C-terminus of heat shock protein 70-interacting protein facilitates degradation of apoptosis signal-regulating kinase 1 and inhibits apoptosis signal-regulating kinase 1-dependent apoptosis

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Abstract Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that is regulated under conditions of cellular stress. ASK1 phosphorylates c-Jun N-terminal kinase (JNK) and elicits an apoptotic response. ASK1 activity is regulated at multiple levels, 1 of which is through inhibition by cytosolic chaperones of the heat shock protein (Hsp) 70 family. Among the proteins that determine Hsp70 function, CHIP (C-terminus of Hsp70-interacting protein) is a cochaperone and ubiquitin ligase that interacts with Hsp70 through an amino-terminal tetratricopeptide repeat (TPR) domain. Prominent among the cellular functions mediated by CHIP is protection against physiologic stress. Because ASK1 is known to contain a TPR-acceptor site, we examined the role of CHIP in regulating ASK1 function. CHIP interacted with ASK1 in a TPR-dependent fashion and induced ubiquitylation and proteasome-dependent degradation of ASK1. Targeting of ASK1 by CHIP inhibited JNK activation in response to oxidative challenge and reduced ASK1-dependent apoptosis, whereas short interfering RNA (siRNA)-dependent depletion of CHIP enhanced JNK activation. Consistent with its ability to reduce cytoplasmic ASK1 levels, CHIP triggered the translocation of ASK1 partner protein death-associated protein (Daxx) into the nucleus, where it is known to activate an antiapoptotic response. These results indicate that CHIP regulates ASK1 activity by inducing its ubiquitylation and degradation, which, together with its effects on Daxx localization, provides a mechanism for the antiapoptotic effects of CHIP observed in the face of cellular and physiologic stress.

INTRODUCTION

The mitogen-activated protein kinase (MAPK) cascades are multifunctional signaling pathways that are evolutionally well conserved in all eukaryotic cells. The MAP kinase cascades are composed of 3 sequentially activating protein kinases: MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs). MAPKKKs activate

MAPKKs, and MAPKKs in turn activate MAPKs. MAPKs regulate pleiotrophic cellular events, including mitogenesis, differentiation, diapedesis, and stress-dependent apoptosis. Two mammalian MAPKs, c-Jun N-terminal kinase (JNK) and p38 MAPK, are activated by environmental stresses such as oxidative stress, ultraviolet radiation, hyperosmolarity, endoplasmic reticulum stress, and proinflammatory cytokines such as tumor necrosis factor, Fas ligand, and interleukin-1 (Nishida and Gotoh 1993; Ichijo et al 1997; Ichijo 1999; Davis 2000; Matsuzawa and Ichijo 2001).

Apoptosis signal-regulating kinase 1 (ASK1) is a ubiq-

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uitously expressed serine–threonine protein kinase that functions as an MAPKKK to activate the JNK and p38 MAPK signaling cascades. ASK1 is activated in response to various stresses including H_2O_2 , serum withdrawal, endoplasmic reticulum stress, Fas ligation, and tumor necrosis factor (Ichijo et al 1997; Tobiume et al 1997; Chang et al 1998; Morita et al 2001). ASK1 plays a critical role in the regulation of signaling in response to oxidative stress, a major contributor to proteotoxic damage and cell death (Saitoh et al 1998; Tobiume et al 2001; Goldman et al 2003). The activity of ASK1 is regulated at multiple steps that include self-dimerization, phosphorylation status, and protein-protein interactions. The activation phase of ASK1 requires dimerization and consequent autophosphorylation, events that are regulated by multiple cellular stressors. At this step, several proteins inhibit ASK1 activity. Thioredoxin binds to ASK1 and inhibits its activity under nonstressed conditions. H_2O_2 in turn triggers the dissociation of thioredoxin from ASK1 and thus is a potent stimulus for its activity (Saitoh et al 1998; Liu and Min 2002). Heat shock protein (Hsp) 70 binds ASK1 at its amino terminus and inhibits ASK1 dimerization induced by thermal stress, which suppresses ASK1-dependent apoptosis (Park et al 2002). Activation of ASK1 can also be inhibited downstream of its dimerization; for example, protein phosphatase 5 specifically binds to phosphorylated ASK1 in response to oxidative stress and inhibits ASK1-dependent apoptosis by catalyzing its dephosphorylation (Morita et al 2001). 14-3-3 proteins suppress ASK1 activity by binding at phosphorylated serine 967 (Zhang et al 1999; Goldman et al 2003). Protein-protein interactions also serve as a mechanism for activation of ASK1-dependent events. ASK1 is activated by Daxx (death-associated protein), a Fas-binding protein, and ASK1 elicits reciprocal relocation of Daxx from the nucleus to the cytoplasm, which is critical to inhibit the nuclear antiapoptotic effects of Daxx (Yang et al 1997; Chang et al 1998; Ko et al 2001; Song and Lee 2003). ASK1 may also be activated by ASK1-interacting protein (which induces ASK1 dissociation from 14-3-3) (Zhang et al 2003) and tumor necrosis factor receptor–associated factor 2 (Nishitoh et al 1998).

