Glucocorticoid-induced Leucine Zipper 1 Stimulates the Epithelial Sodium Channel by Regulating Serum- and Glucocorticoid-induced Kinase 1 Stability and Subcellular Localization*

Received for publication, July 3, 2010, and in revised form, September 20, 2010 Published, JBC Papers in Press, October 14, 2010, DOI 10.1074/jbc.M110.161133

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Serum- and glucocorticoid-induced kinase 1 (SGK1) is a multifunctional protein kinase that markedly influences various cellular processes such as proliferation, apoptosis, glucose metabolism, and sodium (Na⁺) transport via the epithelial Na⁺ channel, ENaC. SGK1 is a short-lived protein, which is predominantly targeted to the endoplasmic reticulum (ER) to undergo rapid proteasome-mediated degradation through the ER-associated degradation (ERAD) system. We show here that the aldosterone-induced chaperone, GILZ1 (glucocorticoid-induced leucine zipper protein-1) selectively decreases SGK1 localization to ER as well as its interaction with ER-associated E3 ubiquitin ligases, HRD1 and CHIP. GILZ1 inhibits SGK1 ubiquitinylination and subsequent proteasome-mediated degradation, thereby prolonging its half-life and increasing its steady-state expression. Furthermore, comparison of the effect of GILZ1 with that of proteasome inhibition (by MG-132) supports the idea that these effects of GILZ1 are secondary to physical interaction of GILZ1 with SGK1 and enhanced recruitment of SGK1 to targets within an “ENaC regulatory complex,” thus making less SGK1 available to the ERAD machinery. Finally, effects of GILZ1 knockdown and overexpression strongly support the idea that these effects of GILZ1 are functionally important for ENaC regulation. These data provide new insight into how the manifold activities of SGK1 are selectively deployed and strengthened through modulation of its molecular interactions, subcellular localization, and stability.

Serum- and glucocorticoid-induced kinase 1 (SGK1), a Ser/Thr protein kinase activated by the PI3K pathway, is expressed in multiple cell types and participates in the regulation of a number of diverse cellular processes including ion transport, stress responses, proliferation, differentiation, and apoptosis (reviewed in Ref. 1). Dysregulation of SGK1 expression and/or function has been implicated in numerous human pathologies including diabetic nephropathy, hypertension, cancer, and cardiac fibrosis (1). An extensive body of literature implicates SGK1 in the modulation of sodium (Na⁺) transport via the epithelial Na⁺ channel, ENaC (reviewed in Refs. 1–4). Given the involvement of SGK1 in the modulation of such a wide variety of physiological functions, understanding the mechanistic basis for SGK1 specificity is extremely important and could lead to new therapeutic strategies for controlling its function in disease states such as salt-sensitive hypertension and the metabolic syndrome.

Unlike most other Ser/Thr kinases, SGK1 is under dual control; both its expression level and specific activity are tightly regulated (reviewed in Refs. 5 and 6). Thus, in ion-transporting epithelia, insulin and other activators of the PI3K pathway stimulate SGK1 activity, whereas its mRNA levels are tightly regulated by transcriptional activation in response to numerous inputs including serum and glucocorticoids (hence its name), aldosterone, and cellular stress (reviewed in Ref. 1). Many tissues and cell types express abundant SGK1 mRNA; however, the protein is barely detectable because its half-life is short (<30 min) (7). SGK1 protein levels appear to be controlled through a rapid and highly regulated proteolytic process. Studies have shown that SGK1 is polyubiquitinylated in vivo and rapidly degraded by the 26 S proteasome (7, 8). Biological activity of SGK1 is tightly controlled through its constitutive degradation by the ubiquitin-proteasome pathway. A six-amino acid hydrophobic motif, GMVAIL, within the N terminus of SGK1 has been shown to be a key determinant of its stability and is responsible for the rapid turnover of this protein (9). This sequence targets SGK1 to the endoplasmic reticulum (ER) where it undergoes ubiquitin modification followed by degradation by the ER-associated degradation system (ERAD) (7, 9). Deletion of this motif disrupts its localization to the ER and prevents its rapid proteasomal degradation. These studies suggest that transport to the ER is a key early step governing SGK1 protein stability. Interestingly, the N terminus of SGK1 (which includes the GMVAIL sequence)
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has been shown to be important for its ENaC stimulatory activities via Nedd4-2 (neural precursor cell expressed, developmentally downregulated protein) inhibition (10, 11): Although the overall expression and kinase activity of SGK1 is greater when the N terminus is deleted, its ability to stimulate ENaC is markedly reduced (10).

