Co-chaperone FKBP38 Promotes HERG Trafficking*

Valerie E. Walker, Roxana Atanasiu, Hung Lam, and Alvin Shrier†
From the Department of Physiology, McGill University, Montreal, Quebec H3G 1Y6, Canada

The Long QT Syndrome is a cardiac disorder associated with ventricular arrhythmias that can lead to syncope and sudden death. One prominent form of the Long QT syndrome has been linked to mutations in the HERG gene (KCNH2) that encodes the voltage-dependent delayed rectifier potassium channel (I\textsubscript{Kr}). In order to search for HERG-interacting proteins important for HERG maturation and trafficking, we conducted a proteomics screen using myc-tagged HERG transfected into cardiac (HL-1) and non-cardiac (human embryonic kidney 293) cell lines. A partial list of putative HERG-interacting proteins includes several known components of the cytosolic chaperone system, including Hsc70 (70-kDa heat shock cognate protein), Hsp90 (90-kDa heat shock protein), Hdj-2, Hop (Hsp-organizing protein), and Bag-2 (BCL-associated athanogene 2). In addition, two membrane-integrated proteins were identified, calnexin and FKBP38 (38-kDa FK506-binding protein, FKBP8). We show that FKBP38 immunoprecipitates and co-localizes with HERG and FKBP38 (38-kDa FK506-binding protein, FKBP8). We propose that FKBP38 is a co-chaperone of HERG and contributes via the Hsc70/Hsp90 chaperone system to the trafficking of wild type and mutant HERG potassium channels.

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† To whom correspondence should be addressed: Dept. of Physiology, McGill University, 3655 Rue Sir William Osler, Montreal, Quebec H3C 1Y6, Canada. Tel.: 514-398-2272; Fax: 514-398-7452; E-mail: alvin.shrier@mcgill.ca.

The abbreviations used are: LQTS, long QT syndrome; HERG, human ether-a-go-go-related gene; WT, wild type; ER, endoplasmic reticulum; Hsc70, 70-kDa heat shock cognate protein; Hsp90, 90-kDa heat shock protein; CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, C-terminal of Hsp70-interacting protein; Bag-2, BCL-associated athanogene 2; Hop, Hsp-organizing protein; FKBP38, 38-kDa FK506-binding protein; TPR, tetratricopeptide repeat; HA, hemagglutinin; HERG-C, C terminus of HERG; HEK, human embryonic kidney; PBS, phosphate-buffered solution; siRNA, small interfering RNA; AU, arbitrary units.
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FKBP38 is a peptidyl prolyl cis-trans isomerase and a member of the FKBP protein family. FKBP38 is important for the trafficking of WT HERG and is capable of rescuing the LQT2 HERG trafficking mutant F805C. Our results indicate that FKBP38 is important for the trafficking of WT HERG and is capable of rescuing the LQT2 HERG trafficking mutant F805C.

EXPERIMENTAL PROCEDURES

Preparation of cDNA Constructs—The generation of N-terminal Myc-tagged and N-terminal hemagglutinin (HA)-tagged HERG has been described previously (26). The HERG missense mutant F805C was engineered using the QuikChange XL site-directed mutagenesis kit (Stratagene) and a HERG-C cassette as PCR template as described previously (26). The cDNA of FKBP38 was kindly provided by Dr. Nakayama (Fukuoka, Japan). The immunoprecipitation samples were incubated with Protein A-Sepharose beads for 2 h after which the beads were washed extensively and then resuspended in sample buffer 2× (12% 0.5 M Tris, pH 6.8, 5% β-mercaptoethanol, 20% glycerol, 20% SDS, Bromphenol blue). Samples were resolved on a 7.5% polyacrylamide SDS gel and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked for 1 h with 5% nonfat dry milk and 0.1% Tween 20 in PBS and then incubated with the appropriate primary antibody for 1 h at room temperature, washed extensively, and then incubated with goat α-mouse/rabbit IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories). After extensive washing the membranes were visualized on x-ray films using the ECL Plus detection kit (Amersham Biosciences).

