LQT1-associated Mutations Increase KCNQ1 Proteasomal Degradation Independently of Derlin-1*

David Peroz1,2,3,4, Shehzadah Dahimene5,6, Isabelle Baro5,6, Gildas Loussouarn5,6, and Jean Merot1,5,6,3,4

From the 1INSERM, UMR915, the 2CNRS, ERL3147, and the 3Université de Nantes, Faculté de Médecine, L’institut du Thorax, Nantes F-44000, France

Mutations in the potassium channel KCNQ1 that determine retention of the mutated proteins in the endoplasmic reticulum (ER) are associated with the autosomal dominant negative Romano–Ward LQT1 cardiac syndrome. In the present study, we have analyzed the consequences and the potential molecular mechanisms involved in the ER retention of three Romano–Ward mutations located in KCNQ1 N terminus (Y111C, L114P, and P117L). We showed that the mutant KCNQ1 proteins exhibited reduced expression levels with respect to wild-type (WT)-KCNQ1. Radiolabeling pulse-chase experiments revealed that the lower expression levels did not result from reduced rate of synthesis. Instead, using a combination of Western blot and pulse-chase experiments, we showed that the mutant channel Y111C-KCNQ1, used as a model, was ubiquitinated and degraded in the proteasome more rapidly ($t_{1/2} = 82$ min) than WT-KCNQ1 channel ($t_{1/2} = 113$ min). On the other hand, KCNQ1 degradation did not appear to involve the GTP-dependent pathway. We also showed that KCNE1 stabilized both wild-type and Y111C proteins. To identify potential actors involved in KCNQ1 degradation, we studied the implication of the ER-resident protein Derlin-1 in KCNQ1 degradation. We showed that although KCNQ1 and Derlin-1 share the same molecular complex and co-immunoprecipitate when co-expressed in HEK293FT cells, Derlin-1 did not affect KCNQ1 steady state expression and degradation. These data were confirmed in T84 cells that express endogenous KCNQ1 and Derlin-1. Small interfering RNA knockdown of Derlin-1 did not modify KCNQ1 expression level, and no interaction between endogenous KCNQ1 and Derlin-1 could be detected.

The long QT syndrome (LQT)5 is a cardiac arrhythmia characterized by the prolongation of the QT interval on the electrocardiogram and a high risk of sudden death (1). Among the different LQTs described, type 1 LQT (LQT1) is caused by mutations in the KCNQ1 gene that result in the reduction of the repolarizing potassium cardiac current $I_{Kr}$. About 250 KCNQ1 mutations have been found in patients suffering from the LQT1 syndrome (2–4), and previous studies showed that these mutations impede KCNQ1 channel function by multiple mechanisms including: abnormal gating or permeation (5), impaired channel assembly (6), and defective protein trafficking (7–10).

As a membrane protein, KCNQ1 is synthesized in the ER and is subjected to a stringent quality control to ensure that only fully functional channels reach the plasma membrane (11–13). If the folding process fails, the channel is retained in the ER and eventually degraded by the ER-associated degradation system. In this context, ER-resident molecular chaperones have the dual role to sense and assist the folding status of the proteins and to dispatch misfolded proteins for destruction. This conformation-sensing system also includes enzymes that tag the misfolded protein with ubiquitin to target them to the proteasome. Then, to reach the cytosolic proteasomal degradation machinery, the transmembrane protein must be retrotranslocated out of the ER phospholipid bilayer (14). The retrotranslocation occurs through an aqueous channel that is not yet clearly identified. Initial works suggested that the Sec61 translocon could constitute the pore (15), but recent works highlighted the role of a macromolecular complex composed of VCP-interacting membrane protein (VIMP), p97, and Derlin-1 in this process (16, 17).

Although many studies were dedicated to the analysis of channel protein trafficking defect and degradation, little is known about the KCNQ1 biosynthetic pathways. One important particularity of KCNQ1 is that it is part of a composite membrane protein complex formed by the association of four $\alpha$-subunits and at least two single transmembrane domain ancillary $\beta$-subunits KCNE1 (18). Although it is recognized that both subunits associate in the ER and that KCNE1s strongly modify channel current kinetics (19), the role of KCNE1 in KCNQ1 biosynthesis and trafficking is less well understood.

