Involvement of Golgin-160 in Cell Surface Transport of Renal ROMK Channel: Co-expression of Golgin-160 Increases ROMK Currents

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Key Words

ROMK • Inward rectifier • Golgin-160 • Yeast two-hybrid • Membrane trafficking • Cell surface transport

Abstract

The weak inward rectifier potassium channel ROMK is important for water and salt reabsorption in the kidney. Here we identified Golgin-160 as a novel interacting partner of the ROMK channel. By using yeast two-hybrid assays and co-immunoprecipitations from transfected cells, we demonstrate that Golgin-160 associates with the ROMK C-terminus. Immunofluorescence microscopy confirmed that both proteins are co-localized in the Golgi region. The interaction was further confirmed by the enhancement of ROMK currents by the co-expressed Golgin-160 in *Xenopus* oocytes. The increase in ROMK current amplitude was due to an increase in cell surface density of ROMK protein. Golgin-160 also stimulated current amplitudes of the related Kir2.1, and of voltage-gated Kv1.5 and Kv4.3 channels, but not the current amplitude of co-expressed HERG channel. These results demonstrate that the Golgi-associated Golgin-160 recognizes the cytoplasmic C-terminus of ROMK, thereby facilitating the transport of ROMK to

the cell surface. However, the stimulatory effect on the activity of more distantly-related potassium channels suggests a more general role of Golgin-160 in the trafficking of plasma membrane proteins.

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Introduction

ROMK (Kir1.1) is an inwardly rectifying K⁺ channel mainly present in the kidney [1, 2]. It shows weak inward-rectification properties and a high open probability and hence is able to conduct significant outward currents necessary for K⁺ secretion at physiological membrane voltages. Several isoforms of the channel with distinct N-termini were cloned [2, 3] and localized to different apical membranes along the nephron [4, 5]. In the thick ascending limb (TAL) ROMK plays an important role in K⁺ recycling across the luminal membrane. This is essential for NaCl reabsorption through the apical Na-K-2Cl cotransporter and the urine diluting mechanism. In the cortical collecting duct (CCD) ROMK provides the major route for K⁺ secretion and thus plays a key role

in body K^+ homeostasis. Accordingly, mutations in ROMK are genetically linked to the antenatal form of Bartter's syndrome [6, 7], causing severe renal salt and water loss, which is also reproduced in knockout mice lacking ROMK [8, 9].

Consistent with a critical role in water and electrolyte homeostasis ROMK channels are regulated by multiple signaling pathways, including several serine-threonine and tyrosine kinases. Phosphorylation by these kinases either directly modulates ROMK channel activity or the number of active channels in the plasma membrane. Dietary K^+ intake modulates ROMK channel activity and hence renal K^+ secretion as such that low K^+ intake decreases, whereas high K^+ intake increases the number of K^+ channels in the CCD [10]. The inhibitory effect of low K^+ is mediated by protein tyrosine kinase-dependent phosphorylation of the channel protein, which stimulates endocytosis of ROMK [10], whereas inhibition of PTK-induced phosphorylation facilitates insertion of ROMK into the cell membrane. Low serum K^+ levels increase expression of Src family protein kinase [11] and co-expression of c-Src reduced ROMK currents in *Xenopus* oocytes [12] suggesting that Src kinases are involved in ROMK internalization. ROMK channels are also a target of protein kinase C and this phosphorylation is essential for expression of ROMK in the plasma membrane in oocytes and HEK293 cells [13]. Enhanced surface expression is also dependent on cAMP-induced direct PKA phosphorylation [14], and underlies regulation of K^+ efflux in CCD cells by the antidiuretic hormone vasopressin. In addition, PKA phosphorylation also directly activates [15], while PKC phosphorylation inactivates the channel [16] by interfering with PIP_2 activation. The aldosterone-induced serine-threonine kinase SGK1 directly phosphorylates ROMK and increases surface expression [17]. This stimulation is enhanced by the PDZ-protein NHERF-2 [18] that directly binds to the very C-terminal end of the channel protein.

Another regulator of the ROMK channel is the serine-threonine kinase WNK4, which is genetically mutated in Pseudohypoaldosteronism type II [19], a disorder characterized by hypertension, increased renal salt reabsorption and impaired K^+ excretion. WNK4 has been shown to negatively regulate surface expression of ROMK by enhancing clathrin-dependent endocytosis [20]. This inhibition does not require kinase activity, but most likely physical interactions with the cytoplasmic C-terminus of ROMK [20]. This region contains the NPXY-like sequence motif necessary for clathrin-dependent endocytosis of ROMK [21], the mutation of which

abolished WNK4-mediated ROMK inhibition in *Xenopus* oocytes [20].

While regulation of ROMK channel activity is largely achieved by controlling channel density at the cell surface, information about the molecular mechanisms controlling the trafficking of ROMK channels is still poor. To further investigate the trafficking mechanisms regulating ROMK function, we used the C-terminal tail of ROMK as bait in a yeast two-hybrid screen to identify interacting proteins. We identify the Golgi-localized protein Golgin-160 as a novel ROMK-associated protein. We show that ROMK and Golgin-160 can be co-immunoprecipitated from mammalian cell extracts and characterize the functional significance of this interaction in *Xenopus* oocytes. Co-injection of Golgin-160 increased ROMK current amplitudes and concomitantly increased the ROMK protein at the cell surface. Moreover, Golgin-160 also stimulated currents of co-expressed Kir2.1, Kv1.5, and Kv4.3, but not of HERG channels. These findings show that Golgin-160 recognizes ROMK in the early secretory pathway and suggest a role for Golgin-160 in the transport of membrane proteins to the cell surface.

