Ubiquitin–proteasome degradation of serum- and glucocorticoid-regulated kinase-1 (SGK-1) is mediated by the chaperone-dependent E3 ligase CHIP

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SGK-1 (serum- and glucocorticoid-regulated kinase-1) is a stress-induced serine/threonine kinase that is phosphorylated and activated downstream of PI3K (phosphoinositide 3-kinase). SGK-1 plays a critical role in insulin signalling, cation transport and cell survival. *SGK-1* mRNA expression is transiently induced following cellular stress, and SGK-1 protein levels are tightly regulated by rapid proteasomal degradation. In the present study we report that SGK-1 forms a complex with the stress-associated E3 ligase CHIP [C-terminus of Hsc (heat-shock cognate protein) 70-interacting protein]; CHIP is required for both the ubiquitin modification and rapid proteasomal degradation of SGK-1. We

also show that CHIP co-localizes with SGK-1 at or near the endoplasmic reticulum. CHIP-mediated regulation of SGK-1 steady-state levels alters SGK-1 kinase activity. These data suggest a model that integrates CHIP function with regulation of the PI3K/SGK-1 pathway in the stress response.

Key words: cell survival, C-terminus of Hsc70 interacting protein (CHIP) E3 ligase, serum and glucocorticoid-regulated kinase-1 (SGK-1), stress response, phosphoinositide 3-kinase (PI3K), ubiquitination.

INTRODUCTION

SGK-1 (serum- and glucocorticoid-regulated kinase-1) is an ‘AGC-family’ serine/threonine kinase that is regulated primarily by transcriptional induction following various environmental stimuli, as well as by post-translational phosphorylation, ubiquitin-modification and proteasomal degradation (reviewed in [1]). Recent genetic experiments in *Caenorhabditis elegans* have demonstrated that SGK-1 is also a critical mediator of insulin signalling via phosphorylation and inactivation of the FOXO3a homologue DAF-16 (decay-accelerating factor 16) [2,3]. SGK-1 mediates a potent survival signal downstream of PI3K (phosphoinositide 3-kinase) activation and is required for glucocorticoid-mediated breast epithelial cell survival [4,5]. In contrast with Akt-1 (protein kinase B), with which SGK-1 shares approx. 50 % amino acid identity in its kinase domain, SGK-1 does not appear to require plasma membrane targeting for activation; rather, SGK-1 is phosphorylated downstream of endogenous PI3K activity regardless of its subcellular location [6]. Therefore the steady-state levels of SGK-1 determine its overall kinase activity and the rapid turnover of SGK-1 via proteasomal degradation plays an important role in limiting SGK-1 activity following stress-induced transcription [7].

Recently, the chaperone-dependent U-box ubiquitin ligase CHIP [C-terminus of Hsc (heat-shock cognate protein) 70-interacting protein] was identified as a protein quality control E3 ligase, whose role along with the Hsps (heat-shock proteins) is to facilitate proper folding of client proteins [8,9]. Either CHIP overexpression or underexpression can cause a defective response to stress, suggesting that CHIP activity is critical for cellular tolerance to adverse conditions. For example, CHIP overexpression in recombinant *Arabidopsis* leads to slowed growth at extremely high and low temperatures [10]. CHIP knock-

out mice show temperature sensitivity manifested by apoptosis in multiple organs after exposure to a temperature of 42 °C [11]. In *C. elegans*, CHIP overexpression causes embryonic lethality, and either CHIP knockout or knockdown causes developmental arrest [12]. In summary, adequate CHIP activity functions to balance proper folding of substrate proteins with their degradation and is important in the cell’s adaptation to environmental challenges. CHIP has also been implicated as an E3 ligase contributing to the ubiquitination and degradation of the androgen receptor [13], glucocorticoid receptor [14], ErbB2 [15] and the transcription factor E2A [16]. In the present study we demonstrate that CHIP forms a complex with SGK-1 resulting in ubiquitin modification and the proteasome-dependent degradation of SGK-1. The results suggest a model in which SGK-1 activity is regulated, at least in part, by CHIP-dependent protein turnover.

EXPERIMENTAL

Cell lines and plasmids

SK-BR-3 and MDA-MB-231 mammary epithelial cancer cell lines, and wild-type (CHIP^{+/+}) and CHIP knockout (CHIP^{-/-}) mouse fibroblasts immortalized with SV40 (simian virus 40) large T-antigen were cultured as described previously [7,11]. Plasmids pLPCX/SGK-1-FLAG, pcDNA3.1/WT (WT, wild-type), H260Q, K30A CHIP-Myc, pSiren/CHIP siRNA (small interfering RNA) and pRBG4/HA-ubiquitin have been described previously [7,15].

Transfections

SK-BR-3 cells were transiently co-transfected with the vectors pLPCX/SGK-1-FLAG, pcDNA3.1/CHIP-Myc, pLPCX,

Abbreviations used: ALLN, *N*-acetyl-L-leucyl-L-leucylnorleucinal; CFTR, cystic fibrosis transmembrane conductance regulator; Hsc, heat-shock cognate protein; CHIP, C-terminus of Hsc70-interacting protein; C_t, threshold cycle; ER, endoplasmic reticulum; GFP, green fluorescent protein; HRP, horseradish peroxidase; Hsp, heat-shock protein; PI3K, phosphoinositide 3-kinase; SGK-1, serum- and glucocorticoid-regulated kinase-1; siRNA, small interfering RNA; SV40, simian virus 40; TPR, tetratricopeptide repeat; WT, wild-type.

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pcDNA3.1 and/or pRBG4/HA-ubiquitin. For some experiments, SK-BR-3 cells stably expressing SGK-1-FLAG and MDA-MB-231 cells stably expressing H260Q CHIP-Myc were selected with puromycin and neomycin respectively. In the experiments with various amounts of SGK-1-FLAG or CHIP-Myc, a relevant empty vector was used to supplement the total DNA amount. In some experiments, ALLN (a peptide aldehyde, *N*-acetyl-L-leucyl-L-leucylnorleucinal; 10 μ M) or vehicle (ethanol) was added overnight 36 h post-transfection.

