Ubiquitin Modification of Serum and Glucocorticoid-induced Protein Kinase-1 (SGK-1)*

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The serum and glucocorticoid-induced protein kinase gene (sgk-1) encodes a multifunctional kinase that can be phosphorylated and activated through a phosphatidylinositol 3-kinase-dependent signaling pathway. In many cell types, endogenous SGK-1 steady-state protein levels are very low but can be acutely up-regulated after glucocorticoid-receptor-mediated transcriptional activation; in breast epithelial and cancer cell lines, this up-regulation is associated with promotion of cell survival. We and others have noted that ectopically introduced full-length SGK-1 is poorly expressed, although SGK-1 lacking the first 60 amino acids (∆60SGK-1) is expressed at much higher-fold protein levels than wild-type SGK-1 in both human embryonic kidney 293T and MCF10A mammary epithelial cells. In this report, we demonstrate for the first time that the low steady-state expression level of SGK-1 is due to polyubiquitination and subsequent degradation by the 26S proteasome. Deletion of the amino-terminal 60 amino acids of SGK-1 results in a mutant SGK-1 protein that is neither efficiently polyubiquitinated nor degraded by the 26S proteasome, accounting for the higher steady-state levels of the truncated protein. We also demonstrate that a subset of SGK-1 localizes to the plasma membrane and that the polyubiquitin-modified SGK-1 localizes to a membrane-associated fraction of the cell. Taken together, these data suggest that a significant fraction of SGK-1 is membrane-associated and ubiquitinated. These findings are consistent with the recently described role of SGK-1 in phosphorylating the membrane-associated protein Nedd4-2 and the integral membrane Na+/H+ exchanger isoform 3 (NHE3) and suggest a novel mechanism of regulation of SGK-1.

Glucocorticoid receptor activation in mammary epithelial cells (1) and hepatocytes (2) initiates a potent antiapoptotic signaling pathway. Activation of the glucocorticoid receptor by ligand binding directly regulates the transcription of several potential downstream mediators of this survival pathway, including SGK-1 (3, 4). SGK-1 is a member of an important subfamily of protein kinases known as the “AGC” subfamily that includes protein kinase A, protein kinase B, and protein kinase G, and protein kinase C isoforms. SGK-1 is 54% identical in its catalytic domain to protein kinase Bo, also known as AKT-1. Two other isoforms of sgk, sgk-2 and sgk-3 (or cytokine-independent survival kinase, cisk), have been identified, and all three products can be phosphorylated and activated following phosphatidylinositol 3-kinase pathway signaling to downstream phosphatidylinositol-dependent kinases including phosphatidylinositol-dependent kinase-1 and -2 (5). Furthermore, phosphatidylinositol-dependent kinase-1 and -2 activation of AKT, SGK-1, and SGK-3/CISK (6) can promote cell survival under a variety of conditions that normally favor apoptosis (for review, see Ref. 7).

In addition to SGK-1 regulation via reversible phosphorylation, sgk-1 transcription is acutely up-regulated after glucocorticoid receptor activation, serum stimulation, and cell stress (8). The role of transcriptional activation in modulating a serine-threonine kinase is unusual and to date has not been implicated in the regulation of either sgk-2 or sgk-3 expression (5).

Another increasingly recognized mechanism of regulation of signaling proteins is through posttranslational modification via covalent addition of one or more ubiquitin molecules (reviewed in Ref. 9). Covalent attachment of ubiquitin, either as a single molecule or as a polyubiquitin chain, can regulate a variety of processes including protein degradation and subcellular trafficking of the substrate protein. For example, activity of the non-receptor tyrosine kinase c-Abl has been recently shown to be down-regulated by a ubiquitin-dependent degradation pathway (10). Similarly, protein kinase C-α is ubiquitinated and down-regulated by the Von Hippel Lindau-Cul 2 E3 ubiquitin ligase (11). Polyubiquitination also directly regulates the ability of TAK1 to phosphorylate MKK6, which in turn activates the c-Jun NH2-terminal kinase-p38 kinase pathway (12). Finally, the PHD domain of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase-1 was recently found to act as an E3 ubiquitin ligase and to thereby mediate ubiquitination and degradation of extracellular signal-regulated kinase 1/2 (13).

