Regulation of the Human Ether-a-go-go-Related Gene (hERG) Channel by Rab4 Through Neural Precursor Cell-expressed Developmentally Downregulated Protein 4-2 (Nedd4-2)*

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Running title: Rab4 regulates hERG via Nedd4-2

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Key words: Ion channel trafficking; LQTS; hERG; Rab4; Nedd4-2

Background: The hERG K+ channel plays an important role in the repolarization of cardiomyocytes where Rab4 is present.

Results: Overexpression of Rab4 decreases the density of mature hERG channels. This effect is mediated through enhanced expression of the ubiquitin ligase Nedd4-2.

Conclusion: Rab4 regulates hERG channel density via Nedd4-2.

Significance: Our data revealed a novel pathway for Nedd4-2 and hERG regulation.

SUMMARY

The human ether-a-go-go-related gene (hERG)2 encodes the pore-forming α-subunit of the rapidly activating delayed rectifier K+ channel (IKr) in the heart, which plays an important role in cardiac action potential repolarization. Dysfunction of IKr causes long QT syndrome (LQTS), a cardiac electrical disorder that predisposes affected individuals to fatal arrhythmias and sudden death. The homeostasis of the hERG channel at the plasma membrane depends on a balance between protein synthesis and degradation. Our recent data indicate that hERG channels undergo enhanced endocytic degradation under low potassium (hypokalemia) conditions. The GTPase Rab4 is known to mediate rapid recycling of various internalized proteins to the plasma membrane. In the present study, we investigated the effect of Rab4 on the expression level of hERG channels. Our data revealed that overexpression of Rab4 decreases the expression level of hERG in the plasma membrane. However, Rab4 does not affect the expression level of the Kv1.5 or EAG K+ channels. Mechanistically, our data demonstrate that overexpression of Rab4 increases the expression level of endogenous Nedd4-2, a ubiquitin ligase that targets the hERG but not Kv1.5 or EAG channel for ubiquitination and degradation. Nedd4-2 undergoes self ubiquitination and degradation, and Rab4 interferes with Nedd4-2 degradation, resulting in an increased expression level of Nedd4-2, which targets hERG. In summary, the present study demonstrates a novel pathway for hERG regulation; Rab4 decreases the hERG density at the plasma membrane by increasing the endogenous Nedd4-2 expression.

The human ether-a-go-go-related gene (hERG)2 encodes the pore-forming subunit of the rapidly activating delayed rectifier K+ channel (IKr) in the heart, which plays an important role in cardiac action potential repolarization (1, 2). A reduction or an increase in IKr can lead to long (LQTS) or short QT syndrome (SQT), both of which predispose affected individuals to fatal arrhythmias and sudden cardiac death (3, 4). Dysfunction of hERG channels can be caused by drugs that interfere with the channel gating. In addition, factors such as hypokalemia (a reduced plasma K+ concentration), and certain drugs such as pentamidine and probucol impair hERG function by decreasing the expression level of the hERG channel (5-7).

The homeostasis of hERG protein at the plasma membrane is a balance of anterograde and
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Retrograde trafficking mechanisms. Recycling of internalized protein to the plasma membrane also plays an important role in this process. The hERG channel is initially synthesized in the endoplasmic reticulum (ER) as the immature core-glycosylated form with a molecular mass of 135 kDa. The immature hERG channel then undergoes full-glycosylation in the Golgi apparatus to become the mature form with a molecular mass of 155 kDa, which is transported to the plasma membrane as functional channels (8). Our previous works have shown that ubiquitination of hERG protein at the plasma membrane triggers internalization of hERG channels under low K+ conditions (5, 9, 10). Furthermore, the ubiquitin (Ub) ligase Nedd4-2 ubiquitinates and degrades mature hERG channels (11-13). However, whether the internalized hERG proteins can be recycled back to the plasma membrane remains to be determined.

Ion channels are targets of small GTPases (14) which play various roles in the regulation of protein trafficking (15-17). It has been shown that Rab1 and Rab2 regulate ER-to-Golgi transport (18, 19); Rab4 is localized at the early sorting endosome and is responsible for rapid/direct recycling from early endosomes to the cell surface (20, 21); Rab5 regulates the fusion between endocytic vesicles and early endosomes, (22, 23); Rab7 has primarily been implicated in the transport from early to late endosomes, and plays an essential role in the maintenance of the perinuclear lysosome compartment (17, 22, 24); and Rab11 is involved in mediating slow recycling from endosomes to the plasma membrane (25).

In the present study, we investigated the regulatory effects of various Rabs on hERG channels. Our data unexpectedly revealed that Rab4 significantly decreases the expression level of hERG at the plasma membrane. Mechanistically, we found that Rab4 decreases the ubiquitination of Nedd4-2, which results in an increase in Nedd4-2 expression. The increased Nedd4-2 then decreases hERG expression at the plasma membrane by targeting the PY-motif in the C-terminus of hERG channels.

