Defects in the trafficking of subunits encoded by the human ether-à-go-go-related gene (*hERG1*) can lead to catastrophic arrhythmias and sudden cardiac death due to a reduction in I_{Kr}-mediated repolarization. Native I_{Kr} channels are composed of two α subunits, hERG1a and 1b. In heterologous expression systems, hERG1b subunits efficiently produce current only in heteromeric combination with hERG1a. We used Western blot analysis and electrophysiological recordings in HEK-293 cells and *Xenopus* oocytes to monitor hERG1b maturation in the secretory pathway and to determine the factors regulating surface expression of hERG1b subunits. We found that 1b subunits expressed alone were largely retained in the endoplasmic reticulum (ER), thus accounting for the poor functional expression of homomeric 1b currents. Association with hERG1a facilitated 1b ER export and surface expression. We show that hERG1b subunits fail to mature because of an “RXXR” ER retention signal specific to the 1b N terminus of the human sequence and not conserved in other species. Mutating the RXXR facilitated maturation and functional expression of homomeric hERG1b channels in a charge-dependent manner. Co-expression of the 1b RXXR mutants with hERG1a did not further enhance 1b maturation, suggesting that hERG1a promotes 1b trafficking by overcoming the RXXR-mediated retention. Thus, selective trafficking mechanisms regulate subunit composition of surface hERG channels.

Voltage-gated potassium (K⁺) channels encoded by the human ether-à-go-go-related gene (*hERG1* or *KCNH2*) mediate the repolarizing cardiac current I_{Kr} (1, 2). Perturbation of I_{Kr} due to mutations in the hERG1 gene or drug block of hERG2 channels can cause sudden cardiac death associated with long QT syndrome (LQTS) (3).

Native I_{Kr} channels are composed of hERG1a and 1b α subunits encoded by alternate transcripts of the hERG1 gene (4). hERG1a and 1b subunits have identical transmembrane and C-terminal sequences but divergent N termini (5, 6), which interact to promote heteromeric assembly early in channel biogenesis (7). When expressed heterologously, hERG1a homomeric and 1a/1b heteromeric channels yield robust currents but with distinct properties because of the divergent N termini of the constituent subunits (5, 8, 9). However, hERG1b homomers produce undetectable or very small currents (5). Why hERG1b functional expression is inefficient, or how hERG1a promotes 1b surface expression, is unknown.

In this study we found that hERG1b protein efficiently exited the ER only in the presence of hERG1a. We tested the hypothesis that hERG1b subunits possess ER retention/retrieval signals that are inactivated upon association with the 1a subunit, thus favoring surface expression of the heteromeric channel. Of two RXXR motifs (two arginines separated by any single residue) in the 1b N terminus, surprisingly only the one motif specific to the human sequence and not conserved in other organisms subserved ER retention. Moreover, mutations within this signal promoted surface expression in a charge-dependent manner. Co-expression of the 1b RXXR mutant with 1a revealed that heteromerization with hERG1a facilitates hERG1b maturation by preventing RXXR-mediated ER retention/retrieval.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—HEK-293 cell culture, transfection, lysis, co-immunoprecipitation procedures, and analyses of Western blots have been described previously (7).

