Coordinated Regulation of Serum- and Glucocorticoid-inducible Kinase 3 by a C-terminal Hydrophobic Motif and Hsp90-Cdc37 Chaperone Complex*

Received for publication, September 16, 2013, and in revised form, December 30, 2013 Published, JBC Papers in Press, December 30, 2013 DOI 10.1074/jbc.M113.518480

Yuanzhong Wang†, Wanping Xu†, Dujin Zhou†, Len Neckers‡, and Shuan Chen†*

From the †Department of Cancer Biology, Beckman Research Institute of the City of Hope, Duarte, California 91010 and ‡Urologic Oncology Branch, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland 20892

The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a critical role in a variety of cellular processes including cell growth and survival. Aberrant PI3K signaling has been implicated in tumorigenesis and tumor progression (1). It is generally believed that dysregulation of PI3K signaling promotes tumorigenesis primarily through Akt. Strikingly, activation of Akt correlates weakly with activating mutations in the α catalytic subunit of PI3K (PIK3CA) in human cancers (2, 3). SGK3 is up-regulated in breast cancer, especially in estrogen receptor (ER)-positive breast cancer (7–10), and promotes estrogen-mediated cell survival (8, 11). Furthermore, Slagsvold et al. (12) reported that SGK3 attenuates the degradation of chemokine receptor CXC4 and promotes breast cancer metastasis.

Serum- and glucocorticoid-inducible kinase 3 (SGK3) mediates a variety of cellular processes including membrane transport, cell proliferation, and survival, and it has been implicated in Akt-independent signaling downstream of oncogenic PIK3CA mutations (activating mutations in the α catalytic subunit of PI3K) in human cancers. However, the regulation of SGK3 is poorly understood. Here we report that SGK3 stability and kinase activation are regulated by the Hsp90-Cdc37 chaperone complex. Hsp90-Cdc37 associates with the kinase domain of SGK3 and acts in concert with a C-terminal hydrophobic motif of SGK3 to prevent Hsp70 association and ubiquitin ligase CHIP (C terminus of Hsc70-interacting protein)-mediated degradation. Phosphorylation of hydrophobic motif triggers release of Cdc37 and concomitant association of 3-phosphoinositide dependent kinase 1 (PDK1) to activate SGK3. Our study provides new insights into regulation of SGK3 stability and activation and the rationale for application of Hsp90 inhibitors in treating SGK3-dependent cancers.

The phosphatidylinositol 3-kinase (PI3K) signaling pathway plays a critical role in a variety of cellular processes including cell growth and survival. Aberrant PI3K signaling has been implicated in tumorigenesis and tumor progression (1). It is generally believed that dysregulation of PI3K signaling promotes tumorigenesis primarily through Akt. Strikingly, activation of Akt correlates weakly with activating mutations in the α catalytic subunit of PI3K (PIK3CA) in breast and ovarian cancers (2, 3). Like many other members of the AGC kinase family (protein kinases A, G, and C), SGK3 requires phosphorylation of two sites for its full activation; one site (Ser-486) in the C-terminal HM and another site (Thr-320) in the activation loop of the kinase domain (16). Ser-486 is a direct substrate of 3-phosphoinositide-dependent kinase 1 (PDK1). In contrast to Akt, SGK3 lacks the pleckstrin homology domain but contains a phox homology domain that binds phosphatidylinositol 3'-monophosphate and targets SGK3 to the early endosome (14, 15).

SGK3 contains three distinguishable domains: a phox homology domain, a kinase domain, and a protein kinases AGC C-terminal domain/hydrophobic motif (HM). Like many other members of the AGC kinase family (protein kinases A, G, and C), SGK3 requires phosphorylation of two sites for its full activation; one site (Ser-486) in the C-terminal HM and another site (Thr-320) in the activation loop of the kinase domain (16). PDK1 phosphorylates SGK3 on Thr-320, whereas the kinase responsible for phosphorylation of Ser-486 is unknown. Tessier and Woodgett (16) have demonstrated that Ser-486 phosphorylation occurs after SGK3 is targeted to endosomes by its phox homology domain, and Ser-486 phosphorylation is a prerequisite for Thr-320 phosphorylation. However, mechanisms regulating SGK3 activation remain poorly understood.

The 90-kDa heat shock protein Hsp90 is a very abundant (~1–2% total cell protein) and highly conserved molecular chaperone complex.* This work was supported, in whole or in part, by National Institutes of Health Grant CA44735 (to S. C.). This work was also supported by a Carr Baird grant (to Y. W.) and by Hope Idol 2012 (to S. C.) and a ThinkCure grant (to S. C.).

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Significance:
The involvement of Hsp90-Cdc37 in SGK3 regulation provides a rationale for Hsp90 inhibition in treating SGK3-dependent cancers.

Conclusion:
Hsp90-Cdc37 complex is essential for SGK3 stability and activation.

Results:
Hsp90-Cdc37 chaperone complex acts in concert with the hydrophobic motif of SGK3 to regulate SGK3 stability and activation.