In the present study we demonstrate that the serine-threonine kinase SGK-1 is modified by polyubiquitination and ultimately degraded by the 26S proteasome. The rapid degradation of SGK-1 suggests that in addition to transcriptional up-regulation and reversible phosphorylation, ubiquitin modification plays an important role in determining the availability of SGK-1 as a kinase. We also demonstrate that the amino terminus of SGK-1 contains a domain that regulates both ubiquitin/proteasome-mediated degradation and efficient association of SGK-1 with the plasma membrane. Furthermore, fractionation studies reveal that although SGK-1 is found in

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1 The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; GFP, green fluorescent protein; HA, hemagglutinin; FCS, fetal calf serum; ALLN, N-acetyl-Leu-Leu-norleucinal; ALLM, N-acetyl-Leu-Leu-normethional; HRP, horseradish peroxidase.
both membrane and cytosolic fractions, the ubiquitin-modified SGK-1 is predominately membrane-associated. Taken together, these findings suggest that SGK-1, a serine-threonine kinase that phosphorylates Nedd4-2 (14, 15) and the Na\(^+\)/H\(^+\) exchanger 3 (NHE3) (16), is negatively regulated by ubiquitin modification and proteasome degradation.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—HA-SGK-1, HA-\(\Delta 60\)-SGK-1, and HA-K127M-SGK-1 were generated by cloning M2-HA-FLAG-HRP antibody conjugate to confirm equal immunoprecipitation between samples. The autoradiogram was scanned on a ChemiImager 5500 densitometer, and data were analyzed using Fluorchem Software (Alpha Innotech Corp.).

**Immunofluorescence**—SK-BR-3 cells were transfected with wild-type HA-SGK-1, HA-\(\Delta 60\)-SGK-1, wild-type SGK-1-GFP or \(\Delta 60\)-SGK-1-GFP-encoding plasmids as described earlier. The following day, transfected cells were transferred to LabTekII glass chamber slides (Nunc) and allowed to adhere overnight. Forty-eight hours after transfection, some cells were treated with 10 \(\mu\)M ALLN for 2–4 h before fixation for 30 min in freshly prepared 4% paraformaldehyde in Hank’s balanced salt solution (Invitrogen). In some experiments, HA-SGK-1-expressing cells were incubated immediately after fixation with a mouse anti-Na\(^+\)/K\(^+\) ATPase \(\beta\) subunit antibody (Affinity Bioreagents), followed by an Alexa Fluor 568 (Molecular Probes) secondary antibody conjugated to a red spectrum Alexa Fluor 488 (Molecular Probes). Cells were washed extensively using the blocking/permeabilization buffer, mounted using Gel Mount (Biomeda), and examined at \(\times600\) using an Olympus Fluoview 200 laser scanning confocal microscope.