Ubiquitylation of proteins proceeds by the sequential action of three types of enzymes (12): E1 (or the “ubiquitin-activating enzyme”) first activates ubiquitin and “charges” a “ubiquitin-conjugating enzyme” or E2 with this ubiquitin moiety. The third set of enzymes in this cascade is referred to as the E3 “ubiquitin ligases,” which provide specificity to this system. These enzymes, of which there are >500 (13), act as adaptors that bind both the target protein and the charged E2 and facilitate the final covalent transfer of ubiquitin from E2 onto the protein substrate. Independent studies have implicated two distinct ER-associated E3 ligases in the ubiquitin modification and proteasome-dependent degradation of SGK1: HRD1 (HMG-CoA reductase degradation protein-1) (7) and CHIP (C terminus of Hsc (heat-shock cognate protein) 70-interacting protein) (14). HRD1 (or synoviolin) is a transmembrane RING finger type E3 ubiquitin ligase of the ER and participates in the ERAD component of the ER protein quality control process (15). CHIP is a chaperone-associated U-box E3 ligase and has also been implicated in protein quality control in association with the ER (16).

In the distal nephron of the kidney, aldosterone is the most notable stimulator of SGK1 expression. Its effects on ENaC are mediated, at least in part, through SGK1-dependent phosphorylation and inhibition of the ENaC inhibitory E3 ligase Nedd4-2. Although Nedd4-2 may also ubiquitylate SGK1, this does not influence ERAD-mediated degradation (17). Aldosterone also coordinately stimulates the expression of a number of other regulatory proteins, including GILZ1 (glucocorticoid-induced leucine zipper protein 1) (18–20). This small chaperone has been shown to stimulate ENaC by physically interacting with and inhibiting Raf-1 and Nedd4-2, which are physically associated with the channel in an ENaC regulatory complex (ERC) (21). In addition to direct inhibition of Raf-1 and Nedd4-2, GILZ1 also physically interacts with SGK1, alters its subcellular localization and selectively recruits it into the ERC (21). Thus, GILZ1 and SGK1 synergize to markedly stimulate ENaC surface expression, by antagonizing the effects of the inhibitory components (21). In the present study, we examine the effects of GILZ1 on SGK1 protein stability, subcellular localization and interactions with CHIP and HRD1. We also establish the relevance of these molecular events to endogenous ENaC activity and endogenous SGK1 expression in a cortical collecting duct cell line. Our results strongly suggest that by interacting with SGK1 and selectively targeting it to the ERC, GILZ1 relocalizes SGK1 away from the ER, and reduces its interactions with the ER-associated E3 ubiquitin ligases HRD1 and CHIP. SGK1 ubiquitylation and subsequent proteasomal degradation are thereby inhibited, and its half-life is prolonged. Our data point to a key role for GILZ1 in controlling SGK1 protein stability and availability in specific subcellular compartments, thus selectively enhancing its regulation of a particular end point, in this case, Na⁺ transport.