Materials and Methods

Molecular Biology

For yeast two-hybrid screening, a bait construct encoding rat ROMK C-terminus (amino acids 167-391, numbering corresponds to ROMK1) was inserted in-frame into the GAL4 DNA binding domain (GAL4 DNA-BD) plasmid pGBKT7 (Clontech, Mountain View, CA, USA). A mutated version of the GCN4 leucine zipper, pLI [22, 23], was fused between ROMK C-terminus and GAL4 DNA-BD. The exact amino acid sequence of this coiled-coil domain and surrounding linkers reads: GGGSGSRMKQIEDKLEELSKLYHIENELARIKKLLGERGGSGSAA. The bait protein is believed to adopt a tetrameric conformation due to the oligomerization of the leucine zipper moiety [23]. For control experiments EGFP was cloned into the same construct instead of the ROMK C-terminus. For expression in mammalian cells hROMK2 and N-terminal FLAG-tagged Golgin-160 were subcloned into pcDNA3.1 (Invitrogen, Karlsruhe, Germany). hROMK C-terminus (167-391) was cloned in-frame into pCMV-HA (Clontech). A hemagglutinin (HA) epitope tag was introduced into the extracellular loop at position 115 of rROMK1, creating a sequence, which reads ^{111} EFYPYDVPDYAPD (ROMK-HA). A protein C (PC) epitope tag was introduced into the extracellular loop at position 119 of hKir2.1, creating a sequence, which reads ^{113} LDTSMSEDQVDPRLIDGKVS (Kir2.1-PC). GenBank™ accession numbers of cDNA clones used in this study are: Golgin-160 (NM_005895), hROMK2 (NM_153764), rROMK1 (NM_017023), GM130 (NM_004486), HERG (NM_000238), hKir2.1 (NM_000891), hKv1.5 (NM_002234), and hKv4.3 (NM_004980). All constructs were

verified by automated sequencing (ABI 3100, Applied Biosystems, Foster City, CA, USA).

Cell Culture

HEK293 and COS-7 cells were maintained at 37°C and 5% CO₂ under standard cell culture conditions in DMEM medium including 4,5 g/l glucose, *GlutaMax*^{TM}, and pyruvate (Invitrogen / Gibco) supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany). Cells were detached by exposure to trypsin-EDTA solution (Biochrom) and transfected by using *Lipofectamine*^{TM}2000 (Invitrogen) and *Opti-MEM* medium (Invitrogen / Gibco) according to the manufacturer's instructions.

Yeast Two-Hybrid Assay

Yeast two-hybrid assay was performed using *Matchmaker GAL4 Two-Hybrid System 3* (Clontech). A construct encoding the C-terminus of rROMK fused to a leucine zipper domain was used as bait to screen a human kidney cDNA library constructed in pGAD10 vector containing GAL4 activation domain (GAL4-AD) (Clontech, catalog number HL4005AB, lot number 3Z037). Bait and library plasmids were successively transformed into *Saccharomyces cerevisiae* strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 :: GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3 :: MEL1_{UAS}-MEL1_{TATA}-lacZ, Clontech). To select for interacting pairs of GAL4 DNA-BD and GAL4-AD fusion proteins, co-transformants were plated onto synthetic dropout medium lacking tryptophan, leucine, and histidine (-Trp/-Leu/-His) and incubated for 4-7 days at 30°C. Colonies were picked and streaked out two consecutive times on dropout medium lacking tryptophan, leucine, histidine, and adenine (-Trp/-Leu/-His/-Ade). Positive colonies were screened for β-galactosidase activity (lacZ reporter) on the same medium including X-gal (-Trp/-Leu/-His/-Ade + X-gal). Library plasmids encoding for potential interacting protein fragments were isolated from yeast cells, amplified in bacteria, and simultaneously re-transformed with bait construct into yeast for binary control experiments. The following negative controls were included: (i) single transformations of bait, prey and empty vectors; (ii) co-transformation of empty GAL4-AD plasmid pGAD10 with the bait construct; (iii) co-transformation of empty DNA-BD plasmid pGBKT7 with the prey construct; (iv) co-transformation of prey construct with EGFP fused to coiled-coil domain in pGBKT7 to eliminate false positives interacting with the leucine zipper. Isolated plasmids encoding for interacting protein fragments were characterized by automated DNA sequencing. Nucleotide sequences were compared to databases by use of the BLAST programs. Protocols were taken from *Matchmaker* user manual (Clontech).