We recently identified a critical trafficking determinant of KCNQ1 in the N terminus of the protein, which is targeted by several mutations (8). This region delineates a critical hotspot in the juxtamembranous domain just preceding its first trans-
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1 The recipient of the "Poste d’Accueil" position from the INSERM.
2 Recipients of tenure positions at the CNRS.
3 Present address: Instituto Nacional de Saúde Dr. Rocardo Jorge, Centro De Genetica Humana, Av Padre Cruz, 1649-016 Lisboa Portugal.
4 The abbreviations used are: LQT, long QT syndrome; ER, endoplasmic reticulum; WT, wild type; siRNA, small interfering RNA; HEK, human embryonic kidney HEK293FT cells; DMEM, Dulbecco’s modified Eagle’s medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ab, antibody; IP, immunoprecipitation; DSP, dithiobis(succinimidyl propionate); CFTR, cystic fibrosis transmembrane conductance regulator; VCP, valosin-containing protein.
membrane domain. Structure prediction analysis revealed that point mutations in this region could introduce potential folding defects and impair trafficking of the channel. We showed that the mutant channels remain trapped in the ER and can exert a dominant negative effect on WT channel subunits, most probably by sequestrating the latter in the ER (8). We initiated the present study to analyze the fate of wild-type KCNQ1 protein along its biosynthetic pathway, the determine role of KCN1 in this process, and to elucidate the potential mechanisms involved in the early ER retention and degradation of the newly synthesized mutant channels.

**EXPERIMENTAL PROCEDURES**

*cdDNA Construct and siRNA*—The human WT and mutant Y111C, P117L, and L114P-KCNQ1 cDNAs were cloned in the pCB6 expression vector as described previously (8). KCNE1* was cloned in pRC/CMV vector. C-terminally FLAG-tagged KCN1 was a kind gift of Dr. AL George (Vanderbilt University, Nashville, TN). Derlin-1* cDNA, kindly provided by Dr. Hidde L. Ploegh (Whitehead Institute, Cambridge, MA), was cloned in pCB6 vector. According to Hegde et al. (20), the siRNA 5’-CGAUUUAAGCCUGCAUUt-3’ targeting nucleotides 445–463 of Derlin-1 mRNA was a Silencer custom-made siRNA from Ambion (Cambridgeshire, UK). siRNA control was a Silencer negative control from Ambion.

*Cell Culture and Transfection*—Human embryonic kidney HEK293FT (HEK) (Invitrogen) and T84 cells (ATCC) were cultured in DMEM and Ham’s F12/DMEM 1:1 (Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin at 37 °C in a humidified 5% CO2 incubator. HEK cells were transiently transfected with 2 μg of total cDNA using FuGENE 6 from Roche Diagnostics following the manufacturer’s instructions. In all the experiments, KCNQ1 cDNA was kept at 660 ng and transfected with equal amounts (660 ng) of KCN1 and/or Derlin-1. CD4 cDNA was used as a ballast to keep total cDNA at 2 μg. Cells were used 24 h after transfection. T84 cells were transfected with the Amaza cell line nucleofector kit from Amaza Inc. (Gaithersburg, MD) following the manufacturer’s instructions. 150 pmol of siRNA were used for 2.106 cells that were harvested 48 h after siRNA transfection.

* Antibodies (Ab) and Chemicals*—Anti-Derlin-1 Ab was obtained from MBL International Corp. (PM018, Nagoya, Japan); anti-GAPDH Ab (SC-32233), anti-rabbit horseradish peroxidase Ab (SC-2054), and anti-mouse horseradish peroxidase Ab (SC-2055) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-KCNQ1 Ab was obtained from Alomone (APC-022, Jerusalem, Israel) or Santa Cruz Biotechnology (SC-C-20); and anti-ubiquitin Ab was obtained from BIOMOL International L.P. (SK2, Plymouth Meeting, PA). All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. MG132 proteasome inhibitor was from BIOMOL International and was used at 25 μM. Mycophenolic acid was from BIOMOL and was used at 2 or 40 μM. Dithiobis(succinimidyl propionate) (DSP) was from Pierce.