**Cellular Fractionation/Ubiquitination Assay**—SK-BR-3 cells were transiently co-transfected with HA-ubiquitin and either pLPCX vector alone, SGK-1-FLAG, or \(\Delta 60\)-SGK-1-FLAG. Forty-eight to 72 h after transfection, cells were treated with ALLN for 4 h and then collected in 1× phosphate-buffered saline with the same inhibitor mixture used for immunoprecipitation experiments. Cells were then lysed manually by 10 passes through a 26-gauge needle and centrifuged at 10,000 \(\times g\) for 10 min to collect unlysed cells and nuclei. The protein concentration of the supernatant was determined by Bradford assay, and equal amounts of protein from the starting cell lysate and the cytoplasmic and membrane (P100) fractions were then separated by SDS-PAGE; one gel was dried and exposed to film, and the other was fractionated. In these experiments, HA-SGK-1 was immunoprecipitated and eluted with high-speed centrifugation at 100,000 \(\times g\) for 1 h at 4°C in a Sorvall RC-M120GX micro-ultracentrifuge. After high-speed centrifugation, the pellet (P100) fraction includes membrane-containing organelles, whereas the supernatant (S100) is cytoplasmic. Equal amounts of protein from the starting cell lysate and the cytoplasmic (S100) and membrane (P100) fractions were then separated by SDS-PAGE, electrophoresis, transfer to nitrocellulose, and analyzed by Western blot using either anti-FLAG-HRP to detect SGK-1, anti-Na\(^+\)/K\(^+\)-ATPase \(\alpha3\) subunit as a control for integral membrane protein localization to the P100 fraction, or mouse anti-\(\alpha\)-tubulin antibody (Oncogene) as a control for accurate fractionation of cytosolic proteins. Immunoreactive protein was detected with ECL (Amersham Biosciences) chemiluminescence per the manufacturer’s instructions. Because of the overwhelming \(\Delta 60\)SGK-1 signal in comparison with the wild-type SGK-1, only one-fifth of the \(\Delta 60\)SGK-1 lysate was examined by Western analysis.

**Protein Concentrations**—Protein concentrations in P100 and S100 cell lysates were then determined, and equal milligram amounts of fractionated whole cell protein were used to immunoprecipitate SGK-1-FLAG as described above. Immunoprecipitated SGK-1 was then eluted with FLAG peptide, separated by SDS-PAGE gel, transferred to nitrocellulose, and analyzed by Western blot using either anti-HA-HRP (to detect the ubiquitinated species of SGK) or anti-FLAG-HRP (to detect the native SGK) antibody conjugates. In some experiments, HA-SGK-1-expressing cells were fractionated. In these experiments, HA-SGK-1 was immunoprecipitated and subjected to immunoblots with a mouse anti-ubiquitin antibody (Stressgen) to detect SGK1 modified by endogenous ubiquitin.

**Western Analysis**—Samples were electrophoresed in either 8% or 9% SDS-PAGE gels and transferred to nitrocellulose membrane (Osmonics). Equal protein loading was confirmed by visual inspection of the membrane by Ponceau S staining. Nitrocellulose was then rinsed with 1× Tris-buffered saline (TBS) and incubated with 5% non-fat dry milk in blocking buffer for 1 h, then pelleted, and the supernatant containing the eluted FLAG-tagged protein was mixed with 5× Laemmli buffer and boiled for 2 min before Western analysis.

**Protein Half-Life**—SK-BR-3 cells were transiently transfected with constructs expressing either SGK-1-FLAG or \(\Delta 60\)SGK-1-FLAG. After 48 h, the cells were starved of methionine and cysteine for 1 h and then labeled with 200 \(\mu\)Ci/ml Redivue Pro-mix [\(^{35}\)S]methionine/cysteine (Amersham Biosciences) for 1 h. Cells were harvested at 0, 30, 60, and 120 min after chasing with Dulbecco’s modified Eagle’s medium (10% FCS) supplemented with 2 mM methionine and 1-cysteine pre-cleared with agarose and normal mouse IgG as described earlier. Each lysate (500 \(\mu\)g) was then immunoprecipitated and eluted with excess FLAG peptide as described above. Lysates were run in duplicate on 9% SDS-PAGE gels; one gel was dried and exposed to film, and the other was transferred to nitrocellulose and analyzed by Western blot probed with either mouse anti-HA or anti-FLAG-HRP.
SGK-1 is substantially degraded by a 26S proteasome-dependent mechanism that requires its amino-terminal domain. 