Background:
Serum- and glucocorticoid-inducible kinase 3 (SGK3) plays a pivotal role in Akt-independent oncogenic signaling.
chaperone involved in the conformational maturation of a specific set of clients participating in signal transduction, including protein kinases, steroid hormone receptors, and transcription factors (17). The chaperone is a validated anti-cancer molecular target with evidence of clinical activity in various cancers (18). Hsp90 function is regulated by ATP binding and hydrolysis and by a number of co-chaperones. Recruitment of protein kinases to Hsp90 is usually mediated by the co-chaperone Cdc37 (also known as p50 in mammals) (19, 20).

In the current study we report that an Hsp90-Cdc37 chaperone complex interacts with the SGK3 kinase domain and acts in concert with the HM of SGK3 to regulate SGK3 stability and activation. Furthermore, we show that Hsp90 inhibition abrogates the anti-estrogen resistance of breast cancer cells mediated by SGK3 overexpression. Our study provides new insights into regulation of SGK3 stability and activation, and it identifies Hsp90 inhibition as a novel strategy to treat SGK3-dependent cancers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T cells, MCF-7 cells, and T47D cells were routinely cultured in minimal Eagle’s medium (MEM) containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 100 units/ml penicillin-streptomycin. LNCaP cells were cultured in RPMI1640 medium containing 10% FBS. MCF-7 cells stably expressing wild-type or mutant SGK3 tagged with or without FLAG were generated by transient transfection with the relevant expression vectors and selected at 200 µg/ml hygromycin B for 1–2 months. These cells were maintained in normal growth medium with 50 µg/ml hygromycin B. CHIP−/− fibroblast cells were derived from CHIP (C terminus of Hsc70-interacting protein) knock-out mice (21), and CHIP+/+ fibroblast cells were generated from wild-type mice (22). Both fibroblast cell lines were maintained in DMEM medium with 10% FBS.

**Antibodies**—Anti-FLAG (M2), anti-HA, anti-FLAG M2 affinity gel, and EZview Red anti-HA affinity gel were purchased from Sigma. Antibodies to Hsp90α/β (H-114), Hsp/Hsc70 (H-300), CHIP (H-231), p62/SQSTM (D-3), and ERα (HC-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit antibodies to Hsp90, Cdc37 (D11A3), Akt, phospho-Akt (Ser-473), and phospho-Akt (Thr-308) (C31E5E), PDK1, phospho-PDK1 (Ser-241) (C49H2), SGK3, phospho-SGK3 (Thr-320) (D30E6), and ubiquitin were purchased from Cell Signaling Technology (Danvers, MA). Mouse monoclonal SGK3 antibody was purchased from AbD SeroTec (Oxford, UK). Horseradish peroxidase-coupled goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Thermo Scientific (Rockford, IL).

**Plasmid Constructs**—pMG-S GK3 vector was described previously (8). To generate pMG-S GK3 and pMG-S GK3-Cdc37, a FLAG tag-encoding sequence was added by PCR to the N terminus of the coding regions of SGK3 and Cdc37, respectively. The resulting DNA fragments were cloned into pMG-H2 vector using BglII-NheI restriction sites. To generate the pMG-S GK3 deletion mutant, DNA fragments coding SGK3 deletion mutants were generated by PCR using pMG-S GK3 as the template, and a FLAG tag-coding sequence was added at the N terminus by PCR. The resulting DNA fragments were then cloned into the BglII-NheI sites of pMG-H2. To generate pMG-S GK3/S486D and pMG-S GK3/S486A, the DNA fragments coding SGK3/S486D and SGK3/S486A were amplified using pMG-S GK3 as the template by a PCR-based site-directed mutagenesis method (QuickChange® site-directed mutagenesis kit, Stratagene) and subcloned into pMG through BglI and NheI sites, respectively. To generate pMG-CHIP, the coding sequence of CHIP was generated by RT-PCR using RNA from MCF-7 cells and then cloned into BglI-NheI restriction sites of pMG-H2 vector. pcDNA3-HA-Cdc37, pcDNA3-HA-Hsp90α, pcDNA3-CHIP-myc-His, pcDNA3-CHIP/K30A-myc-His, and pcDNA3-CHIP/H260Q-myc-His were described previously (21). All the inserts were verified by DNA sequencing.

**Mass Spectrometric Identification of SGK3-interacting Proteins**—293T cells were harvested 24 h after transfection with pMG-H2-FLAG or pMG-FLAG-SGK3 and lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol, 0.2% Nonidet P-40, 0.5 mM DTT, and protease inhibitor mixture). Cell extracts equal to 10 mg of protein in 1 ml of lysis buffer were incubated with 60 µl of anti-FLAG resin overnight at 4 °C on a rocking platform. Immunoprecipitates were washed 4 times with 1 ml of IP lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and complete protease inhibitor mixture). Bound proteins were released by incubating the washed beads in 50 µl of TBS buffer containing 150 µg/ml 3× FLAG peptide (Sigma) for 30 min at 25 °C followed by centrifugation at 1000 × g for 2 min. Five microliters of a 50-µl eluate was loaded on a precast mini-protein gel (Bio-Rad) for analysis by silver staining using the SilverQuest staining kit (Invitrogen). The remaining eluates were resolved on a precast gradient gel, and the gel was stained with the Colloidal Blue staining kit (Invitrogen) according to the manufacturer’s instruction. The gel sections above FLAG-SGK3 band or below the FLAG-SGK3 band were cut out from the stained gel and were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis in Taplin Biological Mass Spectrometry Facility at Harvard Medical School.