**Immunoprecipitation (IP) and Western Blots**—In IP experiments, HEK cells were lysed in 150 mM NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 1% Triton X-100 supplemented with Complete protease inhibitor mixture (Roche Diagnostics); T84 cells were lysed in 150 mM NaCl, 50 mM HEPES, pH 7.5, 0.1% Nonidet P-40, and Complete protease inhibitor mixture. Cell extracts were incubated 30 min at 4 °C under agitation. The cell lysates were centrifuged 15 min at 16 000 × g, and the supernatants were incubated with the appropriate antibody overnight at 4 °C. Immune complexes were isolated with protein G-Sepharose beads (HitTrap protein G HP from GE Healthcare, Chalfont St. Giles, UK), washed extensively with lysis buffer, denatured in Laemmli sample buffer for 10 min at 70 °C, separated by SDS-PAGE on 10% gels, and transferred to nitrocellulose membranes Hybrid C-exra (GE Healthcare). The blots were probed with anti-KCNQ1 (1/4000 for transfected cells or 1/500 for T84), anti-Derlin-1 (1/1000), and anti-GAPDH (1/10 000) antibodies. When anti-ubiquitin Ab (1/500) was used, 2% bovine serum albumin was used to block nitrocellulose membrane instead of 5% fat-free milk. Horseradish peroxidase secondary antibodies were used at 1/10 000. Films were scanned on ImageScanner™ densitometer (GE Healthcare), and the ImageJ software (National Institutes of Health, Bethesda, MD) was used for image quantification. Signals were normalized with respect to GAPDH and are expressed as a ratio of the control condition.

To test KCNQ1 ubiquitination, HEK cells were incubated 4 h with 25 μM MG132 prior lysis, and 10 mM N-ethyl maleimide was added to the IP buffer. Protein concentrations in cell extracts were determined using the D/C assay kit from Bio-Rad. To co-immunoprecipitate KCNQ1 and chaperones, cells were treated with the cross-linker DSP (DSP) for 30 min and then with 100 mM Tris-HCl, pH 7.4, for 20 min and finally washed in phosphate-buffered saline prior to cell lysis.

**Pulse-Chase Assays and Half-life Estimation**—24 h after transfection, transiently transfected HEK cells were starved in methionine and cysteine-free DMEM (Invitrogen) for 30 min and then metabolically labeled using Redvue Pro-mix [35S]Met/Cys (GE Healthcare) 0.5 mCi/ml for 15 min at 37 °C. Cells were washed once with phosphate-buffered saline, washed once with DMEM, and lysed immediately or incubated in complete DMEM for 1, 2, or 4 h at 37 °C. Cells were lysed, and KCNQ1 was immunoprecipitated and separated by SDS-PAGE as described above. Labeled KCNQ1 was revealed by autoradiography on a Typhoon 9410™ scanner (GE Healthcare). The ImageQuant™ software (GE Healthcare) was used for analysis. When used, MG132 (25 μM) and mycophenolic acid (2 or 40 μM) were added just after the 15-min pulse period. To calculate KCNQ1 half-life time, signals at times t (Xt) were normalized with respect to the signal at t = 0 (X0) and plotted according to ln(Xt/X0) = bt. The half-life time (t1/2) was calculated as t1/2 = ln(0.5)/b where b is the slope of the regression curve.

**Statistics**—All experiments were carried out at least three times. All data are presented as means ± S.E., and n indicates the number of experiments. Means were compared with a Student’s t test. Decrease curves were compared with a parallelism
N-terminal KCNQ1 Mutations Exhibit Lower Expression Levels than WT-KCNQ1—In a previous study, we have shown that Romano Ward mutations located in KCNQ1 N terminus determined ER retention of the mutant channel proteins (8). In the present study, we analyzed the mechanisms and the consequences of this ER retention. First, we asked whether WT-KCNQ1 and mutant KCNQ1 exhibited different expression levels. To answer this question, we transiently expressed WT-, Y111C-, L114P-, and P117L-KCNQ1 in HEK cells and quantified channel expression by Western blotting. As illustrated on Fig. 1, A and B, all the mutated proteins were expressed at lower levels than WT-KCNQ1. Also, P117L mutant steady state expression was higher than Y111C and L114P. To ascertain that these differences did not result from undesired mutations in plasmid sequences and/or the quality of DNA preparations, they were entirely sequenced, and the experiments were repeated with three different plasmid preparations. No difference was observed, and the data were pooled in Fig. 1.

