Hsp90 Regulates the Phosphorylation and Activity of Serum- and Glucocorticoid-regulated Kinase-1*

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Larissa Belova1, Deanna R. Brickley1,2, Betty Ky1, Sanjay K. Sharma1, and Suzanne D. Conzen15,2

From the 1Department of Medicine, 2Committee on Cancer Biology, and the 5Ben May Department for Cancer Biology, The University of Chicago, Chicago, Illinois 60637

SGK-1 (serum- and glucocorticoid-regulated kinase-1), a member of the AGC protein kinase family, plays an important role in regulating ion channel expression and contributes to malignant epithelial cell proliferation and survival. SGK-1 activity is regulated on three levels: transcriptional induction following a variety of environmental and intracellular stresses, proteasomal degradation, and phosphorylation. Here we report that phosphoinositide 3-kinase (PI3K)-dependent phosphorylation of SGK-1 requires formation of a complex between SGK-1 and heat-shock protein 90 (Hsp90). Inactivation of Hsp90 by geldanamycin led to decreased SGK-1 phosphorylation independently of increased proteasomal protein degradation, and inhibition of PI3K activity by LY294002 appeared to eliminate SGK-1 phosphorylation at the same residues as those affected by geldanamycin treatment. Interestingly, geldanamycin-targeted phosphorylation sites were not limited to the known conserved PI3K-dependent sites Thr-256 and Ser-422 in SGK-1 but included additional unknown PI3K-dependent residues. Inhibition of Hsp90 also resulted in a complete loss of SGK-1 kinase activity, suggesting that Hsp90 activity is essential for regulating the PI3K/S GK-1 pathway.

To maintain homeostasis, an effective response to environmental challenge is required of all living organisms. Stress-response mechanisms have evolved at both the organismal and cellular levels. One of the important effectors of the cellular stress response that has been identified in several cell types as well as in both simple and complex eukaryotic organisms is SGK-1 (serum- and glucocorticoid-regulated kinase-1). SGK-1 belongs to the “AGC family” of serine-threonine kinases that includes protein kinase B (also known as Akt1), p70 S6 kinase, and protein kinase C. Unlike other members of the AGC family, SGK is transcriptionally induced following various forms of cellular stress, including hyperosmolar or heat shock, UV irradiation, and also following steroid receptor activation (1). After induction, SGK-1 is phosphorylated via a phosphoinositide 3-kinase (PI3K)-dependent pathway at residues Thr-256 and Ser-422 (2, 3). These phosphorylation events activate SGK-1 kinase activity thereby initiating a potent survival signal in epithelial cells; the mechanism downstream of SGK-1 activation is mediated in part via phosphorylation and inactivation of the pro-apoptotic transcription factor FOXO3a (4, 5). Recent findings have similarly demonstrated that SGK-1 functions as a critical component of insulin signaling in Caenorhabditis elegans via phosphorylation and inactivation of the FOXO3a homolog Daf 16 (6, 7). In addition, SGK-1 regulates the activity and abundance of ion channels in the lung (8, 9), kidney (10), and heart (11, 12), thereby integrating sodium homeostasis, fluid balance, blood pressure, and heart function.

In addition to transcriptional induction following a physiological stressor, SGK-1 protein levels are tightly regulated via ubiquitin modification and rapid proteasome-mediated degradation (13). We and others have shown that the endoplasmic reticulum (ER)-associated E3 ligase C terminus of the Hsc70-interacting protein (CHIP) (14) and HRD1 (15) are involved in ubiquitin modification of SGK-1 and contribute to the rapid return of SGK-1 protein expression to base-line levels during recovery from stress. Because many stress-induced proteins require molecular chaperones for proper folding and activity, we hypothesized that SGK-1 activity might be regulated by chaperone interaction. Previously, we showed that heat shock protein 70 (Hsp70) forms a complex with SGK-1 and CHIP (14); here we present evidence that phosphorylation and activity of SGK-1 are also regulated via the ubiquitous molecular chaperone heat shock protein 90 (Hsp90).

Hsp90 plays an important role in maintaining cellular homeostasis by regulating the conformation, activation, and subsequent function of over 100 client proteins. Hsp90 substrates include the glucocorticoid (16–18), androgen (19–21), estrogen (22–24), and progesterone receptors (25–30), tyrosine kinases ErbB2 (31), c-Src (32), v-Src (33–35), BCR-Abl (36, 37), p53 (38), and HIF-1α (39). Hsp90 also regulates several compo-

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1 Both authors contributed equally to this work.

2 To whom correspondence should be addressed: The University of Chicago, 5841 South Maryland Ave., MC 2115, Chicago, IL 60637. Tel.: 773-834-2604; Fax: 773-834-0188; E-mail: sdc@uchicago.edu.