Overexpression and siRNA—For all overexpression experiments, HEK-293 cells grown on 35-mm dishes were transiently transfected at 70–80% confluence with WT or F805C HERG and varying amounts of HA-FKBP38 (0.2, or 0.4 μg) and an appropriate amount of empty vector to equalize total amount of transfected cDNA. After 48 h, cells were lysed as described above and equal amounts of protein were loaded and subjected to SDS-PAGE and Western blot analysis. For siRNA experiments, siRNA oligonucleotides targeting the 5′-AAGAGUG-GCUUGGCAAUUCUGG-3′ sequence in the open reading frame of human FKBP38 mRNA and a control double-stranded RNA with a corresponding scrambled sequence (5′-AAGCGCUGUUGAAGAUUC-3′) were obtained from Dharmaco Research (Lafayette, CO) based on Ref. 27. HEK-293 cells stably expressing Myc-HERG-WT were seeded at low confluence (20–30%) on 35-mm dishes and transfected with peptide mixtures were analyzed by direct on-line liquid chromatography tandem mass spectrometry (LC-MS/MS). The MS/MS spectra were evaluated using Mascot software (Matrix Science) to identify tryptic peptide sequences matched to the National Center for Biotechnology Information (NCBI) non-redundant protein and nucleotide data bases (dbEST) with a confidence level of 95% or greater (30). Peptide sequence tags were generated from the MS/MS spectra as described (31). The location and function of each identified protein were assigned using NCBI, Swiss-Prot, InterProHome, and InterProScan data bases.

Immunoprecipitation and Western Blotting—Twenty four hours post-transfection, cells were washed two times with cold PBS and then incubated in lysis buffer (0.5% Nonidet P-40, 75 mM NaCl, and 50 mM Tris, pH 8) plus a protease inhibitor mixture (Roche Applied Science) for a minimum of 15 min. Cells were homogenized by pipetting, harvested, and then left on ice for an additional 15 min with occasional vortexing. Detergent-insoluble material was sedimented at 16,000 g for 30 min after which the resulting supernatant was collected and the protein concentration was determined with a detergent-compatible assay according to the manufacturer’s instructions (Bio-Rad). For immunoprecipitation, samples of 0.5 to 1 mg of protein were incubated in a volume of 0.5 to 1 ml and incubated overnight at 4 °C with either monoclonal mouse α-Myc (Santa Cruz Biotechnology, Inc.) (1:100), monoclonal mouse α-HA (Covance) (1:100), or polyclonal rabbit α-FKBP38 (1:200) provided by Dr. Nakayama (Fukuoka, Japan). The immunoprecipitation samples were incubated with Protein A-Sepharose beads for 2 h after which the beads were washed extensively and then resuspended in sample buffer 2× (12% 0.5 M Tris, pH 6.8, 5% β-mercaptoethanol, 20% glycerol, 20% SDS, Bromphenol blue). Mass Spectrometry Analysis and Data Base Mining—Four proteomic screens of immunoprecipitated Myc-tagged HERG transfected into cardiac (HL-1) (28, 29) and non-cardiac (HEK-293) cell lines were performed. The Myc-immunoprecipitated HERG-bound complexes were separated by SDS-PAGE and stained by Coomassie blue. Individual bands were destained, excised, reduced, sulfur-alkylated, and digested with trypsin using a robotic digester (Micromass) at the Protein Unit Facility of the Genome Quebec Proteomics Platform. The resultant
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siRNA oligonucleotides according to the manufacturer's instructions using Lipoctamine and Lipofectamine Plus Reagent (Invitrogen). Cells were lysed 2, 4, or 6 days post-transfection and subjected to SDS-PAGE and Western blot analysis as described above.