EXPERIMENTAL PROCEDURES

Molecular Biology — hERG cDNA was provided by Dr. Gail Robertson (University of Wisconsin-Madison); a hERG-expressing human embryonic kidney (HEK) 293 stable cell line (hERG-HEK cells) was provided by Dr. Craig January (University of Wisconsin-Madison). The human ether-a-go-go (EAG) cDNA was provided by Dr. Luis Pardo (Max-Planck Institute of Experimental Medicine, Göttingen, Germany); Kv1.5 cDNA (encoding the cardiac ultra-rapidly activating delayed rectifier K+ channel) was provided by Dr. Michael Tamkun (Colorado State University, Fort Collins, Colorado). GFP-tagged Rab1, Rab4, inactive Rab4 mutant Rab4N121I, Rab5, Rab7, and Rab11 plasmids were obtained from Addgene and Dr. Terry Hébert (McGill University). The scrambled control siRNA and Rab4A siRNA targeting human Rab4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Rab4A siRNA targeting rat Rab4 was purchased from Sigma-Aldrich. The human Nedd4-2 plasmid in pBluescript II was obtained from Kazusa DNA Research Institute (Chiba, Japan). The open reading frame of Nedd4-2 was amplified using polymerase chain reaction (PCR) and cloned into HA-pcDNA3 (Invitrogen) to generate HA-tagged Nedd4-2. The plasmid of the catalytically inactive form of Nedd4-2, Nedd4-2-C801S mutant (Nedd4-2CS), was provided by Dr. Hugues Abriel (University of Bern, Switzerland). To disrupt the Nedd4-2 interaction with hERG, we generated the hERG point mutation Y1078A and C-terminal truncation mutation Δ1073 using PfuUltra Hotstart PCR Master Mix (Agilent Technologies, Santa Clara, CA). The mutations were confirmed by DNA sequencing (Eurofins MWG Operon, Huntsville, AL). Stable cell lines were created and maintained at 37 °C in minimum essential medium (MEM, Life Technology) supplemented with 10% fetal bovine serum (FBS) and 0.4 mg/ml G418 (Invitrogen). For transient transfection, 2 μg of the plasmid of interest was transfected into hERG-HEK or HEK 293 cells growing in a 35-mm dish at 60-70% confluence using Lipofectamine 2000 (Invitrogen). A green fluorescent protein (GFP) plasmid (0.5 μg, pIRES2-EGFP, Clontech) was coexpressed to identify transfected cells in electrophysiological studies. After transfection, cells were cultured in 10% FBS-supplemented MEM for 24 h before experiments.

Neonatal Rat Ventricular Myocyte Isolation — Experimental protocols used for animal studies were approved by the Animal Care Committee of Queen's University. Single cardiomyocytes were
isolated from 1-day-old Sprague-Dawley rats of either sex using enzymatic dissociation methods as described previously (7). Cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Invitrogen, Burlington, OH) with 10% fetal bovine serum. Cardiomyocytes were grown on glass coverslips for electrophysiological and immunocytochemical studies, and in 60-mm dishes for Western blot analysis.

**Electrophysiological Recordings** — For recording the activities of WT and mutant hERG, as well as Kv1.5 and EAG channels stably expressed in HEK 293 cells, the whole-cell patch-clamp method was used. The bath solution contained (in mM): 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4). The pipette solution contained (in mM): 135 KCl, 5 EGTA, 1 MgCl₂, and 10 HEPES (pH 7.2). The hERG current (IhERG), EAG current (IEAG) or Kv1.5 current (IKv1.5) from respective stable HEK cell lines was recorded by depolarizing steps to voltages between −70 mV to +70 mV in 10 mV increments. The tail currents were recorded upon a repolarizing step to −50 mV. The holding potential was −80 mV. For the current amplitude analysis, the peak tail current at −50 mV following the 50 mV depolarizing step for the hERG channel, and the pulse current at the end of the depolarizing step to 50 mV for the Kv1.5 and EAG channels were used. For the recording of native IKr in cultured neonatal rat cardiomyocytes, the pipette solution contained (in mM): 135 CsCl, 5 MgATP, 10 EGTA, and 10 HEPES with pH 7.2 by CsOH. The bath solution contained (in mM): 135 CsCl, 1 MgCl₂, 10 glucose, 10 HEPES, and 10 μM nifedipine with pH 7.4 by CsOH. The current was evoked by depolarizing cells to voltages between −70 mV and 70 mV in 10 mV increments. The current amplitude upon repolarization to the −80 mV holding potential after the depolarizing step of 50 mV was used to measure the amplitude of native IKr (26). Patch-clamp experiments were performed at room temperature (22 ± 1°C).

**Western Blot Analysis and Co-immunoprecipitation (co-IP)** — Following treatments, hERG-HEK cells were cultured in 35-mm dishes for 24 h. Whole-cell proteins were extracted and separated on 8% or 15% sodium dodecyl sulfate polyacrylamide electrophoresis gels, transferred onto PVDF membranes, and blocked for 1 h with 5% non-fat milk. The membranes were incubated with appropriate primary antibodies for 1 h at room temperature, and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies. Actin expression was used for the loading control. The blots were visualized with Fuji film using the ECL detection kit (GE Healthcare). To quantify the Western blot data, the band intensities of proteins of interest in each gel are first normalized to their respective actin intensities; the normalized intensities are then compared with the band intensity from control cells, and expressed as relative values to their controls.

For co-IP, whole-cell proteins (0.5 mg) were incubated with the appropriate primary antibody overnight at 4 °C and then precipitated with protein A/G plus agarose beads (Santa Cruz) for 4 h at 4 °C. The beads were washed 3 times with ice-cold RIPA (radioimmunoprecipitation assay) lysis buffer, resuspended in 2 × Laemmli sample buffer and boiled for 5 min. The samples were centrifuged at 20,000 × g for 5 minutes. The supernatants were collected and analyzed using Western blot analysis.

**Immunofluorescence Microscopy** — hERG-HEK cells were transfected with GFP, GFP-tagged Rab4 or GFP-tagged Rab4N121I. Twenty-four hours after transfection, the cells were fixed with freshly prepared 4% paraformaldehyde for 15 min. The fixed cells were permeabilized with 0.1% Triton-X100 for 10 min and blocked with 5% bovine serum albumin (BSA) for 1 h. The permeabilized cells were immunoblotted with rabbit anti-Nedd4-2 primary antibody and Alexa Fluor 594-conjugated donkey anti-rabbit secondary antibody to detect Nedd4-2. Cultured neonatal rat cardiomyocytes were transfected with GFP-tagged Rab4. Forty eight hours after transfection, cells were fixed and permeabilized. ERG (rat IKr protein) was labeled with anti-hERG primary antibody (C-20) and Alexa Fluor 594-conjugated secondary antibody. The Nedd4-2 was labeled with anti-Nedd4-2 primary antibody and Alexa Fluor 594-conjugated secondary antibody in separate sets of cells. Images were acquired using a Leica TCS SP2 Multi-Photon confocal microscope (Leica, Germany).