**Whole-cell Patch Clamp**—Wild-type hERG1b and the mutant cDNAs (1.5 μg) were transiently expressed along with the enhanced green fluorescent protein, and cells with fluorescence were chosen for electrical recordings. Whole-cell patch clamp recordings were performed using an Axopatch 200B amplifier (Axon Instruments, Inc.). The bath solution contained (in mM) 137 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 glucose, 5 tetraethylammonium, and 10 HEPES (pH 7.4 with NaOH). Tetraethylammonium (5 mM) was included in the bath solution to block endogenous voltage-gated potassium channels. Whole-cell currents were recorded with a fire-polished pipette tip of ~1–2 μm with a resistance of 2–4 megohms. Internal pipette solution contained (in mM) 130 KCl, 1 MgCl₂, 5 EGTA, 5 MgATP, 10 HEPES (pH 7.2 with KOH). Currents were digitized at 2 kHz unfiltered. Series resistance compensation was typically 60–70%, such that voltage errors were less than 5 mV. Leak subtraction was not used. Current recordings were carried out at room temperature (22 °C).
Two-electrode Voltage Clamp—For electrophysiological recordings in oocytes, cRNA preparation and injection were carried out as described previously (8). Briefly, hERG 1b and mutant DNA were cloned into pGHI9 expression vector. cRNA transcripts were purified through a G-50 column (Amerham Biosciences), and 30 ng of cRNA was injected into oocytes. Currents were recorded 2–3 days after injection using a two-electrode voltage clamp. Oocytes were held at a potential of $-80\text{ mV}$. Currents were evoked by 480-ms pulses ranging from $-80\text{ to } -40\text{ mV}$ in increments of $+20\text{ mV}$ followed by a repolarizing step to $-105\text{ mV}$ for 480 ms.

Statistical Analysis—X-ray films of Western blots were quantified by measuring optical density using LabWorks image and acquisition analysis software (Upland, CA). We ensured signals were within a linear range of detection for the ECL reagent and the x-ray film by probing increasing amounts of lysate with the relevant antibody. Data were analyzed using PRISM 2.0 software.
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(GraphPad, San Diego, CA). All Western blotting and electrophysiology data from the RXR mutants were compared with wild-type 1b using one-way analysis of variance and Bonferroni’s multiple comparison post-test. A p value of 0.01 was used to assess significance. In Figs. 1 and 3–6, a single asterisk denotes p < 0.01, and double asterisks denote p < 0.001. A post-test for linear trend was used to assess whether charge-dependent improvement in 1b maturation and function was significant.

RESULTS

hERG 1a Promotes Maturation of 1b—To investigate the biochemical basis for functional rescue of 1b subunits by 1a, we monitored maturation of the 1b protein with or without 1a. Like other glycoproteins (10), both hERG 1a and 1b subunits are detected as two protein bands on a Western blot: a core-glycosylated, immature form and a complexly glycosylated, mature form (4, 11). The mature glycoform represents the fraction of core-glycosylated protein that has exited the ER and has been further processed in the Golgi. When expressed alone, 1b exhibited low levels of maturation, consistent with minimal functional expression reported previously (5). In the presence of 1a, 1b maturation was enhanced ~3-fold (Fig. 1). Thus, hERG 1a facilitates ER exit and Golgi maturation of 1b subunits.

Conserved R22XR Motif Does Not Contribute to Retention of 1b Homomers—We tested the hypothesis that exposed ER retention signals in the divergent 1b N terminus prevent Golgi maturation and trafficking to the cell surface. We identified two RXR motifs in the hERG 1b N terminus: the first, beginning at residue 15, is present only in the human ERG 1b sequence; the second, beginning at residue 22, is conserved across species (Fig. 2A). We mutated the positively charged arginines of the conserved R22XR motif to neutral asparagines (N22XN) to assess its role in preventing maturation of hERG 1b. Western blot analysis of transfected HEK-293 cells revealed no significant improvement in trafficking of N22XN over wild-type 1b (n = 6; *, p < 0.01; **, p < 0.001) (Fig. 2, B and C). Complementary whole-cell patch clamp recordings in HEK-293 cells also failed to exhibit significant functional rescue of 1bN22XN mutants over the typically low wild-type expression levels (Fig. 2, D and E).