3 The abbreviations used are: PI3K, phosphoinositide 3-kinase; ALLN, peptide aldehyde Ac-Leu-Leu-Nle-al; ALLM, Ac-Leu-Leu-methioninal; CHIP, C terminus of Hsc70 interacting protein; ER, endoplasmic reticulum; GA, geldanamycin; Hsp90, heat shock protein 90; AA, aliphatic amino acid; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; WT, wild type; HA, hemagglutinin; UPF, unfolded protein response; ERAD, ER-associated protein degradation; CFTR, cystic fibrosis transmembrane conductance regulator; DTT, dithiothreitol; GAM, goat anti-mouse; MOPS, 4-morpholinepropanesulfonic acid; E3, ubiquitin-protein isopeptide ligase; KD, kinase-dead; OA, okadaic acid; JNK, c-Jun N-terminal kinase.
Hsp90 Regulates SGK-1

In this study we show for the first time that SGK-1 forms a functional complex with Hsp90 that is disrupted following treatment with the Hsp90 inhibitor, geldanamycin (GA). Furthermore, we demonstrate that Hsp90 is required for PI3K-dependent phosphorylation of SGK-1, and that the phosphorylated residues appear to include both previously described Thr-256 and Ser-422 as well as additional unknown amino acid(s). Furthermore, Hsp90-dependent phosphorylation plays a critical role in SGK-1 function because the rapid loss of phosphorylated SGK-1 species following GA treatment completely abrogates SGK-1 kinase activity. Unlike many of the Hsp90 client proteins, including Akt1, SGK-1 is not degraded following GA treatment. In addition, Akt1 is transiently phosphorylated following GA treatment (49), whereas inactivation of Hsp90 by GA leads to a very rapid loss of SGK-1 phosphorylation and subsequent complete inactivation. Taken together, these results suggest that the stress-induced PI3K/SGK-1 pathway is positively regulated by Hsp90 consistent with the distinctive role of SGK-1 in maintaining physiological homeostasis following acute cellular stress.

EXPERIMENTAL PROCEDURES

Cell Culture and Expression Vectors—SK-BR-3 and MDA-MB-231 human breast cancer cell lines and COS-1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Lonza Walkersville Inc., Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (Gemini Bio-Products, West Sacramento, CA) and 1% penicillin/streptomycin (Lonza Walkersville Inc.). Cloning of full-length SGK-1 in-frame with a C-terminal FLAG tag into the retroviral vector pLPCX (Clontech) and creation of SGK-1 mutants S422D, T256A/S422A, and K127M (kinase-dead) SGK-1-FLAG were described previously (13, 50). The mutation of serine 78 to alanine (S78A) was created using the Quick-Change site-directed mutagenesis kit (Biocrest Manufacturing, West Cedar Creek, TX) using the following primers: forward, 5′-CCT TCT CCT CCA GCA GCT CCT TCT CAG CAA ATC-3′, and reverse, 5′-GAT TTG CTG AGA AGG AGC TTG TGG AGG AGA AGG-3′ (51). Myc-tagged constitutively active p110α pCAAX was a kind gift of Dr. J. Downward of Cancer Research UK London Research Institute. HA ubiquitin in the pRbg4 vector was described previously (13). Constitutively active Mek5DD was a kind gift of Dr. Mark Abe of the University of Chicago.

Immunoprecipitation—Typically, 2 × 10⁶ SK-BR-3 cells were transfected with plasmids encoding either SGK-1-FLAG or pLPCX using Polyfect transfection reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Unless otherwise stated, cells were treated with ALLN (a peptide aldehyde (Ac-Leu-Leu-norleucinal)) (10 μM) for 16 h before harvesting. Forty eight hours following transfection, cells were washed with ice-cold phosphate-buffered saline and then lysed in 50 mM Tris, pH 7.5, 120 mM NaCl, 2% Triton X-100, 10 mM EDTA supplemented with protease/proteasome/phosphatase inhibitor mixture: 1 mM benzamidine, 40 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 μM microcystin-LR, 2 mM NaF, 10 μM ALLN, 10 μM ALM (Ac-Leu-Leu-me-thioninal), 0.1 mM sodium orthovanadate, protease mixture (0.25 mM aprotinin, 3 mM leupeptin, 3 mM pepstatin, 13 mM bestatin, 4.5 mM E-64 from Sigma), 1% Nonidet P-40, and a complete tablet of protease inhibitors (Roche Applied Science). Equal amounts of cell protein were immunoprecipitated with FLAG-agarose (Sigma) as described previously (13). SGK-1-FLAG was then eluted from the agarose beads with 5× FLAG peptide (Sigma). Co-immunoprecipitated proteins were resolved using 9% SDS-PAGE and then either silver-stained (Bio-Rad) or analyzed by Western blotting procedure.

Endogenous Hsp90 was immunoprecipitated from either SK-BR-3 cells transiently expressing SGK-1-FLAG and treated with ALLN overnight or subconfluent MDA-MB-231 cells that were starved of all growth factors for 60 h and then stimulated for 10 h with 1 μM dexamethasone, 20% serum, and ALLN. Immunoprecipitation was performed as described previously (52). Briefly, cells were lysed in 25 mM MOPS, pH 7.5, 1 mM EDTA, 0.02% NaN₃, and 10% glycerol (MENG) containing the same protease/proteasome/phosphatase inhibitor mixture as described above. Lysates were passed 10 times through a 25-gauge needle and then centrifuged for 10 min at 10,000 × g, 4 °C. Supernatants were then preclreated for 2 h at 4 °C with 4 μg of mouse IgM (Zymed Laboratories Inc.), 2 μg of goat antimouse (GAM) IgM antibody (Zymed Laboratories Inc.), and 20 μl of protein G-agarose (Sigma). For endogenous SGK-1 co-precipitation, supernatants were preclreated for 30 min at 4 °C with 4 μg of TEPC-183 mouse IgM (tetramethylpentadecane-induced plasmacytoma; Sigma), 2 μg of GAM, and 25 μl of protein G-agarose. Agarose was pelleted by centrifugation for 2
In Vivo Ubiquitination Assay—SK-BR-3 cells stably expressing SGK-1-FLAG were transfected with a plasmid encoding HA-ubiquitin and treated with 1 μM GA. SGK-1 was immunoprecipitated from equal amounts of protein lysate. SGK-1-FLAG species were then immunoblotted with either anti-HA-HRP or anti-SGK-1 antibodies to detect both ubiquitinated and unmodified SGK-1 species, respectively.