Immunoprecipitation and Western Blot Analysis

HEK293 cells were transfected with constructs encoding either both FLAG-tagged Golgin-160 and HA-tagged ROMK C-terminus or one of them. Cells were washed twice, suspended in ice-cold TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) including protease inhibitor cocktail (Roche Applied Sciences,

Mannheim, Germany), and sonicated. Lysates were cleared by centrifugation at 4°C. Protein concentrations of supernatants were determined using Bradford protein assay and equal amounts of protein were used for immunoprecipitation with *EZview*^{TM} Red Anti-HA Affinity Gel and *EZview*^{TM} Red Anti-FLAG M2 Affinity Gel (Sigma, Seelze, Germany). Precipitated proteins were eluted with sample buffer, separated on a 4-12% gradient SDS-PAGE gel (NuPAGE, Invitrogen) and subjected to immunoblot analysis. Membranes were incubated in *Odyssey* blocking buffer (LiCor Biosciences, Bad Homburg, Germany) with additional 0.05% Tween20 at room temperature (RT) for 1 h, incubated with primary antibodies at 4°C overnight, and then with fluorescence-labeled secondary antibodies for 1 h at RT. The samples immunoprecipitated with anti-FLAG antibody were analyzed with primary rabbit anti-ROMK (1:5000, Alomone Labs, Jerusalem, Israel) and secondary Alexa Fluor® 680 goat anti-rabbit antibody (1:2500, Invitrogen / Molecular Probes). Reactions immunoprecipitated with anti-HA antibody were probed with primary mouse anti-FLAG (1:5000, Sigma) and secondary Alexa Fluor® 680 goat anti-mouse antibody (1:2500, Invitrogen / Molecular Probes). Membranes were washed with TBST (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween20, pH 7.4). Blots were analyzed fluorometrically using *Odyssey* imaging system (LiCor Biosciences). Blots of oocytes expressing Kir2.1-PC were incubated with primary anti-PC mouse monoclonal HPC4 (Roche), and secondary HRP-conjugated anti-mouse IgG antibody (1:3000, Jackson Immuno Research Laboratories, West Baltimore Pike, PA, USA). Detection was performed using the ECL system (Amersham Biosciences, Piscataway, NJ, USA).

Immunofluorescence

COS-7 cells were seeded on cover slips and transiently transfected with constructs encoding FLAG-tagged Golgin-160 and ROMK. 36 h after transfection, cells were incubated at 4°C for 5 min in microtubule stabilizing buffer (MTSB, 80 mM Na-Pipes, 1 mM MgCl₂, 1 mM Na-EGTA, 4% polyethyleneglykol 6000, pH 6.9), fixed with 4% paraformaldehyde in MTSB at 4°C for 20 min, permeabilized with 1% Triton X-100 in phosphate-buffered saline pH 7.4 (PBS), blocked with 5% bovine serum albumin (BSA, Sigma) and 3% horse serum (Sigma) in PBS at RT for 1 h, and washed three times with PBS. Cells were incubated for 3 h at RT with primary mouse anti-FLAG (1:1000, Sigma) and rabbit anti-ROMK (1:100, Alomone Labs) antibodies, washed, and incubated with secondary Alexa Fluor® 546 goat anti-rabbit and Alexa Fluor® 488 goat anti-mouse (each 1:1000, Invitrogen / Molecular Probes). Primary antibodies were diluted in PBS buffer including 3% horse serum, and secondary antibodies with PBS including 3% BSA. After final washing, cover slips were mounted on microscope slides using permafluor solution (Immunotech, Marseille, France). Control experiments included immunostaining of cells transfected with empty vectors and co-transfected cells with secondary antibodies only. Immunofluorescence was analyzed by confocal laser scanning microscopy using *Leica TCS SP2 AOBS* equipped with two helium-neon lasers at wavelengths of 543 nm and 633 nm (Leica Microsystems, Mannheim, Germany).

Oocyte Isolation and Injection

Complementary RNA was transcribed *in vitro* from linearized pSGEM plasmids containing the cDNA of interest using T7 RNA polymerase (*mMessage Machine*, Ambion, Austin, TX, USA). cRNA was quantified spectrophotometrically and quality checked by denaturing agarose gel electrophoresis. *Xenopus laevis* oocytes were isolated and injected as described before [24]. The following amounts of cRNA were injected (in ng cRNA/oocyte): ROMK: 0.05, Kir2.1: 0.10, HERG: 9.24, Kv1.5: 0.16, Kv4.3: 0.10, Golgin-160: 1.15 / 5.0, GM130: 5.0, ROMK-HA: 0.50, Kir2.1-PC: 0.5. Oocytes were incubated with gentle shaking at 18°C in ND96 buffer.

Electrophysiology

Two-electrode voltage clamp experiments were carried out as described before [24]. Briefly, recordings were carried out at RT using *Turbo Tec 10CD* amplifier, *ITC-16* interface (NPI, Tamm, Germany) and *Pulse* software (Heka Elektronik, Lambrecht, Germany). During measurements oocytes were permanently perfused with ND96. The microelectrodes were filled with 3 M KCl solution and had a resistance of 0.3–1.5 MΩ. The following voltage clamp protocols were used: ROMK: 500 ms pulses from -120 mV to +40 mV in 20 mV increments, starting from a holding potential of -80 mV and with an inter pulse interval of 5 s. Kir2.1: 500 ms pulses from -160 mV to 0 mV in 20 mV increments, starting from a holding potential of -100 mV and with an inter pulse interval of 5 s. HERG: 1 s prepulses from -120 mV to +80 mV in 20 mV increments, starting from a holding potential of -80 mV, followed by a test pulse to -80 mV and with an inter pulse interval of 10 s. Kv1.5: 500 ms pulses from -100 mV to +100 mV in 20 mV increments, starting from a holding potential of -80 mV and with an inter pulse interval of 5 s. Kv4.3: 1.5 s pulses from -80 mV to +60 mV in 20 mV increments, starting from a holding potential of -80 mV and with an inter pulse interval of 10 s. Current-voltage curves of ROMK, Kir2.1, and Kv1.5 were recorded once for each oocyte. Mean current amplitudes for each potential were determined at an interval between 100 and 400 ms of the test pulses. HERG and Kv4.3 current-voltage curves were recorded three times for each oocyte and averaged. The mean HERG tail currents at the moment of voltage change from different prepulse potentials to -80 mV and Kv4.3 mean peak current amplitude were determined. Current amplitudes were normalized to currents measured at 0 mV (ROMK), -160 mV (Kir2.1), +40 mV (Kv1.5, Kv4.3), and to HERG tail currents at +40 mV. Data were recorded 48–72 h post injection at 1000 Hz (HERG: 2000 Hz) and filtered at 500 Hz.