**Coimmunoprecipitation**—Cells cultured in 6- or 10-cm dishes were lysed in 800 µl or 1 ml of IP lysis buffer A (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and complete protease inhibitor mixture) or IP lysis buffer B (20 mM Hepes, pH 7.2, 100 mM NaCl, 1 mM MgCl2, 0.1% Nonidet P40, 10 mM molybdate, and complete protease inhibitor mixture) on ice for 50 min, respectively. The supernatants were collected by centrifugation at 14,000 rpm for 5 min. After pre-clearing with 30 µl of protein A- or G-agarose by incubation for 3 h at 4 °C on a rocking platform, an equivalent amount of each lysate was incubated with 50 µl of anti-FLAG resin overnight or incubated with antibodies for 1 h followed by the addition of 50 µl of protein G-agarose or protein A-agarose for immunoprecipitation overnight at 4 °C with rotation. Immunoprecipitates were washed 4 times with 900 µl of IP lysis buffer and boiled in SDS loading buffer for subsequent Western blot analysis.

**Western Blotting**—Cells were lysed in radioimmune precipitation lysis buffer (50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 0.5% sodium deoxycholate, and complete protease inhibitor mixture) on ice for 20 min and then sonicated for 30 s.
After centrifugation, the supernatants were collected and considered as the soluble fraction (in this paper, cell extracts refers to the soluble fraction of cell lysate unless otherwise indicated). The pellets were considered as the insoluble fraction and suspended in 5% SDS mixed with 2× sample loading buffer and boiled for 15 min. Protein concentration was quantified using the Bio-Rad Protein Assay. Immunoblotting was performed as described previously (23).

In Vitro Ubiquitination Assay—293T cells were transfected with FLAG-SGK3 expression vector for 24 h and lysed in radio-immune precipitation lysis buffer. FLAG-SGK3 was immunoprecipitated with anti-FLAG resin and served as the substrate of the ubiquitination assay. An in vitro ubiquitination assay was performed as previously described (21).

In Vitro Kinase Activity Assay—Twenty-four hours after 293T cells were transfected with either pMG-FLAG or pMG-FLAG-SGK3, the cells were pretreated with or without 1 µM 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) for 4 h and then treated with or without 50 ng/ml insulin-like growth factor 1 (IGF1) for 15 min. The cell extract equal to 5 mg of protein from each treatment was subjected to immunoprecipitation with anti-FLAG resin as described under “Mass Spectrometric Identification of SGK3-interacting Proteins” above. The immune complex was eluted from the washed beads with 30 µl of TBS buffer containing 150 µg/ml 3× FLAG peptide, and the kinase activity of SGK3, within the immune complex, was determined by in vitro kinase assay using PKB/Akt peptide substrate (CKRPRAASFAE) (SignalChem Co.) according to the activity assay protocol provided by SignalChem. Briefly, 10 µl of a total 30 µl eluate from each immunoprecipitate was incubated with 5 µg of Akt peptide substrate in a 30-µl kinase assay buffer (25 mM MOPS, pH 7.2, 12.5 mM β-glycerolphosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA, and 0.25 mM DTT) containing 0.1 mM ATP and 5 µCi of [γ-32P]ATP at 30 °C for 15 min. The kinase reaction mixture was spotted on P81 chromatography paper strip (Whatman); the P81 papers were washed at least 3 times, 10 min each, in 1% phosphoric acid with constant gentle stirring, and the extent of 32P incorporation was determined by liquid scintillation counting. Results are shown as the corrected cpm (subtract the blank control cpm) and as combined data from three independent experiments.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay—Cells were seeded into 24-well plates at 1 × 105/well. At the indicated time post-treatment, the medium in each well was removed and replaced with 0.5 ml of fresh warm phenol red-free medium containing 0.5 mg/ml MTT and incubated at 37 °C for 1 h. The medium was discarded, and 0.5 ml of DMSO was added to each well to dissolve the formazan dye trapped in the living cells. One hundred microliters of the supernatant were then transferred into a 96-well plate and read in the plate reader at A570.

Statistical Analysis—Data represent the mean ± S.D. of three independent experiments, and Student’s t test was used as the analysis method. A p value < 0.01 was considered statistically significant.