**Reagents and Antibodies** — MEM and FBS were purchased from Invitrogen. Rabbit anti-
Kv11.1 (hERG), anti-Kv10.1 (EAG-1), anti-ubiquitin, mouse anti-actin antibodies, G418, electrolytes, EGTA, HEPES, glucose, protein synthesis inhibitor cycloheximide (CHX), BSA, and proteasome inhibitor ALLN (N-acetyl-leu-leu-norleucinal) were purchased from Sigma-Aldrich. Proteasome inhibitor MG132 (N-CBZ-leu-leu-norleucinal) was purchased from EMD Millipore. Goat anti-hERG (N-20 and C-20), rabbit anti-GAPDH, anti-Kv1.5, anti-Rab4 antibodies, Protein A/G PLUS Agarose, goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Nedd4-2 antibody was purchased from Cell Signaling Technology. Alexa Fluor 594 donkey anti-rabbit and anti-goat secondary antibodies were purchased from Invitrogen.

All data are expressed as the mean ± S.E. One-way analysis of variance followed by Newman–Keuls post hoc tests (GraphPad Prism), and two-tailed paired or unpaired Student's t test was used to determine the statistical significance between control and experimental groups. A p value of 0.05 or less was considered significant.

RESULTS

Overexpression of Rab4 Decreases hERG Expression Level at the Plasma Membrane — Fig. 1 demonstrates the effect of overexpression of various Rab GTPases on the expression level of hERG channels. The hERG proteins extracted from hERG-HEK cells displayed two bands with molecular masses of 155 kDa and 135 kDa, representing the mature, fully-glycosylated form on the plasma membrane and the immature, core-glycosylated form in the ER, respectively (5, 8). Among the Rab GTPases examined (Rab1, Rab4, Rab5, Rab7 and Rab11), only Rab4 significantly reduced the expression level of the 155-kDa hERG protein. This result is surprising, since if Rab4 mediates rapid hERG recycling, an increase in the 155-kDa hERG expression level is expected. As shown in Fig. 1A, overexpression of Rab4 significantly reduced the intensity of the 155-kDa hERG band without affecting the 135-kDa hERG band. Overexpression of Rab4 did not affect the expression level of Kv1.5 or EAG channels stably expressed in HEK 293 cells (Fig. 1C). Consistent with the data obtained using Western blot analysis, Rab4 decreased I_{hERG} but not I_{Kv1.5} or I_{EAG} (Fig. 1B and D). Thus, among potassium channels Kv1.5, EAG, and hERG, Rab4 selectively targets the hERG channel. While Rab4 decreased the hERG current amplitude, it did not affect the biophysical properties of I_{hERG}. The half activation voltage and slope factor of I_{hERG} were −3.1±0.3 mV and 7.1±0.3 mV in control cells, and −6.5±0.9 mV and 7.9±0.7 mV in Rab4-transfected cells (n=4-8 cells, P>0.05).

Overexpression of Rab4 Increases Ubiquitination of hERG Protein and Enhances the Interaction Between hERG and Ub ligase Nedd4-2 — Our data in Fig. 1 show that overexpression of Rab4 leads to a decrease in mature hERG channel levels. Rab4 is natively expressed in HEK cells. To confirm the role of Rab4 in hERG expression, we knocked down endogenous Rab4 in hERG-HEK cells. As shown in Fig. 2A, knockdown of Rab4 led to a significant increase in mature hERG expression.

Ubiquitination is a process involving the covalent binding of Ub to its target proteins. The best-characterized consequence of ubiquitination is the triggering of internalization and/or degradation of the target proteins (27, 28). To determine the involvement of ubiquitination in Rab4 mediated reduction in the 155-kDa hERG expression, we examined the effects of Rab4 overexpression on the Ub-hERG interaction using co-IP analysis. hERG-HEK cells were transfected with pcDNA3 (control) or Rab4 plasmid. To inhibit protein degradation, the proteasome inhibitor ALLN (50 µM) was added to the culture medium. Twenty four hours after transfection, whole-cell proteins were extracted. An anti-Ub antibody was used to immunoprecipitate Ub and its associated proteins. The precipitated proteins were immunoblotted to detect for hERG expression. As shown in Fig. 2B, Rab4 overexpression significantly enhanced the Ub-hERG interaction as evidenced by the more intense ubiquitinated hERG band in Rab4 transfected cells than that in control cells. A hERG band in anti-Ub antibody-precipitated proteins slightly higher than 155 kDa is present, which may reflect mono-ubiquitinated mature hERG channels. The nature of other enhanced bands with molecular masses smaller than 135 kDa in Rab4 transfected cells is unknown and may reflect the fragmented hERG proteins during degradation. Our previous work has also shown that Ub ligase Nedd4-2 only targets the mature (155-kDa) hERG
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on the plasma membrane for degradation (13). Transferring ubiquitin to its target proteins, known as ubiquitination, involves a series of enzymes including Ub ligases such as Nedd4-2 that recognizes and labels target proteins with Ub (29). Nedd4-2 plays a critical role in regulating several plasma membrane proteins including epithelial sodium channel (ENaC), cardiac voltage-gated sodium channel Nav1.5, potassium channel KCNQ1, and neuronal voltage-gated sodium channel (Na+) (30-34). Particularly, we and others have shown that Nedd4-2 interacts with hERG to degrade the mature form (155-kDa) of hERG channels (11-13). To determine whether Nedd4-2 is involved in the Rab4-mediated hERG reduction, we studied the effects of Rab4 overexpression on the hERG-Nedd4-2 interaction. As shown in Fig. 2C, Rab4 overexpression significantly enhanced the hERG-Nedd4-2 interaction as evidenced by a stronger Nedd4-2 band in anti-hERG antibody-precipitated proteins from Rab4-transfected cells than that from control cells. Thus, the Rab4-mediated reduction of mature hERG protein is associated with an enhanced hERG interaction with Nedd4-2.