CHIP (carboxyl terminus of Hsc70-interacting protein) is a ubiquitin ligase (Jiang et al 2001) comprising 3 functional domains: a tetratricopeptide repeat (TPR) at the N-terminus, a U-box domain at the C-terminus, and a highly charged region separating the 2 (Ballinger et al 1999). TPR domains are protein-protein interaction modules that are adapted to function under conditions of cellular stress. CHIP interacts with cytoplasmic molecular chaperones Hsp70 and Hsp90 through this domain. Substitution of a lysine residue at position 30 in the TPR domain of CHIP disrupts Hsp70 interaction by breaking the carboxylate clamp that is required for Hsp-TPR interactions

(Scheufler et al 2000; Xu et al 2002; Dai et al 2003). The U-box domain is a requisite for the ubiquitin ligase activity of CHIP, and many substrates for CHIP-dependent ubiquitylation and degradation are also substrates of the cytoplasmic chaperone machinery: cystic fibrosis transmembrane conductance receptor (Meacham et al 2001), glucocorticoid receptor (Connell et al 2001), and ErbB2 (Xu et al 2002) and thermally denatured proteins (Ballinger et al 1999; McClellan and Frydman 2001; Kampinga et al 2003). Our laboratory has recently reported that CHIP (–/–) mice and cells derived from these mice undergo temperature-sensitive apoptosis in response to thermal and proteotoxic stress (Dai et al 2003). These observations led us to search for a mechanism whereby CHIP might be directly involved in apoptosis in the face of cellular stress. Given the regulation of ASK1 by cytoplasmic chaperones and the presence of TPR-acceptor sites in this MAPKKK, we considered ASK1 a likely candidate for regulation by CHIP. We report that CHIP interacts with ASK1 and, to our surprise, inhibits ASK1-dependent apoptosis by targeting ASK1 for ubiquitylation and proteasome-dependent degradation.

MATERIALS AND METHODS

Cell culture and transfections

COS-7 cells were maintained at 37°C and 5% CO_2 in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and $1 \times$ penicillin and streptomycin. Cells were transiently transfected using FuGene6 reagent (Roche Molecular Biochemicals), according to the manufacturer's instructions. pcDNA3-CHIP and pcDNA3-CHIP H260Q, and pcDNA3-Myc-CHIP and pcDNA3-Myc-CHIP Δ TPR (amino acid residues 32–145 deleted) were transfected with vector expressing hemagglutinin (HA)-tagged ASK1 (generous gift from Howard Y. Chang). In some experiments, transfected cells were treated with MG132 (25 μ M) or vehicle for 8 hours before harvesting. Pulse chase experiments were performed by metabolically labeling cells with ^{35}S -methionine, as we have described previously (Xu et al 2002).

To make the COS-7 cell line stably expressing Myc-CHIP, 1×10^6 cells in a 100-mm dish were transfected with pcDNA3-Myc-CHIP using the FuGene6 method. Five hours after transfection, medium was changed to DMEM growth medium containing 200 μ g/mL G418. Approximately 2 weeks later, G418-resistant colonies were picked and screened for Myc-CHIP expression by Western blotting.