RESULTS

SGK3 Forms a Ternary Complex with Hsp90 and Cdc37—To gain insight into SGK3 regulation, we used an unbiased proteomics approach to identify SGK3-interacting proteins. As shown in Fig. 1A and Table 1, Hsp90 (including two isoforms: Hsp90α and Hsp90β) and its co-chaperone Cdc37 were identified as SGK3-interacting proteins by LC/MS after immunoprecipitation of FLAG tagged-SGK3 from 293T cells. Their interaction was confirmed by several coimmunoprecipitation assays. As shown in Fig. 1B, both Hsp90 and Cdc37 were coimmunoprecipitated by anti-FLAG resin from extracts of MCF-7 cells stably expressing FLAG-SGK3 but not from the extracts of cells that did not express FLAG-SGK3. FLAG-Cdc37 pulled down endogenous SGK3 and Hsp90 from lysates of LNCaP cells expressing FLAG-Cdc37 (Fig. 1C) and hemagglutinin (HA)-tagged Hsp90α coimmunoprecipitated with endogenous SGK3 and Cdc37 from extracts of LNCaP cells expressing HA-Hsp90α (Fig. 1D). To demonstrate endogenous interaction of SGK3 with Hsp90 and Cdc37, we immunoprecipitated the kinase from T47D cells (which express high level of SGK3). As shown in Fig. 1E, both Hsp90 and Cdc37 were coimmunoprecipitated with anti-SGK3 antibody but not with control IgG, confirming that these proteins associate specifically under native conditions.

Hsp90-Cdc37 Chaperone Complex Protects SGK3 from Proteasomal Degradation—To explore the biological relevance of SGK3 interaction with the Hsp90-Cdc37 chaperone complex, we first knocked down Hsp90 or Cdc37 using small interfering RNA (siRNA) in T47D cells and in MCF-7 cells stably expressing FLAG-SGK3 (Fig. 1C) and hemagglutinin (HA)-tagged Hsp90α coimmunoprecipitated with endogenous SGK3 and Cdc37 from extracts of LNCaP cells expressing HA-Hsp90α (Fig. 1D). To demonstrate endogenous interaction of SGK3 with Hsp90 and Cdc37, we immunoprecipitated the kinase from T47D cells (which express high level of SGK3). As shown in Fig. 1E, both Hsp90 and Cdc37 were coimmunoprecipitated with anti-SGK3 antibody but not with control IgG, confirming that these proteins associate specifically under native conditions.

CHIP Mediates SGK3 Ubiquitination and Proteasomal Degradation—The CHIP is a U-box-containing E3 ubiquitin ligase that binds to Hsp90 and Hsp70 through its tetratricopeptide repeat domain. CHIP has been shown to mediate degradation of some Hsp90 client proteins such as ERα (24), glucocorticoid receptor (25), and ErbB2 (21, 26). To test whether CHIP targets SGK3 for proteasomal degradation, we first examined whether alteration of CHIP expression affects ectopically expressed SGK3 levels. As shown in Fig. 3, A and B, knockdown of CHIP increased, whereas overexpression of CHIP decreased FLAG-SGK3 levels, implying that CHIP regulates SGK3 stability. Previous studies have shown that both the tetratricopeptide...
repeat domain and U-box domain, which mediates the ubiquitination reaction, are indispensable for the physiological function of CHIP (21). As shown in Fig. 3B, unlike wild-type CHIP, overexpression of either the tetratricopeptide repeat domain mutant CHIP/K30A or the U-box mutant CHIP/H260Q had little effect on the FLAG-SGK3 level, suggesting that both the association with Hsp70/Hsp90 and E3 ligase activity are required for CHIP to promote SGK3 degradation. Consistent with this result, coexpression of wild-type CHIP, but not K30A or H260Q mutants, with FLAG-SGK3 caused an increase in SGK3 ubiquitination (Fig. 3C). Immunoprecipitation confirmed the association of wild-type and H260Q CHIP, but not K30A CHIP, with SGK3 (Fig. 3C), demonstrating that CHIP associates with SGK3 through its tetratricopeptide repeat domain interaction with Hsp70/Hsp90. An in vitro ubiquitination assay further verified that CHIP ubiquitinates SGK3 (Fig. 3D).

The involvement of CHIP in Hsp90 inhibition-induced SGK3 proteasomal degradation was demonstrated by examining the protein complexes after drug treatment. We showed that 17-DMAG promoted SGK3 dissociation from an Hsp90-Cdc37 chaperone complex while favoring association with an Hsp70-CHIP complex (Fig. 3E), suggesting that chaperone binding to SGK3 is remodeled by Hsp90 inhibitor before SGK3 degradation. Furthermore, overexpression of CHIP significantly accelerated 17-DMAG-induced FLAG-SGK3 degradation (Fig. 3F), whereas knockdown of CHIP attenuated 17-DMAG-induced degradation of endogenous SGK3 (Fig. 3G). Collectively, these data suggest that an Hsp70-CHIP complex mediates Hsp90 inhibitor-induced SGK3 degradation.
**SGK3 Interacts with the Chaperones via Its Kinase Domain**—To provide the molecular basis for interaction of SGK3 with these competing chaperone complexes, we generated a series of plasmids expressing FLAG-tagged SGK3 deletion mutants (Fig. 4A). The expression levels of these deletion mutants varied in 293T cells, although equivalent amounts of the expression vectors were transfected into cells, which implies that these deletion mutants are different in their stability and/or solubility in a cell. Coimmunoprecipitation with anti-FLAG resin revealed that residues 162–320 in the kinase domain were sufficient for interaction with both Hsp90-Cdc37 and Hsp70-CHIP (Fig. 4, B and C). Notably, residues 1–240 or 162–240 could bind Hsp90, Hsp70, and CHIP but not Cdc37, whereas residues 1–320 or 162–320 could interact with all components of these chaperone complexes (Fig. 4, B and C, and data not shown), suggesting that residues 241–320 are required for Cdc37 binding. Interestingly, an HM truncation mutant SGK3ΔHM (residues 1–419) bound many more chaperones (especially Cdc7 and CHIP) than wild-type SGK3 (Fig. 4B), indicating that an intact HM domain regulates SGK3 interactions with these chaperones.