RESULTS

N-terminal KCNQ1 Mutations Do Not Impair Channel Synthesis—The steady state differences we observed could be explained either by different synthesis or by different degradation rates. To test these hypotheses, we analyzed channel synthesis in details. HEK cells transiently transfected with WT or mutants KCNQ1 and KCNE1 cDNAs were pulse-labeled 15 min with 35S-radiolabeled methionine and cysteine. The KCNQ1 proteins newly synthesized during the pulse were then immunoprecipitated and quantified. As shown in Fig. 1, C and D, no significant difference (analysis of variance \( p = 0.3 \)) was observed between WT-KCNQ1 and any of the mutant channels demonstrating equivalent neo-synthesis rates. Taken together, reduced steady state expression levels of the mutants and their similar synthesis rates when compared with WT-KCNQ1 suggested that both WT and mutant proteins followed different degradation kinetics.

KCNQ1 Degradation Is Mediated by the Proteasome—In the following experiments, we analyzed KCNQ1 degradation by the proteasome pathway. We used Y111C-KCNQ1 as a model of KCNQ1 mutant and compared its degradation kinetics with those of WT-KCNQ1. Because ubiquitination is a prerequisite to proteasomal targeting, we first analyzed KCNQ1 ubiquitination. As illustrated in Fig. 2A, both immunoprecipitated WT-KCNQ1 and Y111C-KCNQ1 were labeled by anti-ubiquitin antibodies, and labeling increased when the cells were treated with the proteasome inhibitor MG132 (25 \( \mu \)M for 4 h). Effective proteasomal degradation of KCNQ1 was demonstrated in pulse-chase experiments in the presence of MG132 (Fig. 2, B test. A \( p \) value inferior to 0.05 was considered statistically significant and is reported. The 95% interval of confidence (CI\(_{95}\)) mentioned in parentheses and separated by semicolons (x;x).
and C). Indeed, in cells pulse-labeled for 15 min, only 27% ± 4 (n = 8) of the newly synthesized KCNQ1 remained after a 4-h chase period in the absence of proteasome inhibitor, whereas 84% ± 8 (n = 4) remained after 4 h in the presence of MG132 (25 μM). Y111C mutant degradation was also reduced by the proteasome inhibitor. 16% ± 4 (n = 8) and 54% ± 4 (n = 3) of Y111C remained after the chase period in the absence and the presence of MG132, respectively. Because MG132 efficiency seemed lower for Y111C, we asked whether Y111C could be degraded through alternative lysosomal or GTP-dependent pathways as described recently for CFTR (21). To answer this question, HEK cells transfected with Y111C were pulsed and then chased for 4 h in the presence of the lysosomal or the GTP-dependent degradation pathway inhibitors, chloroquine (100 μM) and mycophenolic acid (2 μM and 40 μM), respectively. None of the inhibitors affected Y111C degradation (data not shown), indicating that lysosomal and GTP-dependent pathways do not play an important role in the degradation of newly synthesized mutant channel. Taken together, these experiments led us to conclude that newly synthesized WT- and Y111C-KCNQ1 degradation was mainly achieved by the proteasome.

**Mutant KCNQ1 Are Less Stable than WT Channel Protein—** We then compared the degradation kinetics of mutant and WT-KCNQ1. Cells were pulse-labeled 15 min, and newly synthesized KCNQ1 proteins remaining after 1, 2, and 4 h of chase were immunoprecipitated and quantified. As shown in Fig. 3, A and B, the Y111C mutant was degraded more rapidly than the WT channel. The mean half-life of Y111C (82 min) was significantly shorter (p = 0.0008, n = 5) than that of WT-KCNQ1 (113 min).