SGK-1 Is Polyubiquitinated in Vivo—Having determined that SGK-1 steady-state levels are dependent on 26S proteasome-mediated degradation, we next asked whether SGK-1 is directly targeted to the proteasome as a consequence of polyubiquitin modification. To test this possibility, either carboxyl-terminal FLAG-tagged wild-type or Δ60SGK-1 along with a HA-tagged ubiquitin construct was transiently co-transfected into HEK293T cells (Fig. 2, A and B). Immunoprecipitation of SGK-FLAG from equivalent milligram amounts of total protein lysates, followed by Western analysis with an anti-FLAG-HRP antibody conjugate, revealed the characteristic significant difference in expression levels between wild-type and Δ60SGK-1 (Fig. 2A). To evaluate in vivo SGK-1 modification by HA-tagged ubiquitin, the immunoprecipitated SGK-1 was analyzed in a parallel Western analysis with an anti-HA-HRP antibody conjugate to detect ubiquitin-modified SGK-1 (Fig. 2B). The anti-HA-HRP blotting revealed abundant polyubiquitinated wild-type SGK-1 (Fig. 2B, lane 2), but significantly less ubiquitinated Δ60SGK-1 (lane 3). The specificity of the anti-FLAG immunoprecipitation and the subsequent in vivo ubiquitination detection was confirmed by the absence of immunoprecipitated HA-reactive protein species in cells transfected with either the empty FLAG (Fig. 2B, lane 1) or the HA-ubiquitin vector alone (lane 4). Taken together, these results demonstrate that SGK-1 is a direct target of polyubiquitin modification and that the efficiency of this modification is altered by deletion of the amino-terminal domain of SGK-1. Because polyubiquitination can serve as a signal for proteasome degradation, decreased ubiquitin modification of Δ60SGK-1 is consistent with the observation of a decreased susceptibility of Δ60SGK-1 to proteasomal degradation, resulting in its longer half-life.

In some cell types, steady-state expression of ectopic SGK-1 appears to be more abundant (14) than in others (21). To rule out a cell type-specific difference in the ability of SGK-1 to undergo efficient ubiquitin modification, the in vivo polyubiquitination assay was repeated in the SK-BR-3 breast cancer line (Fig. 2, C and D), with results similar to those seen in HEK293T cells (Fig. 2, A and B). In these experiments, we also

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**RESULTS**

**SGK-1 Is Degraded by a 26S Proteasome-mediated Mechanism**—Unlike its relatively abundant subfamily member, AKT-1, SGK-1 mRNA steady-state levels are very low in normal mammary epithelial cells but can be acutely up-regulated by glucocorticoid receptor activation (4, 19). However, the rapid induction of SGK-1 mRNA is followed by only a modest increase of endogenous SGK-1 protein levels (8, 19). Interestingly, even after ectopic overexpression of wild-type SGK-1, protein levels remain relatively low in MCF10A cells; however, deletion of the amino-terminal 60 amino acids of SGK-1 (Δ60SGK-1) yields at least 50-fold more protein in both HEK293 and MCF10A-Myc cells (4, 5). Based on these observations, we hypothesized that the amino-terminal domain of SGK-1 may be responsible for low steady-state levels of SGK-1 via rapid protein degradation.

Degradation of cellular proteins is carried out predominantly by the 26S proteasome- and lysosome-mediated pathways. To determine which pathway might be responsible for SGK-1 degradation, MCF10A-Myc cells ectopically expressing either wild-type SGK-1 or Δ60SGK-1 were treated with proteinase or proteasome-specific inhibitors (see Fig. 1). As observed previously (4), ectopically expressed wild-type SGK-1 was barely detectable in MCF10A-Myc cells (Fig. 1A, lane 1). However, in the presence of the proteasome inhibitor ALLN, steady-state levels of two differentially phosphorylated forms of SGK-1 accumulated dramatically (Fig. 1A, lane 2). In contrast, treatment with ALLN, a predominant calpain inhibitor with weak proteasome inhibition, did not lead to a significant accumulation of SGK-1 (Fig. 1A, lane 3). Lactacystin, an irreversible proteasome-specific inhibitor of at least three of the peptidase activities of the 26S proteasome (20) also significantly increased steady-state levels of both forms of SGK-1 (Fig. 1A, lane 4), confirming that SGK-1 is substantially degraded by a 26S proteasome-dependent pathway.