R15XR Causes hERG 1b Retention—We next mutated the arginines of the R15XR motif to neutral asparagine residues (N15XN). Because this RXR motif is not conserved among other species, we were surprised to find the mutation dramatically rescued maturation of hERG 1b (Fig. 3, A, lanes 1 and 2, and B). A double mutation of both RXR motifs in the 1b N terminus (1bN15XN/N22XN) did not further improve maturation of 1b over that of the single N15XN mutation (Fig. 3, A, lane 3, and B). Surface expression and function of N15XN and the double mutant, as assessed by whole-cell patch clamp recordings in HEK-293 cells, paralleled the corresponding biochemical profiles, with an ~3-fold increase in current density attributable to N15XN (Fig. 3, C and D). Thus, mutations in the nonconserved...
R\textsuperscript{15}XR signal enhance 1b maturation and functional surface expression. These findings identify R\textsuperscript{15}XR as a key regulator of 1b trafficking. They also provide the important information that 1b subunits effectively oligomerize and can produce potassium currents in the absence of hERG 1a or heterologously expressed accessory subunits as long as they can traffic to the cell surface.

**Regulation of 1b Trafficking by R\textsuperscript{15}XR Is Determined by Its Charge**—To gain additional insights into the mechanism by which R\textsuperscript{15}XR regulates 1b trafficking, we systematically mutated the positively charged arginine residues to lysine (K\textsuperscript{15}XR, conserved charge), asparagine (N\textsuperscript{15}XR, neutral), and aspartate (D\textsuperscript{15}XR, reversed, negative charge). Compared with wild type, 1bK\textsuperscript{15}XR did not improve maturation significantly, whereas 1bN\textsuperscript{15}XR and 1bD\textsuperscript{15}XR mutations progressively enhanced 1b trafficking up to \(~5\)-fold (Fig. 4, A and B). The increase in maturation of these R\textsuperscript{15}XR mutants was charge-dependent, as evaluated by an analysis of variance post-test for linear trend (see "Experimental Procedures").

We next assessed whether improved maturation in 1b charge mutants translated into greater functional expression at the cell surface. For this we recorded currents using a two-electrode voltage clamp in *Xenopus* oocytes, where the amount of injected cRNA for each construct could be stringently controlled (Fig. 4D). Oocytes expressing mutant 1b produced currents up to \(~4\)-fold higher than wild-type 1b in a charge-dependent manner (Fig. 4, C and E), consistent with their biochemical maturation profiles in HEK-293 cells (Fig. 4B). No differences were apparent in the kinetics or voltage dependence of currents produced by the four 1b constructs, supporting the conclusion that larger currents are due to increased channel number at the cell surface and not to altered gating properties.

**hERG 1a Rescues 1b Function by Overcoming RXR-mediated Retention**—We next asked whether the enhanced maturation of 1b by mutation of R\textsuperscript{15}XR was related to the mechanism by which 1a promotes 1b maturation. An additive effect on maturation of R\textsuperscript{15}XR mutant 1b by co-expression with 1a would reflect regulation by more than one trafficking mechanism. Western blot analysis showed the \(~3\)-fold increase in 1bN\textsuperscript{15}XR maturation was not further enhanced in the presence of 1a (Fig. 5, A and B), suggesting that the mechanism by which 1a and RXR mutations promote 1b trafficking from the ER is the same. The lack of an additive effect on 1bN\textsuperscript{15}XR maturation is not due to a failure of 1a and 1bN\textsuperscript{15}XR to associate, as they can be co-immunoprecipitated using hERG 1a-specific antibody from lysate (Fig. 5C). These results suggest that association with 1a effectively masks the 1b N-terminal RXR retention signal to promote forward trafficking.

We attempted to uncover a region in the 1a N terminus responsible for masking the 1b RXR using deletion analysis (Fig. 6). We generated a series of five constructs deleted progressively by increments of 50 residues. Of these, only the 1aΔ200 protein exhibited a mature protein band comparable with wild-type 1a as assayed via Western blot analysis (Fig. 6A, left panel, lanes 1 and 5). The 1aΔ200 construct was likewise the only construct to express measurable currents (n = 7; \(p < 0.001\)) (Fig. 6, B and C). When co-expressed with hERG 1b, the 1aΔ200 construct effectively promoted 1b maturation (n = 5; \(p < 0.01\)) (Fig. 6A, right panel, lanes 1 and 5). Because 1aΔ200 exhibited maturation and promoted 1b maturation, whereas 1aΔ250 did not (Fig. 6, A–C), we sought to further delineate the masking region between residues 200–250 in the 1a N terminus. Unfortunately, an internal deletion in this region was trafficking-incompetent and therefore also did not improve maturation of 1b (Fig. 6D). We conclude that the masking region in the 1a N terminus lies downstream of residue 200; future experiments using alternative approaches will be required to pinpoint its precise location.