CHIP siRNA preparation

CHIP siRNA was generated using BD Knockout RNAi systems (BD Biosciences). The annealed oligonucleotide

(5'-GGA GCA GGG CAA TCG TCT G-3') was cloned in pSIREN vector and sequence confirmed. pSIREN-DNR containing a scrambled oligo insert served as a control.

Antibodies

The following antibodies were used for immunoblotting and immunoprecipitations: mouse anti-HA (Covance), mouse anti-ubiquitin (Covance), mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Chemicon) mouse anti-Oct-1 (Santa Cruz), mouse anti-Myc (9E10, Santa Cruz), mouse anti-Flag (M2, Sigma), anti-JNK1 (Santa Cruz), and rabbit anti-CHIP.

Western blotting and immunoprecipitations

Transfected cells were harvested 24 hours after transfection and were lysed with radioimmune precipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM ethylene glycol tetra acetic acid (EGTA), 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM Na_3VO_4 , 50 mM NaF) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were clarified by centrifugation at $16\,000 \times g$ for 10 minutes, and protein concentration was determined. For immunoprecipitations, equal amounts of lysate proteins were incubated with 10–15 $\mu\text{L}/\text{mg}$ mouse monoclonal antibodies overnight at 4°C . Antibodies were pulled down using protein A/G-agarose beads (Santa Cruz) incubating for 2 hours at 4°C . The beads were washed 5 times with lysis buffer. Immunoprecipitated proteins or whole cell lysates were mixed with $4\times$ sodium dodecyl sulfate sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed with the appropriate antibodies.

In vitro ubiquitylation reactions

Immunopurified HA-ASK1 from COS-7 was incubated in the presence of 4 μM purified His-tagged CHIP (Jiang et al 2001), 0.1 μM purified rabbit E1 (Calbiochem), 8 μM UBCH4 as an E2, 2.5 mg/mL ubiquitin (Sigma) and 0.1 mg/mL ubiquitin-aldehyde (Calbiochem) in 20 mM 3 (N-morpholino) propanesulfonic acid (MOPS), pH 7.2, 100 mM KCl, 5 mM MgCl_2 , 5 mM adenosine triphosphate (ATP), 10 mM dithiothreitol (DTT), 1 mM PMSF for 2 hours at 30°C . Samples were analyzed by SDS-PAGE and immunoblotting with anti-HA.

In vitro JNK1 kinase assays

COS-7 cells stably transfected with Myc-CHIP were used for this assay. Stably transfected cells were transiently

transfected with HA-ASK1 or vector in 100-mm dishes. Twenty-four hours after transfection, cells were treated with 2 mM H_2O_2 for 20 minutes and lysed with 1 mL kinase lysis buffer containing 20 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid, pH 7.4, 2 mM EGTA, 1 mM DTT, 50 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, and 1 mM PMSF and protease inhibitor cocktail (Roche Molecular Biochemicals). Cell lysates were centrifuged for 10 minutes at 4°C at 14 000 rpm, and the supernatants were used for immunoprecipitation with antibody against JNK1 (Santa Cruz). The immunoprecipitated proteins were then incubated with 1 μg of c-Jun (Santa Cruz) and 1 μCi ^{32}P - γ -ATP in kinase reaction buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β -glycerophosphate, 7.5 mM MgCl_2 , 2 mM EGTA, 0.5 mM NaF, 0.5 mM Na_3VO_4 , and 20 μM ATP) for 20 minutes at 30°C . The reaction was stopped by adding Laemmli sample buffer, boiled for 5 minutes, and resolved on 12% SDS-PAGE. The gel was stained with Coomassie and exposed at -80°C .

For in vitro JNK1 assays using control- and CHIP siRNA-transfected cells, siRNA-expressing plasmids were transiently transfected into COS-7 cells using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were lysed with kinase lysis buffer, and in vitro JNK1 activity assay was performed as described above.