Previous reports have demonstrated that ectopically expressed Δ60SGK-1 steady-state levels can be as much as 100-fold higher than those of wild-type SGK-1 (4, 5). To determine whether the increased steady-state levels of the truncated protein are due to a decreased susceptibility to proteasome-mediated degradation, Δ60SGK-1-expressing MCF10A-Myc cells were exposed to ALLN, ALLM, or lactacystin or vehicle alone. The anti-HA antibody cross-reacting band at 50 kDa (+) also appears in untransfected MCF10A-Myc and co-migrates with the upper band of the differentially phosphorylated species of SGK-1 (Fig. 1C). Autoradiograph and half-life determination of wild-type and Δ60SGK-1 determined by [35S]methionine/cysteine pulse-chase analysis.

In contrast to this, Figure 1D demonstrates that wild-type SGK-1 is substantially degraded by a 26S proteasome-dependent mechanism. Taken together, these data demonstrate for the first time that SGK-1 is a target of 26S proteasome-dependent degradation.

Using available anti-SGK-1 antibodies, we have been unable to efficiently immunoprecipitate [35S]methionine/cysteine-labeled endogenous SGK-1 in epithelial cells, presumably because of the paucity of endogenous protein and/or the quality of available antibodies. Therefore, to determine the half-life of wild-type versus Δ60SGK-1, we transiently transfected wild-type SGK-1 or Δ60SGK-1-FLAG into SK-BR-3 cells and performed a [35S]methionine/cysteine-labeled pulse-chase experiment. As seen in Fig. 1C, wild-type SGK-1 has a half-life of ~30 min. As expected, this rapid degradation requires (either directly or indirectly) the amino-terminal domain of the protein because Δ60SGK-1 has a significantly longer half-life of ~1.5 h. Taken together, these results demonstrate that SGK-1 is degraded by a proteasome-mediated mechanism that requires its amino-terminal domain.

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**Fig. 1. Increase of SGK-1 steady-state levels in the presence of proteasome inhibitors.** Western blot analysis of steady-state wild-type HA-SGK-1 (A) and HA-Δ60SGK-1 (B) levels in MCF10A-Myc cells. Cells were treated overnight with 10 μM ALLN, ALLM, or lactacystin or vehicle alone. The anti-HA antibody cross-reacting band at 50 kDa (+) also appears in untransfected MCF10A-Myc and co-migrates with the upper band of the differentially phosphorylated species of SGK-1. C, autoradiograph and half-life determination of wild-type and Δ60SGK-1 determined by [35S]methionine/cysteine pulse-chase analysis.

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**Ubiquitination of SGK-1**

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**RESULTS**

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**SGK-1 Is Polyubiquitinated in Vivo**—Having determined that SGK-1 steady-state levels are dependent on 26S proteasome-mediated degradation, we next asked whether SGK-1 is directly targeted to the proteasome as a consequence of polyubiquitin modification. To test this possibility, either carboxyl-terminal FLAG-tagged wild-type or Δ60SGK-1 along with a HA-tagged ubiquitin construct was transiently co-transfected into HEK293T cells (Fig. 2, A and B). Immunoprecipitation of SGK-FLAG from equivalent milligram amounts of total protein lysates, followed by Western analysis with an anti-FLAG-HRP antibody conjugate, revealed the characteristic significant difference in expression levels between wild-type and Δ60SGK-1 (Fig. 2A). To evaluate in vivo SGK-1 modification by HA-tagged ubiquitin, the immunoprecipitated SGK-1 was analyzed in a parallel Western analysis with an anti-HA-HRP antibody conjugate to detect ubiquitin-modified SGK-1 (Fig. 2B). The anti-HA-HRP blotting revealed abundant polyubiquitinated wild-type SGK-1 (Fig. 2B, lane 2), but significantly less ubiquitinated Δ60SGK-1 (lane 3). The specificity of the anti-FLAG immunoprecipitation and the subsequent in vivo ubiquitination detection was confirmed by the absence of immunoprecipitated HA-reactive protein species in cells transfected with either the empty FLAG (Fig. 2B, lane 1) or the HA-ubiquitin vector alone (lane 4). Taken together, these results demonstrate that SGK-1 is a direct target of polyubiquitin modification and that the efficiency of this modification is altered by deletion of the amino-terminal domain of SGK-1. Because polyubiquitination can serve as a signal for proteasome degradation, decreased ubiquitin modification of Δ60SGK-1 is consistent with the observation of a decreased susceptibility of Δ60SGK-1 to proteasomal degradation, resulting in its longer half-life.