Disrupting the hERG-Nedd4-2 Interaction Eliminates the Effect of Rab4 on hERG Channels

To determine if Rab4 regulates hERG expression via Nedd4-2, we investigated the effects of Rab4 overexpression on mutant hERG channels whose Nedd4-2 binding motif is disrupted. As shown in Fig. 3A, both the point mutation Y1078A (PY-motif disrupted) and C-terminal truncation mutation Δ1073 (PY-motif removed) were essentially able to remove the effects of Nedd4-2 on the expression of hERG channels. Also, we have previously shown that Nedd4-2 overexpression decreases I_{hERG}, and both Y1078A and Δ1073 mutations eliminate the Nedd4-2-induced I_{hERG} reduction (13). Disrupting the Nedd4-2 binding motif also abolished the effects of Rab4 overexpression on hERG channels (Fig. 3B). Consistent with the Western blot analyses, electrophysiology experiments revealed that while Rab4 overexpression decreased WT I_{hERG} by 50% (from 1.39±0.14 nA, n=18 to 0.70±0.09 nA, n=14; P<0.01), it did not significantly affect Δ1073 I_{hERG} (0.94±0.13 nA, n=14, in control, versus 0.92±0.16 nA, n=9, in Rab4-transfected cells. P=0.05). Thus, the Rab4-mediated decrease in hERG expression in the plasma membrane requires Nedd4-2.

Overexpression of Rab4 Increases Nedd4-2 Expression – To investigate the mechanisms of Nedd4-2 involvement in the Rab4-mediated reduction in hERG expression, we examined the effect of Rab4 on the expression of Nedd4-2. As shown in Fig. 4, overexpression of Rab4 significantly enhanced the expression level of Nedd4-2 as revealed by Western blot (Fig. 4A) and immunocytochemical analyses (Fig. 4B). To further confirm the effects of Rab4 on Nedd4-2 expression, we also knocked down Rab4 using siRNA transfection. Knockdown of Rab4 in HEK cells resulted in a significant decrease in the Nedd4-2 expression level (Fig. 4C). In our blots with the Nedd4-2 antibody from Cell Signaling, endogenous Nedd4-2 extracted from HEK cells displayed two bands with molecular masses of 110 kDa and 120 kDa (Fig. 3A, 4A and C, 5A and B). While the nature of these two bands warrants further investigation and may represent a mixture of Nedd4-2 and its modified forms (ubiquitinated or apo-Nedd4-2), two reasons prompted us to focus on the 110-kDa form of Nedd4-2: first, as shown in Fig. 2C, when the interaction between hERG and Nedd4-2 was examined using co-IP analysis in hERG-HEK cells, only the 110-kDa band was detected in proteins precipitated with an anti-hERG antibody; second, as shown in Fig. 3A, transfection of Nedd4-2 plasmid into hERG-HEK cells led to Nedd4-2 overexpression which displayed a molecular mass of 110 kDa.

Rab4 Increases Nedd4-2 Expression by Inhibiting Nedd4-2 Degradation – Nedd4-2 binds to the PY motif of target proteins to mediate protein ubiquitination/degradation (30). Interestingly, Nedd4-2 itself also possesses a PY motif within its catalytic HECT (Homologous to E6-associated protein C-Terminus) domain. When Nedd4-2 is not interacting with its substrates, the PY motif of Nedd4-2 weakly binds to the WW domain of other Nedd4-2 proteins, leading to self-ubiquitination and degradation (35, 36). To test the possibility that Rab4 increases Nedd4-2 by interfering with Nedd4-2 degradation, we treated cells with proteasome inhibitors. We have previously shown that proteasome inhibition can impede Ub-
mediated degradation of hERG channels (10). Treatment of hERG-HEK cells with 50 μM ALLN (or 10 μM MG132, data not shown) increased the level of Nedd4-2 expression, likely by preventing proteasomal degradation. On this elevated background, Rab4 overexpression no longer significantly increased Nedd4-2 expression levels (Fig. 5A).

To directly investigate the effects of Rab4 on the degradation rate of Nedd4-2, we blocked protein synthesis using cycloheximide (CHX, 10 μg/ml) and monitored the Nedd4-2 degradation in control as well as in Rab4-transfected cells. As shown in Fig. 5B, Rab4 overexpression significantly decreased the Nedd4-2 decay rate; after 12 h treatment with CHX, while the Nedd4-2 expression decreased by more than 50% in control cells, it only decayed by 15% in Rab4-transfected cells.

Catalytic activity of Nedd4-2 is reported to be required for its self ubiquitination (35). To confirm this, we examined the Nedd4-2 ubiquitination and the role of catalytic activity in Nedd4-2 degradation by comparing the expression levels of ubiquitinated Nedd4-2 between cells expressing wild-type (WT) Nedd4-2 and those expressing a catalytically inactive Nedd4-2 mutant, Nedd4-2-C801S. It has been shown that due to the lack of catalytic activity, Nedd4-2-C801S neither catalyzes its substrates nor undergoes self-ubiquitination (35). As shown in Fig. 6A, WT Nedd4-2 but not Nedd4-2-C801S experienced significant ubiquitination. In particular, besides the smeared background which may indicate poly-ubiquitination, a single band of Ub-precipitated Nedd4-2 which is slightly higher than the Nedd4-2 band in Western blot analysis is present, which should reflect the mono-ubiquitinated Nedd4-2. Ubiquitination appears to be a prerequisite for the Rab4-mediated increase in Nedd4-2 expression. As shown in Fig. 6B, while Rab4 overexpression significantly increased the expression level of Nedd4-2, it failed to increase the expression level of Nedd4-2-C801S. These data raised the possibility that Rab4 preferentially interacts with ubiquitinated Nedd4-2, interferes with its degradation and promotes its recycling, resulting in an increased expression level of Nedd4-2. Our data shown in Fig. 6C directly support this notion; between Nedd4-2 and Nedd4-2-C801S, Rab4 preferentially co-precipitated with Nedd4-2. Furthermore, the Rab4-precipitated Nedd4-2 band is slightly higher than the Nedd4-2 band in Western blot analysis, suggesting that Rab4 interacts with mono-ubiquitinated Nedd4-2. Finally, overexpression of Rab4 decreased the ubiquitinated Nedd4-2 (Fig. 6D), which may underlie the slowed degradation, and enhanced expression of Nedd4-2.