Apoptosis assays

COS-7 cells were transiently transfected with pEGFP and plasmids encoding HA-ASK1, Myc-CHIP or empty vector. Twenty-four hours after transfection, cells were incubated with serum-free DMEM for another 24 hours. The cells were then fixed with 4% formaldehyde and stained with 4', 6'-diamidino-2-phenylindole dihydrochloride (DAPI). The DAPI-stained nuclei of green fluorescence protein (GFP)-positive cells were analyzed for apoptotic morphology by fluorescence microscopy. The percentage of apoptotic cells was calculated as the number of GFP-positive cells with apoptotic nuclei divided by the total number of GFP-positive cells. Specific apoptosis was calculated as the percentage of apoptotic cells in each experimental condition minus the percentage of apoptotic cells of the vector control as described previously (Morita et al 2001). For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate, and then incubated for 60 minutes at 37°C with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and tetramethylrhodamine red (TMR) red-labeled dUTP provided by an in situ cell death detection kit (Roche Molecular Biochemicals). TUNEL-positive cells as

Fig 1. ASK1 expression is reduced in the presence of overexpressed CHIP. COS-7 cells were transiently transfected with HA-ASK1 with or without CHIP (wild type and H260Q). Twenty-four hours after transfection, cells were lysed in radioimmune precipitation buffer, and the cell lysates were blotted with anti-HA, anti-CHIP, and anti- β -actin, as indicated. IB, immunoblotting.

well as GFP-positive cells were detected by fluorescence microscopy.

Subcellular localization studies

COS-7 cells were transiently transfected with Flag-tagged Daxx (generous gift from Howard Y. Chang) either with wild-type CHIP, CHIP H260Q, or empty vector as described above in 100-mm dishes. For subcellular fractionation, we used NE-PERTM Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the protocol of the manufacturer. The same amount of proteins from each subcellular fractionation sample was loaded onto SDS-PAGE gels for Western blot analysis.

RESULTS

Regulation of ASK1 expression by CHIP

We recently demonstrated that mice deficient in CHIP develop widespread apoptosis after thermal stress (Dai et al 2003), which led us to consider the stress-activated apoptotic pathways that are regulated by CHIP. Three lines of reasoning led us to consider ASK1 as a potential target. First, ASK1 is noted for inducing apoptosis in response to environmental stress (Ichijo et al 1997; Matsuzawa and Ichijo 2001; Takeda et al 2003); second, ASK1 activity is negatively regulated by members of the molecular chaperone family (Charette et al 2000; Park et al 2002); and third, protein phosphatase 5 inhibits ASK1-dependent apoptosis by interactions with the TPR repeats of protein phosphatase 5 (Morita et al 2001), which is structurally similar to the TPR domain at the amino terminus of CHIP. To test this possibility, HA-tagged ASK1 (HA-ASK1) was transiently transfected with or without CHIP into COS-7 cells, and the cell lysates were probed by Western blotting for ASK1. Increased expression of CHIP decreased steady-state HA-ASK1 levels by $72 \pm 9\%$ ($n = 3$, Fig 1). In contrast, the CHIP H260Q mutant, which lacks ubiquitin ligase activity (Xu et al 2002), had negligible effects on HA-ASK1 levels, indicating that CHIP and

ASK1 might functionally interact and that ASK1 might serve as a substrate for the ubiquitin ligase activity of CHIP. Consistent with these observations, we found in ³⁵S-labeling experiments that the half-life of ASK1 followed first-order kinetics and was 70 ± 16 minutes and was reduced to 41 ± 9 minutes in the presence of CHIP.

CHIP stimulates ASK1 ubiquitylation

To determine whether the CHIP-dependent decrease in steady-state protein levels of ASK1 were due to ubiquitin-dependent proteasomal degradation, COS-7 cells were co-transfected with HA-ASK1 and CHIP and treated with or without MG132, a proteasome inhibitor, followed by Western blot analysis. Reduced expression of ASK1 by CHIP was partially recovered after treatment with MG132 (Fig 2A), which suggests that the effects of CHIP on ASK1 are proteasome dependent. Because ASK1 is known to undergo ubiquitin-dependent proteasomal degradation, we examined whether ubiquitylation of ASK1 is increased by CHIP. HA-tagged ASK1 was immunoprecipitated from cells with or without coexpression of CHIP using an anti-HA antibody, and ubiquitylated forms of ASK1 were detected by blotting ASK1 immunoprecipitates with an anti-ubiquitin antibody. This analysis demonstrated that ubiquitylation of ASK1 was enhanced by CHIP (Fig 2B).