Surface Expression Assay

Surface expression of externally HA-tagged rROMK1 and protein C-tagged hKir2.1 channels was measured in single oocytes following procedures as described [25] with slight modifications. Briefly, each oocyte was injected with 0.5 ng cRNA encoding tagged channels, or 1.15 ng Golgin-160, or both and incubated for 48 h in ND96. The following steps were performed at 4°C in ND96 containing 1% BSA. Oocytes were blocked for 30 min, labeled for 1 h with primary anti-HA monoclonal antibody from rat (0.5 µg/ml, Roche) or monoclonal

mouse HPC4 antibody (1 µg/ml, Roche), washed four times (total time 1 h), incubated for 1 h with secondary goat anti-rat HRP-coupled whole antibody (1:500, Amersham Biosciences) or HRP-conjugated goat anti-mouse IgG antibody (Jackson Immuno Research Laboratories) and washed six times (total time 1 h). The last two washing steps were performed without BSA. Individual oocytes were placed in white 96-well plate, containing 50 µl ND96 per well. Luminescence of single oocytes was measured automatically for 1 s after addition of 200 µl *SuperSignal ELISA Stable Peroxidase Solution* (Pierce, Rockford, IL, USA) and incubation for 1 min in a *LumiStar* luminometer (BMG Labtech, Offenburg, Germany). Luminescence was reported as relative light units per s (RLU/s).

Data Analysis

Data acquisition and statistical analysis was performed using *Pulse* (Heka) and *SigmaPlot 2001 for Windows Version 7.0* (Systat Software Inc., Point Richmond, CA, USA). Paired t-test was used to verify significance. Values of at least $P < 0.05$ were considered significant. The concentration required for 50% block of current (IC_{50}) was determined from Hill plots using 5 concentrations and at least 3 oocytes per concentration. Data are given as means $\pm$ SEM (standard error of mean).

Results

The C-terminus of ROMK was used as bait in a yeast two-hybrid screen of a human kidney cDNA library. The ROMK tail was expressed as a fusion protein with a leucine zipper, a coiled-coil domain that drives tetramerization, and thereby mimicks the homotetrameric assembly of the native channel [22, 23]. We isolated a 708 base pair cDNA clone, coding for a 236 amino acid fragment of Golgin-160 (corresponding amino acids 614–849) [26]. It reacted positive in all selection systems, namely growth on synthetic dropout medium lacking tryptophan, leucine, histidine, and adenine (-Trp/-Leu/-His/-Ade) and generation of blue colonies in β -Gal assay. Since the isolated region belongs to a coiled-coil domain of the Golgi protein [27], we tested for a possible interaction via the coiled-coil domains. However, no interaction was found when the Golgin-160 fragment was co-expressed with an EGFP leucine zipper fusion protein, demonstrating that the observed interaction was specific for ROMK and Golgin-160 (Table 1).

We also studied the ability of 10 C-terminal ROMK mutations underlying Bartter syndrome to bind to Golgin-160 in binary tests in yeast. With exception of mutation R311W, which alters pH-dependent gating [28] the other mutations disrupt normal cell surface trafficking of the ROMK channel in both *Xenopus* oocytes and mammalian

Table 1. Yeast two-hybrid assay.

Plasmids	Selective media		
	-Trp/-Leu	-Trp/-Leu/-His/-Ade	-Trp/-Leu/-His/-Ade + X-Gal
pGBKT7 (empty) + pGAD10 (empty)	+	-	-
Golgin-160(614-849) / pGAD10	-	-	-
Golgin-160(614-849) / pGAD10 + pGBKT7 (empty)	+	-	-
ROMK-Cterm leucine zipper / pGBKT7	-	-	-
ROMK-Cterm leucine zipper / pGBKT7 + pGAD10 (empty)	+	-	-
ROMK-Cterm leucine zipper / pGBKT7 + Golgin-160(614-849) / pGAD10	+	+	blue
EGFP leucine zipper / pGBKT7	-	-	-
EGFP leucine zipper / pGBKT7 + pGAD10 (empty)	+	-	-
EGFP leucine zipper / pGBKT7 + Golgin-160(614-849) / pGAD10	+	-	-

pGBKT7: GAL4 DNA binding domain plasmid containing *TRP1* gene; pGAD10: GAL4 activation domain plasmid containing *LEU2* gene; - Trp/-Leu/His/-Ade: synthetic dropout media lacking at least two of the specific nutrients tryptophan, leucine, histidine, and adenine; + indicates growth on selective media; blue indicates activation of β -galactosidase reporter gene detected on medium containing X-Gal.