The Effect of Rab4 on the Expression and Function of I_Kr in Neonatal Rat Cardiomyocytes – Rab4 is natively expressed in cardiomyocytes and serves important functions. By transgenic expression of dominant negative Rab4 S27N in mice, Odley et al. showed that Rab4 mediates recycling of internalized β-adrenergic receptors, which is necessary for normal cardiac catecholamine responsiveness and resensitization after agonist exposure (37). Rab4 expression levels have been shown to be altered in certain pathological conditions. Transgenic overexpression of β2-adrenergic receptors in mouse hearts leads to heart failure with augmented Rab4 expression levels (38). Increased Rab4 expression is also observed in Akt2-deficiency-induced cardiomyopathy which is similar to type 2 diabetic cardiomyopathy (39). Transgenic overexpression of Rab4 in the mouse myocardium induces concentric cardiac hypertrophy (40). To determine whether Rab4 expression affects I_Kr in cardiomyocytes, we altered Rab4 expression levels by overexpressing Rab4 as well as knocking down Rab4 in neonatal rat cardiomyocytes. As shown in Fig. 7A-C, overexpression of Rab4 decreased the expression level of mature form of ERG (I_Kr protein in rats) whereas knockdown of Rab4 increased the expression level. As well, overexpression of Rab4 significantly decreased the I_Kr recorded by whole-cell patch clamp using Cs+ permeation to isolate I_Kr from other K+ currents (Fig. 7D). To confirm the involvement of Nedd4-2 in Rab4-mediated changes in ERG, immunofluorescence microscopy was used to examine the effects of Rab4 overexpression on ERG and Nedd4-2 levels. As shown in Fig. 7E, overexpression of Rab4-GFP led to a decrease in ERG expression and an increase in Nedd4-2 expression.

**DISCUSSION**

The hERG potassium channel plays a critical role in the repolarization of the cardiac action
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potential. The whole-cell current of any type of ion channel is determined by single channel activities (gating, i.e. open versus closed) and the total number of functional channels at the plasma membrane. Studies have shown that mutations in hERG or drugs can decrease the hERG density at the plasma membrane, leading to a diminished I_hERG, which causes LQTS (7, 41). However, it is unknown how the density of hERG channels on the plasma membrane is regulated. We previously demonstrated that hERG channels undergo endocytic degradation which is accelerated under hypokalemic conditions (5). Since Rab4 has been shown to mediate the rapid recycling of various endocytosed membrane proteins (20, 21), we investigated the effects of various Rab GTPases, especially Rab4, on hERG expression. Our data show that Rab4 selectively decreases the plasma membrane-localized 155-kDa hERG proteins (Fig. 1A and 3B). We demonstrated a novel regulatory mechanism where Rab4 decreases the expression of hERG potassium channels through enhancing the expression level of the Ub ligase Nedd4-2, which mediates hERG ubiquitination and degradation.

Covalent attachment of single or multiple Ub molecules to a target protein is known as ubiquitination. The WW domain of Nedd4-2 binds to the PY-motif of the target protein to mediate protein ubiquitination. Interestingly, although both immature (135-kDa) and mature (155-kDa) hERG channels possess the PY motif, our present as well as previous studies demonstrate that Nedd4-2 selectively targets and induces the degradation of the mature (155-kDa) hERG channels located at the plasma membrane (13). Similar results were also published by Albesa et al. (12). The mechanisms for Nedd4-2 to selectively reduce the 155-kDa form of hERG remain to be fully understood, and the following two mechanisms may be involved. First, membrane adaptor proteins such as NDFIP2 (Nedd4 family-interacting protein 2) function as both a recruiter and a strong activator of the Nedd4 family (42). We have also previously shown that caveolin-3 recruits Nedd4-2 to the plasma membrane to interact with mature hERG channels (13). Second, Nedd4-2 possesses a C2 domain in its N-terminus, which binds to the cellular membrane in a Ca^{2+}-dependent manner (43). The C2 domain of Nedd4-2 binds to its HECT domain in the inactive state. Calcium-dependent binding of the C2 domain to the membrane phospholipids dissociates the C2 domain from the HECT domain, leading to the activation of Nedd4-2 (36). Thus, C2 domain-mediated translocation of Nedd4-2 to the cellular membrane could be responsible for the selective targeting of Nedd4-2 to the plasma membrane-localized mature hERG channels.

Nedd4-2 also possesses a PY-motif in its catalytic HECT domain (35). In the absence of other substrates, the PY-motif (LpXY) of Nedd4-2 weakly binds to the WW domains of Nedd4-2 to prevent Nedd4-2 from ubiquitination by neighbouring Nedd4-2 molecules. However, when Nedd4-2 interacts with its target proteins such as hERG, a conformational change of Nedd4-2 exposes the PY-motif to neighbouring Nedd4-2 molecules, leading to the ubiquitination and degradation of Nedd4-2 (35). Our data demonstrate that Nedd4-2 indeed experiences self-ubiquitination and Rab4 overexpression significantly decreases ubiquitinated Nedd4-2, slows down Nedd4-2 degradation, and increases expression of Nedd4-2 (Fig. 4-6). Our data further show that Rab4 likely interacts with the ubiquitinated Nedd4-2 (Fig. 6C). These findings support the notion that Rab4 mediates Nedd4-2 recycling and that deubiquitination must also occur during the recycling (Fig. 8).