Inhibitor Treatments—SK-BR-3 or MDA-MB-231 cells were treated with 1 μM GA or vehicle (DMSO) for 3 h or as otherwise indicated. In some experiments, 50 nM okadaic acid (EMD Chemicals, Inc.) was added for 2 h prior to GA treatment. In others, either 10 μM ALLN or MG132 was added during GA treatment for 16 or 2.5 h, respectively. To inhibit potential kinases acting upstream of SGK-1, either 100 μM U0126 (Cell Signaling Technology), 10 μM SB203580 (Sigma), 10 μM JNK inhibitor II (EMD Chemicals, Inc.), 100 nM staurosporine (Sigma), or 50 μM LY294002 (LC Laboratories, Woburn, MA) was added to cells stably expressing SGK-1-FLAG for 3 h.

Kinase Assay—Preparation of SGK-1 immunocomplexes and in vitro kinase assays were performed according to recommendations of the SGK-1 kinase assay protocol (Millipore, Research Park, MO) with minor modifications. Ectopically expressed wild type (WT), K127M (kinase-dead (KD)) or S422D SGK-1 was immunoprecipitated using 25 μl of FLAG-agarose from 2.5 μg of SK-BR-3 cell lysates prepared in TNE lysis buffer (50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 5 mM EGTA, 1% Nonidet P-40, 1% Triton X-100, 1 mM DTT containing inhibitors listed above). Immunoprecipitated SGK-1 was washed three times with 0.5 ml of buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Nonidet P-40, 1% Triton X-100, 0.1% β-mercaptoethanol, and the inhibitors as in TNE lysis buffer), two times with 0.5 ml of buffer B (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, phosphatase inhibitor mixture I), and two times with 0.2 ml of kinase buffer (20 mM MOPS, pH 7.2, 5 mM EGTA, 5 mM DTT, 25 mM β-glycerol phosphate, and phosphatase inhibitor mixture I). Immunoprecipitated SGK-1 was mixed with 40 μl of reaction mixture (kinase buffer containing 62.5 μM SGKtide, 17 mM MgCl₂, 112.5 mM ATP, 10 μCi [γ-32P]ATP (3000 Ci/mmol) (GE Healthcare), 5 μM cAMP-dependent protein kinase (EMD Chemicals, Inc.), protein kinase C (Millipore) inhibitor peptides, and 5 μM calmidazolium chloride (EMD Chemicals, Inc.) and then incubated for 40 min at 30 °C. Supernatant (40 μl) was then absorbed onto P81 phosphocellulose paper (Millipore), washed six times with 0.75% phosphoric acid, and once with acetone. Incorporated radioactivity was determined using a Beckman scintillation counter LS 6500 (Beckman Coulter, Fullerton, CA).

In Vitro Dephosphorylation—SK-BR-3 cells transiently expressing SGK-1-FLAG were lysed in either buffer A or B. Buffer A contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EGTA, 2 mM MgCl₂, 2 mM DTT, 1% Nonidet P-40, 10 μM ALLN, 10 μM ALLM, and protease mixture. Buffer B contained 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 5 mM DTT, 1% Nonidet P-40, 10 μM ALLN, 10 μM ALLM, and protease mixture. Equal amounts of lysates (100 μg) were either left untreated or mixed with 1000 units of λ-phosphatase (New England Biolabs, Ipswich, MA; Buffer A) or YOP phosphatase...
Hsp90 Regulates SGK-1

Identification of Hsp90 as an SGK-1-associated Protein—In comparison with the regulation of its closely related family member, Akt1, modulation of SGK-1 activity is less well understood. To identify SGK-1-associated proteins, we immunoprecipitated ectopically expressed SGK-1-FLAG, resolved the co-precipitated proteins on a denaturing gel, and used MALDI-TOF mass spectrometry to identify silver-stained proteins that specifically co-precipitated with SGK-1. As seen in Fig. 1A, an ~90-kDa protein appeared to be specific to the SGK-1 lane; sequence analysis of eight peptides from this band identified it as Hsp90.

To confirm that Hsp90 forms a specific complex with SGK-1, we performed immunoprecipitation of ectopically expressed SGK-1-FLAG from membrane and cytosolic fractions of SK-BR-3 cells using an anti-FLAG antibody followed by immunoblotting with an anti-Hsp90 antibody. As shown in Fig. 1B, SGK-1 was found in both the cytosolic (S100) and membrane (P100) fractions, consistent with our previous observations (13). However, endogenous Hsp90 associated with SGK-1 primarily in the cytosolic fraction, consistent with the predominantly cytosolic localization of Hsp90 (53).

