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MEKK1 Mediates the Ubiquitination and Degradation of c-Jun in Response to Osmotic Stress▽

Yan Xia,[1] Ji Wang,[2] Shuichan Xu,[3] Gary L. Johnson,[4] Tony Hunter,[5] and Zhimin Lu[1,6,7]*

Brain Tumor Center and Department of Neuro-Oncology,[1] Department of Thoracic and Cardiovascular Surgery,[2] and Department of Molecular Genetics,[6] The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030; Signal Pharmaceuticals, LLC, San Diego, California 92121[3]; Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599[4]; Molecular and Cell Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037[5]; and The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas 77030[7]

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c-Jun, a major transcription factor in the activating protein 1 family of regulatory proteins, is activated by many physiologic and pathological stimuli. We show here that c-Jun was downregulated in response to osmotic stress via ubiquitination-dependent degradation by the PHD/RING finger domain of MEKK1, which exhibited E3 ubiquitin ligase activity toward c-Jun in vitro and in vivo. The reduced c-Jun protein level resulting from exogenous expression of wild-type MEKK1 and the opposite effect induced by expression of a MEKK1 PHD/RING finger domain mutant were consistent with a higher level of c-Jun protein in MEKK1$^{-/-}$ cells than in corresponding wild-type cells. The deficiency of MEKK1 blocked posttranslational downregulation of c-Jun in response to osmotic stress. Furthermore, apoptosis induced by osmotic stress was suppressed by overexpression of c-Jun, indicating that the downregulation of c-Jun promotes apoptosis.

Activating protein 1 (AP-1) transcription factors are basic region-leucine zipper proteins and are composed of the Jun (c-Jun, JunB, and JunD), Fos (c-Fos, FosB, Fra-1, and Fra-2), Maf (c-Maf, MafB, MafA, MafG/F/K, and Nrl), and ATF (ATF2, LRF1/ATF3, B-ATF, JDP1, and JDP2) subfamilies (1, 5, 6). AP-1 members form homodimers and/or heterodimers through their leucine zipper domains and recognize 12-*O*-tetradecanoylphorbol-13-acetate (TPA) response elements [5′-TGA(G/C)TCA-3′], cyclic AMP response elements (5′-TGACGTCA-3′), MAF recognition elements [5′-TGCTGAC(G)TCAGCA-3′], or antioxidant response elements (5′-RTGACnnnGC-3′) (15, 24, 36, 40). Various physiologic and pathological stimuli, including phorbol ester, growth factors, oncoproteins (e.g., Src and Ras), proinflammatory cytokines, chemotherapeutic drugs, osmotic stress, and UV radiation, can activate c-Jun and other AP-1 transcription factors (2, 27, 31, 39). As a major component of AP-1, the proto-oncogene c-*jun* is the cellular homologue of v-*jun*, the transforming oncogene in the genome of avian sarcoma virus 17 (42). c-Jun activation is instrumental in cell growth and differentiation, apoptosis, cell transformation, tissue morphogenesis, and inflammatory responses (19). Mouse embryos lacking c-Jun die at mid- to late gestation and exhibit impaired hepatogenesis, altered fetal liver erythropoiesis, and generalized edema (18, 22).

The activity of individual AP-1 components can be controlled by transcriptionally regulated protein expression, interaction with other proteins, and posttranslational modifications, such as phosphorylation, ubiquitination, and sumoylation (32, 33). c-Jun binds to AP-1 binding sites of the c-*jun* enhancer and engages in a positive autoregulatory loop (42). c-Jun activity can be posttranslationally regulated by a mitogen-activated protein (MAP) kinase signaling pathway. This pathway is a three-tiered cascade: a MAP kinase is activated by a MAP kinase/ERK kinase (MEK) or a MAP kinase kinase (MKK), which is in turn activated by a MEK kinase (MEKK) or MAP kinase kinase kinase (MKKK) (27). The classic MAP kinase family consists of three subfamilies: extracellular signal-regulated kinases (ERK; ERK1 and ERK2), c-Jun NH_2-terminal kinases (JNK; JNK1, JNK2, and JNK3), and p38-MAP kinases (α, β, δ, and γ) (27). In response to stress stimuli, c-Jun is activated by JNK phosphorylation at the serine 63 (Ser63) and Ser73 residues in the transactivation domain near its N terminus (42). Acting as a MAP kinase kinase kinase, MEKK1 phosphorylates several different MKKs; its strongest activity is directed toward MKK4, an upstream kinase for JNKs (47).