The preceding observations are consistent with the known ubiquitin ligase activity of CHIP (Jiang et al 2001). To confirm that CHIP directly targets ASK1 for ubiquitylation, ASK1 ubiquitylation was recapitulated in an *in vitro* system using immunoprecipitated HA-ASK1 and recombinant E1, E2 (UBCH4), and His-tagged CHIP (Fig 2C). Slowly migrating ubiquitylated forms of ASK1 were detected in these reactions when ubiquitin, E1, UBCH5, and CHIP were present, and deletion of any component abolished their assembly. These data demonstrated that ASK1 is a substrate for the ubiquitin ligase activity of CHIP and suggested that CHIP may regulate ASK1 activity through ubiquitin-dependent degradation.

Fig 2. CHIP stimulates ubiquitylation of ASK1. (A) COS-7 cells were transiently transfected with HA-ASK1 with or without Myc-CHIP and treated with 25 μ M MG132 or vehicle for 8 hours before harvesting. Cell lysates were immunoblotted for HA-ASK1, Myc-CHIP, and β -actin to normalize protein amounts. (B) COS-7 cells were transfected as in panel A and immunoprecipitated with anti-HA antibody. Immunoprecipitated proteins were immunoblotted with anti-ubiquitin antibody. (C) In vitro ubiquitylation assay was performed using HA-ASK1 as a substrate with the indicated components of the ubiquitylation reaction. Ub-ASK1, ubiquitylated ASK1; WCL, whole cell lysates; IP, immunoprecipitation.

CHIP interacts with ASK1 through the TPR domain

We performed coimmunoprecipitation assays to determine whether ASK1 and CHIP directly interact. Because protein phosphatase 5 interacts with ASK1 through its

TPR domain, we were particularly interested in whether the TPR region of CHIP is required to bind and degrade ASK1. HA-ASK1 was coexpressed with Myc-tagged wild-type CHIP or a similar construct lacking the TPR domain (Myc-CHIP Δ TPR) in COS-7 cells. The cell lysates were

Fig 3. CHIP interacts with ASK1 via its TPR domain. COS-7 cells were transiently transfected with HA-ASK1 and either vector, wild-type Myc-CHIP (Myc-CHIP WT) or Myc-CHIP lacking the TPR domain (Myc-CHIP Δ TPR). Twenty-four hours after transfection, cell lysates were immunoprecipitated with anti-Myc. Whole cell lysates and the immunoprecipitated proteins were immunoblotted with anti-HA, anti-Myc, and anti- β -actin, as indicated.

immunoprecipitated with an antibody recognizing the Myc epitope. Full-length CHIP, but not the mutant protein lacking the TPR domain, efficiently coimmunoprecipitated ASK1 (Fig 3, left panels), indicating an *in vivo* interaction between these proteins. Interestingly, CHIP Δ TPR also failed to induce ASK1 degradation (Fig 3, right panels), which demonstrates that degradation of ASK1 by CHIP requires specific interactions between the 2 proteins.