RESULTS

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To further investigate the specificity of association of SGK-1 with Hsp90, we performed the reciprocal immunoprecipitation of endogenous Hsp90 from equal amounts of SK-BR-3 cell protein lysates. Co-precipitated proteins were resolved with SDS-PAGE and examined by immunoblotting using an anti-FLAG antibody to detect SGK-1. As shown in Fig. 1C, SGK-1 was detected in a complex with Hsp90, providing further evidence that Hsp90 associates with SGK-1.

Finally, to determine whether endogenous Hsp90 and SGK-1 associate, we immunoprecipitated Hsp90 from MDA-MB-231 cells after inducing SGK-1 expression with serum and glucocorticoid stimulation. Fig. 1D illustrates the specific immunoprecipitation of Hsp90 followed by detection of endogenous SGK-1 using an anti-SGK-1 antibody directly conjugated to horseradish peroxidase. When SGK-1 was not induced by serum and glucocorticoid stimulation, the interaction could be barely detected, presumably because of the very low levels of SGK-1. However, in stimulated cells, endogenous SGK-1 clearly co-precipitates with Hsp90. Thus, we show here that both endogenous and ectopic SGK-1 form an in vivo complex with endogenous Hsp90.

Hsp90 Inhibition Abolishes Slow Mobility Species of SGK-1 Independently of Proteasome-mediated Degradation—Hsp90-specific inhibitors have been shown to be useful tools for studying Hsp90 function. An irreversible Hsp90 inhibitor, GA, binds specifically to the ATP-binding pocket of Hsp90, which has a unique conformation compared with other chaperones (e.g. Hsp70) (54). GA thereby interferes specifically with the ATP-dependent association of Hsp90 and its client proteins (55). Because the affinity of GA for Hsp90 is within the micromolar range (56), we examined whether inhibition of Hsp90 by 1 μM GA would result in its dissociation from SGK-1. We treated SK-BR-3 cells stably expressing SGK-1-FLAG with GA for 3 h and then performed an anti-FLAG immunoprecipitation. As seen in Fig. 2A, Hsp90-β, as well as Hsp90-α (data not shown), co-immunoprecipitated with SGK-1. However, following treatment with GA, Hsp90 was no longer detected in the SGK-1 immunoprecipitation, further suggesting that SGK-1 and Hsp90 binding in vivo is ATP-dependent. Notably, GA treatment resulted only in the loss of the slowly migrating bands of SGK-1, suggesting that Hsp90 inhibition affects the post-translational modification of SGK-1.

Hsp90 has been reported to stabilize a wide variety of client proteins (for review see Ref. 57). We therefore examined the effect of GA on endogenous SGK-1 steady-state levels in MDA-
MB-231 breast cancer cells. To induce SGK-1 expression, we
starved cells for 24 h prior to overnight treatment with 1 μM
dexamethasone and 20% serum. In parallel, cells were treated
with ALLN for 4 h prior to cell lysis. Fig. 2B (left panel) shows
that endogenous SGK-1 levels significantly increased following
ALLN treatment, consistent with the known proteasome-me-
diated degradation of SGK-1 (13, 50). Between 3 and 6 h, GA
cause the complete disappearance of slow mobility SGK-1
species (Fig. 2B, right panel). Surprisingly, ALLN did not rescue
the disappearance of these slow mobility species, suggesting
that increased protein degradation was not the cause of the
GA-induced loss of the upper species. At a later time point of
GA treatment (22 h), an overall reduction in endogenous
SGK-1 levels was seen that could be due to the predicted loss
of glucocorticoid receptor expression leading to decreased tran-
scriptional induction of SGK. Similar results were obtained in
SK-BR-3 breast cancer cells (data not shown).

We next investigated whether the more specific proteasome
inhibitor MG132 could reverse the disappearance of the slowly
migrating species of SGK-1. SK-BR-3 cells transiently expressing
SGK-1-FLAG were treated with GA for 6 and 9 h. Parallel
cell cultures were treated with or without MG132 for 2.5 h
before each collection time point. SGK-1-FLAG accumulated
following MG132 treatment (Fig. 2C, left), which is consistent
with accumulation of endogenous SGK-1 following protea-
some inhibition. Once again, the slow mobility species of
SGK-1 largely disappeared following 3 h of GA treatment either
in the presence or absence of MG132 (Fig. 2C, right). In addi-
tion, we observed that GA treatment increased the accumula-
tion of the faster mobility species of SGK-1, suggesting that
these species are not degraded following GA treatment. Similar
results were obtained in HEK 293 cells (data not shown). Taken
together, these data suggest that the slow mobility species of
both endogenous and ectopic SGK-1 are affected by GA in a
similar manner in different cell types, and that their rapid dis-
appearance is not the result of an increase in proteasome-me-
diated protein degradation.