The HM Domain Is Required for SGK3 Stability and Acts in Concert with Hsp90-Cdc37 to Protect SGK3 from CHIP-mediated Degradation—We noticed that SGK3ΔHM is less stable than wild-type SGK3 (Fig. 5A), although it binds more Hsp90 and Cdc37, and the proteasome inhibitor MG132 blocked its degradation (Fig. 5B). Consistent with these results, SGK3ΔHM had higher steady-state ubiquitination levels compared with the wild-type SGK3 (Fig. 5C), suggesting that the HM domain is critical for SGK3 stability and protects SGK3 from proteasomal degradation. Interestingly, 17-DMAG did not cause any additional increase in ubiquitination and degradation of SGK3ΔHM (Fig. 5, C and D), although it rapidly disrupted the interaction between SGK3ΔHM and the Hsp90-Cdc37 complex (Fig. 5E). The data suggest that the interaction with Hsp90-Cdc37 complex does not stabilize SGK3ΔHM; in other words, the HM domain is required for SGK3 to be stabilized by Hsp90-Cdc37. Consistent with these observations and in contrast to wild-type SGK3, 17-DMAG did not promote further interaction of SGK3ΔHM with the Hsp70-CHIP complex, further supporting that the HM domain regulates SGK3 interaction with these chaperones, and Hsp90 inhibition does not induce CHIP-dependent SGK3ΔHM degradation. Given that SGK3ΔHM is prone to degradation and displays a dramatic increase in CHIP binding in the absence of Hsp90 inhibition, we hypothesized that the HM domain also helps stabilize SGK3 by preventing de-regulated CHIP-mediated ubiquitination. Supporting this hypothesis, SGK3ΔHM is much more stable in CHIP−/− cells than in CHIP+/+ cells (Fig. 5F). Together, these data suggest that the HM domain and Hsp90-Cdc37 complex coordinately regulate SGK3 stability by protecting SGK3 from CHIP-mediated proteasomal degradation.

**SGK3 Phosphorylation and Activation Require Hsp90 Activity**—SGK3 becomes fully activated after being phosphorylated on both Ser-486 in the HM domain and Thr-320 in the activation loop, and Ser-486 phosphorylation is a prerequisite for phosphorylation of Thr-320 by PDK1 (16, 27). As the Hsp90-Cdc37 complex interacts with the SGK3 kinase domain, we asked whether the chaperones are involved in SGK3 activation. Because it has been reported that IGF1 activates SGK3 (13, 14), we first examined whether Hsp90 inhibition affects IGF1-induced SGK3 phosphorylation. Fig. 6A showed that IGF1 significantly increased SGK3 Thr-320 phosphorylation, and this effect could be blocked by pretreatment with 17-DMAG for 4 h in T47D cells. Similar results were obtained in MCF-7 cells.
overexpressing SGK3 (MCF-7/SGK3) (Fig. 6B). In contrast, IGF1 robustly induced Akt degradation in addition to phosphorylation (Fig. 6, A and B). Moreover, 17-DMAG pretreatment did not inhibit IGF1-induced Akt phosphorylation on either Thr-308 or Ser-473, although Akt Thr-308 phosphorylation is also mediated by PDK1. These data suggest that 17-DMAG specifically inhibits SGK3 phosphorylation by disrupting Hsp90/Cdc37 association. To further confirm this result, we pretreated 293T cells expressing FLAG-SGK3 with 17-DMAG for 4 h before adding IGF1 for 15 min. FLAG-SGK3 was immunoprecipitated from cell lysates with anti-FLAG resin and subjected to in vitro kinase assay. As shown in Fig. 6C, IGF1 significantly increased SGK3 kinase activity, and this effect was blocked by pretreatment with 17-DMAG, confirming that Hsp90 association is required for PI3K-dependent SGK3 activation.