Immunolocalization—HEK-293 cells stably expressing WT HERG-Myc were seeded onto gelatin/fibronectin-coated coverslips. 24 h post-seeding, cells were washed with PBS and then fixed for 15 min with 4% formaldehyde at room temperature on an orbital shaker. Cells were then washed with PBS and permeabilized with 0.5% Triton X-100 in PBS for 5 min. Nonspecific antibody binding was blocked by PBS containing 10% goat serum for 30 min at room temperature after which cells were incubated with blocking solution containing either α-Myc (1:1000), α-KDEL (1:600), or α-FKBP38 (1:1000) antibody for 1 h. Coverslips were extensively washed before being incubated with blocking solution containing either Alexa Fluor 488-conjugated mouse secondary antibody or Alexa Fluor 633-conjugated rabbit secondary antibody at 1:400. After washing with PBS the coverslips were mounted onto glass slides using Immuno-Fluo Mounting Medium (ICN Biomedicals). Images were acquired with a Zeiss Axiovert 200 automated inverted microscope.

Denisitometry and Statistical Analysis—Densitometric analysis was carried out using the digital imaging program ImageJ (National Institutes of Health). Each band was quantified by a mean pixel value after subtraction of background. Pixel values for the upper and lower HERG bands and the FKBP38 band were normalized to the loading control protein PP2A. For both the siRNA and overexpression experiments, HERG trafficking efficiency is represented by the ratio of the normalized HERG upper band to the total normalized HERG intensity (HERG upper + HERG lower). All experiments were repeated in triplicate. Paired t tests were used to compare groups of data to determine significant.

RESULTS

HERG Interacts with Cytosolic Chaperones—To search for other possible chaperones or co-chaperones that interact with HERG we conducted a total of four proteomic screens of immunoprecipitated Myc-tagged HERG transfected into cardiac and non-cardiac cell lines. The Myc-immunoprecipitated HERG-bound complexes were separated by SDS-PAGE, excised, subjected to tryptic digestion, and then analyzed by tandem mass spectrometry at the Montreal Genome Centre. Identified HERG-interacting chaperones and co-chaperones include Hsc70, Hsp90, Hdj2, Hop, Bag-2, and calnexin (Table 1). In addition to these abundant cytosolic chaperones we identified the immunophilin FKBP38, a putative co-chaperone that may interact with the Hsc70/Hsp90 chaperone network involved in the HERG processing pathway.

HERG and FKBP38 Co-immunoprecipitate and Co-localize—Although FKBP38 is ubiquitously expressed, it is found in greatest abundance in the brain and the heart (24). To confirm that there is an interaction between HERG and FKBP38 we conducted co-immunoprecipitation experiments. First, HEK-293 cells were transiently transfected with HA-tagged WT HERG and then lysed and immunoprecipitated with an antibody against endogenous FKBP38. As shown in Fig. 1A, both the mature and immature forms of HERG were immunoprecipitated with the α-FKBP38 antibody. It remains unclear whether FKBP38 truly interacts with both forms of the HERG protein, given that most chaperones release their substrate once it has reached its native state. It seems conceivable that FKBP38 is bound to HERG even after its release from the ER. This might be expected if FKBP38 is involved in a late HERG folding stage or in the ER exit of HERG. Second, we transiently transfected HEK-293 cells with HA vector alone, HA-tagged WT HERG, Myc vector alone, or Myc-tagged WT HERG and then lysed and immunoprecipitated with either α-HA or α-Myc antibody. Fig. 1B shows the presence of a band at ~62 kDa that corresponds to FKBP38, indicating that endogenous FKBP38 is immunoprecipitated with both Myc-tagged and HA-tagged WT HERG but not with the vectors alone. Finally, immunoprecipitation studies were conducted in HEK-293 cells alone or stably expressing Myc-tagged WT HERG and in AP-1 cells stably expressing the HA-tagged sodium-hydrogen exchanger NheI. Fig. 1C shows that stably expressed Myc-tagged HERG can precipitate endogenous FKBP38 whereas the stably expressed HA-tagged sodium-hydrogen exchanger NheI cannot. Taken together these results indicate that HERG and FKBP38 do indeed interact and predict that the two proteins are present in the same cellular compartments.