Despite the lack of proteasome-mediated degradation of
SGK-1 following GA treatment, we hypothesized that GA
might still affect SGK-1 ubiquitin modification (58–61). We
therefore examined whether the efficiency of the ubiquitin-
modification of SGK-1 was altered following GA treatment.
SK-BR-3 cells stably expressing SGK-1-FLAG were transiently
transfected with a plasmid encoding HA-ubiquitin. Cells
treated with GA or vehicle were lysed, and SGK-1 was immu-
noprecipitated. Levels of ubiquitinated SGK-1 were deter-
mined by immunoblotting with anti-HA antibody. As shown in
Fig. 2D, slowly migrating species of immunoprecipitated
SGK-1 disappeared following 1 h of GA treatment (bottom
panel, lane 8). However, overall SGK-1 ubiquitination
increased after 2 h of GA treatment (Fig. 2D, upper panel,
lane 9). Therefore, the disappearance of the upper band of SGK-1
(1 h) precedes the increase in overall ubiquitin modification (2 h),
suggesting that the loss of the slowly migrating SGK-1 species is
not a result of increased ubiquitin modification. Following lon-
ger GA treatment, the accumulation of the faster migrating
species of SGK-1 (Fig. 2D, bottom panel, lanes 9–12) was
accompanied by an increase in ubiquitin-modified SGK-1
(upper panel, lanes 9–12). The ratios of ubiquitinated
versus unmodified SGK-1 actually decreased over time as measured by
densitometry (data not shown), suggesting that inhibiting
Hsp90 activity decreases the efficiency of SGK-1 ubiquitina-

**FIGURE 2. Inhibition of the Hsp90 and SGK-1 complex abolishes slow mobility species of SGK-1.** A, GA disrupts the SGK-1 complex with Hsp90. SGK-1-FLAG/SK-BR-3 cells were treated with GA for 3 h; SGK-1-FLAG was immunoprecipitated (IP), and precipitated proteins were probed with respective antibodies. B, Hsp90 inhibition with GA abolishes slow mobility species of endogenous SGK-1. MDA-MB-231 cells were starved for 24 h, stimulated with dexamethasone and serum for 16 h, and then treated with GA. Endogenous SGK-1 from whole cell lysates was immunoblotted (IB) using anti-SGK-1 antibody. In a parallel culture, cells were pretreated with ALLN overnight. C, GA abolishes the slow mobility species of ectopic SGK-1. SK-BR-3 cells stably expressing SGK-1-FLAG were treated with or without GA for the indicated times. MG132 was then added for the last 2.5 h of incubation. Whole cell lysates were resolved on SDS-PAGE, and SGK-1 was detected with anti-FLAG antibody. D, efficiency of ubiquitin modification of SGK-1 decreases following GA treatment. SK-BR-3 cells stably expressing SGK-1-FLAG were transiently transfected with a plasmid encoding HA-ubiquitin. Cells treated with GA or vehicle were lysed, and SGK-1 was immunoprecipitated. Levels of ubiquitinated SGK-1 were determined by immunoblotting with anti-HA antibody. As shown in Fig. 2D, slowly migrating species of immunoprecipitated SGK-1 disappeared following 1 h of GA treatment (bottom panel, lane 8). However, overall SGK-1 ubiquitination increased after 2 h of GA treatment (Fig. 2D, upper panel, lane 9). Therefore, the disappearance of the upper band of SGK-1 (1 h) precedes the increase in overall ubiquitin modification (2 h), suggesting that the loss of the slowly migrating SGK-1 species is not a result of increased ubiquitin modification. Following lon-
ger GA treatment, the accumulation of the faster migrating
species of SGK-1 (Fig. 2D, bottom panel, lanes 9–12) was
accompanied by an increase in ubiquitin-modified SGK-1
(upper panel, lanes 9–12).
To further investigate Hsp90-dependent phosphorylation of SGK-1, we directly examined whether Hsp90 inhibition interferes with in vivo SGK-1 phosphorylation. We labeled SK-BR-3/SGK-1-FLAG cells with $^{32}$Porthophosphate in the presence of either vehicle or GA, immunoprecipitated SGK-1, and examined the levels of either total or phosphorylated SGK-1 using immunoblotting and autoradiography, respectively. As seen in Fig. 3B, the slowly migrating SGK-1 species had electrophoretic mobility similar to the radiolabeled SGK-1 (lanes 1 and 3), strongly suggesting that these species are phosphorylated. In the presence of GA, these upper radiolabeled SGK-1 species almost completely disappeared (Fig. 3B, lane 4) consistent with the depletion of slow moving species (lane 2). These results strongly suggest that Hsp90 activity is required for SGK-1 phosphorylation in vivo.

It has been shown that Hsp90 can enhance phosphorylation of Akt1 by inhibiting Akt1-directed phosphatase activity (62–64). We therefore examined whether the phosphorylated SGK-1 species that disappear following GA treatment might consist of differentially phosphorylated species of SGK-1 that are phosphatase-sensitive. We treated SK-BR-3 cells stably expressing SGK-1-FLAG with 50 nm of the phosphatase inhibitor okadaic acid (OA) for 2 h followed by GA treatment. Whole cell lysates were collected and analyzed by immunoblotting. As shown in Fig. 4A, 30 min of GA treatment completely depleted the slowly migrating phospho-SGK-1 bands, and longer treatment caused further accumulation of the faster migrating, unmodified SGK-1 bands (Fig. 4A, top). As expected, OA, which inhibits both protein phosphatases 1 and 2A (PP1 and PP2A) at the concentrations used, caused a relative accumulation of phosphorylated SGK-1 over time as seen in Fig. 4A (bottom left). However, OA did not rescue the disappearance of the phosphorylated species of SGK-1 following GA treatment (Fig. 4A, bottom right), suggesting that GA does not increase phosphatase activity toward SGK-1.