In addition to functioning as a Ser/threonine protein kinase, MEKK1 acts as a ubiquitin (Ub) E3 ligase via its N-terminal plant homeodomain (PHD) (29, 46), the structure of which is closely related to the RING finger domain, with seven cysteines (Cys) and a histidine (His) spatially arranged in a C_4HC_3 consensus sequence (8). Because of this striking similarity, the MEKK1 PHD domain is regarded as an atypical RING finger domain (3) and is currently designated a PHD/RING finger domain. Unlike the enzymatic HECT (homologous to the E6-AP C terminus) domain E3s, the PHD/RING finger domain, the RING finger domain, and the U box E3 ligases do not form a thioester with Ub or transfer Ub directly to substrates but rather function as adaptors, facilitating the interaction between various distinct substrates and a Ub-conjugating enzyme (E2) (4, 8). Polyubiquitination mediated by a Ub-activating enzyme (E1), an E2, and an E3 targets substrate proteins for proteasome-mediated degradation.

In this report, we demonstrate that c-Jun is downregulated

* Corresponding author. Mailing address: Department of Neuro-Oncology, Unit 1002, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030. Phone: (713) 834-6231. Fax: (713) 834-6230. E-mail: zhiminlu@mdanderson.org.

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in response to osmotic stress and that MEKK1, which exhibits E3 Ub ligase activity toward c-Jun, plays an important role in osmotic stress-induced and Ub/proteasome-dependent c-Jun degradation.

MATERIALS AND METHODS

Cells and cell culture conditions. NIH 3T3 mouse fibroblasts, 3Y1 rat fibroblasts, MEKK1$^{+/+}$ and MEKK1$^{-/-}$ mouse 3T3 fibroblasts, and 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

Cell stimulation with osmotic stress. Cells were grown in medium containing 0.5% serum for 16 h and then treated with sorbitol (500 mM).

Materials. MG132 was from Calbiochem (San Diego, CA). Polyclonal antibody against JNK1 (C-17) and hemagglutinin (HA) and monoclonal antibodies against phospho-JNK (G-7) and phospho-c-Jun (KM-1) were from Santa Cruz Biotechnology (Santa Cruz, CA). Cycloheximide, TPA, sorbitol, and monoclonal antibodies against actin, poly-His, and tubulin were from Sigma (St. Louis, MO). Monoclonal anti-Ub antibody was from Zymed Laboratories (South San Francisco, CA). Hoechst 33342, Alexa Fluor 488 goat anti-mouse antibody, and Alexa Fluor 594 goat anti-rabbit antibody were from Molecular Probes (Eugene, OR). Epidermal growth factor (EGF) was from Upstate Biotechnology (Lake Placid, NY). Anti-c-Jun monoclonal antibody was from BD Biosciences (San Jose, CA).

DNA constructs and mutagenesis. A PCR-amplified human c-*jun* cDNA was cloned into pHis8 vector between BamHI and HindIII. Plasmids for the glutathione *S*-transferase (GST)-MEKK1 PHD/RING finger, GST-MEKK1 PHD/RING finger C433A, HA-MEKK1, HA-MEKK1 C433/478A, HA-MEKK1 D1369A, and pCep4 were described previously (29).

Immunoprecipitation and immunoblotting analysis. Extraction of proteins with a modified buffer from cultured cells was followed by immunoprecipitation and immunoblotting with corresponding antibodies as described previously (28). The immunoblotting analysis with the anti-Ub antibody was carried out after the membranes had been preincubated with denaturing buffer (6 M guanidine-HCl, 20 mM Tris-HCl [pH 7.5], 5 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride) for 30 to 60 min at 4°C followed by extensive washing in phosphate-buffered saline.

Purification of recombinant proteins. The wild-type (WT) or mutant GST-MEKK1 PHD/RING finger protein, His-E1, His-E2 (UbcH5B), GST-Ub, and His-c-Jun were expressed in bacteria and purified as described previously (29).

In vitro ubiquitination assays. In vitro ubiquitination reactions were carried out as described previously (21). Approximately 3 μg of WT or mutant GST-MEKK1 PHD/RING finger and His-c-Jun was incubated with 50 to 500 nM His-E1, 0.5 to 5 μM His-UbcH5B, 10 μM GST-Ub, and 2 mM ATP in reaction buffer (50 mM Tris-HCl [pH 7.5], 2.5 mM $MgCl_2$, and 0.5 mM dithiothreitol).