TABLE 1
Identified HERG-interacting chaperones and co-chaperones

Four proteomic screens of immunoprecipitated Myc-tagged HERG transfected into cardiac and non-cardiac cell lines were performed. The Myc-immunoprecipitated HERG-bound complexes were separated by SDS-PAGE. Individual bands were digested at the Protein Unit Facility of the Genome Quebec Proteomics Platform. The resultant peptide mixtures were analyzed by direct on-line liquid chromatography tandem mass spectrometry (LC-MS/MS). The MS/MS spectra were evaluated using Mascot software to identify tryptic peptide sequences matched to the NCBI non-redundant protein and nucleotide data bases (dbEST) with a confidence level of 95% or greater. The location and function of each identified protein were assigned using NCBI, Swiss-Prot, InterProHome and InterProScan data bases.

| Protein     | Other names         | Species | Accession number (NCBI) | Localization   | Domains            |
|-------------|---------------------|---------|-------------------------|----------------|--------------------|
| Hsc70       | 70-kDa heat shock   | Human   | NP_005588               | Cytosol        | J domain           |
|             | cognate protein     |         |                         |                |                    |
| Hsp90       | 90-kDa heat shock   | Human   | NP_00107963             | Cytosol        |                    |
|             | protein             |         |                         |                |                    |
| HOP         | STP1; Hsp-organizing | Mouse   | NP_034610               | Cytosol        | 2 TPR domains      |
|             | protein             |         |                         |                |                    |
| BAG-2       | RCL2-associated     | Human   | NP_004273               | Cytosol        | BAG domain         |
|             | athanogene 2        |         |                         |                |                    |
| FKBP38      | 38-kDa FK506-binding| Human   | Q14318                  | ER membrane    | PPI, TPR, calmodulin binding motif |
|             | protein, FKBP8      |         |                         |                |                    |
| Calnexin    | Major histocompatibility | Human | AAH42843              | ER membrane    | Calreticulin domain, calcium binding domain |
|             | class I antigen-binding protein p88 |       |                         |                |                    |

a Human protein was found in immunoprecipitations conducted in HEK-293 cells. Mouse protein was found in immunoprecipitations conducted in HL-1 cells.

b ppi, peptidylprolyl cis/trans isomerase.
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FKBP38 primarily localizes to the outer membrane of the mitochondria through its C-terminal membrane anchor where it is proposed to play a role in the regulation of apoptosis through its interaction with Bcl-2 (27). Recent immunofluorescence and cell fractionation data indicate that FKBP38 resides at both the ER and mitochondrial membranes (27, 32, 33). To first confirm the ER localization of FKBP38 in our system we performed immunolocalization experiments in HEK-293 and HL-1 cells. As shown in Fig. 2 there is overlap between the ER marker (α-KDEL antibody) and FKBP38 indicating that FKBP38 is localized to the ER as well as the mitochondria. To determine whether HERG and FKBP38 co-localize in our cell system we co-stained HEK-293 cells stably expressing Myc-tagged HERG and an α-myHC antibody. FKBP38 knock down on day 2 led to a statistically significant diminution of FKBP38 expression by 2 days post-transfection whereas a double-stranded scrambled oligonucleotide had no effect on the expression level of FKBP38 (Fig. 4A). Importantly, this FKBP38 knock down on day 2 led to a statistically signifi-
cant reduction in the HERG trafficking efficiency relative to control (HG upper/HG total) on day 4 (15.0 ± 1.8 relative to control 36.8 ± 8.4 arbitrary units (AU); p = 0.03) and day 6 (24.6 ± 1.24 relative to control 41.5 ± 5.5 AU; p = 0.02; Fig. 4B). We attribute the delay in the siRNA-induced FKB38 reduction of HERG trafficking efficiency to the relative stabilities of FKB38 and HERG. HERG is a stable protein with a half-life of ~12–16 h at the cell surface; thus, a maximal diminution of mature HERG would not be expected on day 2 when FKB38 levels are minimal but rather at the next time point, on day 4 as observed. Thus, the reduction of FKB38 expression results in a decrease in the efficiency of HERG maturation.