We next asked whether Hsp90-dependent phosphorylation of SGK-1 involves a specific kinase or activated pathway upstream of SGK-1. We treated SK-BR-3 cells stably expressing SGK-1-FLAG with a panel of kinase inhibitors that target the major intracellular signaling pathways. Neither U0126 (a dual Erk1/2 and Erk5 inhibitor at the concentrations used), SB203580 (a p38 inhibitor), a JNK inhibitor II, nor staurosporine (a myosin light chain kinase inhibitor) demonstrated any significant effect on the slowly migrating phosphorylated species of SGK-1 (Fig. 4B, lanes 4–7). In contrast, only the PI3K inhibitor LY294002 eliminated the two most slowly migrating species of SGK-1, resulting in a loss of the same SGK-1 species that disappear following Hsp90 inhibition (Fig. 4B, lanes 2 and 3).

To confirm that PI3K activity plays a role in the Hsp90-dependent phosphorylation of SGK-1, we co-expressed SGK-1-FLAG and the constitutively active catalytic subunit of PI3K p110α (p110 CAAX) in COS-1 cells. Following GA treatment in control cells, the slower migrating species of SGK-1 disappeared, and the faster migrating SGK-1 species rapidly accumulated as in previous experiments (Fig. 4C, lanes 1–4). Expression of p110 CAAX both significantly increased SGK-1 basal levels and impaired the depletion of the slowly migrating phos-

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**FIGURE 3.** Hsp90-dependent species of SGK-1 are phosphorylated. A, equal amounts of SGK-1-FLAG/SK-BR-3 cell lysates were treated in vitro with or without either phosphatase $\lambda$ or YOP for 3 h, resolved on SDS-PAGE, and immunoblotted (IB) with an anti-SGK-1 antibody. In parallel, a cell lysate of GA-treated cells is shown. B, GA interferes with phosphorylation of SGK-1 in vivo. SK-BR-3 cells were starved of all growth factors in phosphate-free media, pretreated with 1 μM GA, and then stimulated with 20% serum in the presence of 1 μCi of $^{32}$P orthophosphate. SGK-1 was immunoprecipitated and immunoblotted with SGK-1-HRP antibody, and the levels of phosphate incorporation were determined by autoradiography. Fifty-kDa marker and phosphorylated SGK-1 are indicated.

**A**

| IB: α-SGK-1 | 1 | 2 | 3 | 4 | 5 |
|-------------|---|---|---|---|---|
| 50          |   |   |   |   |   |

**B**

| IB: α-SGK-1 | 1 | 2 | 3 | 4 |
|--------------|---|---|---|---|
| 50           |   |   |   |   |

**IP: α-FLAG**

| IB: α-SGK-1 | 1 | 2 | 3 | 4 |
|--------------|---|---|---|---|
| 50           |   |   |   |   |

**3P**

| phosho-SGK-1-FLAG | 1 | 2 | 3 | 4 |
|-------------------|---|---|---|---|
|                  |   |   |   |   |
phorylated species of SGK-1 following GA treatment (Fig. 4C, lanes 6–8). These results suggest that very high levels of the PI3K pathway activation by p110 CAAX can overcome a loss of Hsp90 function, perhaps by allowing SGK-1 to be phosphorylated despite suboptimal protein folding. Interestingly, in a similar experiment using constitutively active Mek5DD that should activate phosphorylation of SGK-1, SK-BR-3 cells stably expressing SGK-1-FLAG were treated with various kinase inhibitors. Whole cell lysates were collected, resolved on SDS-PAGE, and immunoblotted with anti-SGK-1 antibody. PI3K-p110 activity reverses GA-mediated loss of SGK-1 phosphorylation. COS-1 cells were transiently transfected with SGK-1-FLAG and p110 CAAX and treated with GA. Whole cell lysates were collected, resolved on SDS-PAGE, and immunoblotted with antibodies as indicated. The SGK-1 blot is shown in two different exposures: 2.5 min for GFP co-expressed group and 30 s for p110 CAAX group. Fifty- and 150-kDa markers are indicated.

**FIGURE 5.** Hsp90-dependent phosphorylation of SGK-1 includes but is not limited to Ser-422. A, phosphorylation of Ser-422 is Hsp90-dependent. SGK-1-FLAG was immunoprecipitated (IP) from SK-BR-3 cells transiently expressing either WT or the double alanine mutant T256A/S422A SGK-1-FLAG were treated with 1 μM GA and immunoblotted with an anti-SGK-1 antibody. B, T256A/S422A SGK-1 phosphorylation is decreased following Hsp90 inhibition. In vivo labeling of WT and T256A/S422A SGK-1 were performed as described in Fig. 3B legend. Fifty-kDa marker is indicated.
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S422A mutant should not lose the slowly migrating bands following GA treatment. We examined WT and T256A/S422A SGK-1-FLAG in SK-BR-3 cells treated with vehicle or 1 μM GA for 4 h by immunoblotting using an anti-SGK-1 antibody. Unexpectedly, T256A/S422A SGK-1 had virtually the same banding pattern as WT SGK-1 (Fig. 5B, lanes 1 and 3), suggesting that the double alanine SGK-1 mutant is still highly phosphorylated. Moreover, the slowly migrating phosphorylated species of T256A/S422A SGK-1 disappeared following GA treatment similarly to the wild type (Fig. 5B, lanes 1 and 2 versus 3 and 4), further suggesting that Thr-256 and Ser-422 are not the only residues affected by GA-mediated dephosphorylation and that additional phosphorylation sites are involved in Hsp90-mediated phosphorylation of SGK-1.

