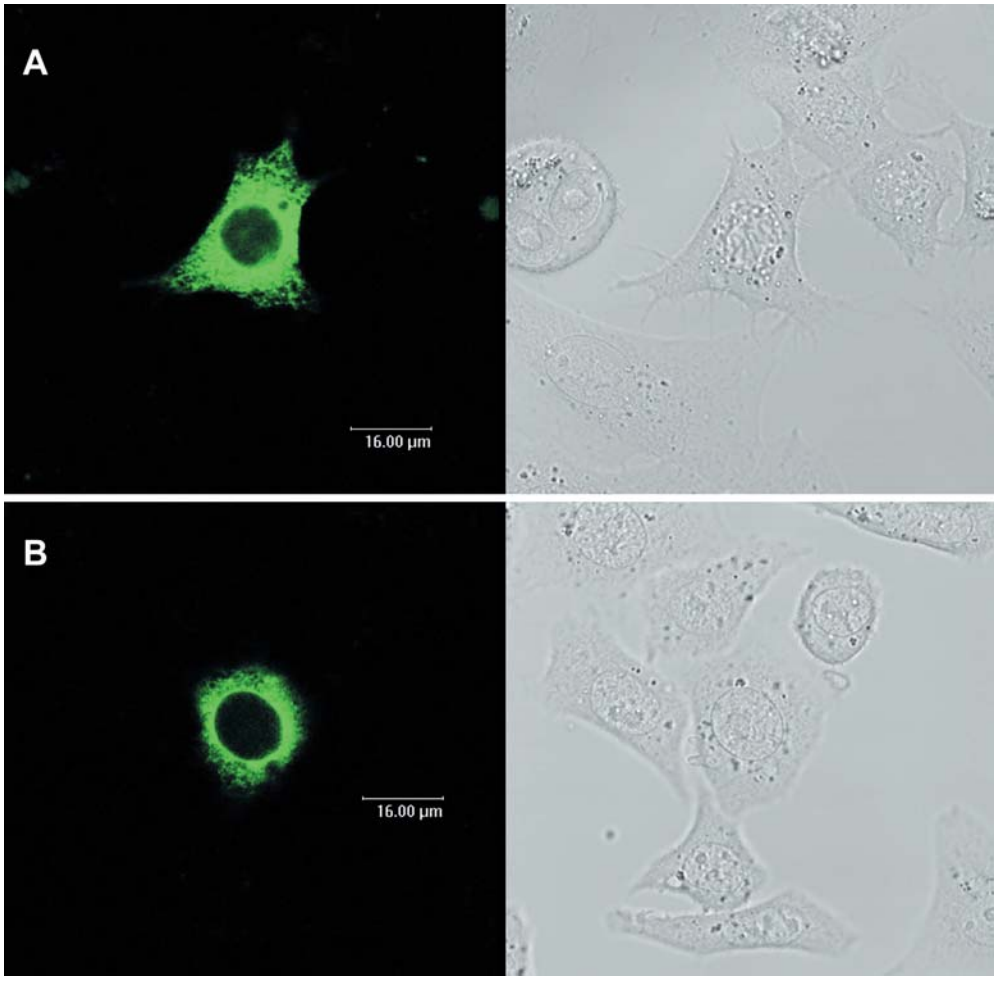

Fig. 4A, B Localization of WT and R863X mutant HERG in living cells by GFP fluorescence. **A** The GFP signal of WT protein was located in the cytoplasm and the plasma membrane. The unstained region within the cell is the nucleus. **B** The GFP signal of R863X mutant protein was located in the cytoplasm, but not in the plasma membrane

increased slightly compared with that for WT (5.97±0.65, *P*<0.05).

To analyze the deactivation time course accurately long hyperpolarizing test pulses were applied after a depolarizing conditioning pulse. Deactivating currents during test pulses could be fitted to a double exponential function. At all test potentials, the fast and slow time constants for R863X/WT were not different from that for WT (data not shown).

The inactivation time course of expressed currents was analyzed by applying brief hyperpolarizing pulses to allow the HERG channel to recover from inactivation after an initial long depolarizing pulse, and then depolarizing test pulses were applied to record inactivating currents. The time course of inactivating currents could be fitted by a single-exponential function. The inactivation time constants of R863X/WT channels (10.97±1.28 ms at −50 mV, and 7.12±0.47 ms at 10 mV, n=10) were decreased slightly compared with WT channels (13.88±1.52 ms at −50 mV, P=0.001 and 8.08±0.40 ms at 10 mV, n=10, P<0.01) (Fig. 3). The results revealed that R863X *HERG* could accelerate the channel inactivation when coexpressed with WT.