Western blot analysis and immunoprecipitation

For Western blot analysis, cells were lysed in SDS/PAGE sample buffer, and equal amounts of lysates were separated by SDS/PAGE (12 % gels). Resolved proteins were then immunoblotted with anti-FLAG-HRP (horseradish peroxidase; Sigma), anti-Myc-HRP (Santa Cruz Biotechnology), anti-SGK-1-HRP [5] (conjugated using the EZ link conjugation kit; Pierce), anti-Akt-1 (Cell Signaling Technology), anti-phospho-Akt-1 (S473; Cell Signaling Technology) or anti-CHIP antibodies (a gift from Dr L. Petrucelli and Dr M. Hutton, Mayo Clinic, Jacksonville, FL, U.S.A.). An anti-rabbit HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology) was used. Primary and HRP-conjugated antibodies were diluted 1:1000. An anti-tubulin antibody (Calbiochem) was used at 1:10 000, followed by an anti-mouse HRP-conjugated secondary antibody diluted 1:10 000 (Santa Cruz Biotechnology).

SGK-1-FLAG immunoprecipitation was performed using equal amounts of cell lysates as described previously [7] with minor modifications. Cells were lysed in a buffer containing 50 mM Tris/HCl (pH 7.5), 250 mM NaCl, 10 mM EDTA and 2 % (v/v) Triton X-100; anti-FLAG M2-agarose (Sigma) was coated with an excess of BSA prior to immunoprecipitation. Precipitated proteins were eluted with 0.5 mg/ml FLAG-peptide (Sigma), resolved on SDS/PAGE (10 % gels), and immunoblotted with anti-FLAG-HRP, anti-Myc-HRP, anti-CHIP (Calbiochem), anti-SGK-1 or anti-Hsp70 (Stressgen) antibodies (1:1000) followed by either anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology). CHIP immunoprecipitation was performed essentially as described above except Protein G-agarose (Invitrogen) coated with anti-CHIP (Calbiochem) or rabbit IgG (Sigma) antibodies was used for immunoprecipitation. Immunoblotting was performed with anti-FLAG-HRP and anti-CHIP antibodies followed by an anti-rabbit HRP-conjugated secondary antibody.

Immunofluorescence

At 48 h post-transfection with a plasmid encoding SGK-1 fused to GFP (green fluorescent protein), CHIP-Myc and/or an ER (endoplasmic reticulum) marker pDsRed2-ER (Clontech), COS-7 cells were seeded on coverslips and treated with ALLN (10 μ M) overnight. Cells were then fixed in 4 % paraformaldehyde, blocked with 1 % foetal calf serum and permeabilized with 0.01 % saponin as described previously [4]. SGK-1 or DsRed2-ER was then detected directly using GFP or DsRed2 fluorescence. CHIP-Myc was detected using an anti-Myc antibody (1:100; Santa Cruz Biotechnology) followed by an anti-mouse Alexa Fluor[®] 488-conjugated secondary antibody (1:1500, green; Molecular Probes). Endogenous SGK-1 was detected using a C-terminus chicken anti-SGK-1 antibody (1:100; US Biologicals) followed by an anti-chicken Alexa Fluor[®] 488-conjugated secondary antibody (1:1500). Endogenous CHIP was detected using a rabbit anti-CHIP antibody (1:100; Calbiochem) followed by an anti-rabbit Alexa Fluor[®] 488-conjugated secondary antibody (1:1500). Cells were then examined at 630 $\times$ magnification with a

Zeiss Axiovert 200 fluorescent microscope, using a cooled Orca ER digital camera (Hamamatsu). Images were captured and analysed using Openlab[®] software. For co-localization studies, two approaches were used. First, SGK-1-GFP and CHIP-Myc were detected as described above, except that the anti-Myc antibody was followed by an anti-mouse Alexa Fluor[®] 568-conjugated secondary antibody (1:1500, red). Secondly, endogenous SGK-1 and CHIP were detected using chicken anti-SGK-1 (1:100) and rabbit anti-CHIP (1:100) antibodies followed by an anti-chicken Alexa Fluor[®] 488-conjugated secondary antibody (1:1500) or an anti-rabbit Alexa Fluor[®] 568-conjugated secondary antibody (1:1500) respectively.

In vivo ubiquitination assay

SK-BR-3 cells stably expressing SGK-1-FLAG were transfected with plasmids encoding HA-ubiquitin (1 μ g) and WT CHIP-Myc (0.25–1 μ g). SGK-1 was immunoprecipitated from equal amounts of protein lysate as described previously [7]. SGK-1 species were then immunoblotted with either anti-HA or anti-SGK-1 HRP-conjugated antibodies. CHIP expression in the original cell lysates was detected using Western blotting with the anti-Myc-HRP antibody (1:1000).

In vitro kinase assay

The *in vitro* kinase activity of SGK-1-FLAG expressed either in the presence of WT CHIP-Myc or CHIP siRNA was determined following immunoprecipitation from equal amounts of total cell lysate by using a peptide called 'Sgktide' (KKRNRRLSVA) as a substrate [17]. The experiment was performed six times for SGK-1 in the presence of WT CHIP and four times for SGK-1 in the presence of CHIP siRNA. Ratios of SGK-1 activity in the presence of WT CHIP-Myc or CHIP siRNA compared with SGK-1 activity alone were determined. Values are expressed as the means $\pm$ S.E.M. Student's *t* test was used for statistical evaluation and *P* < 0.05 was considered significant. Equal volumes of residual beads from the kinase assay were boiled in sample buffer, run on SDS/PAGE and then probed for SGK-1 using Western blot analysis. The expression of CHIP and tubulin from equivalent amounts of total cell lysates was detected using appropriate antibodies.