EXPERIMENTAL PROCEDURES

Co-immunoprecipitation/Immunoblotting Assays in HEK293T Cells—Human embryonic kidney (HEK 293T) cells were regularly maintained in plastic tissue culture flasks at 37 °C in DMEM supplemented with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin. For co-immunoprecipitation experiments, cells were seeded on 10-cm dishes (3 × 10⁶ cells/dish) and allowed to grow overnight in antibiotic-free medium. They were then transfected with 1.5 μg of untagged/HA-tagged mGILZ1, 2 μg of mSGK1/S422D-HA/FLAG and 500 ng of FLAG-mRaf-1 per dish as specified, using Lipofectamine according to the manufacturer’s instructions (Invitrogen). Following 48 h of transfection, cell lysates were harvested for protein analysis. For certain experiments (see Figs. 1, 3, 4), cells were treated with cycloheximide (100 μg/ml, Sigma) or MG-132 (Sigma) for specified time periods prior to harvest. Immunoprecipitations were performed using protein A-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-GILZ antibody (described in Ref. 22) or anti-CHIP antibody (Cell Signaling Technology), immobilized anti-FLAG (Sigma) or anti-HA beads (Roche Applied Science), and immunoblotting using our anti-GILZ antibody (22), anti-FLAG-HRP antibody (Sigma), anti-HA-HRP antibody (Roche), anti-CHIP antibody, anti-HRD1 antibody (Novus Biologicals, Inc., Littleton, CO), or anti-ubiquitin antibody (Covance, Emeryville, CA). Total GAPDH was used as a loading control. Anti-GAPDH antibody was purchased from Chemicon, Inc. (Temecula, CA). Each experiment was repeated at least three independent times with similar results.

For densitometric analyses of immunoblots, signal intensities of specified bands were determined using NIH ImageJ software and normalized to that of the internal control as described previously (21). Values so obtained were used to determine mean ± S.E. for a graphical representation.

Confocal Immunofluorescence Microscopy—HEK 293 cells were grown on poly-L-lysine-coated coverslips in six-well plates in DMEM supplemented with 10% FBS to optimal cell density, followed by transfection with the following plasmids in combinations as specified (see Fig. 2A): untagged mGILZ1 (250 ng), mSGK1-FLAG (500 ng), pDsRed2-ER (300 ng; gift from Dr. Mark VonZastrow, University of California, San Francisco) and pMO (vector control) per well. Transfections were performed using Lipofectamine according to the manufacturer’s instructions (Invitrogen). Following 24 h of transfection, cells were fixed with 3.7% formaldehyde and 3% sucrose + 0.1% Triton X-100. Cells were then washed in 1 × Tris-buffered saline solution containing 1 mM CaCl₂. The primary antibody used was mouse monoclonal anti-FLAG M2 (1:600 dilution, Sigma). The secondary antibody used was FITC-conjugated donkey anti-mouse IgG (1:300 dilution, Jackson ImmunoResearch Laboratories), followed by nuclear co-staining with DAPI (Vectashield, Vector Laboratories). Cells were then examined with a Zeiss LSM 510 META confocal microscope (Confocal Microscopy Core Facility, University of California, San Fran-
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FIGURE 1. GILZ1 protects SGK1 from rapid degradation. A, immunoblot assays showing total cellular expression of SGK1 protein in transfected HEK 293T kidney epithelial cells treated with cycloheximide (CHX; 100 μg/ml) for varying time periods (as specified) in the presence and absence of GILZ1. Also depicted are blots showing total cellular expression of GILZ1 and GAPDH. GAPDH was used as a loading control. IP, immunoprecipitation; IB, immunoblot; WCL, whole cell lysate. B, graphical representation of the above data showing relative rate of degradation of SGK1 protein in the presence and absence of GILZ1 (n = 4).

cisco). These experiments were repeated five independent times with similar results.