FKB38 Rescues Trafficking of F805C—Given that siRNA knock down of FKB38 reduced HERG maturation, we questioned whether overexpression of FKB38 would augment HERG maturation and/or rescue a HERG trafficking mutant. To test these possibilities, we overexpressed HA-tagged FKB38 in the presence of either WT HERG or the HERG trafficking mutant F805C. The F805C mutation is present in the cyclic nucleotide binding domain (34) and can be rescued by a reduction in temperature (35) or by the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase inhibitor thapsigargin (34). As shown in Fig. 5A, FKB38 (0.2 or 0.4 µg of cDNA) or empty vector were transfected along with WT HERG or F805C into HEK-293 cells and the resulting HERG expression was analyzed by Western blotting.

FIGURE 5. Overexpression of FKB38 partially rescues the trafficking of LQT2 trafficking-deficient mutants. A HEK-293 cells were transiently transfected with HA-tagged WT HERG or F805C and indicated amounts of FKB38 or empty vector. Two days post-transfection, cells were lysed and the expression of HERG was analyzed by Western blotting. B, corresponding mean data (n = 3 experiments/condition). *, p < 0.01 versus F805C 0.0.

DISCUSSION

The major conclusion of this work is that FKB38 interacts with the HERG potassium channel and influences its trafficking efficiency. This influence on trafficking is evident in two ways. Most importantly, overexpression of FKB38 partially rescues the LQT2 trafficking mutant F805C. In addition, when FKB38 protein expression levels are reduced by targeted siRNA, there is a corresponding reduction in WT HERG trafficking efficiency.
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Ciency. We suggest that HERG and FKBP38 interact in the ER membrane late during HERG biogenesis and that this interaction is necessary for successful exit of HERG from the ER.

We propose a model in which FKBP38 acts to present HERG to the ER export machinery. The hypothesis that FKBP38 is involved in the late stage of the folding pathway of HERG and may be involved in ER export is supported by the observation that the majority of FKBP38 interacts with the immature ER form of HERG and there is a relatively minor interaction with the fully glycosylated form of HERG. The fact that a small amount of fully glycosylated HERG interacts with FKBP38 suggests that the two proteins are still bound as HERG is exported to the Golgi where complex glycosylation occurs. If FKBP38 facilitates the process by which HERG reaches its final native conformation, then it seems plausible that it may not immediately dissociate from HERG. To support this contention, Fig. 3 demonstrates that the overlap between FKBP38 and HERG may not be solely restricted to the ER.

To date, most published accounts of FKBP38 describe its role in the regulation of apoptosis through its interaction with Bcl-2 (27, 32, 33, 36, 37). However, it has been proposed that some immunophilins can act as chaperones by binding unfolded polypeptides (38). As stated above, we are proposing a role for FKBP38 as a co-chaperone of the Hsc70/Hsp90 chaperone system that differ in their interactions with ΔF508 and WT CFTR, including the co-chaperone FKBP38. Interestingly, there are differences between their results regarding the effect of FKBP38 on the trafficking of CFTR-ΔF508 and our results on the trafficking of HERG-F805C. Wang et al. (12) found that siRNA knock down of FKBP38 resulted in a reduction in both the ER and Golgi bands of CFTR-ΔF508. This is similar to the effect that siRNA knock down had on WT HERG trafficking. Interestingly, they found that overexpression of FKBP38 also caused a reduction in both ER and Golgi bands of CFTR-ΔF508. These results led them to postulate that FKBP38 function and expression are tied to the steady-state concentration of Hsp90 and assure optimal performance of the CFTR-specific Hsp90-client folding pathway. They concluded that FKBP38 influences the stability of CFTR-ΔF508 in the ER whereas we show that it affects HERG trafficking efficiency. To reconcile the differences in the effect of FKBP38 overexpression on CFTR-ΔF508 versus HERG-F805C, we suggest that the levels of co-chaperones needed for successful folding and export are protein- and possibly cell type-dependent. It would then follow that the same co-chaperones could have varying effects on different client proteins (i.e. HERG and CFTR).