Apoptosis assay

SK-BR-3 cells stably expressing SGK-1-FLAG were transiently transfected with a plasmid encoding CHIP siRNA for 24 h and then treated with UV (100 J/m²). Cells were fixed 48 h after UV-treatment using 4 % formaldehyde and stained with anti-(cleaved caspase 3) Alexa Fluor[®] 488-conjugated antibody according to the manufacturer's protocol (Cell Signaling Technology). Flow cytometry analysis of 10 000 cells was performed on a four-colour FACSCalibur equipped with an argon-ion laser (488 nm excitation) and CellQuest software (Becton Dickinson). Cells were gated using forward- and side-scatter properties and fluorescence intensity. The quantification of cleaved caspase-3-positive populations was performed using FlowJo 8.0.1 software. Each experiment was performed in triplicate; results are presented as means $\pm$ S.E.M.

SGK-1 mRNA stability assay

CHIP^{+/+} and CHIP^{-/-} mouse lung fibroblasts were treated with 5 μ g/ml of actinomycin D to inhibit transcription, and the cells were collected 0.5, 1, 1.5, 2, 3 and 4 h after treatment. Total mRNA was isolated using the RNeasy mini kit (Qiagen) and was then transcribed to cDNA with a TaqMan reverse transcription

kit using a random hexamer primer (Applied Biosystems). Real-time PCR of mouse *SGK-1* and *Actb* (β -actin) cDNAs was performed in triplicate with ABI Prism 7700 or 7300 instruments (Applied Biosystems) using the SYBR[®] Green PCR master mix (Applied Biosystems). The following primers were used: *SGK-1* sense 5'AGGCCCATCCTTCTCTGTTT 3' and antisense 5'TTCACTGCTCCCCTCAGTCT 3', and *Actb* sense 5'AA-TGGGGTACTTCAGGGTCA 3' and antisense 5'GATATCGC-TGCGCTGGTC 3'. The C_t (threshold cycle) values for *SGK-1* and *Actb* were calculated from the obtained standard curves, and *SGK-1* C_t values were then normalized to the C_t value for *Actb*. To detect genomic DNA contamination, real-time PCR was also performed in mRNA control samples in the absence of reverse transcriptase during cDNA synthesis. Each experiment was performed at least three times, and results are reported as means $\pm$ S.E.M.

RESULTS

CHIP E3 ligase activity demonstrates an inverse correlation with SGK-1 steady-state levels

During a candidate search for an E3 ligase that targets SGK-1 for degradation, we examined the effect of ectopically expressed WT or mutant CHIP proteins on steady-state SGK-1 levels. Figure 1(A) is a diagram of CHIP depicting the location of the mutations used in the present study. A U-box domain mutation, H260Q, renders CHIP unable to bind its cognate E2 ligase and is therefore inactive as an E3 ligase [15]. A TPR (tetratricopeptide repeat) domain mutation, K30A, renders CHIP unable to bind Hsp/Hsc70, thereby indirectly abolishing the E3 ligase activity of CHIP [14,15]. An equal number of SK-BR-3 breast cancer cells were transiently transfected with a plasmid encoding SGK-1-FLAG or in combination with increasing amounts of plasmid DNA encoding WT, H260Q or K30A CHIP-Myc. Parallel cell cultures were incubated in the presence or absence of the proteasome inhibitor ALLN; SGK-1 and CHIP steady-state protein levels were determined by Western blot. Figure 1(B) shows that SGK-1 steady-state levels were barely detectable in the absence of ALLN, although protein levels accumulated following ALLN treatment consistent with proteasome-mediated degradation as shown previously [7]. When WT CHIP was co-expressed, SGK-1 steady-state levels accumulated slightly in the presence of ALLN (Figure 1C, top panels). Interestingly, SGK-1 protein expression decreased as WT CHIP expression increased, suggesting that proteasome inhibition was not sufficient to override augmented SGK-1 turnover. This may be due to the incomplete inhibition of the proteasome by ALLN [18].

H260Q CHIP is a dominant-negative protein due to a single point mutation that abrogates E2 ligase interaction and yet allows substrate sequestration from endogenous CHIP [15]. As expected, increasing amounts of H260Q CHIP correlated with increased SGK-1 steady-state levels, best demonstrated in the presence of ALLN. In the absence of ALLN, SGK-1 steady-state levels were also increased with the highest expression of H260Q CHIP (Figure 1C, middle panels). These results suggest that CHIP's U-box domain and its accompanying E2 ligase are likely to be involved in the rapid turnover of SGK-1 [7]. Interestingly, expression of K30A CHIP, a mutant CHIP protein unable to bind Hsp/Hsc70, resulted in slightly increased SGK-1 levels in the presence of ALLN (Figure 1C, bottom panels). This suggests that the chaperone-deficient K30A CHIP may also interfere with SGK-1 degradation, although not as efficiently as the H260Q mutant CHIP protein.

To study further the relationship between CHIP and SGK-1, we examined cells transfected with a fixed amount of ectopic

Figure 1 CHIP E3 ligase activity promotes the down-regulation of SGK-1

(A) Domain structure of WT and mutant CHIP. (B) SK-BR-3 cells were co-transfected with SGK-1-FLAG DNA (1.5 μ g) and pcDNA3.1 (1.5 μ g). At 36 h post-transfection, ALLN or vehicle (ethanol) were added overnight. The cells were lysed and subjected to immunoblotting with anti-FLAG antibodies to detect SGK-1. (C) The plasmids encoding SGK-1 (1.5 μ g) and increasing amounts of WT, H260Q and K30A CHIP-Myc (0.1, 0.2, 0.38 and 0.75 μ g) were expressed in SK-BR-3 cells. ALLN was added as above, and the cells were lysed and subjected to Western blot analysis with the indicated antibodies. (D) Increasing amounts of SGK-1-FLAG-encoding plasmids (0.5, 1.0 and 2.0 μ g) were co-transfected with WT and mutant CHIP constructs into SK-BR-3 cells and SGK-1 expression was determined by Western blot analysis. (E) MDA-MB-231 cells stably expressing H260Q CHIP or the empty pcDNA3.1 vector were treated with ALLN or vehicle overnight and then examined by Western blotting for endogenous SGK-1 and ectopic CHIP expression. IB, immunoblot.