We propose that the ubiquitination of Nedd4-2 takes place at the plasma membrane when it interacts with and mediates ubiquitination of hERG channels. Ubiquitinated Nedd4-2 can be either degraded along the degradation pathways or recycled back to the plasma membrane (Fig. 8). Rab4 is known to be associated with early endosomal recycling and the transport of internalized proteins back to the plasma membrane (37, 44). Thus, Rab4 may facilitate the recycling of Nedd4-2, leading to decreased degradation and increased expression. The increased Nedd4-2 consequently leads to an enhanced degradation of mature hERG channels (Fig. 8). Our data show that disrupting the interaction between Nedd4-2 and hERG completely abolishes the effects of Rab4 on hERG channels, indicating that Nedd4-2-mediated regulation is the primary pathway for Rab4 to regulate hERG, and the direct effects of Rab4 on hERG trafficking seem to be negligible.

Our findings may have broad implications for the Nedd4-2 target proteins which possess the PY-
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motif. For example, previous studies have demonstrated that Rab4 overexpression decreases the plasma membrane-expression of epithelial sodium channel (ENaC) (45) and cystic fibrosis transmembrane conductance regulator (CFTR) (46). However, the mechanisms for Rab4 in these regulations are not known (45, 46). Because both ENaC and CFTR are Nedd4-2 substrates (30, 47), it is likely that Rab4 also regulates these two channels via Nedd4-2. In addition, cardiac and neuronal Na⁺ channels, cardiac K⁺ channel KCNQ1, and neuronal K⁺ channels KCNQ2/3/5 are among the substrates of Nedd4-2 (32-34, 48). Besides the direct effects on these channels, Rab4 is expected to regulate these channels via altered Nedd4-2 expression levels. Finally, Nedd4-2 is involved in a variety of cellular processes including neuronal development and cell growth (49, 50). Thus, the Rab4-mediated regulation of Nedd4-2 may have a wide impact on various cellular processes.

Rab4 expression is highly variable and regulated in cardiomyocytes (37-39). Our data demonstrate that Rab4-mediated alterations in ERG expression also exist in cardiomyocytes (Fig. 7). A previous study has demonstrated that cardiac Rab4 is upregulated in a dilated cardiomyopathy model overexpressing β₂-adrenergic receptors (38). Upregulation of Rab4 expression is also observed in ventricular tissues of Akt2-knockout mice which develop a syndrome similar to diabetes mellitus type 2 cardiomyopathy (39). Transgenic overexpression of Rab4 in the mouse myocardium induces concentric cardiac hypertrophy (40). Cardiomyopathy as well as heart failure are closely associated with QT prolongation and ventricular arrhythmias (51-55). Specifically, a link between depressed hERG channel function and abnormal QT prolongation in diabetic rabbits was also reported (56). Thus, our study raised the possibility that elevated Rab4-mediated reduction in hERG expression may play a role in the development of QT prolongation in patients with cardiomyopathy and heart failure.

In summary, the present study has revealed a novel mechanism for Nedd4-2 and hERG regulation: Rab4 increases Nedd4-2 expression which consequently decreases the expression of mature hERG channels. The Rab4-mediated Nedd4-2 regulation could impact other cellular processes that are regulated by Nedd4-2.

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**FOOTNOTES**

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The abbreviations used are: hERG, the human ether-a-go-go-related gene; IKr, the rapidly activating delayed rectifier potassium channel; Nedd4-2, neural precursor cell expressed developmentally down-regulated protein 4 subtype 2; Ub, ubiquitin; LQTS, long QT syndrome; HECT domain, Homologous to E6-associated protein C-Terminus domain.

**FIGURES AND LEGENDS**

**FIGURE 1.** Rab4 decreases the expression level of mature hERG protein and I_hERG. *A*, Effects of Rab GTPases on hERG expression levels. The relative band intensities (Intensity-Rel) of the 155-kDa hERG bands from cells transfected with various Rabs were normalized to the value of the control (Ctrl) cells in each gel, summarized and plotted against each treatment (n=3-5). ** P<0.01 vs. control. *B*, hERG currents recorded from pcDNA3- (control: Ctrl), Rab4- or inactive Rab4N121I-transfected cells. The summarized tail current-voltage relationships (n=4-8) are shown beneath the current traces. *C*, Effects of
Rab4 regulates hERG via Nedd4-2

Rab4 on the expression levels of the EAG or Kv1.5 channel. The band intensities of the respective channel proteins from Rab4-transfected cells were normalized to the values from control cells and summarized (n=3, respectively). D, Effects of Rab4 on the EAG or Kv1.5 current. Families of EAG or Kv1.5 currents in pcDNA3- (Ctrl) or Rab4-transfected cells are shown along with the current amplitudes upon the 50-mV depolarization step. The numbers in the parentheses above each bar indicate the number of cells tested from at least three independent experiments.

FIGURE 2. Rab4 enhances hERG ubiquitination and increases the hERG-Nedd4-2 interaction. A, Knockdown of Rab4 increases hERG expression in hERG-HEK cells. hERG-HEK cells were transfected with control siRNA or Rab4A siRNA for 24 h. Whole-cell lysates were obtained and analyzed using Western blot analysis. The relative 155-kDa band intensities (Intensity-Rel) compared to the control is shown beneath the Western blots (** P<0.01 vs. control, n=4). B, Rab4 enhances hERG ubiquitination. hERG-HEK cells were transfected with pcDNA3 (Ctrl) or Rab4 for 24 h. The cells were then treated with the proteasome inhibitor ALLN (50 µM) for 24 h to prevent hERG degradation. The whole-cell lysates were precipitated with anti-Ub antibody and immunoblotted with anti-hERG antibody (n=7). To confirm that Rab4 enhanced Ub-hERG interaction, GAPDH was used as a control for the anti-Ub antibody (left panel) in the immunoprecipitation assay. As well, HEK cells were used as control for hERG-HEK cells (right panel). C, Rab4 enhances the hERG-Nedd4-2 interaction. hERG-HEK cells were transfected with pcDNA3 (Ctrl) or Rab4, then cultured for 24 h. Whole-cell lysates were immunoprecipitated with anti-hERG antibody (N-20) and the precipitates were immunoblotted with anti-Nedd4-2 antibody (n=3).