The HM Domain Phosphorylation Promotes Release of Cdc37, Leading to PDK1-mediated SGK3 Phosphorylation in the Activation Loop—The requirement of Hsp90 for SGK3 phosphorylation and the regulatory role of the HM domain in chaperone association raised the possibility that SGK3 activation is regulated coordinately by the HM domain and the Hsp90-Cdc37 chaperone complex. To address this question, we examined chaperone interaction with SGK3 Ser-486 mutants (S486D and S486A). As predicted, the phospho-mimic S486D mutant was hyper-phosphorylated on Ser-320, and the unphosphorylatable S486A mutant was hypophosphorylated on this residue compared with wild-type SGK3 (Fig. 7A). Not surprisingly, more activated PDK1 was found to be associated with SGK3/S486D mutant (Fig. 7B). Interestingly, although both mutants bound comparable levels of Hsp90, SGK3/S486D bound significantly less Cdc37 (Fig. 7, B and C), suggesting that

**FIGURE 3.** CHIP mediates SGK3 proteasomal degradation. A, MCF-7/FLAG-SGK3 cells were transfected with siRNA negative control or CHIP siRNA for 72 h. The cell extracts were immunoblotted with the relevant antibodies. B, MCF-7/FLAG-SGK3 cells were transfected with empty vector or vectors expressing either wild-type or mutant CHIP for 48 h. The cell extracts were immunoblotted with anti-FLAG, anti-CHIP, and anti-β-actin antibodies. C, 293T cells were cotransfected with the vector expressing FLAG-SGK3 and the empty vector or the vector expressing either wild-type or mutant CHIP tagged with myc-His for 40 h. Cell extracts were immunoprecipitated with anti-FLAG resin, and the precipitated proteins as well as whole cell extracts (WCE) were immunoblotted (IB) with the indicated antibodies. D, *in vitro* ubiquitination assay of SGK3 by CHIP. E, 293T cells transfected with empty vector or FLAG-SGK3 expression vector were treated with or without 2 μM 17-DMAG for the indicated times. The cell extracts were immunoprecipitated with anti-FLAG resin and the precipitated proteins were immunoblotted with the relevant antibodies. F, MCF-7/FLAG-SGK3 cells were transfected with empty vector or CHIP expression vector for 40 h and then treated with 1 μM 17-DMAG for the indicated time. The cell extracts were immunoblotted for SGK3, CHIP, and β-actin (upper panel). The band intensities of SGK3 were quantitated using NIH ImageJ 1.47 software and normalized to β-actin. Quantitation of the results was shown in the lower panel. G, MCF-7 cells were transfected with siRNA negative control or CHIP siRNA for 72 h and then treated with 0.5 μM 17-DMAG for the indicated times. The cell extracts were immunoblotted with the relevant antibodies (upper panel). Quantitation of the results was shown in the lower panel.
Cdc37 interaction might negatively regulate Thr-320 phosphorylation. Indeed, an inhibitory role of Cdc37 in SGK3 Thr-320 phosphorylation was confirmed by siRNA-mediated specific knockdown where Thr-320 was phosphorylated to such a high level that it could not be further increased by IGF1 stimulation (Fig. 7D). These data support a model in which SGK3 phosphorylation on Ser-486 promotes Cdc37 dissociation, allowing Hsp90 to facilitate PDK1 association and subsequent Thr-320 phosphorylation leading to SGK3 activation (Fig. 7E).

**DISCUSSION**

SGK3 is a member of the SGK family, which also includes SGK1 and SGK2. Their amino acid sequences share extensive identity (~80%) in the kinase domain but vary significantly in the N- and C-terminal regions (13). SGK1 is an unstable protein with a half-life of 30 min, and the N terminus of SGK1 serves as a signal for ubiquitination and subsequent proteasomal degradation (29–32). However, very little is known about factors affecting the stability and degradation of SGK2 and SGK3. Here we have shown that, in contrast to SGK1, SGK3 is stable. We found that this stability results from a HM in the C terminus and constitutive association of SGK3 with an Hsp90-Cdc37 chaperone complex, both of which protect the kinase from CHIP-mediated proteasomal degradation.

17-DMAG Abrogates Anti-estrogen Resistance Conferred by SGK3 Overexpression in Breast Cancer Cells—We have shown previously that overexpression of SGK3 confers ER-positive MCF-7 breast cancer cells with resistance to the anti-estrogen ICI 182,780 (8). Because we demonstrated above that Hsp90 inhibition results in inactivation and depletion of SGK3, we tested whether Hsp90 inhibitor could abrogate SGK3-mediated anti-estrogen resistance. For this purpose we used another ER-positive breast cancer cell line, T47D, to generate cells with doxycycline-inducible SGK3 expression, designated T47D/TetOnSGK3. As shown in Fig. 8A, the addition of doxycycline induced high expression of SGK3 and increased phosphorylation of glycogen synthase kinase 3β (GSK3β), which is a known SGK3 substrate (28). Consistent with our previous study using MCF-7 cells (8), induction of SGK3 expression by doxycycline conferred resistance to ICI182,780 in T47D/TetOnSGK3 cells but did not affect cell growth in the absence of ICI182,780 (Fig. 8B). 17-DMAG treatment dramatically decreased doxycycline-induced SGK3 expression and depleted phospho-SGK3 as well as phospho-GSK3β (Fig. 8A). Moreover, Hsp90 inhibition totally abrogated ICI182,780 resistance resulting from SGK3 induction (Fig. 8B).
Hsp90 and Cdc37 were identified by mass spectrometry of cellular SGK3 immunoprecipitates and were confirmed to interact with SGK3 under native conditions, suggesting the importance of these chaperones in the normal function of SGK3. Of note, SGK3 was recently identified in a high throughput screen of Hsp90-interacting proteins (20). In the current study we demonstrated that SGK3 binds to Hsp90-Cdc37 via its kinase domain, and silencing of either Hsp90 or Cdc37 negatively affects SGK3 stability. Furthermore, we identified a region in the small lobe of the SGK3 kinase domain to be involved in interacting with both chaperones, although a deletion mutant (amino acids 1–240 or 161–240) that completely lost Cdc37 association retained weak Hsp90 association. These findings suggest that Hsp90 and Cdc37 bind to SGK3 via overlapping but distinct motifs. Indeed, for other Hsp90-dependent kinases, the ATP binding loop in the small lobe of the kinase domain was shown to be important for Cdc37 association (33), whereas a loop between the α-C helix and β4 strand in the small lobe was shown to be required for association of Hsp90 (34). We found that SGK3 phosphorylation on Ser-486 in the HM domain diminishes Cdc37 association without affecting Hsp90 interaction. We believe that such distinct modulation of the binding of the two chaperones by phosphorylation of the HM domain creates a mechanism for SGK3 to be activated by upstream effectors, as will be discussed below.