Biochemical Fractionation of Endoplasmic Reticulum (ER)-enriched Microsomes—HEK 293T cells grown on 15-cm dishes were transfected with appropriate expression vectors as specified in Fig. 2B (4 μg of mSGK1/S422D-HA with/without 2 μg of untagged-mGILZ1 per dish), using Lipofectamine 2000. Viral supernatants were harvested 48 h after transfection. Viral titers were determined as described previously (23). A viral titer of 5 × 10^7/ml was routinely observed by examining cells for turbo-GFP fluorescence. Turbo-GFP is an improved variant of the green fluorescent protein copGFP cloned from the copepoda Pontellina plumata. mkpCCD_c14 cells were infected in 10 cm dishes at a multiplicity of infection of 2. Infected cells were plated on Transwell filters and allowed to grow to high resistance. ENaC-dependent Na^+ currents were measured upon aldosterone treatment (10^{-7} M) as described above. Aldosterone-induced SGK1 expression was determined by immunoblotting using a commercial antibody purchased from Sigma. An anti-calnexin antibody was typically used as an ER marker, using a commercially available kit (Sigma) according to the manufacturer’s instructions (Invitrogen). Following 48 h of transfection, the cell layers were exposed to a water-soluble membrane-permeable cross-linking agent, DTBP (dimethyl 3,3’-dithiobispropionimidate-2 HCl, 2 mM) (Thermo Fisher Scientific) for 30 min at room temperature. Calnexin-enriched ER-containing microsomes were then isolated from these cells using a commercially available kit (Sigma) according to the manufacturer’s instructions. The isolation process was monitored by measuring the activity of NAPDH cytochrome c reductase, an ER membrane enzyme that is commonly used as an ER marker, using a commercially available kit (Sigma) according to the manufacturer’s instructions (data not shown). Anti-calnexin antibody for confirming ER-enrichment by immunoblotting was purchased from Cell Signaling Technology. The ER marker calnexin was typically enriched ~3.72-fold in isolated microsomes compared with whole cell lysate. Each experiment was repeated at least three independent times with similar results.

mkpCCD_c14 Cell Culture, Transfection, and Electrophysiological Measurements—mkpCCD_c14 cells were regularly maintained and grown on Transwell filters as described previously (20, 22). Cells were transfected with appropriate expression vectors (Myc/FLAG-mGILZ1 and mSGK1/S422D-HA) or the pMO vector control using a high efficiency electroporation protocol (nucleofection, Amaxa Biosystems, Inc.) as described previously (20). For certain experiments (see Figs. 5 and 6), cell monolayers were treated with aldosterone (10^{-7} or 10^{-6} M) for 2 h prior to electrophysiological measurements. All experiments were performed in parallel with appropriate vehicle controls (aldosterone was dissolved in ethanol). Transepithelial resistance and potential difference across the cell monolayers were measured using a minivolt-ohmmeter (MilliCell ERS, Millipore), and equivalent short-circuit current (I_{sc}) calculated as described previously (20). All experiments were repeated at least three independent times with similar results.

Generation of Recombinant Lentiviruses Harboring GILZ shRNA and Knockdown of Endogenous GILZ in mpkCCD_c14 Cells—MISSION turbo-GFP-expressing plasmids harboring shRNA for GILZ and a scrambled nontarget sequence were obtained from Sigma. These plasmids were co-transfected into HEK 293T packaging cells according to the manufacturer’s instructions using Lipofectamine 2000. Viral supernatants were harvested 48 h after transfection. Viral titer was determined as described above (23). A viral titer of 5 × 10^7/ml was routinely observed by examining cells for turbo-GFP fluorescence. Turbo-GFP is an improved variant of the green fluorescent protein copGFP cloned from the copepoda Pontellina plumata. mkpCCD_c14 cells were infected in 10 cm dishes at a multiplicity of infection of 2. Infected cells were plated on Transwell filters and allowed to grow to high resistance. ENaC-dependent Na^+ currents were measured upon aldosterone treatment (10^{-7} M) as described above. Aldosterone-induced SGK1 expression was determined by immunoblotting using a commercial antibody purchased from Sigma. α-Tubulin was used as a loading control.

For densitometric analyses of immunoblots, signal intensities of specified bands were determined using NIH ImageJ software and normalized to that of the internal control as described previously (21). For quantitation of GILZ blots, signal intensities corresponding to both the detectable endogenous isoforms of GILZ, GILZ1, and GILZ2 (22) were added to pro-
vide one value. Values so obtained were used to determine mean ± S.E. for a graphical representation relative to control. These experiments were repeated at least four independent times with similar results.

Statistical Analyses—Data are represented as mean ± S.E. In all experiments involving statistical analyses, three to six samples were tested, and at least three independent experiments were performed with the same treatment protocol. Unless otherwise specified, all statistical comparisons were evaluated using a Student’s unpaired two-tailed t test, and significance was defined as a p value < 0.05.