Derlin-1 Associates with KCNQ1 in HEK Cells—To characterize the mechanisms involved in KCNQ1 control quality and the ER-associated degradation system, we have analyzed the role of the ER-resident protein Derlin-1 that has been implicated in the retrotranslocation from the ER to the cytosol of CFTR (22) and Kc_a.2.3 and Kc_a.3.1 (23) channels. We first analyzed whether KCNQ1 and Derlin-1 are part of the same molecular complex. HEK cells were transiently transfected with WT- or Y111C-KCNQ1, KCNE1, and Derlin-1, and KCNQ1 was immunoprecipitated. As shown in Fig. 4A, Derlin-1 was efficiently co-immunoprecipitated with both WT-KCNQ1 and Y111C-KCNQ1. We then analyzed whether this association had any functional effect on KCNQ1 expression. Contrarily to
what has been described for CFTR and \( K_{Ca} \)(s). Derlin-1 overexpression did not significantly modify WT- and Y111C-KCNQ1 steady state expression levels (Fig. 4, B and C) or the degradation kinetics of the newly synthesized proteins (Fig. 4, D and E). To ensure that these contradictory observations did not result from the overwhelming of the biosynthetic and/or degradation pathways in the \textit{in vitro} expression systems used here, a new series of experiments was conducted in T84 cells that endogenously express both KCNQ1 and Derlin-1. As illustrated in Fig. 5, endogenous Derlin-1 did not co-immunoprecipitate with either endogenous KCNQ1 (Fig. 5A) or overexpressed WT- or Y111C-KCNQ1 (Fig. 5B). Tunicamycin, used to induce ER stress and to favor Derlin-1 association with its substrates, as shown previously for CFTR and p97 (24), did not increase Derlin-1 interaction with KCNQ1 either (data not shown). Moreover, decreasing Derlin-1 expression using specific siRNA did not increase KCNQ1 steady state expression either (Fig. 5, C and D). Together our data indicate that Derlin-1 is not functionally involved in KCNQ1 retrotranslocation.

\textbf{DISCUSSION}

In the present study, we have analyzed the biosynthesis pathways of the WT- and LQT1-associated KCNQ1 channel...
Proteasomal Degradation of KCNQ1 LQT1 Mutants

Mutants. To our knowledge, our data represent the first study of KCNQ1 channel processing in the early secretory pathway. In the heart, KCNQ1 subunits associate with the ancillary subunits KCNNE1 to carry the slow component of the delayed rectifier potassium current (\(I_{\text{Ks}}\)) (19). In a previous study, we showed that LQT1-associated mutations located in the N terminus of the protein may introduce sufficient protein structure alterations to determine the ER retention of the mutant channels (8). The lower steady state expression levels of mutant KCNQ1 channels, which we observed with respect to WT protein, prompted us to analyze the fate of newly synthesized channels. As expected, in the in vitro re-expression system, we did not detect significant differences between the synthesis rates of the different constructs. Moreover, preliminary co-immunoprecipitation, fractionation, and solubilization studies did not reveal differential aggregation properties between WT and any of the mutants (data not shown), suggesting that they shared the same pathways and cellular compartments. Indeed, as with all the membrane proteins, KCNQ1 undergoes a strict quality control in the ER. In this compartment, molecular chaperones scrutinize the structure of nascent proteins and help them to acquire a native conformation and serve to dispose of those that do not have a proper one (11). We tested a panel of cytosolic and ER chaperones including Hsp70, Hsp90, BiP, calnexin, and calreticulin for their interactions with WT- and Y111C-KCNQ1. Neither lectin chaperone, calnexin nor calreticulin, was found to bind the channel. Although this is consistent with the absence of core glycosylation in KCNQ1, more surprisingly, interaction was not detected in the presence of glycosylated KCNNE1 despite the fact that KCNNE1 was efficiently co-immunoprecipitated with KCNQ1. On the other hand, Hsp70 was found to physically interact with both mutant and WT-KCNQ1. Our observation is consistent with previous studies showing that Hsp70 interacts with a subset of proteins that are believed to have difficulties to reach their final conformation (27). Indeed, as far as ionic channel channels are concerned, Hsp70 was shown to bind Herg channel (26) and CFTR (25). However, one should note that, in contrast to Herg, Hsp70-KCNQ1 interaction was only detected in the presence of the cross-linking agent DSP, suggesting that the interaction was transient and weak. This is consistent with the lack of effect of Hsp70 overexpression or knockdown (using siRNA) on KCNQ1 expression level (data not shown).