WT or mutant CHIP and increasing amounts of the SGK-1-FLAG plasmid. Without ectopic CHIP expression, SGK-1 steady-state levels increased according to transfected DNA amounts, and were further increased by ALLN (Figure 1D, top panels). As

expected, SGK-1 levels decreased with WT CHIP co-expression and were stabilized by ALLN treatment. In contrast, H260Q CHIP co-expression dramatically increased steady-state SGK-1 levels even in the absence of ALLN, further suggesting that H260Q CHIP interferes with ubiquitin-mediated degradation of SGK-1. Expression of the K30A CHIP mutant did not significantly change SGK-1 levels in comparison with expression of the empty vector (Figure 1D, bottom panels). This suggests that the K30A CHIP dose-dependent stabilization of SGK-1 observed in Figure 1(C) may be due to an indirect mechanism (e.g. heterodimerization and inactivation of endogenous CHIP when expressed at high concentrations) rather than to the binding and sequestration of SGK-1 from endogenous CHIP [19].

We then asked whether the E3 ligase activity of CHIP mediates down-regulation of endogenous SGK-1. To this end, we attempted to generate MDA-MB-231 breast cancer cells stably expressing WT or H260Q CHIP. However, stable overexpression of WT CHIP was not tolerated during the antibiotic selection process despite multiple attempts. This phenomenon is reminiscent of the temperature intolerance seen in *Arabidopsis* stably overexpressing WT CHIP [10]. Stably expressing H260Q CHIP cells were successfully generated. Pools of these cells were starved of all growth factors overnight and then stimulated with 1 μ M dexamethasone for 16 h to induce endogenous SGK-1 expression. As shown in Figure 1(E), stable expression of H260Q CHIP dramatically increased endogenous SGK-1 steady-state levels, even in the absence of ALLN. These results suggest that the E3 ligase activity of CHIP contributes to rapid SGK-1 turnover, and that constitutively increased CHIP activity is not compatible with cell survival.

SGK-1 associates with CHIP *in vivo*

To determine whether CHIP interacts with SGK-1 *in vivo*, we co-transfected SK-BR-3 cells with Myc-tagged WT, H260Q or K30A CHIP and SGK-1-FLAG. SGK-1-FLAG was immunoprecipitated with an anti-FLAG antibody, and co-precipitating proteins were analysed by immunoblotting. There was no background CHIP co-precipitated in the absence of SGK-1-FLAG (Figure 2A, lanes 1 and 2), and the anti-FLAG antibody efficiently precipitated SGK-1-FLAG (Figure 2A, lanes 3 and 4). WT CHIP co-precipitated with SGK-1, but only in the presence of ALLN (Figure 2A, lane 6). Presumably, proteasome inhibition by ALLN allows SGK-1-CHIP complexes to accumulate to a level sufficient for detection; in the absence of proteasome inhibition, SGK-1-CHIP complexes may not be detectable owing to their rapid degradation. In contrast with WT, H260Q CHIP co-precipitated both in the presence and absence of ALLN, suggesting that the lack of E3 ligase activity allowed the accumulation of H260Q SGK-1-CHIP complexes (Figure 2A, lanes 7 and 8). K30A CHIP, which is impaired in chaperone binding, did not co-precipitate with SGK-1, suggesting that Hsp binding to CHIP is required for the SGK-1-CHIP interaction (Figure 2A, lanes 9 and 10). It is not clear at this point whether CHIP directly interacts with SGK-1 or whether the CHIP-SGK-1 interaction is mediated by Hsp tethering of the two proteins (Figure 7).

The chaperones Hsc70 and Hsp70, in association with CHIP, are known to participate in protein quality control. When proteins are misfolded, Hsp70 replaces Hsp90 in a complex with client proteins and interacts with CHIP via its TPR-domains [15]. Therefore the CHIP-Hsp70 complex can target misfolded proteins for degradation by the proteasome. Because SGK-1 appeared to co-precipitate with CHIP, we next examined whether or not Hsp70 is part of the SGK-1-CHIP complex. SK-BR-3 cells stably expressing SGK-1-FLAG were transfected with a

Figure 2 CHIP and Hsp/Hsc70 interact with SGK-1 *in vivo*

(A) SGK-1-FLAG was expressed in SK-BR-3 cells and immunoprecipitated followed by Western blotting in the presence or absence of WT and mutant CHIP-Myc expression. (B) SGK-1 was immunoprecipitated from SK-BR-3 cells stably expressing SGK-1-FLAG and transiently expressing WT CHIP-Myc. Immunoblotting was then performed to detect SGK-1 and Hsp/Hsc70 or CHIP using the appropriate antibody. (C) WT or H260Q CHIP was immunoprecipitated from SK-BR-3 cells transiently transfected with SGK-1-FLAG and CHIP constructs and subjected to Western blot analysis to detect SGK-1. IB, immunoblot.

plasmid encoding WT CHIP, treated with or without ALLN, and lysates were subjected to FLAG-immunoprecipitation followed by immunoblotting with an anti-CHIP or an anti-Hsp70 antibody. In this experiment, the anti-CHIP antibody detects both endogenous and ectopic CHIP co-precipitated with SGK-1 even in the absence of ALLN, suggesting that stable overexpression of SGK-1 increased the ability to detect SGK-1-CHIP complexes (Figure 2B, left panel compared with Figure 2A, lane 5). Hsp70 and Hsc70 were also detected in the presence of ectopic CHIP, suggesting that the amount of Hsp/Hsc70 in the complex with SGK-1 may be dependent on CHIP expression levels. In the presence of ALLN, Hsp70 co-precipitated with SGK-1 even though endogenous CHIP could not be detected. However, with additional ectopic CHIP expression, both Hsp70 and CHIP were detectable (Figure 2B, right panel).