RESULTS

GILZ1 Protects SGK1 from Rapid Degradation—SGK1 is a short-lived protein and is degraded rapidly in cells (7, 8). Given that GILZ1 dramatically augments SGK1 function with respect to stimulation of ENaC surface expression (21), we wanted to determine whether GILZ1 influences SGK1 protein stability per se. We transfected HEK 293T cells with epitope-tagged SGK1, treated them with the protein synthesis inhibitor cycloheximide, and determined the stability of existing SGK1 protein in the presence and absence of GILZ1 (Fig. 1, A and B). As previously reported by others (7), we also observed a rapid loss in SGK1 steady-state protein levels in the presence of cycloheximide. Interestingly, however, in the presence of GILZ1, this rapid loss was dramatically prevented (Fig. 1, A and B), suggesting that GILZ1 protects SGK1 from rapid degradation.

GILZ1 Decreases SGK1 Accumulation in ER—SGK1 targeting to the ER has been causally implicated in its rapid degradation by the ER-associated degradation system (or
ERAD) in both mammalian cells and yeast (7, 9, 14). Our observations thus far suggested that GILZ1 increases the half-life of SGK1 protein (Fig. 1), and we found previously that GILZ1 increases SGK1 colocalization with Raf-1 and Nedd4-2 (21), neither of which is heavily localized to the ER (24–26). We were therefore interested in determining whether GILZ1 alters the subcellular localization of SGK1. We performed immunofluorescence analyses to co-localize SGK1 protein with the pDsRed2-ER marker in the presence and absence of GILZ1. pDsRed2-ER contains the ER targeting sequence of calreticulin as well as the ER retention sequence KDEL (27, 28) and provides strong fluorescent labeling of ER. Fig. 2A shows representative confocal images of HEK 293T cells transfected with expression vectors as shown. As can be seen in the merged images, in the absence of GILZ1, SGK1 is strongly colocalized with the ER marker (yellow signal), and indeed little SGK1 is visualized in non-ER locations (green signal). In contrast, in GILZ1-expressing cells, there is a marked increase in SGK1 outside the ER. As an independent confirmation, we also performed Western blot analyses on calnexin-enriched ER microsomal fractions isolated from these cells. As shown in Fig. 2B (representative immunoblots) and Fig. 2C (densitometric quantitation), SGK1 expression in ER fractions is markedly reduced in the presence of GILZ1. These data confirm and extend previous observations that suggest that SGK1 predominantly localizes to the ER under baseline conditions (7, 9, 14) and strongly support the idea that GILZ1 causes a marked redistribution of SGK1 from the ER to a non-ER compartment (Fig. 2, A–C). Importantly, we did not observe any reduction in the total cellular SGK1 protein in the presence of GILZ1. In fact total SGK1 expression was even increased in the presence of GILZ1 (Fig. 2B, WCL-IB HA/SGK1; Fig. 3A, lanes 3 and 4, and SGK1 expression; and Fig. 4, lanes 1 and 2, SGK1 expression). Together, these observations are consistent with the idea that GILZ1 triggers the relocalization of SGK1 from the ER to a compartment rich in Nedd4-2 and Raf-1 (which are themselves colocalized (21)) and consequently decreases its availability to the ERAD system.
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![Image](image_url)

**FIGURE 4. GILZ1 recruits SGK1 into the ENaC regulatory complex while markedly reducing the ubiquitinylation and subsequent proteasome-mediated degradation of SGK1.** HEK 293T kidney epithelial cells were transfected as shown and treated with increasing concentrations of the proteasome inhibitor MG-132. A, co-immunoprecipitation assays demonstrating the ability of GILZ1 to induce the interaction of SGK1 with one of its key targets, Raf-1 (an integral component of the ENaC regulatory complex), in the presence of increasing concentrations of MG-132 and simultaneously decreasing the ubiquitinylation status of SGK1. Blots showing total cellular expression of SGK1 and GAPDH are also depicted. GAPDH was used as a loading control. *IP: HA, immunoblot; WCL, whole cell lysate. Also shown are graphical representations of the above data demonstrating the relative ability of GILZ1 and MG-132 in inducing SGK1-Raf-1 interaction (B) and decreasing SGK1 ubiquitinylation (C). Data are shown normalized to total SGK1 expression (n = 4). *, p < 0.05; **, p < 0.01 compared with respective controls.