Immunofluorescence analysis. Cells were fixed and incubated with primary antibodies, Alexa Fluor dye-conjugated secondary antibodies, and Hoechst 33342 according to standard protocols. Cells were examined using a fluorescent deconvolutional microscope (Zeiss, Thornwood, NY) with a 60-Å oil immersion objective. Axio Vision software from Zeiss was used to deconvolve Z-series images.

Cell apoptosis analysis. Apoptosis was measured using the annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit from BD Biosciences according to the manufacturer's instructions. Cells were treated with or without sorbitol for 12 h. Both adherent and floating cells were collected and resuspended in 1× cold binding buffer (10 mmol/liter HEPES [pH 7.4], 150 mmol/liter NaCl, 2.5 mmol/liter $CaCl_2$, 1 mmol/liter $MgCl_2$, and 4% bovine serum albumin) for analysis. Cells were also stained with propidium iodide to detect dead cells. Analysis was done on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro software (BD Biosciences). Unstained cells were classified as "live or survival" cells and those stained with annexin V only as "early apoptotic," while cells stained with both annexin V and propidium iodide were classified as "late apoptotic" and those stained with propidium iodide only as "dead."

RESULTS

Osmotic stress-induced Ub/proteasome-mediated c-Jun degradation. Extracellular stimuli such as tumor necrosis factor alpha, UV B irradiation, EGF, nerve growth factor, and basic fibroblast growth factor increase c-*jun* mRNA and protein levels (7, 11, 20, 41). To examine the effect of osmotic stress on c-Jun expression, we stimulated 3Y1 rat fibroblasts with sorbitol for up to 6 h. Immunoblotting with an anti-c-Jun antibody revealed that c-Jun protein was downregulated at 6 h of sorbitol osmotic stimulation, whereas tubulin levels remained constant (Fig. 1A, left panel). Similar results were obtained with NIH 3T3 mouse fibroblasts (data not shown). c-Jun was phosphorylated at Ser63 within 1 h of sorbitol treatment, and prolonged sorbitol treatment reduced the amount of phosphorylated c-Jun to an extent corresponding to the decrease in c-Jun protein (Fig. 1A, left panel). In contrast, stimulation of NIH 3T3 cells with serum, EGF, or TPA for 1 or 6 h resulted in phosphorylation of c-Jun and sustained upregulation of c-Jun expression. These data indicate that that the level of c-Jun protein is regulated differentially depending on the extracellular stimuli and cellular milieu involved (Fig. 1A, right panel).

Pretreatment with the proteasome inhibitor MG132 in the presence of the de novo protein synthesis inhibitor cycloheximide (CHX) reduced the sorbitol-induced c-Jun downregulation (Fig. 1B), suggesting that proteasome activity is involved in c-Jun downregulation. To examine the role of phosphorylation in sorbitol-induced posttranslational c-Jun downregulation, 293T cells were transiently transfected with WT His-c-Jun or a His-c-Jun S63/73A mutant, in which JNK phosphorylation sites Ser63 and Ser73 are mutated to alanine (Ala). Pretreatment with CHX before sorbitol revealed that the mutant, acting similarly to its WT counterpart, was downregulated (Fig. 1C). CHX treatment alone resulted in strong phosphorylation of WT c-Jun but not of the mutant. Immunoblotting of immunoprecipitated WT His-c-Jun or the S63/73A mutant from 293T cells with an anti-Ub antibody demonstrated sorbitol-induced polyubiquitination of both the WT and mutant proteins, as shown by the enhanced smear of the more slowly migrating bands (Fig. 1D). These data indicate that the process of Ub/proteasome-mediated protein degradation is involved in the sorbitol-induced downregulation of c-Jun, which is independent of its phosphorylation at S63/73 by JNK.

MEKK1 PHD/RING finger domain-dependent c-Jun ubiquitination. Sorbitol treatment activates MEKK1 (29) and in turn the MAP kinase cascade, leading to c-Jun phosphorylation by JNK1/2 (47). The MEKK1 PHD/RING finger domain possesses E3 ligase activity (29, 46). To determine whether MEKK1 has E3 ligase activity toward c-Jun, we performed an in vitro ubiquitination assay. Purified bacterially expressed His-c-Jun was incubated with purified E1, E2 (UbcH5B), Ub, and GST-MEKK1 PHD/RING finger domain WT protein or the C433A mutant protein, in which the first Cys in the PHD/RING finger domain is mutated to Ala, thereby abolishing its E3 ligase activity (29). Immunoblotting with an anti-c-Jun antibody revealed that WT protein but not the C433A mutant stimulated oligoubiquitination of c-Jun (Fig. 2A). Thus, the MEKK1 PHD/RING finger domain has E3 ligase activity toward nonphosphorylated c-Jun in vitro.