To further characterize SGK-1-CHIP interactions, we performed the reciprocal immunoprecipitation of ectopic WT or H260Q CHIP in SK-BR-3 cells transiently expressing SGK-1-FLAG. Both WT and H260Q CHIP were specifically immunoprecipitated with an anti-CHIP antibody (Figure 2C, upper panel, lanes 2, 4, 6 and 8). As expected, SGK-1 co-precipitated with both WT and H260Q CHIP, while a control IgG did not precipitate either CHIP or SGK-1 (Figure 2C, bottom panel). Taken together, these results suggest that SGK-1 can form a complex with Hsp/Hsc70 and CHIP that facilitates the chaperone-dependent proteasome-mediated degradation of SGK-1.

CHIP mediates SGK-1 protein down-regulation and ubiquitin modification

We next asked whether the absence of CHIP would increase endogenous SGK-1 steady-state levels. To address this, we examined SGK-1 protein levels in CHIP^{+/+} or CHIP^{-/-} mouse lung fibroblasts immortalized with SV40 large T-antigen [11]. Equal numbers of cells were treated with or without ALLN and then harvested in SDS/PAGE sample buffer. As shown in Figure 3(A), SGK-1 was barely detectable in the absence of ALLN but accumulated following ALLN treatment in CHIP^{+/+} cells, whereas in CHIP^{-/-} cells, there was significantly more endogenous SGK-1 expressed without ALLN. In addition, SGK-1 steady-state levels did not change significantly in CHIP^{-/-} cells following ALLN treatment, further suggesting that endogenous CHIP down-regulates SGK-1 via a proteasome-dependent process. Interestingly, neither total Akt-1 nor phosphorylated Akt-1 (phosphorylated on Ser⁴⁷³) levels changed in CHIP^{-/-} cells, suggesting that CHIP-mediated down-regulation is relatively specific to SGK-1.

We next determined whether differences in mRNA stability might contribute to the increased SGK-1 protein expression observed in CHIP^{-/-} cells. CHIP^{+/+} and CHIP^{-/-} cells were treated with actinomycin D for various periods of time, RNA was isolated and *SGK-1* mRNA was quantified using real-time PCR. As shown in Figure 3(B), the stability of *SGK-1* mRNA in both CHIP^{+/+} and CHIP^{-/-} cells was similar, suggesting that the increased SGK-1 protein levels observed in CHIP^{-/-} cells are due to a post-transcriptional mechanism rather than to differences in stability of *SGK-1* mRNA.

To further study the effect of CHIP knockdown on SGK-1 steady-state levels in another cell line, we examined endogenous SGK-1 protein levels in SK-BR-3 cells stably transfected with CHIP siRNA. As shown in Figure 3(C) (middle panel and histogram), endogenous CHIP levels decreased by approx. 50% following CHIP siRNA expression, and endogenous SGK-1 levels increased moderately (Figure 3C, upper panel). The relatively small accumulation of SGK-1 following CHIP knockdown in SK-BR-3 cells compared with CHIP^{-/-} cells is probably due to the incomplete knockdown of CHIP with siRNA.

Finally we asked whether SGK-1 is an *in vivo* substrate for CHIP-mediated E3 ubiquitin ligase activity. Increasing amounts of WT CHIP and a single concentration of the HA-ubiquitin plasmids were co-transfected into SK-BR-3 cells stably expressing SGK-1-FLAG. Following immunoprecipitation of SGK-1-FLAG, ubiquitinated SGK-1 was detected with an anti-HA antibody. Ubiquitinated SGK-1 species increased following WT CHIP overexpression and further increased with greater WT CHIP expression (Figure 3D, lane 2 compared with lanes 3–5). Concurrently, unmodified SGK-1 steady-state levels decreased slightly, providing additional evidence that CHIP targets SGK-1 for degradation via ubiquitin modification (Figure 3D, lanes 3, 4 and 5). Taken together, these experiments suggest that CHIP E3 ligase activity decreases SGK-1 steady-state levels via a proteasome-dependent pathway.

CHIP and SGK-1 co-localize at the ER

SGK-1 has been shown by biochemical fractionation to exhibit approximately equivalent cytoplasmic and membrane-associated subcellular localization [7]. Interestingly, the membrane-associated fraction of SGK-1 is highly polyubiquitinated, whereas the soluble cytoplasmic fraction is largely unmodified by ubiquitin [7]. CHIP has also been shown to be predominantly cytoplasmic, with some localization to the ER [11,20]. We therefore

Figure 3 CHIP is required for SGK-1 protein stability and ubiquitin modification

(A) CHIP^{+/+} and CHIP^{-/-} mouse fibroblasts were treated overnight with ALLN or vehicle, lysed, and then examined by Western blotting for endogenous SGK-1, CHIP, Akt-1 and phospho-Akt-1 proteins. (B) Mouse fibroblasts were treated with 1 μ g/ml of actinomycin D for indicated time periods. Total RNA was then isolated, and *SGK-1* mRNA was quantified using quantitative real-time PCR. The ratio of *SGK-1* to *Actb* mRNAs was calculated at each time point, and then expressed as a percentage of *SGK-1/Actb* ratio at 60 min. Data points represent the means for three independent experiments. S.E.M. and the calculated half-life are shown. (C) SK-BR-3 cells were stably transfected with CHIP siRNA and subjected to immunoblotting to examine endogenous SGK-1, CHIP and tubulin levels. The representative immunoblot from one of three independent experiments is shown. Quantification of the representative immunoblot shows CHIP protein levels relative to those of tubulin. (D) SK-BR-3 cells stably expressing SGK-1-FLAG were transfected with HA-ubiquitin plasmid and increasing amounts of WT CHIP plasmid. Immunoprecipitated SGK-1 was subjected to immunoblotting with anti-SGK-1 and anti-HA antibodies. CHIP expression was evaluated by Western blot analysis in the total lysate. The blot is representative of three independent experiments. IB, immunoblot.