**GILZ1 Physically Interacts with SGK1, Markedly Reduces SGK1 Interaction with ER-associated E3 Ubiquitin Ligases CHIP and HRD1, and also Prevents Subsequent Polyubiquitinylation and Proteasome-mediated Degradation of SGK1**—Once targeted to the ER, SGK1 is polyubiquitinylated and rapidly degraded by the ER-associated ubiquitin-proteasomal machinery (7, 14). Two major ER-associated E3 ubiquitin ligases have been implicated in this process: HRD1 (7) and CHIP (14). Independent studies have shown that both CHIP and HRD1 physically interact with SGK1, result in its polyubiquitinylation, and accelerate its subsequent proteasomal degradation. With the above observations in mind, we next asked whether GILZ1 influences the interaction of SGK1 with these ER-associated E3 ubiquitin ligases, which are known to play a key role in determining its steady-state expression levels. We performed a series of co-immunoprecipitation experiments using lysates prepared from HEK 293T cells transfected with expression vectors for SGK1 and its regulators and treated with the proteasome-inhibitor MG-132 (10 μM) for 16 h (Fig. 3).

As reported previously (21), GILZ1 interacted strongly with SGK1 (Fig. 3A). We also observed specific interactions of SGK1 with endogenous HRD1 as well as CHIP, which were markedly reduced by GILZ1 (Fig. 3, A–C). This was accompanied by an equally dramatic reduction in the ubiquitinylation status of SGK1 protein (Fig. 3, A and D), strongly suggesting that GILZ1 blocks ubiquitinylation of SGK1 per se and that its effect to increase SGK1 half-life is upstream of proteasome-mediated degradation. Interestingly, we were not able to detect any interaction of GILZ1 with either HRD1 or CHIP (data not shown).

**GILZ1 Recruitment of SGK1 into ERC May Account for Markedly Reduced Ubiquitinylation and Subsequent Proteasome-mediated Degradation of SGK1**—We next sought to directly determine whether GILZ1 recruitment of SGK1 into the ERC was the cause or consequence of reduced SGK1 degradation in the presence of GILZ1. To address these questions, we compared the effects of GILZ1 and MG-132 on the interaction of SGK1 with one of its target proteins, Raf-1. MG-132 markedly increases SGK1 levels by blocking its rapid proteasome-dependent degradation (7) and would be predicted to increase SGK1-Raf-1 interaction by mass action. We performed a series of co-immunoprecipitation experiments using lysates prepared from HEK 293T cells transfected with expression vectors as shown in Fig. 4, in the presence of increasing doses of MG-132. Our data reveal that lower concen-
trations of MG-132 (while being sufficient to significantly enhance cellular SGK1 protein levels) do not significantly enhance the interaction of SGK1 with its target protein, Raf-1 (Fig. 4, A and B). Only at high concentrations of MG-132 (5 μM), which cause marked elevation in SGK1 expression, did we observe any appreciable stimulatory effect on the SGK1-Raf-1 interaction. In contrast, GILZ1 significantly enhanced the SGK1-Raf-1 interaction, even in the absence of MG-132 (Fig. 4, A and B), when SGK1 expression was only modestly increased. Furthermore, in contrast to MG-132, which potently disrupts the proteolytic activity of the 26S proteasome and consequently increases the accumulation of ubiquitylated forms of target proteins (29), GILZ1 markedly blunted accumulation of ubiquitylated SGK1 (Fig. 4, A and C), suggesting that GILZ1 acts at or upstream of the ubiquitylation step. These data further support the conclusion that GILZ1 increases SGK1 stability and function as a consequence of physically associating with SGK1 and enhancing its recruitment into the ERC, thereby restricting access to ER and associated ubiquitin ligases CHIP and HRD1.