To test whether MEKK1 is an E3 ligase for c-Jun ubiquitination in vivo, 293T cells were transfected with HA-tagged WT MEKK1 or the C433/478A MEKK1 E3 ligase mutant, in which both the first and penultimate Cys in the PHD/RING finger domain are mutated to Ala. Immunoprecipitation of c-Jun followed by immunoblotting with an anti-Ub antibody showed that the overexpression of WT MEKK1 but not the C433/478A mutant resulted in polyubiquitination and reduced

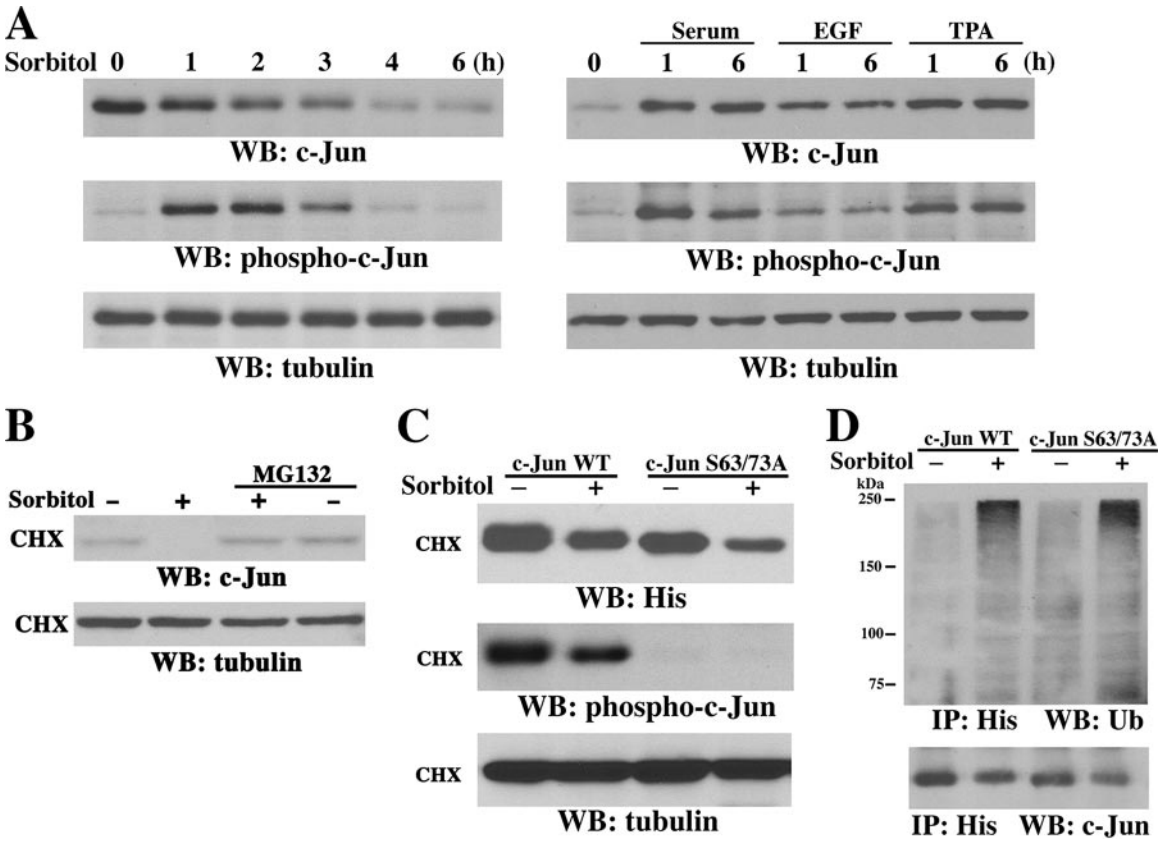

FIG. 1. Osmotic stress-induced Ub/proteasome-mediated c-Jun degradation. Immunoblotting (Western blotting [WB]) analyses were carried out with the indicated antibodies. Tubulin was used as an input control. (A) Serum-starved 3Y1 cells (left panel) or NIH 3T3 cells (right panel) were treated with sorbitol (500 mM), 20% fetal bovine serum, EGF (100 ng/ml), or TPA (400 nM) for the indicated times. (B) 3Y1 cells were treated with CHX (200 μg/ml) for 30 min, then MG132 (50 μM) for 30 min, and finally sorbitol with CHX and MG132 for 6 h. (C) 293T cells transiently transfected with WT pMT35-His-c-Jun or pMT35-His-c-Jun S63/73A were treated with CHX (200 μg/ml) for 30 min and then with or without sorbitol for 6 h. (D) Immunoprecipitation (IP) of WT His-c-Jun or His-c-Jun S63/73A from 293T cells with or without sorbitol treatment for 1 h.