To further confirm that GA causes SGK-1 dephosphorylation beyond the canonical Thr-256 and Ser-422 residues, we performed in vivo 32P labeling of both WT and T256A/S422A SGK-1 in the presence of GA. We reasoned that if Thr-256 and Ser-422 were the only two Hsp90-dependent phosphorylation sites, T256A/S422A SGK-1 should demonstrate less efficient phosphorylation and decreased sensitivity to GA treatment. However, both WT and the double alanine mutant SGK-1 were similarly phosphorylated in vivo (Fig. 5C, lane 1 versus 3), and furthermore, T256A/S422A SGK-1 lost significant phosphorylation following GA treatment (lane 3 versus 4), strongly suggesting that Hsp90-dependent phosphorylation of SGK-1 takes place at additional phosphorylation sites beyond Thr-256 and Ser-422.

Geldanamycin Treatment Results in Decreased SGK-1 Kinase Activity—Having established that Hsp90 inhibition causes the disappearance of hyperphosphorylated SGK-1 species involving Thr-256 and Ser-422, we next examined how Hsp90 function affects overall SGK-1 kinase activity. SK-BR-3 cells were transfected with the kinase-dead (KD) and WT SGK-1-FLAG plasmids and then treated with GA or vehicle for 3.5 h before cell lysis. SGK-1 was immunoprecipitated, and an in vitro kinase assay was performed using “SGKtide” as described previously (3). Using the immunoprecipitates from equal amounts of cell lysate, we found that WT SGK-1 activity was abolished following GA treatment (Fig. 6A, left). A parallel Western analysis of SGK-1 revealed that although the levels of the upper species of WT SGK-1 were reduced following GA treatment, significant amounts of total SGK-1 remained that were devoid of kinase activity (Fig. 6A, right).

Based on our previous results, we hypothesized that Hsp90-dependent phosphorylation of SGK-1 takes place at the conserved (Thr-256 and Ser-422) sites as well as additional site(s). To investigate this hypothesis, we examined whether the activation mutation of Ser-422 to aspartic acid (S422D) (3) is sufficient to overcome the GA-mediated inhibition of kinase activity. Surprisingly, S422D SGK-1-FLAG lost 50% of its kinase activity following GA treatment (Fig. 6B, left), further confirming that Hsp90-dependent phosphorylation includes additional unknown residues beyond Ser-422 and Thr-256 that are required for full kinase activity. The results of these kinase assays suggest that the effect of GA on SGK-1 activity takes place through both the canonical PI3K sites Thr-256 and Ser-422 and additional unknown site(s).

Hsp90 Has Distinctive Roles in the Phosphorylation of SGK-1 Versus Akt1—SGK-1 shares ~50% homology with the catalytic domain of Akt1, and Akt1 has also been shown to be a client protein for Hsp90 chaperone activity. Because both kinases are phosphorylated downstream of the PI3K/PDK pathway, we examined whether or not SGK-1 and Akt1 phosphorylation is differentially regulated by Hsp90. MDA-MB-231 cells expressing either an empty vector or SGK-1-FLAG were treated with 1 μM GA, and the levels of SGK-1, endogenous phospho-Akt1, and total Akt1 were determined by immunoblotting. As shown in Fig. 7 (top panel, lanes 1–5), endogenous SGK-1 was barely
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Although Hsp90 affects Akt1 indirectly via modulating PI3K activity, SGK-1 phosphorylation is directly dependent on Hsp90 binding, which we propose to be a limiting step for SGK-1 phosphorylation. For example, we observed that Akt1 is transiently phosphorylated at Ser-473 following a short 30-min exposure to GA. This observation is consistent with a previous report (49) suggesting that GA rapidly disrupts Src association with Hsp90, allowing activated Src to phosphorylate Cbl, which recruits the p85 subunit of PI3K and activates Akt1. However, although we saw rapid phosphorylation of Akt1 at Ser-473, we concurrently saw a loss of SGK-1 phosphorylation, suggesting that GA affects SGK-1 activation via a direct disruption of the association of Hsp90 with SGK-1. Unexpectedly, following overexpression of SGK-1, we observed even higher Akt1 phosphorylation in the presence of GA. We speculate that GA allows Hsp90 to dissociate from Src but that residual active Hsp90 may be sequestered by the highly overexpressed SGK-1, thereby increasing Akt1 phosphorylation. Alternatively, overexpressed SGK-1 may feed back to activate the PI3K pathway. Another possibility is that Akt1 and SGK-1 form a complex that makes Akt1 more accessible for phosphorylation at Ser-473 (6).