GILZ1 Synergizes with Aldosterone and SGK1 to Stimulate ENaC Function in Polarized Cortical Collecting Duct Cells—To determine whether the above effects of GILZ1 on SGK1 stability and subcellular localization have functional implications, we studied the effect of GILZ1 and SGK1 overexpression on ENaC-mediated Na\(^+\) transport in polarized kidney epithelial cells, using transient transfection in mpkCCD\(_{14}\) cells as described previously (20). We first determined how GILZ1 affects aldosterone action per se in these cells (Fig. 5A). As previously reported (20), we consistently observed a significant increase in ENaC activity in the presence of GILZ1, even in the absence of aldosterone. In addition, we observed a marked increase in aldosterone-induced ENaC-mediated Na\(^+\) current in the presence of GILZ1 (Fig. 5A), suggesting that GILZ1 and aldosterone synergize to stimulate ENaC activity.

We next co-transfected GILZ1 with low levels of SGK1, insufficient to stimulate Na\(^+\) current, and repeated the above experiments in the absence of aldosterone (Fig. 5B). Under these experimental conditions, SGK1 and GILZ1 had a synergistic effect, suggesting that the cooperative effect of GILZ1 and aldosterone was due to synergy of GILZ1 and SGK1.

Knockdown of Endogenous GILZ Adversely Affects Endogenous SGK1 Protein Levels and ENaC Activity—To confirm the role of endogenous GILZ in SGK1 protein stability and ENaC activity, we performed lentiviral shRNA-mediated knockdown of endogenous GILZ in mpkCCD\(_{14}\) cells. Aldosterone-induced expression of GILZ and SGK1 in this cell line has been well demonstrated previously (22). Using lentivirus-mediated transduction, we were able to achieve a ~60% knockdown in GILZ expression with GILZ-specific shRNA, whereas control- and scrambled (nontarget)-shRNA had no significant effect (Fig. 6, A and B). Concomitantly, endogenous SGK1 expression was markedly reduced by GILZ shRNA but not by control shRNA (Fig. 6, A and C), suggesting that GILZ indeed contributes to SGK1 protein stability and expression level. Reduction in endogenous GILZ and SGK1 protein levels was also accompanied by a significant decrease in aldosterone-induced ENaC-mediated Na\(^+\) transport (Fig. 6D), suggesting an important role for the GILZ-SGK1 synergy in this process.

DISCUSSION

Aldosterone-stimulated Na\(^+\) transport via ENaC in tight epithelia involves the cooperation of two important early gene products, SGK1 and GILZ1, among others. We recently reported the identification of a signaling complex containing both these proteins, which selectively modulates the cell surface expression of ENaC (21). Raf-1 and the ubiquitin ligase Nedd4-2, are constitutively expressed inhibitory components of this complex, which we have termed the ERC. The activities of Nedd4-2 and Raf-1 are cooperatively inhibited by the

![Graph A](image1.png)

**Figure 5.** GILZ1 synergizes with aldosterone (**A**) and SGK1 (**B**) to stimulate ENaC function in polarized CCD cells. mpkCCD\(_{14}\) cells were transfected with appropriate expression vectors (as indicated) using nucleofection (see “Experimental Procedures” for details), and grown to confluence on Transwell filters. In A, one set of cells was treated with aldosterone (10⁻⁶ M for 2 h), and the other with ethanol (vehicle control) prior to determination of equivalent short circuit current (I\(_{sc}\)) as a measure of ENaC activity. Shown is a graphical representation of the electrophysiological readings obtained. **, p < 0.01; ***, p < 0.001 compared with respective controls (n = 3). B, shown is the relative ability of GILZ1 and SGK1 to stimulate ENaC activity in mpkCCD\(_{14}\) cells, when expressed either alone or together. *, p < 0.05; **, p < 0.01 (n = 4).
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aldosterone-stimulated components of this complex, SGK1 and GILZ1. GILZ1 physically interacts with SGK1 and increases its colocalization and physical interaction with both Nedd4-2 and Raf-1, thereby increasing surface expression of ENaC (21). In the present manuscript, we demonstrate the functional significance of the ERC and GILZ1 recruitment of SGK1 to the ERC in protecting SGK1 from rapid ER-associated proteasome-mediated degradation.