hypothesized that CHIP and SGK-1 might co-localize to a membranous compartment in the cytoplasm, where SGK-1 is subsequently ubiquitinated. To test this hypothesis, we first

Figure 4 SGK-1 and CHIP co-localize to the ER

(A) COS-7 cells were transfected with the plasmids encoding SGK-1-GFP, WT-CHIP-Myc or both (top, middle and bottom panels respectively) and the ER marker, pDsRed2-ER (which encodes calreticulin and an ER-retention signal fused to DsRed2). CHIP was detected using an anti-Myc antibody and a secondary anti-mouse antibody conjugated to Alexa Fluor[®] 488 (middle and bottom panels). (B) COS-7 cells were transfected with pDsRed2-ER vector (top and middle panels). Endogenous SGK-1 was detected with an anti-SGK-1 chicken antibody followed by an anti-chicken Alexa Fluor[®] 488-conjugated secondary antibody (top and bottom panels). Endogenous CHIP was detected with anti-CHIP rabbit antibody followed by anti-rabbit Alexa Fluor[®] 488-conjugated secondary antibody (middle panel) or anti-rabbit Alexa Fluor[®] 568-conjugated secondary antibody (bottom panel). Co-localization is shown in the merge column in yellow.

determined whether either CHIP or SGK-1 co-localizes with an ER marker, calreticulin. SGK-1-GFP or CHIP-Myc was co-transfected into COS-7 cells along with a plasmid encoding the ER-targeting sequence of calreticulin and an ER-retention sequence (KDEL) fused to DsRed2 (pDsRed-ER; Clontech). CHIP-Myc was detected using an anti-Myc antibody followed by an anti-mouse Alexa Fluor[®] 488-conjugated secondary antibody (green). SGK-1-GFP appeared to co-localize with the ER-targeted red marker in the majority of the cells (Figure 4A, upper panel), although a mitochondrial localization pattern was also seen in approx. 15% of SGK-1-GFP-expressing cells (results not shown). In agreement with a previous report [20], CHIP-Myc also localized to the ER (Figure 4A, middle panel).

In order to determine the extent of co-localization, SGK-1-GFP and CHIP-Myc were co-transfected into COS-7 cells, and CHIP was detected as indicated above, except that the anti-Myc antibody was followed by an anti-mouse Alexa Fluor[®] 568-conjugated secondary antibody (red). SGK-1-GFP (green) and CHIP-Myc (red) showed co-localization (yellow), although there was clearly some cytoplasmic SGK-1-GFP that did not appear to be associated with CHIP-Myc (Figure 4A, bottom panel). These results suggest that ectopic SGK-1 and CHIP co-localize at or near the ER, and provide an explanation for our previous findings that polyubiquitin modified SGK-1 is found exclusively in a membrane-associated fraction [7].

We further examined whether localization of endogenous SGK-1 and CHIP is similar to that of ectopically expressed proteins. We expressed the pDsRed2-ER marker in COS-7 cells and then detected endogenous SGK-1 with a chicken anti-SGK-1 antibody

followed by an anti-chicken Alexa Fluor[®] 488-conjugated IgG. The localization pattern of endogenous SGK-1 (Figure 4B, top panel) resembled the localization of ectopic SGK-1-GFP. Endogenous CHIP was probed with a rabbit anti-CHIP antibody followed by an anti-rabbit Alexa Fluor[®] 488-conjugated IgG. We found that endogenous CHIP (green) also localized to the ER (red) in a pattern similar to that of ectopically expressed CHIP-Myc (Figure 4B, middle panel). In co-localization studies, which were performed with the antibodies mentioned above except that the anti-chicken IgG was conjugated to Alexa Fluor[®] 488 and anti-rabbit to Alexa Fluor[®] 568, endogenous SGK-1 (green) and endogenous CHIP (red) also showed significant co-localization (yellow; Figure 4B, bottom panel).

CHIP expression levels alter SGK-1 kinase activity *in vitro*

We then asked whether, as a consequence of regulating SGK-1 expression, CHIP affects overall SGK-1 kinase activity. To address this, we immunoprecipitated SGK-1-FLAG from normally growing unstimulated SK-BR-3 cells transiently transfected with WT CHIP or CHIP siRNA and measured SGK-1 kinase activity using an *in vitro* kinase assay (Figure 5). Following WT CHIP expression, SGK-1 kinase activity decreased relative to SGK-1 activity in the absence of ectopic CHIP (Figure 5A, left-hand panel). Consistent with our previous results, steady-state SGK-1 levels also decreased (Figure 5B, left-hand panel). In contrast, knockdown of CHIP was associated with slightly increased SGK-1 activity in the absence of serum stimulation (Figure 5A, right-hand panel) and increased SGK-1 expression

Figure 5 CHIP expression levels regulate SGK-1 *in vitro* kinase activity

(A) Following transient transfection of SK-BR-3 cells with the plasmids encoding SGK-1-FLAG and either CHIP-Myc or CHIP siRNA, SGK-1 was immunoprecipitated and used in an *in vitro* kinase assay as previously described [17]. Ratios of SGK-1 activity in the presence of CHIP-Myc or CHIP siRNA to SGK-1 activity alone are shown as a mean of six or four independent experiments respectively $\pm$ S.E.M. * $P < 0.05$ compared with SGK-1 activity alone. (B) Following the kinase assay, levels of immunoprecipitated SGK-1 were determined by Western blot analysis. In the total lysates, ectopic CHIP was detected with an anti-Myc antibody, while endogenous CHIP and tubulin were determined using an anti-CHIP and an anti-tubulin antibody respectively. IP, immunoprecipitation; IB, immunoblot.