CHIP inhibits JNK1 kinase activity and apoptosis

To determine the biological consequences of ASK1 ubiquitylation and degradation by CHIP, we examined effects of CHIP on JNK1 activity, a downstream target of the ASK1 signaling pathway that is a proximate effector of apoptosis by stresses, such as H_2O_2 and serum starvation, that induce ASK1 activity (Saitoh et al 1998; Morita et al 2001; Takeda et al 2003). We established COS-7 cells stably expressing Myc-CHIP (or control COS-7 cells) for these assays. These cells were transiently transfected with HA-ASK1 (or a control plasmid) and stimulated with H_2O_2 to induce JNK1 activity in an ASK1-dependent fashion. Western blot analysis indicated that ASK1 levels were lower in COS-7 cells expressing Myc-CHIP (Fig 4A), as expected. Cell lysates were subsequently immunoprecipitated using an antibody against JNK1. The immunoprecipitated proteins were assayed for *in vitro* JNK1 kinase activity in the presence of ^{32}P - γ -ATP, using purified c-Jun protein as a substrate (Fig 4B, upper panel). JNK1 activity was increased by H_2O_2 treatment in an ASK1-dependent fashion, but JNK1 activity was attenuated in COS-7 cells expressing Myc-CHIP. JNK1 protein levels were not decreased by CHIP (Fig 4B, lower panel), but a slower migrating band likely representing phosphorylated JNK1 was not apparent in these cells. We confirmed this result by transfecting CHIP siRNA into COS-7 cells to knock down endogenous CHIP and performed *in vitro* JNK1 kinase assays. Under the conditions of our assays, CHIP protein levels were decreased by 80% using siRNA-mediated silencing. Reduction of CHIP expression en-

hanced JNK1 activity under basal conditions and after H_2O_2 treatment (Fig 5). Because JNK1 is a known target of ASK1 kinase activity, the observations in these studies indicate that CHIP inhibits JNK1 activity through its effects on ASK1 steady-state levels, although an additional direct effect on ASK1 activity cannot be excluded.

To test the cellular consequences of CHIP's effects on ASK1, we examined the effects of CHIP on stress-dependent apoptosis using 2 different methods: TUNEL staining and nuclear morphology, as described previously (Zhang et al 1999; Morita et al 2001; Park et al 2002). For these assays, COS-7 cells were transiently transfected with plasmids expressing HA-ASK1 and either Myc-CHIP or empty vector along with pEGFP (to identify transfected cells). Transfected cells were serum-starved for 24 hours to induce ASK1-dependent apoptosis (Zhang et al 1999). After fixation, GFP-expressing cells were identified to indicate transfected cells, and around 400 GFP-expressing cells were counted in each experiment and sample. Cells demonstrating apoptotic nuclear morphology (cell shrinkage and chromatin condensation) were counted. Under these conditions, $26 \pm 7\%$ of cells underwent apoptosis, but cells expressing CHIP were protected from serum starvation-dependent apoptosis ($11 \pm 1\%$), a difference that is statistically significant ($P < 0.01$). Similar results were also apparent by TUNEL staining (data not shown). These apoptosis assays are consistent with the effects of CHIP on *in vitro* JNK1 activity (Fig 4). Therefore, we conclude that CHIP reduces stress-dependent apoptosis mediated by ASK1 through the ubiquitylation and degradation of ASK1.

CHIP elicits nuclear relocalization of the apoptotic regulator Daxx

CHIP exists mainly in the cytoplasm, although it can accumulate in the nucleus under conditions of stress (Ballinger et al 1999; Dai et al 2003). ASK1 also exists exclusively in the cytoplasm (Ko et al 2001; Song and Lee 2003). Daxx

A. Western Blot

B. In vitro JNK1 assay

Fig 4. Ubiquitylation and degradation of ASK1 mediated by CHIP reduces JNK1 activity under stress. (A) COS-7 cells stably transfected with Myc-CHIP (COS-7/Myc-CHIP). COS-7/Myc-CHIP as well as COS-7 cells were transiently transfected with either HA-ASK1 or vector. Twenty-four hours after transfection, cells were treated with or without 2 mM H₂O₂ for 20 minutes before harvesting. Cell lysates were blotted with anti-HA, anti-Myc, and anti- β -actin. Quantitative densitometric analysis of JNK1 activity ($n = 3$) is shown in the bar graph. (B) The same cell lysates as in panel A were immunoprecipitated with anti-JNK1 antibody and immunoprecipitated proteins were subjected to an in vitro JNK1 activity assay using c-Jun (79) as a substrate as described in Materials and Methods. ³²P-radiolabeled c-Jun was observed after separation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and exposure to film (upper panel). The same cell lysates were also used for immunoblotting JNK1 expression level (lower panel) with anti-JNK1 antibody.