The proposed co-chaperone role for FKBP38 is motivated in part by consideration of a similar immunophilin, FKBP52, which plays a role in steroid receptor activation (39). In the case of the steroid receptors, Hsc70 and Hsp90 work together with several nonessential, TPR domain-containing co-chaperones including FKBP52 to open a hydrophobic cleft in the native state of the steroid receptor (40). As shown in the left panel of Fig. 6, steroid receptors are bound early in biogenesis by both Hsc70 and Hsp90, at which point Hsp90 binds to Hsc70. The activated receptor complex changes conformation such that it can bind Hsp90 and Hop. As the steroid binding cleft is opened, Hsp90 achieves its ATP-dependent conformation, Hop dissociates from the receptor-bound Hsp90, and p23 binds to Hsp90 (14). The dissociation of Hop from the receptor complex frees a TPR domain acceptor site on Hsp90 to bind the TPR domain of the immunophilin FKBP52. It is proposed that FKBP52 is
responsible for the trafficking of steroid receptors to the nucleus upon steroid binding by acting to link them to cytoplasmic dynein for transport along the cytoskeleton into the nucleus (41–44). Given that many of the same chaperones and co-chaperones described above for FKBP52 were immunoprecipitated with HERG, we suggest that FKBP38 acts as an analogous Hsp90 co-chaperone in the WT HERG maturation pathway (Fig. 6, right panel). Specifically, we hypothesize that WT HERG is released from its final chaperone complex in a native folded state and exits the ER, possibly still in a complex with FKBP38. All interactions occur transiently and at no point are any of the chaperones or co-chaperones limiting to the process. FKBP38 could play a role in the attachment of HERG to the motor protein kinesin, as FKBP52 does with the steroid receptors and dynein, for transport to the plasma membrane via an interaction with the TPR domain of FKBP38 and the TPR domain at the C terminus of the kinesin light chain (45–47). Investigations are currently underway to test this hypothesis.

In contrast to WT HERG, F805C progresses through a similar processing pathway except that its interaction with the initial chaperone complex of Hdj-2, Hsc70, Hop, and Hsp90 is likely prolonged (8, 9). The F805C mutant is recognized by these chaperones as non-native, and additional attempts are likely made to properly fold the protein. If these attempts to fold the protein fail, then it is expected that CHIP would replace Hop on Hsc70, which then targets the mutant protein for degradation as is known to occur with the trafficking-defective CFTR mutant ΔF508 (20, 21). Recent work has demonstrated for the first time that FKBP38 interacts with Hsp90 through its TPR domain and that FKBP38 is inhibited by Hsp90 (37). One speculation is that FKBP38 and Hsp90 function in opposition with respect to the trafficking of HERG mutants and that the interaction of FKBP38 with HERG and Hsp90 somehow facilitates the release of the channel.

The fact that FKBP38 overexpression rescued the trafficking of F805C suggests that it may be a limiting co-chaperone in this pathway. Of interest is the fact that although FKBP38 overexpression rescued the trafficking of F805C, it had no effect on the trafficking efficiency of WT HERG. Precedence exists in the literature demonstrating that rescue of a trafficking mutant does not always correlate with an increased efficiency of WT trafficking. Ficker et al. (35) showed that E4031, cisapride, and quinidine all rescued the trafficking of the HERG trafficking mutant G601S, but none of the compounds had an effect on the trafficking efficiency of WT HERG.

Given the fact that no compound currently exists that is capable of rescuing all HERG trafficking mutants, it is likely that HERG has multiple ER exit strategies. The current research provides one example of a HERG trafficking mutant that can be rescued by overexpression of FKBP38. It is entirely possible that a different balance of co-chaperones is required to rescue different HERG trafficking mutants.

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