(Figure 5B, right-hand panel). These results suggest that CHIP modulates SGK-1 expression and kinase activity even in the absence of PI3K stimulation, consistent with previous models suggesting a primary role for SGK-1 expression levels in determining SGK-1 kinase activity [6].

CHIP functions in both SGK-1-dependent and -independent cell survival pathways

SGK-1 has been shown to play a prominent anti-apoptotic role in breast cancer cells subjected to cellular stresses including chemotherapy [5], growth factor withdrawal [4,6] and UV [6]. CHIP has also been shown to regulate the stress response, and CHIP knockout causes increased apoptosis in mouse fibroblasts and physiological intolerance to heat shock in mice [11]. In the present study, because we observed that CHIP expression inversely correlates with SGK-1 steady-state levels and activity, we examined the relative roles of CHIP and SGK-1 expression in mediating cell survival. We exposed SK-BR-3 cells expressing CHIP siRNA, SGK-1-FLAG or both to UV and determined the percentage of apoptotic cells positively staining for cleaved caspase-3 using flow cytometry. The cells expressing ectopic SGK-1 had approx. 40% less apoptosis than control cells (Figure 6A, white bar compared with black bar), suggesting a role for SGK-1 in cell survival following UV damage. This observation is similar to the anti-apoptotic effect of ectopically expressed SGK-1 observed in non-tumorigenic rat mammary epithelial cells following UV [6]. The representative scatter plots shown in

Figure 6 SGK-1 and CHIP have both overlapping and independent anti-apoptotic roles

(A) SK-BR-3 cells stably expressing SGK-1-FLAG or empty vector were transiently transfected with CHIP siRNA DNA. At 48 h post UV-irradiation (100 J/m²), the percentage of apoptotic cells was determined using flow cytometric detection of cells staining for cleaved caspase-3 using the respective Alexa Fluor® 488-conjugated antibody. Results represent the means of the positively stained cell population $\pm$ S.E.M. for three independent experiments. (B) Representative flow cytometric plots depict the percentage of cleaved caspase-3-positively stained cells in the experimental groups.

Figure 6(B) illustrate the differences in cleaved caspase-3-positive stained cells in the control compared with SGK-1-expressing groups.

We also observed that despite the effect of CHIP siRNA on increasing endogenous SGK-1 expression (experiment in Figure 3C), CHIP knockdown significantly increased apoptosis in SK-BR-3 cells following UV stress (Figures 6A, grey bar compared with black bar, and 6B). Therefore down-regulation of CHIP appears to override the anti-apoptotic effect expected by increasing endogenous SGK-1, suggesting that CHIP knockdown also facilitates SGK-1-independent apoptotic pathways. We next examined whether additional ectopic SGK-1 expression would decrease apoptosis following CHIP siRNA, reasoning that more

SGK-1 expression would provide an enhanced pro-survival effect. Indeed, we found that additional SGK-1 expression partially rescued apoptosis caused by CHIP knockdown (Figures 6A, hatched bar compared with grey bar, and 6B). However, SGK-1 ectopic expression in the presence of CHIP siRNA did not protect cells from apoptosis as effectively as expression of ectopic SGK-1 alone (Figure 6A, hatched bar compared with white bar). This suggests that down-regulation of CHIP affects both SGK-1-dependent and SGK-1-independent anti-apoptotic pathways. This model is supported by previous findings that CHIP knockdown increases apoptosis via up-regulation of the apoptosis signal-regulating kinase 1 [21]. In summary, the study of the relative effects of CHIP compared with SGK-1 expression on apoptosis suggests that CHIP functions in multiple ways to fine-tune the cellular response to stress; the multitude of CHIP substrates identified to date suggests a complex regulation between CHIP and both pro- and anti-apoptotic proteins.

DISCUSSION

It has been well-documented that *SGK-1* is transcriptionally up-regulated following various stress stimuli, thereby providing a transient increase in SGK-1 protein levels and a survival signal to the cell [5,6]. However, the stress-induced expression and activity of SGK-1 are usually not sustained because of the short half-life of both its mRNA and protein product [7]. Additional mechanisms that limit the duration of SGK-1 activity following stress induction are not completely understood. In the present study we show that the E3 ligase CHIP plays an important role in the rapid protein degradation of SGK-1. The co-localization of SGK-1 and CHIP at the ER is consistent with our recent finding that SGK-1 degradation requires a hydrophobic amino-acid sequence (amino acids 19–24) that is also required for the localization of SGK-1 to the ER [22]. Interestingly, CHIP also targets several other ubiquitin-modified stress-induced signalling proteins including ErbB2 [15], CFTR (cystic fibrosis transmembrane conductance regulator) [20], p53 [23], apoptosis signal-regulating kinase 1 [21] and c-Raf [24]. Our findings further extend the function of CHIP to the regulation of the PI3K pathway. Although CHIP expression down-regulates SGK-1 steady-state levels and activity, CHIP knockdown results in increased SGK-1 expression and activity. Although SGK-1 and Akt-1 are closely related kinases, we found that Akt-1 protein levels and phosphorylation were not affected in CHIP knockout cells, suggesting that CHIP-mediated regulation of the PI3K pathway is relatively specific to SGK-1.

CHIP and related chaperone-associated proteins have been implicated in protein quality control in association with the ER [20]. We now report that both CHIP and SGK-1 co-localize to the ER, suggesting that CHIP, in complex with Hsps, assists in proper SGK-1 folding. This folding may allow for full activation of SGK-1 similar to the regulatory mechanism of the binding of Akt-1 to phosphatidylinositol 3,4,5-trisphosphate prior to its activation as a kinase.