Among other potential KCNQ1 molecular chaperones, we analyzed the role of the ancillary subunit KCNNE1 in the fate of the channel complex. Using pulse-chase experiments, we showed that KCNNE1 increased the stability of both WT-KCNQ1 and mutant KCNQ1 (Fig. 6). These results further sustain the idea that KCNNE1 and KCNQ1 associate early in the secretory pathways during channel biogenesis and assign a new role to KCNNE1 in the macromolecular KCNQ1 channel complex. Indeed, previous studies clearly showed that KCNNE1 increased KCNQ1 current and modified the electrophysiological properties of the KCNQ1 channel (half-activation potential and kinetics of inactivation) (19, 28–30). In addition, although more contradictory, previous studies also reported increased trafficking of KCNQ1 when co-expressed with KCNNE1 (31). Finally, the chaperone role that we propose for KCNNE1 is consistent with previous publications showing that \(\beta\)-subunits stabilize and enhance the trafficking of other channels (32, 33). Together our data assign a new role to the KCNNE1 subunit in channel stability and biogenesis. Most probably, other molecular chaperones assist KCNQ1 channel folding and will be unveiled in future investigations.

We showed that WT and mutant KCNQ1 are ubiquitinated. Furthermore, when MG132 was added during pulse-chase experiments, the KCNQ1 remaining after 4 h of chase was increased. These results are consistent with the notion that KCNQ1 degradation is mediated by the ubiquitin-proteasome pathway. In our experiments, 84 and 54% of WT- and Y111C-KCNQ1 remain in the presence of the MG132, respectively. The strong effect on WT channel is in agreement with the 80% reduction of the proteasome activity reported by Zafar et al. (34). Whether part of the Y111C mutant follows an additional degradation pathway remains to be determined. In any case, lysosomal or GTP-dependent degradation pathways do not seem to be involved in this process as either chloroquine or mycophenolic acid affected Y111C degradation (21, 35).

To be degraded in the proteasome, membrane proteins must be retrotranslocated toward the cytoplasm (14). Recently, the ER-resident Derlin-1, in association with Vimp1 and p97 (16), was shown to promote the degradation of CFTR (22), KcA3.1, KcA2.3 (23), and the heavy chain of major histocompatibility complex I (16, 17). We thus questioned whether Derlin-1 was also implicated in KCNQ1 retrotranslocation. Our results show that Derlin-1 has no essential role in KCNQ1 degradation. Whatever the amount of WT- and Y111C-KCNQ1 plasmid cDNA we used to limit channel expression and avoid overwhelming of degradation pathways, and despite the fact that overexpressed proteins could be co-immunoprecipitated, we could not detect any effect of Derlin-1 on channel steady state expression level or degradation. These data were confirmed in T84 cells that endogenously express both KCNQ1 and Derlin-1. Indeed, siRNA knock-down of Derlin-1 had no effect on KCNQ1 expression level in T84 cells. Whether other pathways are involved in KCNQ1 retrotranslocation remains to be determined. We suspect that the two additional Derlins (Derlin-2 and Derlin-3) described in mammalians are not involved in this process (36, 37). Indeed, Derlin-2 seems to be restricted to the retrotranslocation of luminal ER substrates, whereas Derlin-3 facilitates the disposal of misfolded glycosylated substrates (36, 37). On the other hand, the Derlin-1-independent and ubiquitin- and proteasome-dependent HIP pathway (for Hrd-independent proteolysis) described in yeast by Haynes et al. (38) may be a good candidate for KCNQ1 retrotranslocation and degradation. This alternative pathway will have to be tested in future studies.

In conclusion, we have shown that LQT1-associated mutations determine increased degradation of the mutant proteins by the proteasome and that the ancillary subunit KCNNE1 participates to the stability and the biogenesis of KCNQ1 channel complex.

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Proteasomal Degradation of KCNQ1 LQT1 Mutants

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