R863X *HERG* does not translocate to the plasma membrane

Several LQT_2 mutations have been found to cause reduced I_{kr} due to defective transport of the mutant protein to the plasma membrane or through enhanced degradation of the protein. To examine whether the R863X mutant proteins could be translocated to the plasma membrane, we visualized the location of HERG protein in CHO cells using GFP fusion proteins for both WT and R863X *HERG*. The GFP signal of the R863X mutant protein was located mainly in the cytoplasm and was not found in the cell plasma membrane, whereas the GFP signal of the WT protein was distributed both in the cytoplasm and in the plasma membrane (Fig. 4), indicating that the R863X protein could not be effectively translocated to the plasma membrane.

Discussion

In this study, a novel mutation, R863X, in the HERG C-terminus was identified in a Chinese LQTS family. Carriers of this mutation have a high risk of developing lethal arrhythmias. In comparsion with other LQT_2 C terminus mutations (Fig. 5), R863X exhibited unique electrophysiological properties. R863X *HERG* alone failed to form functional HERG channels, consistent with other reported C-terminus mutations [23, 24, 25]. However, coexpression of R863X *HERG* with WT reduced the current densities and accelerated the inactivation of WT-HERG channels, which was not the case for mutants in the cyclic nucleotide binding domain (CNBD) and Y667X. The latter has no influence on the current amplitudes and the properties of HERG channels when coexpressed with WT, whereas coexpression of CNBD mutants with WT has not been found to alter current amplitudes significantly, butchanged the half voltage of activation ($V_{1/2}$). The R863X mutation is positioned just downstream of the CNBD region with a deletion of 296 amino acids (residues 863–1159) in the HERG C-terminus. Interestingly, it has been reported that the residues 863–1159 in the HERG C-terminus may contain

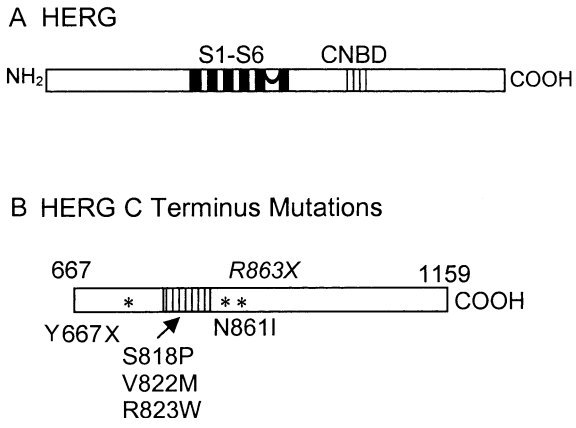

Fig. 5 The C-terminal mutations of the HERG protein

a domain involving the inactivation process of the channel.

Failure of cell surface expression has now been identified as a mechanism of ion channel dysfunction in several human familial arrhythmias, including LQTS and Brugada syndrome [11, 12, 13, 26]. Many of the mutant HERG proteins fail to generate HERG current because of trafficking problems [11, 12, 13]. Aydar and Palmer [27] have reported that HERG proteins with a C-terminal truncation of 311 or more amino acid residues cannot form functional channels. Moreover, Akhavan et al. [28] have recently identified a region between residues 860–899 that is critical for trafficking, and have shown that truncations or deletion of residues 860–899 result in a decreased expression level and an absence of the mature glycosylated form of the HERG protein. Our study revealed that the mutant R863X alone failed to form functional channels due to ineffective trafficking to the plasma membrane. These results demonstrated that C-terminus of the HERG protein may play an important role in HERG trafficking and channel maturation.

Coexpression of R863X *HERG* with WT reduced the current densities of HERG channels, and speeded up their inactivation slightly. This faster inactivation contributed to a lower current density. However, the maximal current densities of R863X/WT channels were lower than those of WT channels when all of the channels were open, which suggested that R863X may also interfere with WT protein trafficking and/or accelerate its degradation. The decrease in current density by R863X may result in the longer QT intervals documented in patients with this mutation.

A recent study has showed that the 14-3-3 protein family, interact with HERG channels and alter the effect of β-adrenergic signaling upon HERG activity [29]. This may provide a mechanism for plasticity in cardiac electrophysiological response to stress. Members of the 14-3-3 family are highly conserved and are present in all eukaryotic organisms. HERG associates with 14-3-3ε and potentiates the cAMP/PKA effect upon HERG. 14-3-3 accelerates and enhances HERG activation, an effect that

requires phosphorylation of HERG at S283 and S1137. The mutant R863X lacks the S1137 PKA site. Therefore, the R863X/WT HERG channel can not efficiently interact with 14-3-3, which may weaken cAMP/PKA effects on HERG, leading to an increased risk for fatal cardiac arrhythmias in the face of stress. This could be the mechanism for the clinical events seen in this family.

In summary, we have identified a novel mutation, R863X, in the *HERG* gene. The mutation results in a deletion of 296 amino acids. The R863X mutant can not translocate normally to the cell surface. Coexpression of WT and R863X *HERG* showed reduced current densities and faster inactivation. These may contribute to a prolongation of QT intervals and thus to LQT_2. The results indicate that these residues are necessary for the expression of functional HERG channels.

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