protein levels of c-Jun (Fig. 2B). Relatively similar amounts of c-Jun protein in the cells transfected with the vector and the C433/478A mutant were detected in the immunoprecipitates (Fig. 2B), in contrast to the enhanced level of total c-Jun observed by immunoblotting whole-cell protein from the cells expressing the C433/478A mutant (Fig. 3). This disparity occurred because the amount of anti-c-Jun antibody used for the immunoprecipitation was limiting and the antibodies were saturated with c-Jun.

Immunoblotting of immunoprecipitated MEKK1 with an anti-c-Jun antibody revealed a weak association between endogenous c-Jun and WT HA-MEKK1 or the D1369A kinase-dead mutant and an enhanced interaction between endogenous c-Jun and the C433/478A mutant (Fig. 2C). Thus, the regulation of c-Jun ubiquitination in vivo depends on the integrity of the MEKK1 PHD/RING finger domain, mutation of which enhances the association between MEKK1 and c-Jun. This enhanced association could be due to an increased amount of c-Jun protein or to an increased stability of the complex caused by the PHD/RING finger domain mutation. Immunofluorescence studies demonstrated that although MEKK1 was localized primarily in the cytosol, colocalization of MEKK1 with c-Jun in the nucleus could be observed (Fig. 2D). This finding is consistent with the detection of nuclearly localized MEKK1 by nucleus fractionation analyses (data not shown). These results imply that c-Jun ubiquitination by MEKK1 occurs in the nucleus.

MEKK1 PHD/RING finger domain-dependent c-Jun degradation. To test whether MEKK1 regulates c-Jun expression in vivo, 293T cells were transfected with HA-tagged WT MEKK1, the C433/478A MEKK1 E3 ligase mutant, or the D1369A kinase-dead mutant. As detected by immunoblotting analysis, expression of WT MEKK1 or the D1369A mutant moderately downregulated c-Jun, whereas the PHD/RING finger domain mutant significantly upregulated c-Jun (Fig. 3). In contrast, the level of JNK1 protein was unaffected. Expression of both WT MEKK1 and the C433/478A mutant activated JNK, as shown by immunoblotting with an anti-phospho-JNK antibody, whereas only the C433/478A mutant hampered c-Jun degradation. Furthermore, the kinase-dead mutant, which did not activate JNK, was still able to reduce c-Jun expression. These results indicate that expression of the MEKK1 E3 ligase mutant affects normal c-Jun turnover and strongly suggest that c-Jun downregulation is independent of JNK activation.

MEKK1 deficiency blocked posttranslational downregulation of c-Jun in response to osmotic stress. To examine the