HEK cells [29]. However none of the single point mutations A198T, L220F, A306T, R311W, Y314C, L320P, R324L, F325C, as well as the two truncation mutations 362X and 368X lacking the last 29 and 23 C-terminal amino acids, respectively, disrupted ROMK/Golgin-160 interaction (not shown).

The interaction between Golgin-160 and ROMK C-terminus was further confirmed by co-immunoprecipitations in HEK293 cells. Therefore a FLAG-tagged full-length Golgin-160 was co-transfected with a HA-tagged ROMK C-terminal tail. Both proteins were precipitated from protein extracts with antibodies specific for the individual epitopes. The immunoprecipitates were then analyzed in a Western blot and the associated proteins were detected. In both cases, single protein bands of the expected molecular weight were detected. Co-migrating protein bands were not detected in cells expressing the C-terminus of ROMK or Golgin-160 alone, which served as negative controls, again demonstrating the specificity of the identified interaction (Fig. 1A, B). Together these results demonstrate that Golgin-160 protein interacts with the ROMK C-terminus in mammalian cells.

FLAG-tagged Golgin-160 and ROMK wild-type overlap at the subcellular level when visualized by fluorescence confocal microscopy of COS-7 cells transfected with both cDNAs. While bulk ROMK signal mainly occurs within the endoplasmic reticulum (ER) and the Golgi in transfected mammalian cells (Fig. 1C) [17, 30], Golgin-160 is localized to the Golgi complex (Fig. 1D) [27], which is the site where both proteins co-localize,

as evidenced by the yellow signal in the merged images (Fig. 1E). Incubation of untransfected cells with primary and secondary antibody or incubation of transfected cells with secondary antibody only did not show any signal (not shown).

We investigated possible effects of interacting Golgin-160 on co-expressed ROMK channels using two-electrode voltage clamp in *Xenopus* oocytes. Golgin-160 alone (Fig. 2A) did not generate any currents different from control oocytes injected with water, but when co-injected with ROMK, Golgin-160 increased ROMK currents over the tested voltage range from -120 mV to +40 mV. Golgin-160 increased the ROMK current amplitudes analyzed at a potential of 0 mV by about 2-fold from a mean of 1.55 μ A ($\pm$ 0.27 μ A, n = 12) to 3.08 μ A ($\pm$ 0.33 μ A, n = 16, P < 0.01). No differences in kinetics were observed between macroscopic ROMK currents recorded in oocytes expressing ROMK alone or together with Golgin-160. The stimulation of ROMK currents by co-expressed Golgin-160 was reproducibly observed using different batches of cRNA and oocytes isolated from different frogs. We performed several control experiments to further corroborate the obtained results: (i) the concentration of barium required to reduce current amplitudes by 50% (IC_{50}) was 430 μ M ($\pm$ 25 μ M) in ROMK-expressing oocytes, and 359 μ M ($\pm$ 28 μ M) for co-expressed currents, demonstrating that Golgin-160 increased ROMK currents and not endogenous currents, and that Golgin-160 did not change blocking properties of barium. (ii) we controlled the effect of co-expressed Golgin-160 on ROMK currents over incubation times.

Fig. 1. Interaction between ROMK and Golgin-160 in mammalian cells. *A, B*, co-immunoprecipitation of ROMK and Golgin-160 from lysates of HEK293 cells expressing FLAG-tagged Golgin-160 and HA-tagged C-terminus of ROMK (lanes 1 and 3) but not from lysates of cells expressing either HA-tagged C-terminus of ROMK (lane 2) or FLAG-tagged Golgin-160 only (lane 4). Immunoprecipitation (IP) and immunoblots (WB) were performed with antibodies recognizing the respective epitopes or ROMK protein. All lanes are from the same blot. *C-E*, Confocal images of COS-7 cells co-expressing FLAG-tagged Golgin-160 and ROMK. *C*, intracellular distribution of ROMK. *D*, Golgin-160 localization at the Golgi. *E*, overlap of both signals (in yellow) is consistent with co-localization in the Golgi. White bar represents 10 μ m.

We found that the stimulatory effect of co-expressed Golgin-160 occurred already between 12 h and 24 h post injection and that the maximal stimulatory effect was reached after 48h (not shown). (iii) the effect of Golgin-160 on ROMK current was analyzed in dependence of the amount of injected cRNAs. Stimulation of ROMK currents was observed with low (1.15 ng cRNA / oocyte) and high (5.0 ng cRNA/oocyte) amounts of co-injected Golgin-160 cRNA. (iv) finally, to exclude that increase of ROMK currents was due to an overall stimulation of the oocyte protein translation by co-injected Golgin-160 cRNA, ROMK was co-expressed with a nonfunctional MaxiK α subunit. This trafficking-defective mutant channel protein possesses a similar molecular weight (120 kDa) and does not generate currents (own unpublished results), and thus served as a suitable control for the intracellular Golgi protein. However, the co-expressed mutant MaxiK protein did not alter ROMK currents (not shown), demonstrating that the increase in current amplitude by Golgin-160 was due to a specific interaction with the ROMK C-terminus and not by interference with the protein translation machinery.