Another indirect Hsp90-mediated mechanism of Akt1 activation that has been proposed is via stabilization of ErbB2 (a known activation pathway of PI3K). This mechanism does not seem to apply to SGK-1/Hsp90 interaction either. For example, we found that loss of SGK-1 phosphorylation following inhibition of Hsp90 in MDA-MB-231 cells (moderate PI3K activity because of low ErbB2) occurred with similar kinetics (data not shown). This is in contrast to Akt1 phosphorylation, where Hsp90 inhibition by a GA derivative, 17(allylamino)-17-demethoxygeldanamycin, results in a more rapid dephosphorylation of Akt1 in high ErbB2-expressing cells compared with low ErbB2 cells (64). In these studies, Hsp90 inhibition resulted in degradation of ErbB2, lower PI3K activity, and in the release of ErbB2-mediated repression of the phosphatase PP1, so that Akt1 becomes less phosphorylated (44, 68). In contrast, we find that GA-mediated dephosphorylation of SGK-1 appears to be independent of ErbB2 levels and of phosphatase activity, suggesting that Hsp90 is more directly involved in SGK-1 phosphorylation.

What are the implications of the distinct Hsp90-mediated regulation of SGK-1 versus Akt1? SGK-1 is a transcriptionally induced protein kinase whose levels are increased by various stress-related extra- and intracellular factors. Therefore, its regulation should be rapid in response to acute stress to provide a strong but transient signal. SGK-1 protein levels are negatively regulated via post-translational ubiquitin modification rendering SGK-1 rapidly degraded. Positive regulation of SGK-1 is provided by phosphorylation, which we show here requires Hsp90 binding for maximal activation. Akt1, on the other hand, is a constitutively expressed protein that needs to be precisely tuned in response to insulin. This goal is achieved by the dual role Hsp90 plays in the functioning of Akt as follows: positive regulation maintaining Akt1 stability and phosphorylation and negative regulation limiting the unnecessary phosphorylation and sequential activation of Akt1. By integrating the regulation of both SGK-1 and Akt1, Hsp90 plays an impor-
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In vitro treatment as well as following bands and loses the slowly migrating upper band following GA SGK-1 lacking the first 60 amino acids expresses as two distinct (49 kDa). However, we have also found that ectopically expressed protein; they are therefore likely to be modified full-length SGK-1 majority of the Hsp90-dependent phospho-SGK-1 species have the effect of GA treatment. We hypothesize that Hsp90 inhibition may limit the phosphorylation of SGK-1 in the presence of physiological PI3K activity, but the requirement for Hsp90 is overcome as a consequence of the constitutively active membrane-bound, p110α CAAX. Therefore, tumors with highly active PI3K and overexpressed SGK-1 might be treated more effectively with a combination of drugs targeting both Hsp90 and PI3K.

One possible interpretation of our data could be that GA actually affects the activity of the immediate upstream kinase, PDK1, instead of directly affecting SGK-1. However, it has been shown previously that Hsp90 inhibition only decreases protein levels of PDK1 after 12 h (59, 71). Given the much more rapid (within 1 h) loss of the phosphorylation of SGK-1 following GA treatment, it is unlikely that decreased PDK1 expression at 12 h contributes to the rapid and complete loss of the phosphorylated species of SGK-1. Furthermore, PDK1-mediated phosphorylation of Thr-256 has been shown to require Ser-422 phosphorylation, suggesting that Ser-422 plays a more critical role in Hsp90-dependent activation of SGK-1. The most likely explanation of our data is that a loss of Hsp90 function prevents SGK-1 from optimal protein conformation and inhibits phosphorylation of Ser-422 and additional site(s).

It was recently published that SGK-1 has alternative translational start site products with masses ranging from 42 to 49 kDa (72). In this study, we observed multiple bands of different electrophoretic mobilities when examining both endogenous and ectopic SGK-1 expression. It is possible that these bands consist of a mixture of both full-length and truncated SGK-1 proteins because neither Hsp90 nor PI3K inhibition completely resolved the multiplicity of the banding pattern of SGK-1. It should be noted that the majority of the Hsp90-dependent phospho-SGK-1 species have apparent molecular masses greater than the full-length SGK-1 protein; they are therefore likely to be modified full-length SGK-1 (49 kDa). However, we have also found that ectopically expressed SGK-1 lacking the first 60 amino acids expresses as two distinct bands and loses the slowly migrating upper band following GA treatment as well as following in vitro phosphatase treatment (data not shown). Therefore, we predict that the recently described alternatively translated forms of SGK-1 are also likely dependent on Hsp90 for phosphorylation.

In this study we have shown for the first time that the activity of SGK-1 requires Hsp90 binding. Through this interaction, Hsp90 aids in PI3K-dependent phosphorylation and activation of SGK-1 independently of increasing SGK-1 protein stability (Fig. 8). Interestingly, the requirement of Hsp90 to phosphorylate SGK-1 is not limited to the known conserved sites Thr-256 and Ser-422 but appears to include additional phosphorylated residues. Taken together with previous data on the role of Hsp90 in regulating Akt1 activity, our findings suggest that Hsp90 has an inhibitory effect on Akt1 phosphorylation while allowing SGK-1 phosphorylation. Because SGK-1 function appears to be an important component of the response of a cell to environmental stress, we hypothesize that rapid Hsp90-dependent SGK-1 activation (and concomitant Akt1 inactivation) may serve to fine-tune the relative activities of PI3K-regulated signaling, thereby optimizing the response of a cell to acute stress (Fig. 8).

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