In some cell types, steady-state expression of ectopic SGK-1 appears to be more abundant (14) than in others (21). To rule out a cell type-specific difference in the ability of SGK-1 to undergo efficient ubiquitin modification, the in vivo polyubiquitination assay was repeated in the SK-BR-3 breast cancer line (Fig. 2, C and D), with results similar to those seen in HEK293T cells (Fig. 2, A and B). In these experiments, we also
asked whether inhibition of 26S proteasome activity by ALLN would lead to the accumulation of the ubiquitinated wild-type SGK-1 species that otherwise would have undergone proteasome-mediated degradation. Indeed, ALLN treatment of cells would lead to the accumulation of the ubiquitinated wild-type SGK-1. Taken together, these results suggest that the amino terminus of SGK-1 contributes to ubiquitination or proteasome-mediated degradation of SGK-1 (data not shown). This observation is consistent with an indirect functional or structural requirement of the amino terminus for ubiquitin modification of SGK-1.

The Amino Terminus of SGK-1 Is Required for Efficient Membrane Localization of SGK-1—The markedly different susceptibility of SGK-1 versus Δ60SGK-1 to polyubiquitin modification and proteasome-mediated degradation suggested the possibility that the amino terminus might serve to localize the wild-type protein to an appropriate intracellular location for ubiquitin modification. Previous experiments in a rat mammary cell line have suggested that endogenous SGK-1 is alternatively cytoplasmic or nuclear, depending on the absence or presence of serum (19). To investigate the subcellular localization of SGK-1, SGK-1-GFP fusion proteins were expressed in several cell types. Confocal microscopy of SGK-1-GFP in HEK293T and Cos cells (data not shown) as well as in SK-BR-3 cells (Fig. 3A) revealed cytoplasmic and plasma membrane localization, with rare cells exhibiting nuclear fluorescence in either the absence or presence of FCS. In contrast, Δ60SGK-1-GFP was detected in a homogeneous distribution throughout the cells under both serum conditions. These results suggest that the amino terminus of SGK-1 contributes to a localization pattern that appears predominantly cytoplasmic and membrane-associated; the absence of subcellular translocation with FCS may reflect cell type-specific differences and has also been reported in Madin-Darby canine kidney cells ectopically expressing GFP-SGK-1 (22).

To confirm that the SGK-1 localization in SK-BR-3 cells was not an artifact of the GFP fusion protein, cells expressing HA-SGK-1 and HA-Δ60SGK-1 were examined with an anti-HA antibody conjugated directly to the green spectrum Alexa Fluor dye 488 (Fig. 3B). Consistent with the SGK-1-GFP localization, HA-SGK-1 was detected predominantly in the cytoplasm and...
plasma membrane of SK-BR-3 cells, whereas HA-Δ60SGK-1 was found homogeneously throughout the entire cell. To better define the association of SGK-1 with the plasma membrane, the integral membrane protein Na+/K+-ATPase was detected by indirect immunofluorescence using a mouse anti-Na+/K+-ATPase and HA-ubiquitin and then treated with or without ALLN for the last 4 h of the experiment. Seventy-two h after transfection, cells were lysed, and equal amounts of total cell lysate were fractionated and evaluated for SGK-1 localization by immunoblotting with anti-FLAG-HRP-conjugated antibody. A, SGK-1 was then immunoprecipitated from equal amounts (100 μg) of the S100 and P100 fractions. Immunoprecipitated SGK-1 was then analyzed by immunoblotting with anti-FLAG-HRP. B, Western analysis of immunoprecipitated HA-SGK-1 using an anti-ubiquitin antibody to detect polyubiquitinated SGK-1. C, relative kinase activity of total membrane-associated SGK-1 compared with total (native and ubiquitin-modified) cytosolic SGK-1. Kinase activity is measured as %cpm detected over a baseline (kinase-dead HA-K127M-SGK-1) and represents the average of two experiments (± S.E.).