**DISCUSSION**

In this study we discovered a mechanism by which heteromeric association of subunits overcomes an N-terminal ER retention motif to regulate subunit composition of hERG surface channels. We ascribe ER retention of hERG 1b subunits to a single N-terminal RXR motif, R\textsuperscript{15}XR. The efficacy of R\textsuperscript{15}XR-mediated retention is dictated by its charge, as demonstrated by progressive improvements in 1b maturation and surface expression as the charge is respectively neutralized and reversed. Because a simple point mutation of the RXR motif in hERG 1b promotes trafficking and functional surface expression, it seems unlikely that gross misfolding of the 1b subunits contributes to its ER retention. Instead, fully formed homomeric 1b channels are prevented from trafficking. Heteromerization with 1a overcomes the RXR-mediated ER retention and promotes 1b maturation. Thus, a mechanism for promoting surface expression of hERG 1a/1b heteromers is encoded in the
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![Image of functional rescue experiment](image)

**FIGURE 6. Analysis of 1a N-terminal deletions on hERG 1b trafficking.** A, left panel, Western blot showing maturation profiles of sequential N-terminal deletions of 1a probed with C-terminal pan-hERG antibody (α-CT). Lysate from cells expressing hERG 1a or 1b is shown for comparison. Only 1a and 1aΔ200 proteins exhibited a mature protein band; the other four deletions failed to exit the ER. Right panel, Western blot analysis of 5 μg of lysate from cells co-expressing hERG 1b with the 1a N-terminal deletions. 5 μg of lysate from cells co-expressing 1b with 1a is shown as a positive control. An increased quantity (25 μg) of lysate from cells expressing only 1b (lost lane) is also shown for comparison of mature (M) and immature (I) 1b protein bands. The mature/total 1b ratio failed to improve when 1b was co-expressed with the trafficking-impaired 1a N-terminal deletion constructs. Co-expression of 1b with the only trafficking-competent N-terminal deletion, 1aΔ200, improved 1b maturation significantly (n = 5; p < 0.01). The protein for 1aΔ250 migrated at the same size as mature 1b protein; expression studies were used to assess maturation in this case (see B and C; B, two-electrode voltage clamp recordings from *Xenopus* oocytes show 1aΔ200 expressed efficiently, whereas 1aΔ250 did not. C, histogram comparing the peak tail current from 1aΔ200 and 1aΔ250 shows significant differences in expression levels (n = 7; **, p < 0.001). D, based on the above data from 1aΔ200 and 1aΔ250, an internal deletion between residues 200 and 250 of the 1a N terminus was made to determine whether this region contained the masking domain for the 1b N terminus. Unfortunately, the internally deleted 1aΔ203–228 protein was itself trafficking-incompetent and therefore also did not improve maturation of 1b. α-PDI, protein-disulfide isomerase antibody.

limited part of the N terminus that uniquely characterizes hERG 1b. We propose that the 1a N terminus masks the 1b ER retention signal or otherwise causes a conformational change that protects it from the ER retrieval machinery. Such a masking effect could result from a direct interaction, consistent with our previous finding that the 1a and 1b N termini interact in a direct, dose-dependent manner, as demonstrated by in vitro binding of purified N-terminal polypeptides (7).

Arginine-based ER retention signals have also been identified in K_{ATP} channels and γ-aminobutyric acid type B (GABA_{B}) and N-methyl-d-aspartate (NMDA) receptors, where subunit heteromultimerization masks ER retention/retrieval signals exposed in homomeric proteins (12–14). We demonstrate that hERG 1a subunits missing the first 200 residues are able to traffic and promote 1b maturation. Attempts to further map a masking region in the hERG 1a N terminus were hindered by the failure of downstream 1a deletion mutants to traffic. Perhaps the 1a N-terminal masking domain also acts homotypically, such that in its absence both 1a and 1b RXR signals are exposed and the subunits are retained/retrieved to the ER.