is an apoptotic regulator that shuttles between the nucleus and the cytoplasm. When localized in the cytoplasm, Daxx interacts with and activates ASK1 (Chang et al 1998; Charette et al 2000; Ko et al 2001; Song and Lee 2003); in contrast, when ASK1 levels are suppressed, Daxx relocates to the nucleus and exerts antiapoptotic effects (Ko et al 2001). Therefore, we tested subcellular localization of Daxx in COS-7 cells expressing Flag-Daxx alone or with either wild-type CHIP or CHIP H260Q. In the absence of CHIP

expression and in the overexpression of CHIP H260Q, Daxx was uniformly distributed between the nucleus and cytoplasm (Fig 6). Expression of wild-type CHIP induced a dramatic relocation of Daxx into the nucleus. Thus, degradation of ASK1 by CHIP reduces cytoplasmic (and proapoptotic) Daxx and increases nuclear (and antiapoptotic) Daxx, indicating that CHIP regulates multiple steps in the ASK1-dependent apoptotic pathway to protect against stress-dependent cell death.

Fig 5. Regulation of JNK1 activity by endogenous CHIP levels. COS-7 cells were transiently transfected with CHIP and control siRNA expression vectors. Cells were treated with or without 2 mM H_2O_2 for 20 minutes before harvesting, and JNK1 activity was measured. Quantitative densitometric analysis of JNK1 activity ($n = 3$) is shown in the bar graph.

DISCUSSION

ASK1 is well known as a proapoptotic, stress-activated signaling molecule, and it is under tight regulation at multiple levels. In this report, we demonstrate that the cochaperone/ubiquitin ligase CHIP associates with and induces ubiquitin-dependent degradation of the proapoptotic MAPKKK ASK1. This event leads to inhibition of stress-induced, ASK1-dependent JNK activation and apoptosis and, concomitantly, nuclear translocation of the ASK1 partner protein Daxx. Given the central role of CHIP in coordination of the cellular stress response through its activity as a cofactor for cytoplasmic chaperones, as well as its ability to induce proteasome-dependent degradation of damaged proteins, it makes sense that CHIP would also exert functions that counter a cell's propensity to activate the apoptotic program. Direct convergence and counter regulation of CHIP-dependent cell survival mechanisms and ASK1-dependent apoptosis therefore provides a plausible and perhaps necessary means to balance cellular life or death decisions when homeostatic mechanisms are imbalanced.

It is well known that molecular chaperones and co-chaperones play a protective role under conditions of cellular stress by facilitating both refolding and degradation of misfolded proteins (Gabai et al 1997; Beere et al 2000; Song et al 2001). In addition, Hsps directly modulate stress-dependent signaling pathways to attenuate cell damage and repress apoptotic events during the response to stress. Figuring prominently among these pathways, it

is now well accepted that ASK1 signaling cascades are regulated by molecular chaperones. Hsp70 affects this pathway at sequential steps by direct interactions with JNK (through its peptide-binding domain) and ASK1 (through the N-terminal ATP-binding domain) (Park et al 2001, 2002). These interactions inhibit JNK and p38 MAP kinase activities and block ASK1-dependent apoptosis. Hsp27, which like Hsp70 is a stress-inducible chaperone, inhibits Fas-induced ASK1-dependent apoptosis by preventing translocation of Daxx into the cytoplasm and blocking its interaction with ASK1 (Charette et al 2000), demonstrating yet another chaperone-dependent mechanism for regulating ASK1-dependent signaling and apoptosis. The interactions described in the present studies provide another means by which protective chaperone-dependent events regulate ASK1 activation.