**Membrane-associated, but not Cytosolic, SGK-1 Is Polyubiquitinated**—Our data thus far have demonstrated that wild-type SGK-1 is more efficiently polyubiquitinated and membrane-associated than Δ60SGK-1. Therefore, we next asked whether the ubiquitin-modified SGK-1 might localize differently from the total protein. SGK-1-FLAG and HA-ubiquitin were co-expressed in SK-BR-3 cells in the presence or absence of ALLN. Biochemical fractionation revealed the previously observed pattern of wild-type and Δ60SGK-1 expression (Fig. 4A). To determine ubiquitin modification of SGK-1-FLAG in the cytosol, wild-type SGK-1 was consistently found in both the membrane and cytosolic fractions, whereas deletion of the amino terminus resulted in a much smaller proportion of SGK-1 localized to the membrane fraction. Taken together, these experiments suggest that the amino-terminal domain of SGK-1 is required for the efficient plasma membrane association of SGK-1.

**Fig. 5.** Subcellular distribution of HA-SGK-1 protein and kinase activity in SK-BR-3 cells. SK-BR-3 cells were transiently transfected with either HA-SGK-1 or HA-K127M-SGK-1. Seventy-two h later, cell lysates were fractionated and immunoprecipitated with anti-HA antibody. One-third of the immunoprecipitated SGK-1 was used for Western analysis, and two-thirds were used in an *in vitro* kinase assay with SGK-tide as the substrate (18). The background activity of the kinase-dead mutant was similar to a mock-transfected control and was subtracted from the activity of SGK-1. A, relative amounts of nonubiquitinated HA-SGK-1 as analyzed by immunoblotting of immunoprecipitated SGK-1 with anti-HA-HRP. B, Western analysis of immunoprecipitated HA-SGK-1 using an anti-ubiquitin antibody to detect endogenous ubiquitin modification of SGK-1. C, relative kinase activity of total membrane-associated SGK-1 compared with total (native and ubiquitin-modified) cytosolic SGK-1. Kinase activity is measured as %cpm detected over a baseline (kinase-dead HA-K127M-SGK-1) and represents the average of two experiments (± S.E.).
plasmic versus the membrane compartments, the S100 and P100 fractions were then individually immunoprecipitated with the anti-FLAG M2 antibody (Fig. 4B). Immunoprecipitated SGK-1-FLAG was then analyzed by Western blotting with either anti-FLAG-HRP antibody conjugate to confirm the efficiency of the immunoprecipitation or anti-HA-HRP antibody conjugate to detect HA-ubiquitin-modified SGK-1 species (Fig. 4C). Polyubiquitinated species of SGK-1 were detected almost exclusively in the membrane-associated P100 fraction. In addition, the membrane-associated fraction of Δ60SGK-1 also contained some polyubiquitinated species, suggesting that the amino-terminal domain, per se, is not absolutely required for ubiquitin modification of SGK-1. Furthermore, ALLN treatment, which prevents proteasome degradation of polyubiquitinated SGK-1, caused accumulation of ubiquitinated SGK-1 species in the membrane and not in the cytosolic fraction (Fig. 4C). Taken together, the fractionation/ubiquitination assays suggest that the amino terminus of SGK-1 is required for efficient membrane localization and polyubiquitination of SGK-1.