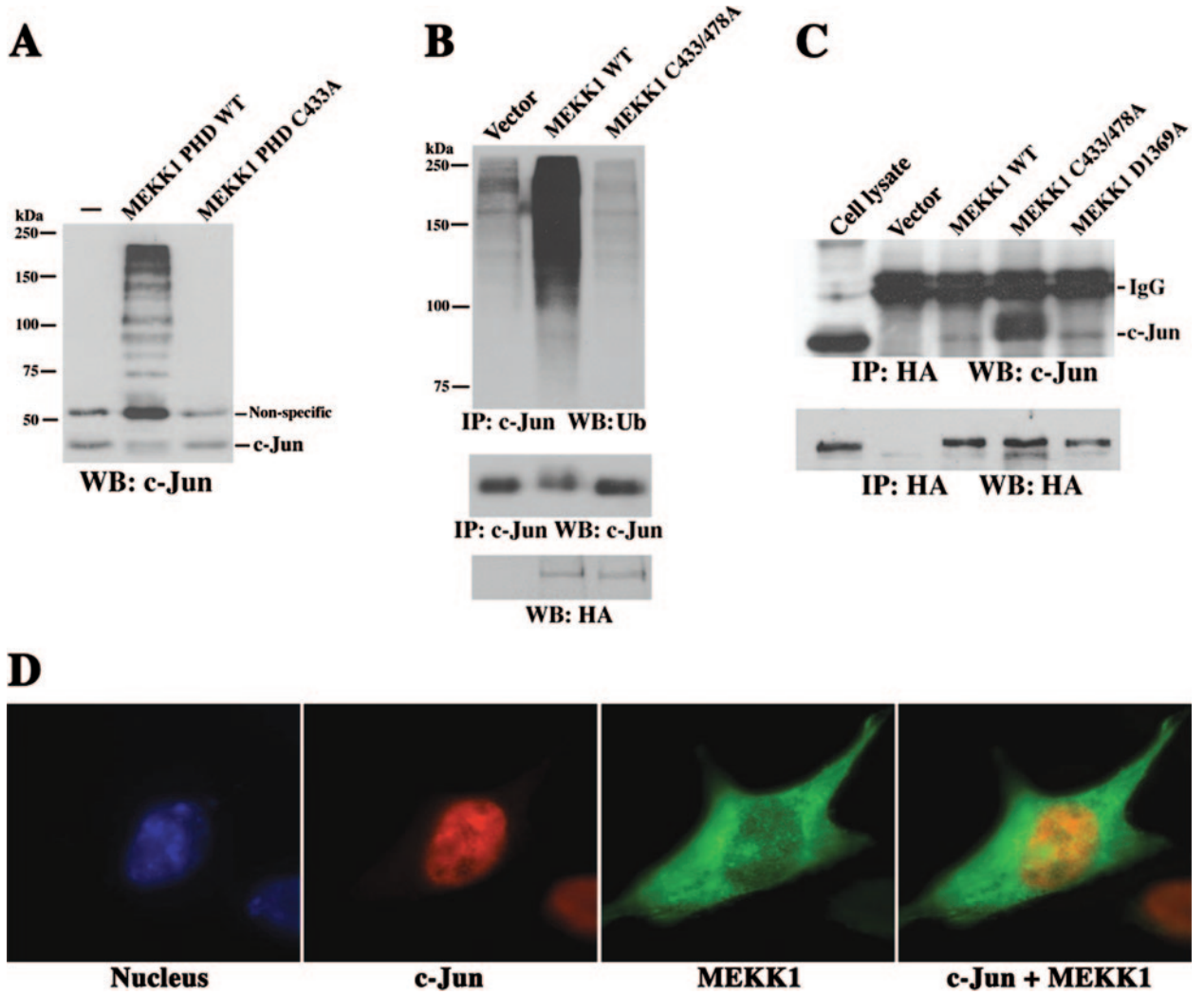

FIG. 2. MEKK1 PHD/RING finger domain-dependent c-Jun ubiquitination. Immunoblotting and immunofluorescence analyses were carried out with the indicated antibodies. (A) In vitro ubiquitination reactions with purified His-c-Jun with or without the WT GST-MEKK1 PHD/RING finger or the GST-MEKK1 PHD/RING finger C433A mutant protein. (B) 293T cells transiently transfected with pCep4 (vector), WT HA-MEKK1, or HA-MEKK1 C433/478A for 48 h were used for immunoprecipitation with an anti-c-Jun antibody. (C) 293T cells transiently expressing WT HA-MEKK1, HA-MEKK1 C433/478A, or HA-MEKK1 D1369A for 48 h were used for immunoprecipitation with an anti-HA antibody. (D) NIH 3T3 cells expressing HA-MEKK1 were stained with an anti-c-Jun antibody (red), an anti-HA antibody (green), or Hoechst 33342 (blue). Yellow indicates overlap of red and green.

effect of MEKK1 deficiency on c-Jun expression, MEKK1$^{-/-}$ and MEKK1$^{+/+}$ fibroblasts were treated with sorbitol for 6 h or left untreated. Consistent with the posttranslational regulation of c-Jun by MEKK1, the level of c-Jun protein (Fig. 4A), but not the level of c-*jun* mRNA measured by real-time quantitative reverse transcription-PCR analysis (data not shown), was higher in untreated MEKK1$^{-/-}$ fibroblasts than in MEKK1$^{+/+}$ cells. Sorbitol stimulation reduced c-Jun levels in both MEKK1$^{-/-}$ and MEKK1$^{+/+}$ cells.