To test whether the increase in current amplitude was due to an increased channel conductance or resulted from increased cell surface expression of ROMK channel protein, a cDNA construct encoding a ROMK protein containing a hemagglutinin epitope (HA-tag) in the extracellular loop was expressed alone or together with

Golgin-160 in oocytes. Since current levels of HA-tagged ROMK (ROMK-HA) were lower compared to wild-type ROMK, a higher amount of ROMK-HA cRNA (0.5 ng cRNA / oocyte) was injected. The HA-tagged ROMK generated currents identical to wild-type ROMK with respect to inward rectification and barium sensitivity (not shown) that also were stimulated by co-expressed Golgin-160 (Fig. 2B). Cell surface density of ROMK protein was extrapolated from the chemoluminescence signal (RLU/oocyte/s) resulting from binding of an HA epitope-directed antibody to oocytes expressing either ROMK-HA, Golgin-160, or both proteins [25]. The normalized cell surface expression was increased by co-expressed Golgin-160 from $1.0 (\pm 0.11, n = 24)$ to $3.5 (\pm 0.39, n = 20)$ (Fig. 2B). The 3.5-fold increase of cell surface density of HA-tagged ROMK protein is consistent with the observed increase in current amplitude (Fig. 2B) obtained in voltage clamp measurements. Taking together these data strongly suggested that Golgin-160 increased cell surface density of ROMK protein resulting in higher current amplitudes.

As next we tested the effects of Golgin-160 on the related Kir2.1 channel. Again, co-expressed Golgin-160 stimulated Kir2.1 currents over the whole tested voltage range (Fig. 3A). At -160 mV Kir2.1 current amplitude was stimulated 2.2 fold (± 0.24). Similar to ROMK, also the surface-expressed Kir2.1 protein was increased by a factor of $1.8 (\pm 0.08)$. In contrast, the amount of total

Fig. 2. Co-expressed Golgin-160 increased current amplitude and cell surface expression of ROMK. *A*, representative macroscopic current-voltage relationships of ROMK and Golgin-160 expressed singly or together. Inset shows pulse protocol. *B*, current amplitude at a potential of 0 mV (white bars), and surface expression (black bars) of extracellular HA-

tagged ROMK (ROMK-HA) expressed alone or together with Golgin-160. Data were normalized to the mean of ROMK-HA expressed alone. Asterisks indicate significant difference from ROMK-HA. Data were determined using two-electrode voltage clamp and surface expression assay (** $P < 0.01$, *** $P < 0.001$; numbers (n) of oocytes are indicated above the columns).

Fig. 3. Co-expressed Golgin-160 increased current amplitude and cell surface expression but not total protein of Kir2.1. *A*, representative macroscopic current-voltage relationships of Kir2.1 expressed alone or together with Golgin-160. Inset shows pulse protocol. *B*, current amplitude at a potential of -160 mV (white bars), and surface expression (black bars) of extracellular

protein C (PC) epitope-tagged Kir2.1 (Kir2.1-PC) expressed alone or together with Golgin-160. Data were normalized to the mean of Kir2.1-PC expressed alone. Asterisks indicate significant difference from Kir2.1-PC. Inset shows Western blot of total PC-tagged protein in oocyte homogenates (*** $P < 0.001$; numbers (n) of oocytes are indicated above the columns).

Fig. 4. Differential effects of co-expressed golgin proteins on HERG and ROMK currents. *A*, representative macroscopic current-voltage relationships of HERG expressed alone, or together with Golgin-160 or GM130. Tail currents were measured at the moment of voltage change from prepulse potential to -80 mV (see arrow in inset). *B*, tail currents from *A* analyzed after prepulse to +40 mV. *C*, representative macroscopic current-voltage relationships of ROMK expressed alone, or together with Golgin-160 or GM130. *D*, currents from *C* analyzed at a potential of 0 mV. Asterisks show significant differences between singly expressed and co-expressed channels (** $P < 0.01$; *** $P < 0.001$; *n.s.* = not significant; numbers (*n*) of oocytes are indicated above the columns).

Kir2.1 protein was not significantly different in oocytes expressing either Kir2.1 or Kir2.1 together with Golgin-160 (Fig. 3B, inset).

GM130 was the first Golgi protein reported to be involved in the vesicular transport of the HERG potassium channel. In contrast to the results with ROMK, co-expression of GM130 in *Xenopus* oocytes suppressed HERG current amplitudes [31]. To test whether the stimulatory effect of Golgin-160 on ROMK currents is also seen with HERG and whether GM130 also exerts an inhibitory effect on ROMK, we co-expressed both channels with the individual Golgi protein (Fig. 4). In accordance with the results reported by Roti Roti and coworkers [31], GM130 suppressed HERG current amplitude by about 35% from a mean of 2.28 μA ($\pm 0.23 \mu\text{A}$, $n = 16$) to 1.49 μA ($\pm 0.11 \mu\text{A}$, $n = 22$, $P < 0.01$) at a potential of +40 mV (Fig. 4A, B). However, we did not observe any statistically significant changes in HERG currents after co-expression with Golgin-160. In contrast to its effect on HERG, co-expressed GM130 increased ROMK current amplitude by about 1.6-fold. In the representative experiment shown in Fig. 4C, D the mean ROMK current determined at 0 mV increased from

1.84 μA ($\pm 0.30 \mu\text{A}$, $n = 12$) to 2.94 μA ($\pm 0.27 \mu\text{A}$, $n = 12$, $P < 0.01$), while in the same batch of oocytes, Golgin-160 increased co-expressed ROMK currents by about 1.9-fold to a mean current of 3.43 μA ($\pm 0.41 \mu\text{A}$, $n = 16$, $P < 0.01$) (Fig. 4C, D). As was the case with Golgin-160, GM130 alone did not induce a current (Fig. 4C, D). These data suggest that Golgin-160 and GM130 may play different roles in the cell surface transport of ROMK and HERG channels.