Cytoplasmic and Membrane-associated SGK-1 Have Similar Kinase Activities—Because ubiquitinated SGK-1 is located predominantly in the membrane fraction, we next asked whether there might be a difference in the relative kinase activities between the cytosolic and membrane-associated SGK-1 fractions. In these experiments, SK-BR-3 cells were transfected with either HA-S GK-1 or, as a control for kinase activity, kinase-dead HA-K127M-S GK-1. To determine the relative SGK-1 kinase activities of the two fractions, HA-S GK-1 was immunoprecipitated from the S100 and P100 fractions as described. One-third of the immunoprecipitated HA-S GK-1 was analyzed by Western blot using anti-HA-HRP, and the remaining fraction was employed in an in vitro kinase assay (18). Western analysis revealed that unmodified HA-S GK-1 was found in approximately equal amounts in both the cytosolic and membrane fractions (Fig. 5A). Endogenous ubiquitin modification of the immunoprecipitated HA-S GK-1 was detected using an anti-ubiquitin antibody. A significantly greater amount of high molecular weight polyubiquitinated SGK-1 was detected in the membrane versus the cytosolic compartment (Fig. 5B), although exact quantification of the relative amounts of ubiquitin-modified versus unmodified SGK-1 is not possible due to the multiple ubiquitin epitopes that are likely to amplify the anti-ubiquitin antibody signal in the polyubiquitinated species. The in vitro kinase assay revealed that the overall kinase activity of the two SGK-1 fractions was approximately equal (Fig. 5C). Taken together, these findings suggest that the relatively high expression of polyubiquitinated SGK-1 in the membrane-associated versus cytosolic fraction does not correlate with a significantly different overall SGK-1 kinase activity in the membrane-associated compartment.

DISCUSSION

Although it is well-established that sgk-1 mRNA is up-regulated as an immediate early response to serum and glucocorticoid stimulation, the regulation of SGK-1 kinase activity is only partly understood. It has been previously established that SGK-1 is phosphorylated and activated by a phosphatidylinositol 3-kinase-dependent pathway on conserved serine and threonine residues homologous to those found in AKT-1. However, in most cell types, steady-state levels of SGK-1 are far less abundant than AKT-1 (8). In this report, we demonstrate for the first time that in addition to the transcriptional regulation of sgk-1, SGK-1 protein levels are regulated by the ubiquitin-proteasome pathway, adding another dimension to the mechanism of regulation of this unusual serine-threonine kinase. We also show that polyubiquitin-modified SGK-1 is predominantly localized to the membrane-associated fraction of the cell. These observations suggest that SGK-1 is ubiquitinated at or near the membrane rather than in the cytosol. Alternatively, deubiquitinating enzymes may preferentially act on SGK-1 in the cytoplasmic compartment, thereby resulting in accumulation of polyubiquitinated SGK-1 in the membrane fraction.

While these studies were ongoing, SGK-1 was shown to directly phosphorylate and inactivate Nedd4-2, a known membrane-associated protein that is the E3 ligase responsible for ubiquitination and degradation of the epithelial sodium channel (ENaC) (14, 15). This raises the interesting possibility that SGK-1 may both phosphorylate and act as an E3 substrate of Nedd4-2 (or another E3 ligase), thereby achieving a tight negative feedback of SGK-1 activity. Our finding that the majority of ubiquitinated SGK-1 is associated with the plasma membrane is consistent with this proposed model (see Fig. 6).

Unlike SGK-3 (CISK), which contains a complete phosphatidylinositol phosphate-binding PX domain that is required to localize SGK-3 to early endosomes (24), SGK-1 contains only the α-helix-2 domain arginine residues that are predicted to be required for binding phosphatidylinositol (25) does not alter SGK-1 membrane localization or kinase activity. Therefore, SGK-1 appears to use an independent mechanism, perhaps via a protein-protein interaction requiring the amino terminus, for translocation to the cell membrane.

In summary, we have demonstrated that the steady-state protein levels and kinase activity of SGK-1 are down-regulated via polyubiquitin modification of the membrane-associated fraction of SGK-1. Regulation of serine-threonine kinases via ubiquitin modification underscores the potential importance of phosphorylation-independent mechanisms in the regulation of signaling molecules. In cell types or disease conditions in which phosphatidylinositol 3-kinase activity is constitutively high (e.g. with HER-2/new amplification, Ras mutations, or PTEN deletions), the availability of downstream targets is likely to be of critical importance to the activity of these pathways.

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