Because c-Jun could in principle be regulated at both the posttranslational and protein synthesis levels, sorbitol-induced c-Jun downregulation in MEKK1$^{-/-}$ cells might be due to reduced protein synthesis. Treatment with CHX, which blocks protein synthesis and eliminates the effects of transcriptional regulation, abrogated sorbitol-induced c-Jun downregulation in MEKK1$^{-/-}$ cells but not in MEKK1$^{+/+}$ cells, although it should be noted that the overall level of c-Jun was lower in the CHX-treated cells as expected (Fig. 4A). These data demonstrate that MEKK1 is necessary for sorbitol-induced posttranslational degradation of c-Jun. The fact that sorbitol still induced a decrease in c-Jun expression in MEKK1$^{-/-}$ cells suggests that nonposttranslational regulation is another important mechanism for regulating c-Jun levels. Reconstituting the expression of WT MEKK1 or the C433/478A mutant in MEKK1$^{-/-}$ cells by stable transfection showed that the expression of WT MEKK1, but not its mutant lacking E3 ligase functionality, reduced c-Jun protein expression in the absence of sorbitol (Fig. 4B). These results further corroborate the function of MEKK1 as an E3 ligase regulating c-Jun protein levels.

Antiapoptotic function of c-Jun in sorbitol-induced apoptosis. Prolonged stress stimuli, such as sorbitol treatment, can

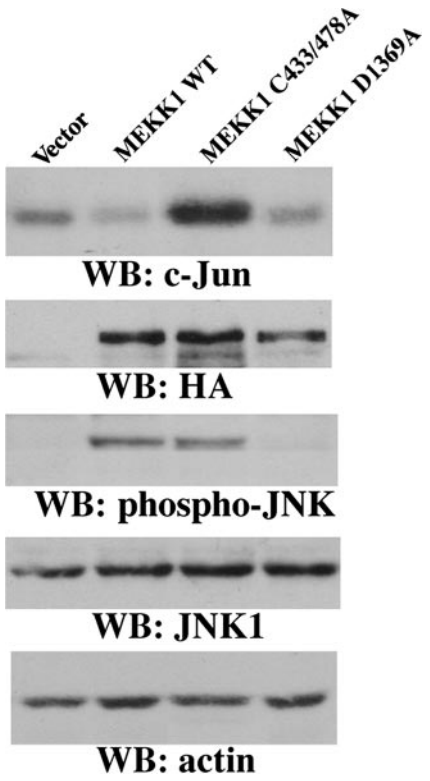

FIG. 3. MEKK1 PHD/RING finger domain-dependent c-Jun degradation. 293T cells transiently expressing WT HA-MEKK1, HA-MEKK1 C433/478A, or HA-MEKK1 D1369A for 48 h were used for immunoblotting analysis with the indicated antibodies. Actin was used as an input control.

cause apoptosis (12, 30). MEKK1$^{-/-}$ cells reconstituted for expression of the C433/478A MEKK1 mutant showed increased resistance to sorbitol-induced apoptosis, in contrast to MEKK1$^{-/-}$ cells reconstituted for expression of WT MEKK1, implying that c-Jun promotes cell survival against osmotic stress (Fig. 5A). To further clarify the role of c-Jun in sorbitol-induced apoptosis, NIH 3T3 cells transiently expressing His-c-Jun were treated with or without sorbitol for 12 h. NIH 3T3 cells overexpressing c-Jun showed an increased resistance to sorbitol-induced apoptosis, in contrast to untransfected cells, which displayed condensed and fragmented nuclei (Fig. 5B and C). These results indicate that c-Jun has antiapoptotic effects and that downregulation of c-Jun by MEKK1 promotes sorbitol-induced apoptosis.

DISCUSSION

In its role as a major AP-1 transcription factor, c-Jun, phosphorylated and activated in response to diverse extracellular stimuli, regulates gene expression. Stimulation with serum, EGF, or TPA induced c-Jun phosphorylation and increased its expression. In contrast, sorbitol-induced c-Jun phosphorylation diminished after prolonged treatment and was accompanied by reduced c-Jun expression. These results indicate that c-Jun activity is dynamically regulated via the dual mechanisms of phosphorylation and protein expression and that the activation of c-Jun is transient or sustained depending on the nature of the extracellular stimuli to which the cells are exposed.