FIGURE 3. Nedd4-2 is involved in the Rab4-mediated reduction in mature hERG expression. A, Effects of Nedd4-2 on the expression of WT and mutant hERG channels. Disrupting Nedd4-2 binding site in hERG by mutations eliminated the Nedd4-2-induced reduction in the mature (upper-band) hERG expression (n=4-6). B, Effects of Rab4 on the expression of WT and mutant hERG channels. Disrupting the Nedd4-2 binding site in hERG by mutations abolished the Rab4-induced reduction in the mature hERG expression (n=5-7). In both A and B, the band intensities of the mature hERG bands from Nedd4-2- or Rab4-transfected cells were normalized to their respective controls and summarized. ** P<0.01 vs. control.

FIGURE 4. Rab4 increases the expression level of endogenous Nedd4-2. A, Effects of Rab4 or inactive Rab4N121I on the expression level of Nedd4-2. The hERG-HEK cells were transfected with pcDNA3, GFP-tagged Rab4 or GFP-tagged Rab4N121I. Twenty four hours after transfection, whole-cell lysates were extracted and analyzed using western blot analysis (n=4). B, Confocal images showing that overexpression of Rab4 but not inactive Rab4N121I enhances the expression of endogenous Nedd4-2. hERG-HEK cells were transfected with GFP, GFP-tagged Rab4 or GFP-tagged Rab4N121I (green). Nedd4-2 was labelled with anti-Nedd4-2 primary antibody and Alexa Fluor 594-conjugated (red) secondary antibody. C, Knockdown of Rab4 decreases the expression of endogenous Nedd4-2. The hERG-HEK cells were transfected with scrambled control siRNA and Rab4A siRNA. Twenty four hours after transfection, whole-cell lysates were extracted and analyzed using Western blot analysis (n=4). For analyses in A and C, the intensities of the 110-kDa band of Nedd4-2 from cells in experimental groups were normalized to their respective controls and plotted as relative values. * P<0.05, ** P<0.01 vs. control.

FIGURE 5. Rab4 interferes with the degradation process to increase Nedd4-2 expression. A, Inhibition of Nedd4-2 degradation eliminates the Rab4-mediated increase in Nedd4-2 expression. HEK 293 cells were transfected with pcDNA3 (Ctrl) or Rab4. Twenty four hours after transfection, cells were treated with or without the proteasome inhibitor ALLN (50 µM) for an additional 24 h. Cells were then collected for Western blot analysis. The intensities of the 110-kDa band of Nedd4-2 from cells under various treatments were normalized to the control in the absence of ALLN, and plotted as relative values (n= 4). * P<0.05, N.S.: no significant difference. B, Rab4 slows down the degradation rate of Nedd4-2.
HEK 293 cells transfected with pcDNA3 (Ctrl) or Rab4 were treated with cycloheximide (CHX, 10 µg/ml) to inhibit protein synthesis. Nedd4-2 expression levels (110-kDa) at various time points following CHX treatment were normalized to the initial value (time 0), and summarized for control and Rab4-transfected cells (n=4). ** P<0.01 vs. control.

FIGURE 6. Ubiquitination of Nedd4-2 is required for the Rab4-Nedd4-2 interaction and the Rab4-mediated Nedd4-2 increase. A, Ubiquitination of Nedd4-2 and catalytically inactive Nedd4-2-C801S (Nedd4-2CS). Whole-cell lysates from HEK 293 cells transfected with Nedd4-2 or Nedd4-2-C801S were immunoprecipitated with anti-Ub antibody. The precipitates were immunoblotted with anti-Nedd4-2 antibody to detect ubiquitinated Nedd4-2 (n=3). B, Effects of Rab4 on the expression levels of Nedd4-2 and catalytically inactive Nedd4-2-C801S (Nedd4-2CS). HEK 293 cells transfected with either Nedd4-2 or Nedd4-2-C801S were co-transfected with pcDNA3 (Ctrl) or Rab4. Western blot analyses were performed 24 h after transfection. The intensities of Nedd4-2 in Rab4-transfected cells were normalized to their respective controls and expressed as relative values (n=3). * P<0.05 vs. control. C, Rab4 interacts with Nedd4-2 but not Nedd4-2-C801S (Nedd4-2CS). Whole-cell lysates from HEK 293 cells transfected with Nedd4-2 or Nedd4-2-C801S were immunoprecipitated with anti-Rab4 antibody. The precipitates were immunoblotted with anti-Nedd4-2 antibody (n=4). D, Effects of Rab4 on Ub-Nedd4-2 interactions. Whole-cell proteins from HEK 293 cells co-transfected with Nedd4-2 plus pcDNA3 (Ctrl) or Nedd4-2 plus Rab4 were immunoprecipitated with anti-Ub antibody. The precipitates were immunoblotted with anti-Nedd4-2 antibody (n=6). Rab4 overexpression significantly decreased the ubiquitinated Nedd4-2. In A, C and D, a fraction of protein from Nedd4-2-transfected cells was immunoblotted with anti-Nedd4-2 antibody to show Nedd4-2 expression as a positive control.