The finding that Golgin-160 can exert distinct effects on ion channel trafficking prompted us to determine the range of action of Golgin-160 in more detail. For this, we co-expressed it with the voltage-gated potassium channels Kv1.5, and Kv4.3. As illustrated in Fig. 5 co-expression of Golgin-160 also stimulated these K^+ channels and increased currents of Kv1.5 by 1.9-fold (± 0.18), and of Kv4.3 by 1.7-fold (± 0.31). Again, we never observed changes in kinetics of the various currents (see insets in Fig. 5). To investigate whether Golgin-160 can also affect surface transport of other membrane proteins, we co-expressed it with the muscarinic M2 receptor and analyzed its cell surface density. However number of surface-expressed M2 receptors was not different in

Fig. 5. Golgin-160 increased current amplitudes of voltage-activated Kv1.5 and Kv4.3 channels. Fold increase of mean current amplitudes of ROMK, HERG (tail currents), Kir2.1, Kv1.5, and Kv4.3 after co-expression with Golgin-160. Error bars indicate SEM in absence and presence of Golgin-160. Asterisks show significant differences of unpaired observations (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; $n=10$). Representative macroscopic currents of channels co-expressed with Golgin-160 are shown above. Arrows and lines indicate time point and period used for data analysis.

presence or absence of Golgin-160 (not shown). The combined data show for the first time a role of Golgin-160 in the Golgi to cell surface transport of several ion channel proteins.

Discussion

The cytoplasmic C-terminus of ROMK comprises more than half of the total protein. This region is identical in all channel isoforms and involved in regulation of the channel by many intracellular factors and second messengers [32]. It contains 2 histidine residues involved in pH-dependent gating, 2 sites for phosphorylation by PKA, 1 site for phosphorylation by PKC, as well as a tyrosine kinase site. Phosphorylation of these sites either directly affects channel activity or its expression on the cell surface [32]. Similar to other inward rectifiers binding of PIP_2 to a positively charged residue in the ROMK C-terminus is required for its activation [33]. The extreme

ROMK C-terminus constitutes a canonical type I PDZ binding motif and its interaction with Na/H exchange regulatory factors, NHERF-1 and NHERF-2, is required for efficient channel expression on the cell surface [34]. The C-terminus also contains signals for endocytic internalisation [21] and ER export [35]. Many Bartter mutations cluster in the C-terminal tail and result in alterations of these regulatory pathways, and several of them generate trafficking defective channel proteins [29].

From there, we tried to identify novel proteins interacting with the ROMK C-terminus that are involved in channel regulation and plasma membrane trafficking. For this we used a modified yeast two-hybrid system, in which the ROMK C-terminus was fused to an artificial oligomerization domain that forms parallel tetramers [22]. The resulting tetrameric bait protein thus imitates the homotetrameric architecture of the native ROMK channel protein. One of the proteins identified in the two-hybrid screen of a human kidney cDNA library and confirmed by co-immunoprecipitation in mammalian cells was Golgin-160, a member of the golgin family of Golgi-localized proteins, which includes integral membrane proteins, but also peripheral membrane proteins, such as Golgin-160. Golgins play a role in the structural organization of the Golgi apparatus and in tethering of vesicles to the Golgi membrane [36].

Golgin-160 is a ubiquitously expressed protein of 1498 amino acids, and like other golgins is characterized by the presence of an extensive C-terminal coiled-coil structure. This rod-like structure is predicted to extend from residues 394 to 1459, and is only interrupted by a short non-coil stretch between residues 597 and 645. The interacting Golgin-160 clone encoded a protein fragment of 236 amino acids, corresponding to residues 614 to 849 in the full-length protein, and thus confines the ROMK interacting region to the short intervening non-coil segment and the first part of the adjacent coiled-coil domain. The short non coiled-coil N-terminal head domain of Golgin-160 contains a Golgi targeting signal, but surprisingly also a cryptic nuclear localization signal. The latter signal is unmasked [37] during programmed cell death through cleavage of the head domain by caspase enzymes, which is required for efficient apoptotic disassembly of the Golgi, a process which is promoted by phosphorylation of the head domain by the mixed-lineage-kinase-3 [38]. The N-terminal head domain has been shown to interact with another golgin, GCP16, which is anchored to the Golgi membrane by acylation with palmitic acid [39]. When overexpressed the acylated form of GCP16 inhibited the transport of the viral VSV-G protein to the cell surface

[39], suggesting a role of the Golgin-160/GCP16 complex in vesicle transport from the Golgi to the cell surface.