We have recently reported that CHIP induces trimerization and transcriptional activation of heat shock transcription factor (Hsf1), which in turn leads to induction of stress-responsive chaperones such as Hsp70 and Hsp27 (Dai et al 2003). In addition, stress-induced apoptosis is increased in CHIP (-/-) fibroblasts in vitro and in thermally challenged CHIP (-/-) mice in vivo; taken together, these studies suggest that CHIP may be an important molecular bridge between protein folding systems and stress-induced apoptosis. The present studies extend these observations by demonstrating the physical and functional interactions of CHIP with the stress-regulated apoptotic protein, ASK1. The TPR domain of CHIP

Fig 6. CHIP triggers translocation of Daxx into nucleus. COS-7 cells were transiently transfected with Flag-Daxx with wild-type CHIP, CHIP H260Q mutant, or the empty vector. Twenty-four hours after transfection, cells were fractionated to nucleus and cytoplasm, and localization of Flag-Daxx and CHIP were determined by immunoblotting using antibodies against Flag and CHIP, respectively. Anti-Oct-1 and anti-GAPDH were used for quantitating nuclear and cytoplasmic proteins, respectively. Nu, nucleus; Cy, cytoplasm; Vec, empty vector (pcDNA3); WT, wild-type CHIP; H260Q, CHIP H260Q mutant.

is required for its interactions with both Hsp70 (Dai et al 2003) and ASK1 (Fig 3), which raises the question of whether competitive interactions exist among these proteins. Coimmunoprecipitation of ASK1 and CHIP indicates that Hsp70 binding to ASK1 is enhanced in the presence of CHIP (J. Hwang and C. Patterson, unpublished observations). This suggests that ASK1 binding to CHIP is not competitive with Hsp70 for the TPR domain and is consistent with both CHIP and Hsp70 having suppressive effects on ASK1 activity. It is plausible that CHIP may have a facilitative effect on the interaction between ASK1 and Hsp70, such that ASK1 activity is inhibited by Hsp70 interactions and ASK1 itself is subject to ubiquitin-dependent degradation that is activated by the ubiquitin ligase activity of CHIP.

Concomitant with its effects on ASK1 activity, CHIP inhibits translocation of Daxx from the nucleus to the cytoplasm (Fig 6). Although it is not clear how CHIP affects the localization of Daxx, it is likely that reduction of endogenous cytoplasmic ASK1 levels by CHIP may cause the ASK1 partner protein Daxx to accumulate in the nucleus. Daxx has also recently been shown to interact with and activate Hsf1 activity (Boellmann et al 2004). Therefore, it is also possible that CHIP-dependent Hsf1 nuclear translocation (Dai et al 2003) may cause Daxx to accumulate in the nucleus, where it may enhance the transcriptional activity of Hsf1 to induce Hsp70 expression level. Finally, Charette et al. (2000) reported that Hsp27 blocks Fas-induced translocation of Daxx from the nucleus to the cytoplasm. Because Hsp27 expression is induced by CHIP (Dai et al 2003), it is also plausible that CHIP's effect on Daxx localization may be mediated in part through effects on Hsp27. In any event, retention of Daxx within the nucleus by CHIP provides another layer of

antiapoptotic activity because cytoplasmic Daxx exerts proapoptotic effects by binding and activating ASK1.

We propose a model that accounts for the ability of CHIP to modulate ASK1-dependent signaling and apoptosis at multiple levels. CHIP exerts direct effects through ubiquitylation and degradation of ASK1 that suppress ASK1-dependent apoptosis in the setting of stress. In addition, CHIP causes Daxx to accumulate within the nucleus (as a consequence of reduced levels of ASK1 in the cytoplasm), and this may in turn enhance activation of Hsf1. Increased Hsf1 activity would lead to expression of Hsp70 (an inhibitor of ASK1 and JNK [Park et al 2001, 2002]) and Hsp27 (preventing the binding of Daxx to ASK1 and thus inhibiting apoptosis [Charette et al 2000]). These activities allow CHIP to regulate ASK1-dependent signaling and apoptosis at multiple levels downstream of the kinase. Taken together, these observations provide a potential mechanism for the ability of CHIP to inhibit stress-dependent apoptosis, which we have described previously (Dai et al 2003). Also, we cannot exclude the possibility that CHIP might regulate apoptosis through other parallel mechanisms. Further identification of other pro- or antiapoptotic proteins regulated by CHIP (which may also be substrates for CHIP's ubiquitin ligase activity) will help us understand CHIP's functional effects on the coordination of decisions about cellular life and death in response to stress.

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