FIGURE 7. Effects of Rab4 on the function and expression of I\(_{Kr}\) in neonatal rat cardiomyocytes. A and B, Overexpression of Rab4 decreases whereas knockdown of Rab4 increases the expression of ERG in neonatal rat cardiomyocytes. Cultured neonatal rat cardiomyocytes were transfected with pcDNA3 or Rab4-GFP; control siRNA or Rab4A siRNA. Forty eight hours after transfection, whole-cell lysates were extracted and analyzed using Western blot analysis. Whole-cell proteins from hERG-HEK cells were also loaded in the Western blots to show hERG expression. C, Summarized relative intensities (Intensity-Rel) of the mature ERG band compared to their respective controls (n=4-6, * P<0.05). D, Overexpression of Rab4 decreases I\(_{Kr}\). Cultured neonatal rat cardiomyocytes were transfected with GFP or GFP-tagged Rab4 (Rab4-GFP). Cells expressing GFP were selected for recording Cs\(^+\)-mediated I\(_{Kr}\) (I\(_{Kr-Ca}\)). The summarized amplitudes of the tail currents of I\(_{Kr-Ca}\) were plotted (n=21 in control and 22 in Rab4-transfected cells). * P<0.05 vs. control. E, Confocal image showing that overexpression of Rab4 decreases the native ERG expression and increases the Nedd4-2 expression. Cultured neonatal rat cardiomyocytes were transfected with GFP-tagged Rab4 (green). ERG was labeled with anti-hERG (C-20) primary antibody and Alexa Fluor 594-conjugated (red) secondary antibody. Nedd4-2 was labeled with anti-Nedd4-2 primary antibody and Alexa Fluor 594-conjugated (red) secondary antibody. Detection of ERG or Nedd4-2 was performed independently.

FIGURE 8. A proposed scheme illustrating that Rab4 increases Nedd4-2 expression by facilitating Nedd4-2 recycling. Prior to targeting its substrates, Nedd4-2 remains inactive with its WW domain binding to the PY motif within the catalytic HECT domain of the same molecule. Nedd4-2 binding to the PY motif of hERG channels with its WW domain in the plasma membrane leads to Nedd4-2 activation which mediates hERG ubiquitination and endocytic degradation. Meanwhile, activation of Nedd4-2 leads to the expose of its own PY motif within HECT domain to other Nedd4-2 molecules, resulting in the ubiquitination of Nedd4-2. The ubiquitinated Nedd4-2 can be either degraded along the degradation pathways or recycled back to the plasma membrane. Rab4 facilitates the recycling and consequently reduces degradation of Nedd4-2, resulting in an increased Nedd4-2 level. The increased Nedd4-2 level causes a decreased expression of mature hERG channels at the plasma membrane.
Rab4 regulates hERG via Nedd4-2

Figure 1
Figure 2

Rab4 regulates hERG via Nedd4-2

A

Ctrl siRNA Rab4 siRNA
hERG 155 kDa
Rab4 135
Actin 25

Intensity-Rel (155 kDa)

Ctrl siRNA Rab4 siRNA

B

WB: hERG Ctrl Rab4
155
135

IgG

IP: Ub GAPDH

IB: hERG

C

HEK Ctrl Rab4
155
135

IgG

IP: hERG

IB: Nedd4-2
### Figure 3

**A**

|        | WT      | Δ1073   | Y1078A  |
|--------|---------|---------|---------|
| hERG   | Ctrl Nedd4-2 | Ctrl Nedd4-2 | Ctrl Nedd4-2 |
|        | ![hERG band](image) | ![hERG band](image) | ![hERG band](image) |
|        | 155 kDa | 135     |         |
| Nedd4-2| Ctrl Nedd4-2 | Ctrl Nedd4-2 | Ctrl Nedd4-2 |
|        | ![Nedd4-2 band](image) | ![Nedd4-2 band](image) | ![Nedd4-2 band](image) |
|        | 110     | 42      |         |
| Actin  | Ctrl Nedd4-2 | Ctrl Nedd4-2 | Ctrl Nedd4-2 |
|        | ![Actin band](image) | ![Actin band](image) | ![Actin band](image) |
|        | 42      | 42      |         |

**B**

|        | WT      | Δ1073   | Y1078A  |
|--------|---------|---------|---------|
| hERG   | Ctrl Rab4 | Ctrl Rab4 | Ctrl Rab4 |
|        | ![hERG band](image) | ![hERG band](image) | ![hERG band](image) |
|        | 155     | 135     |         |
| Rab4-GFP | Ctrl Rab4 | Ctrl Rab4 | Ctrl Rab4 |
|        | ![Rab4 band](image) | ![Rab4 band](image) | ![Rab4 band](image) |
|        | 50      |         |         |
| Actin  | Ctrl Rab4 | Ctrl Rab4 | Ctrl Rab4 |
|        | ![Actin band](image) | ![Actin band](image) | ![Actin band](image) |
|        | 42      |         |         |

**Intensity-Rel (upper band)**

- **WT**: Ctrl Nedd4-2, Δ1073, Y1078A
- **Δ1073**: Ctrl Nedd4-2, Δ1073, Y1078A
- **Y1078A**: Ctrl Nedd4-2, Δ1073, Y1078A

**Notes:**
- **WT** control condition.
- **Δ1073** condition with deletion of amino acids 1073.
- **Y1078A** condition with mutation at amino acid 1078.
Rab4 regulates hERG via Nedd4-2

Figure 4

A

B

C

10 µm
Figure 5

A

B

CHX (h) 0 3 6 9 12

Nedd4-2 120 110

Actin 42

Rab4-2 120 110

Actin 42

Intensity-Rel (110 kDa)

0.0 1.0 2.0

0.0 0.5 1.0

CHX Chase Time (h)

ALLN

Ctrl Rab4 Ctrl Rab4

Ctrl Rab4 Ctrl Rab4

* N.S.
Figure 6

A

B

Nedd4-2

110 kDa

50

IP: GAPDH Ub

IB: Nedd4-2

Nedd4-2

110

50

Ctrl

Rab4

Ctrl

Rab4

Nedd4-2

Rab4-GFP

Actin

Intensity-Rel (110 kDa)

* * *

C

D

Nedd4-2

110

50

IP: GAPDH Rab4

IB: Nedd4-2

Nedd4-2

110

50

IP: GAPDH Ub

IB: Nedd4-2
Rab4 regulates hERG via Nedd4-2

Figure 8
Regulation of the Human Ether-a-go-go-Related Gene (hERG) Channel by Rab4 Through Neural Precursor Cell-expressed Developmentally Downregulated Protein 4-2 (Nedd4-2)
Zhi Cui and Shetuan Zhang

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