SGK1 is an important signaling effector of the PI3K pathway and influences a variety of cellular processes. The rapid turnover of SGK1 via proteasomal degradation is believed to play an important role in limiting SGK1 activity. Cycloheximide decay experiments (Fig. 1) suggest that GILZ1 protects SGK1 from rapid degradation. Immunofluorescence and biochemical fractionation studies (Fig. 2) clearly demonstrate that GILZ1 alters the subcellular localization of SGK1 away from the ER, where it is otherwise subjected to rapid degradation through the ERAD system. We also observed that GILZ1, upon interacting with SGK1, markedly reduces the interaction of SGK1 with ER-associated E3 ubiquitin ligases HRD1 and CHIP and prevents its subsequent polyubiquitinylation and proteasome-mediated degradation (Fig. 3). In order to enable detection of polyubiquitinylated species of SGK1, we treated cells with MG-132, a proteasome inhibitor. Both MG-132 and GILZ1 increase SGK1 steady state levels (Figs. 1, 3, and 4). Interestingly however, our results suggest that augmenting SGK1 protein stability is only a secondary effect of GILZ1 and that its primary role is to “recruit” SGK1 to the ERC and help stabilize the interaction of SGK1 with components of the ERC (such as Raf-1), consequently redirecting it away from the ER.

The following key observations favor the “recruitment” hypothesis: 1) GILZ1 markedly stimulates SGK1-Raf-1 interaction even in the absence of MG-132 (Fig. 4). On the other hand, in the absence of GILZ1, large concentrations of MG-132 (5 μM) are required to achieve a nearly similar effect. 2) Typically, in the presence of MG-132, there is accumulation of end stage degradation complexes/“aggresomes,” in other
words, polyubiquitinated species of target proteins, and end stage degradation of these already polyubiquitinated species is prevented. Interestingly, in the presence of GILZ1, SGK1 polyubiquitination is itself reduced (Figs. 3 and 4), suggesting that GILZ1 acts upstream of the proteasome to stabilize SGK1. 3) GILZ1 does not affect the expression levels of either HRD1 or CHIP (Fig. 3A, HRD1 and CHIP expression). On the other hand, MG-132 uniformly increases the expression levels of SGK1, CHIP, and HRD1 (data not shown), suggesting a more general inhibition of the common proteasome machinery. (Note that CHIP has been shown to undergo autoubiquitination (30) and is therefore itself a target for proteasomal degradation; GILZ1 has no appreciable effect on CHIP expression.) 4) GILZ1 and SGK1 synergize to cooperatively stabilize ENaC activity (Fig. 6), lending further support to the functional significance of these effects. 5) Finally, knockdown of endogenous GILZ significantly reduces aldosterone-induced SGK1 protein expression and ENaC activity (Fig. 6), lending further support to the functional significance of these effects.

Taken together with earlier work (20, 21), these data point to GILZ1 as a key molecular determinant of intracellular signaling specificity governing SGK1 function and epithelial Na+ transport. In this regard, it is notable that an unrelated activity of SGK1, inhibition of FOXO-mediated gene transcription, is unaffected by GILZ1 (21). The present findings lend support to the growing view that subcellular localization is a critical factor controlling SGK1 protein stability and further suggest that GILZ1 is a key determinant of where SGK1 localizes within the cell and with which proteins it interacts. Our current data strongly suggest that GILZ1 stabilizes SGK1 by recruiting it to the ERC. However, our experiments do not firmly exclude the alternative possibility that SGK1 interaction with the ERC is increased because it is stabilized by GILZ1. Future studies will address these issues in further detail. How exactly GILZ1 controls SGK1 localization will also be the subject of future investigation. The molecular identities of players that facilitate this process and the specific subcellular compartments wherein these interactions occur also remain to be determined. Understanding the mechanistic details of this process will shed light on an important question in cell biology, which is determining how cells achieve signaling specificity.

Acknowledgments—We thank Editha Setiawan for technical assistance and Vivek Bhalla for suggestions on the gene knockdown experiments in mpkCCD cells.

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