Although the 1b N terminus contains two RXR motifs, only one is conserved across species, but the other, nonconserved R^{15}XR mediates ER retention. In other species, R^{15}XR is replaced by QXR (Fig. 2A). Given that mouse ERG 1b subunits are similarly inefficient at producing current (5), it is reasonable to predict its QXR acts like the human RXR to retain the 1b subunit in the ER. Indeed, a similar motif in SUR2 subunits, RKQ, serves as an ER retention signal (15). Furthermore, the nonconserved R^{15}XR in hERG 1b is preceded by a leucine residue predicted, based on previous studies in K_{ATP} channels, to strengthen the retention capability of an RXR signal (12).

The observation that the effects on 1b maturation by R^{15}XR mutations and co-expression with 1a are not additive suggests that heteromerization with 1a overcomes ER retention of 1b. It could be argued that association of 1bN^{15}XN with 1a produces channels in numbers that fully occupy the Golgi glycosylation machinery, obscuring a potential additive effect contributed by the 1a subunits. However, observations on the charge dependence of maturation suggest that the 1bN^{15}XN mutant has not reached its full maturation potential (D^{15}XD exhibits more maturation), yet it does not further mature when associated with 1a. Interestingly, our D^{15}XD mutation closely resembles an ER export signal, DXE (16); we speculate that although both the NXN mutation and the presence of 1a overcome the retention checkpoint in 1b, the mutation to DXE might serve an additional function as a forward trafficking motif.

More than 290 LQTS mutations have been mapped to the hERG 1a sequence. These represent almost half of all the LQTS mutations identified to date in known target genes (Gene Connection for the Heart database). Most of the hERG1 mutations examined thus far fail to mature (17–19). Although the underlying mechanisms are unknown in most cases, some muta-
tions lead to protein misfolding and trigger the ER quality control system (20, 21). In one LQTS mutant, truncation of the C terminus by a nonsense mutation exposes an otherwise hidden C-terminal RXR motif, providing a molecular mechanism of ER retention for LQTS pathogenesis (22). However, in contrast to hERG 1b, wild-type 1a subunits readily form functional homomeric channels, and therefore it is likely that 1a RXR motifs are either inactive or serve as reporters of 1a subunit misfolding during normal biogenesis. To what extent exposure of ER retention motifs accounts for other LQTS disease phenotypes remains to be determined.

The role of hERG 1b in normal function and disease has not been fully explored. A specific knock-out of mouse 1b, which spares 1a, evokes cardiac arrhythmia (23), implying a critical role for the 1b subunit in normal cardiac function. Heteromeric 1a/1b currents exhibit less rectification and correspondingly larger currents in response to a voltage clamp command that mimics the ventricular action potential, suggesting the loss of 1b in native tissues is likely to result in reduced I_{Kr} amplitude.3

It will therefore be important to search for disease mutations in the hERG 1b-specific exon and to study phenotypes of hERG 1a-specific mutations in the presence of hERG 1b. We do not know yet the stoichiometry of the hERG 1a and 1b subunits in channels expressed in heterologous systems or native tissues. As an alternate transcript (5, 6), 1b may be subject to independent transcriptional control that alters stoichiometry under certain developmental, hormonal, or disease conditions. Future studies of hERG 1b will shed light on normal IKr function and the mechanisms by which its perturbation causes cardiac arrhythmia.

Acknowledgments—We thank Robert Zinkel, Tiffany Ekey, and Kristen DeCarlo for technical support; Drs. Cynthia Czajkowski, Akhil Bhalla, Andrew Boileau, and members of the Robertson laboratory for helpful discussion; and Dr. Barry Ganetzky for reading an earlier version of the manuscript.

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