It has been postulated that Hsp90 protects client proteins from degradation by preventing the interaction of Hsp70. Although switching from an Hsp90 complex to an Hsp70 complex in the presence of Hsp90 inhibitor has been observed for many Hsp90 clients, it is not clear whether the two complexes interact with the client in overlapping or distinct regions. Here we showed that Hsp70 binds to a similar but expanded region on SGK3 compared with Hsp90. Both chaperones interact with the small lobe of the kinase domain, but Hsp70 also interacts with an SGK3 deletion mutant (amino acids 241–496) that does not interact with Hsp90. The existence of shared interaction regions for Hsp90 and Hsp70 on SGK3 supports a model in which Hsp90 protects its clients from Hsp70 association by steric hindrance. More importantly, we demonstrated that SGK3 interaction with chaperones is further regulated by its HM. We found that HM deletion dramatically increased the association of SGK3 with Hsp70-CHIP, suggesting that the HM interaction.

**FIGURE 5.** The HM domain is required for SGK3 stability and acts in concert with Hsp90-Cdc37 to protect SGK3 from CHIP-mediated degradation. **A,** 293T cells expressing either FLAG-SGK3 or FLAG-SGK3HM were treated with 100 µg/ml cycloheximide (CHX) for the indicated times. The cell extracts were immunoblotted with anti-FLAG and anti-β-actin antibodies. **B,** 293T cells expressing FLAG-SGK3HM were treated with or without 100 µg/ml cycloheximide alone or plus 15 µM MG132 for 11 h. The same amount of cell extracts was immunoblotted with the relevant antibodies. **C,** 293T cells expressing either FLAG-SGK3 or FLAG-SGK3HM were treated with 15 µM MG132 alone or plus 2 µM 17-DMAG for the indicated times. The cell extracts were immunoprecipitated with anti-FLAG resin, and the precipitated proteins were immunoblotted with anti-ubiquitin (Ub) and anti-FLAG antibodies. **D,** 293T cells expressing either FLAG-SGK3 or FLAG-SGK3HM were treated with the increasing concentrations of 17-DMAG for 24 h. The cell extracts were immunoblotted with the relevant antibodies. **E,** 293T cells were transfected with empty vector or FLAG-SGK3HM expression vector for 24 h and then treated with 2 µM 17-DMAG for the indicated times. The cell extracts were immunoprecipitated with anti-FLAG resin, and the precipitated proteins were immunoblotted with the indicated antibodies. **F,** CHIP−/− fibroblast cells and CHIP+/+ fibroblast cells were transfected with the same amount of FLAG-SGK3HM expression vector for 30 h and then treated with 100 µg/ml cycloheximide for the indicated time. The cell extracts were immunoblotted with the relevant antibodies.
domain also helps mask the Hsp70-CHIP- interacting region on SGK3.

Hsp70 promotes protein degradation by recruiting CHIP, an E3 ubiquitin ligase. We found that SGK3 interacts with Hsp70 as well as CHIP upon Hsp90 inhibition. We also showed that CHIP association is via Hsp70, as the CHIP/K30A, which loses interaction with Hsp70, was not detected in SGK3 immunoprecipitates. CHIP mediates the ubiquitination and eventual degradation of SGK3, supported by the following evidence: first, increased SGK3 ubiquitination occurred in parallel with elevated association of wild-type CHIP; second, mutant CHIP lacking E3 ligase activity failed to induce SGK3 ubiquitination; third, wild-type CHIP ubiquitinated SGK3 in vitro; fourth, the unstable deletion mutant SGKΔHM was expressed to a significantly higher level in CHIP−/− cells than that in CHIP+/+ cells.