Both SGK-1 and CHIP have been implicated in the response to cellular stress. *SGK-1* transcriptional up-regulation is required for PI3K-mediated cell survival signalling following hyperosmotic shock, temperature stress, UV irradiation and endocrine factor stimulation [5,6]. SGK-1 overexpression has been associated with renal, lung and cardiac pathophysiology via regulation of ion channels (reviewed in [25]). For example, SGK-1 increases the abundance and activity of the epithelium sodium channel and the potassium channel ROMK1 [26,27]. This enhanced activity appears to increase sodium reabsorption, potassium secretion,

water retention, and as a result, blood pressure. In lung tissue, SGK-1 up-regulates epithelium sodium channel and CFTR [28,29], both of which enhance fluid secretion and mucus clearance in the lung. In cystic fibrosis, fluid secretion and mucus clearance are impaired due to defective CFTR and increased sodium channel activity mediated, in part by SGK-1 [29].

In cardiac muscle, SGK-1 up-regulates the voltage-gated sodium channel SCN5A [30,31] and the potassium channels KCNE1/KCNQ1 and Kv 4.3 [32,33], which participate in the generation and propagation of cardiac potential and repolarization. The importance of SGK-1 in the heart has also been demonstrated in cardiomyocytes that are subjected to hypoxia as well as mechanical, hormonal and oxidative stresses [34,35,36]. All of these factors increase SGK-1 expression, which can mediate phosphorylation of GSK3 β [37], CTGF (connective tissue growth factor) [35,37] and ANF (atrial natriuretic factor), a marker of the hypertrophic response [34]. Deregulation of SGK-1-mediated events is likely to underlie cardiac myocyte hypertrophy and myocardial fibrosis. In addition, the pro-survival activity of SGK-1 in cardiomyocytes following stress may further aggravate hypertrophy [34]. These findings suggest a central role for SGK-1 in the integrated regulation of sodium homeostasis, fluid secretion, blood pressure and heart function.

The exact role of CHIP in the response to physiological stress is just beginning to be understood. Interestingly, CHIP and SGK-1 play similar roles in multiple diseases. For example, following heat shock, CHIP contributes to cardiomyocyte survival via trimerization and transcriptional activation of HSF1 (heat-shock factor 1), which in turn up-regulates Hsp70 protein levels [11,38]. CHIP then attenuates Hsp70 levels via ubiquitin modification, allowing a recovery from stress [39]. The most striking illustration of a global role for CHIP in the regulation of the stress response comes from the recent study of the CHIP^{-/-} mouse response to cardiac ischaemia and reperfusion injury. Mice that lack CHIP have decreased survival and an increased incidence of arrhythmias and myocardial infarction during reperfusion. This pathology is accompanied by impaired up-regulation of the chaperone Hsp70 and increased apoptosis in cardiomyocytes and vascular endothelial cells [40]. Like SGK-1, CHIP also participates in maturation of the glycoprotein CFTR in epithelial cells, suggesting that SGK-1-mediated activation of CFTR may also be regulated by CHIP activity. In neurons, the chaperone and E3 ligase activities of CHIP regulate folding and degradation of the misfolded polyglutamine proteins, tau and α -synuclein, thereby preventing their aggregation [41–43]. This function links CHIP to the neurodegenerative diseases Huntington's disease, Parkinson's disease and Alzheimer's disease; the list of neurodegenerative diseases associated with CHIP is likely to increase as identification of CHIP substrates grows. Interestingly, SGK-1 activity has also been implicated in Parkinson's disease and ALS (amyotrophic lateral sclerosis) via regulation of glutamate transporters, ion channels and increased neuronal cell survival [44].

In the present study we show for the first time that the PI3K/SGK-1 signalling pathway appears to be regulated by the chaperone-dependent E3 ligase machinery. Under conditions that enhance PI3K/SGK-1 signalling, such as glucocorticoid stimulation or environmental stress, SGK-1 provides a strong survival signal to epithelial cells (Figure 6). SGK-1 signalling can be either down-regulated by the E3 ligase activity of CHIP or might remain active if the SGK-1–CHIP interaction is compromised. Because of the multifunctional nature of CHIP, distinguishing the regulation of SGK-1 by CHIP from the other roles of CHIP will require mapping the interaction domains of both SGK-1 and CHIP.

In summary, the activity of both CHIP and SGK-1 appear to be important components of the physiological stress response in

Figure 7 Proposed model of CHIP-mediated regulation of SGK-1 function

SGK-1 is induced as part of the stress response and is then available to be phosphorylated and activated downstream of PI3K. Upon either a direct or indirect interaction with CHIP at or near the ER, we hypothesize that SGK-1 becomes properly folded and fully active, thereby promoting cell survival. CHIP-mediated ubiquitin (Ub) modification of SGK-1, however, also allows for the subsequent rapid degradation and return to baseline of SGK-1 levels.

epithelial cells, and in the present study we demonstrate a physical and functional link between these two proteins. We propose that under conditions such as DNA damage, SGK-1 expression and activity are up-regulated. CHIP–Hsp70–Hsc70 complexes might then assist in the proper folding and full activation of SGK-1 followed by ubiquitin modification and increased SGK-1 turnover, thereby moderating the stress response (Figure 7). Because continuous SGK-1 up-regulation could be deleterious for cells, SGK-1 expression probably requires a mechanism for negative regulation that includes ubiquitin modification and proteasomal degradation. Deregulated SGK-1 expression has been associated with breast cancer [45,46] and diabetic nephropathy [47]. The relationship between SGK-1 and CHIP in those diseases associated with physiological stress (e.g. DNA damage, oxidative damage and hyperosmolarity) remains the subject of future studies.

Note added in proof (received 28 September 2006)

While this manuscript was under review, Arteaga et al. [48] have published findings consistent with our results that SGK-1 is ubiquitin-modified in association with an ER-associated ubiquitin ligase complex.

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