This prompted us to investigate the effect of co-expressed Golgin-160 on ROMK cell surface transport. Since ROMK largely accumulates within the ER in mammalian expression systems [17], but is effectively targeted to the cell surface in *Xenopus* oocytes, we used the oocyte system to investigate a possible functional interaction of both proteins. A further reason was that this system allowed us to control their expression levels by varying the amount of injected RNA and incubation time. Co-expression of both proteins reproducibly increased ROMK current amplitude. The magnitude of current stimulation was 2-fold and in some experiments even 3-fold. Parallel measurements revealed a similar fold increase in cell surface protein, strongly indicating that enhancement of currents was due to an increase in the number of channels at the plasma membrane and not due to an increase in single channel conductance or open probability. The stimulated currents showed the same sensitivity against barium ruling out that Golgin-160 up-regulated an endogenous current.

The stimulatory effect of Golgin-160 on ROMK current expression is in contrast to several other studies, where overexpression of different Golgi proteins, including also other members of the golgin family, like GCP60 and GM130, generally resulted in an inhibitory effect on cell surface transport [40, 31]. The golgin GM130 (Golgin-95) is a component of a vesicle-tethering complex at the cis-side of the Golgi [41], and also was identified in a yeast two-hybrid screen of a human heart library to interact with the C-terminal tail of the HERG potassium channel. Co-expression of GM130 with HERG consistently reduced HERG currents in *Xenopus* oocytes [31], implicating it in HERG regulation.

The Golgin-160 binding region on the ROMK protein does not include the extreme C-terminal tail that participates in regulation of ROMK cell surface expression via endocytic internalisation and NHERF protein interaction, since corresponding C-terminally truncated ROMK mutants as well as other single point mutations found in Bartter patients were still able to bind to Golgin-160. Our studies do not rule out that some disease-causing ROMK mutations might interfere with Golgin-160 binding. However, for the tested ones this seems rather unlikely.

Because of the considerable sequence similarity between the ROMK and Kir2.1 C-termini, the observed stimulatory effect of Golgin-160 on Kir2.1 currents could be expected. Although we did not prove that Kir2.1

directly binds to Golgin-160 this seems likely to us for the same reason. However, the finding that overexpression of Golgin-160 also stimulated the distantly related Kv1.5 and Kv4.3 potassium channels was rather surprising, since there is no obvious sequence similarity present in the cytoplasmic tails of these inward rectifiers and voltage-gated channels. This rather might indicate a different mechanism of action. It is also intriguing that Golgin-160 and GM130 both stimulate ROMK, but exert different effects on HERG. While we could quantitatively reproduce the inhibitory effect of co-expressed GM130 on HERG currents as reported by Roti Roti and coworkers [31], co-expressed GM130 on the contrary increased ROMK current amplitudes, whereas Golgin-160 had no effect on co-expressed HERG. However the Golgin-160 and GM130 complexes in addition to proteins involved in vesicle tethering and fusion may also contain other proteins able to modify these interactions in response to signaling pathways. During preparation of this manuscript, Hicks and Machamer [42] reported the PDZ protein PIST (PDZ domain containing protein interacting specifically with TC10), as a novel interactor of Golgin-160. The PDZ domain of PIST, also known as GOPC (Golgi-associated PDZ and coiled-coil motif-containing protein) and CAL (CFTR-associated ligand), has been reported to interact with several membrane proteins including the intracellular CLC-3 [43] and plasma membrane CFTR [44] chloride channels. The site required for PIST binding is located in the N-terminal head domain of Golgin-160 and includes the caspase-3 cleavage site D139, and therefore might be located immediately adjacent to or even overlapping with the binding site of GCP16 (residues 137-169) [39]. Overexpression of PIST was demonstrated to reduce the total cellular amount of both co-expressed chloride channel proteins [43], possibly by directing trafficking to the degradative lysosomal pathway [44]. PIST originally was identified by its binding to TC10, a small Rho GTPase, which is involved in membrane fusion events. When TC10 is co-expressed it can oppose the negative effect of PIST and even increase CFTR at the cell surface [45]. This example shows that different ways of regulation might result by recruitment of other proteins into these protein complexes, and that overexpression of particular components might result in different effects on membrane expression.

Interestingly, the CFTR chloride channel is a possible regulator of the native ROMK secretory channel, and both co-localize in kidney epithelia. It has been shown that co-expression of CFTR in oocytes increases the sensitivity of ROMK currents to Mg-ATP [46] rendering

the recombinant ROMK channel as sensitive as the native secretory K⁺ channel in the principal cells [47]. Moreover, CFTR [48], like ROMK, interacts with NHERF-1/2 PDZ proteins, an interaction that stimulates ROMK cell surface expression and also facilitates ROMK/CFTR interaction [30]. CFTR is also believed to couple the increased sodium reabsorption through the epithelial ENaC sodium channel that is produced by vasopressin and mineralocorticoid hormones in the CCD to the stimulation of K⁺ secretion by the increase of ROMK currents and surface expression [49], suggesting that these ion channels may be functionally coupled in a multiprotein complex. The novel finding of a direct interaction between Golgin-160 and the C-terminus of ROMK, together with those of others showing also direct interactions between Golgin-160 and PIST [42], and between PIST and CFTR [43, 44], supports this view. Such a complex thus may already exist in the early secretory pathway, with Golgin-160

playing a critical role in its assembly and cell surface trafficking.

Here we show for the first time, that Golgin-160 is directly involved in cargo interactions at the Golgi compartment and regulates the cell surface expression of ROMK and of other potassium channels. Taken together, our results and those of others suggest that Golgin-160 plays an important role in the assembly and membrane trafficking of multiprotein complexes.

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