Importantly, the Hsp90 chaperone complex not only maintains SGK3 stability but also regulates its activation. IGF1 failed to induce SGK3 activation in the presence of 17-DMAG, evidenced by no increase in either Thr-320 phosphorylation or its kinase activity. This indicates that normal Hsp90 function is required for SGK3 activation by IGF1. Surprisingly, Cdc37 functions as a negative regulator of SGK3 activation, as steady-state Thr-320 was phosphorylated to higher levels upon Cdc37 silencing, and under these conditions Thr-320 phosphorylation was not further increased by IGF1. Cdc37 likely inhibits SGK3 activation by blocking PDK1 association and/or hindering access to Thr-320, which is contained within the Cdc37-interacting motif of SGK3. We found that the phosphomimic S486D mutant interacts normally with Hsp90 but substantially less with Cdc37, with a concomitant increase in the association of activated PDK1. Because PDK1 binding is facilitated by Ser-486 phosphorylation (16, 27), our findings suggest a model in which Ser-486 phosphorylation triggers the release of Cdc37 from the heterotrimer SGK3-Hsp90-Cdc37, allowing access to PDK1, which catalyzes Thr-320 phosphorylation in the activation loop of the kinase domain, leading to SGK3 activation (as depicted in Fig. 7E).

In the current study both Hsp90α and Hsp90β were identified by mass spectrometry of cellular SGK3 immunoprecipitates. We have not compared their functional difference in reg-
ulation of SGK3. It is likely that both isoforms are involved in regulation of SGK3 stability and activation, as they exhibit similar interactions with cochaperones and have functional similarities. It should be noted that in addition to stability and activation, Hsp90 inhibition might reduce SGK3 transcription, as SGK3 is transcriptionally regulated by ERα (8), a known Hsp90 client protein. Through mass spectrometry of cellular SGK3 immunoprecipitates, we also identified several other proteins (e.g. SLC25A11) that have not been reported to interact with SGK3, but their interactions with SGK3 need to be further verified.

Taken together, our data provide a model in which SGK3 stability and activation are coordinately regulated by its HM and Hsp90-Cdc37. Constitutive association of the kinase domain with the chaperones Hsp90 and Cdc37 stabilizes SGK3 conformation, and the HM domain folds back to mask the kinase domain and block interaction with Hsp70/CHIP. HM domain phosphorylation on Ser-486 triggers Cdc37 release, allowing PDK1 to bind. PDK1 association, which is likely facilitated by Hsp90, results in Thr-320 phosphorylation and SGK3 activation. Hsp90 inhibition dissociates the Hsp90-Cdc37 complex, promoting the interaction of SGK3 with Hsp70 and the E3 ligase CHIP, which ubiquitinates SGK3, leading to its proteasome-mediated degradation. Thus, our findings show that SGK3 interaction with the Hsp90-Cdc37 chaperone complex serves a dual purpose, that of maintaining SGK3 stability and modulating its activation. Given its role as an Akt-independent oncogenic kinase, inhibiting the stability and activation of SGK3 as a consequence of Hsp90 inhibition represents a potentially attractive approach to treating SGK3-dependent cancers.

FIGURE 7. HM phosphorylation promotes release of Cdc37, leading to PDK1-mediated SGK3 phosphorylation in the activation loop. A, MCF-7 cells stably transfected with empty vector (Vec) or the vectors expressing wild-type or mutant SGK3 were treated with or without 100 ng/ml IGF-1 for 1 h. The same amount of cell extracts was immunoblotted with the relevant antibodies. B, 293T cells were transfected with empty vector or the vectors expressing FLAG tagged wild-type or mutant SGK3 for 24 h. Cell extracts were immunoprecipitated with anti-FLAG resin and the precipitated proteins were immunoblotted (IB) with the relevant antibodies. WCE, whole cell extracts. C, 293T cells were cotransfected with HA-Cdc37 expression vector and either empty vector or the vectors expressing FLAG-tagged wild-type or mutant SGK3. Cell extracts were immunoprecipitated with anti-HA resin, and the precipitated proteins were immunoblotted with anti-FLAG, anti-Hsp90, and anti-HA antibodies. The same amount of whole cell extracts were immunoblotted to evaluate the expression levels of HA-Cdc37 and FLAG-tagged wild-type or mutant SGK3 in these transfected cells. D, MCF-7/SGK3 cells were transfected with siRNA negative control (NC) or Cdc37 siRNA for 72 h and then treated with or without 100 ng/ml IGF1 for 30 min. The cell extracts were immunoblotted with the relevant antibodies. E, the diagram of the proposed model for regulation of SGK3 activation by Hsp90-Cdc37. KD, kinase domain.
proliferation was measured by the MTT assay. Dox - +     +

FIGURE 8. 17-DMAG abrogates anti-estrogen resistance conferred by SGK3 overexpression. A, T47D/TetON/SGK3 cells were added with or without 500 ng/ml doxycycline (Dox) alone or plus 1 μM 17-DMAG for 48 h. Cell extracts were immunoblotted with the indicated antibodies. B, T47D/TetON/SGK3 cells were grown with or without 500 ng/ml doxycycline and then treated with or without 100 nm ICI 182,780 alone or plus 1 μM 17-DMAG for the indicated times. Cell proliferation was measured by the MTT assay. Error bars represent standard deviation of three independent experiments.

Acknowledgments—We thank Dr. Ross Tomaino in Taplin Mass Spectrometry Facility at Harvard Medical School for mass spectral analysis of SGK3-interacting proteins and Karineh Petrossian for English proofreading of the manuscript.

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