The mutation of JNK phosphorylation sites in c-Jun did not hamper sorbitol-induced c-Jun ubiquitination and downregulation. Furthermore, MEKK1 ubiquitinated nonphosphorylated c-Jun in vitro. These findings emphasize that JNK-dependent c-Jun phosphorylation is not required for sorbitol-induced ubiquitination and downregulation of c-Jun. It was previously shown that the FBW7-containing Skp1/cullin/F-box protein complex, an E3 ligase, recognizes and degrades S63/73-phosphorylated c-Jun but not nonphosphorylated c-Jun in a neuronal system (34). However, this observation was not supported by a more recent report that showed that in the absence of extracellular stimulation, phosphorylation of Thr239 and Ser243 by GSK3, but not of Ser63/73, of c-Jun is required for FBW7-mediated c-Jun degradation. v-Jun containing a Ser243-to-phenylalanine

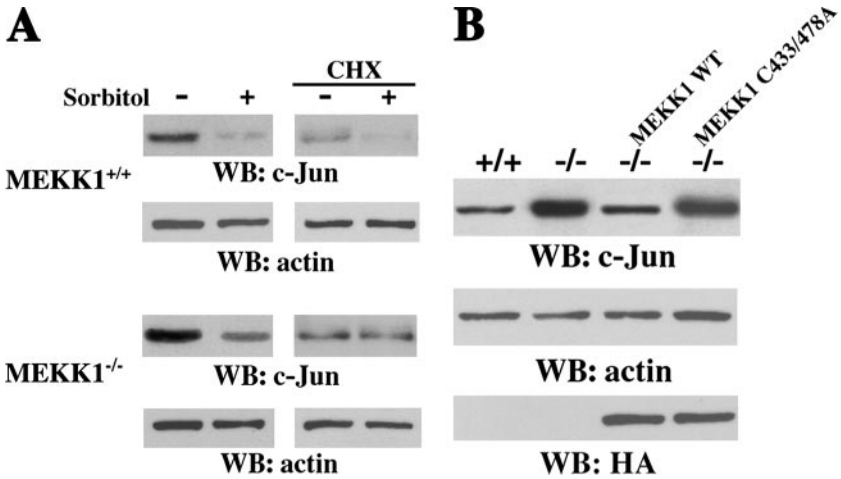

FIG. 4. MEKK1 deficiency blocked posttranslational downregulation of c-Jun in response to osmotic stress. Immunoblotting analyses were carried out with the indicated antibodies. Actin was used as an input control. (A) MEKK1$^{+/+}$ and MEKK1$^{-/-}$ cells were treated with or without sorbitol in the presence or absence of CHX (200 μg/ml) for 6 h. (B) Cell lysates of MEKK1$^{+/+}$ cells, MEKK1$^{-/-}$ cells, and MEKK1$^{-/-}$ cells with reconstituted expression of WT MEKK1 or MEKK1 C433/478A.

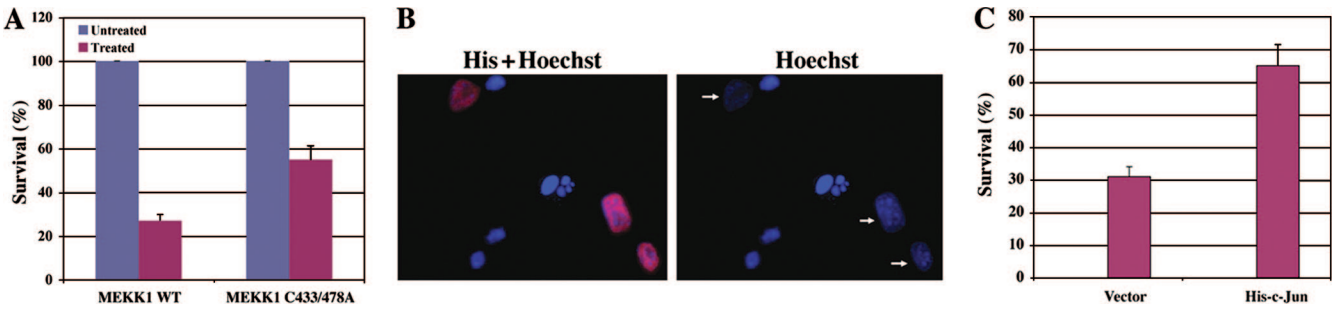

FIG. 5. Antiapoptotic function of c-Jun in sorbitol-induced apoptosis. (A) MEKK1$^{-/-}$ cells with reconstituted expression of WT MEKK1 or MEKK1 C433/478A were treated with or without sorbitol (500 mM) for 12 h, followed by staining with annexin V-FITC and propidium iodide, before being analyzed by flow cytometry. The relative levels of surviving cells were normalized to the levels of untreated cells. Data are means plus standard deviations for three independent experiments. (B) Representative photomicrographs of immunofluorescence analyses. Two days after transfection of NIH 3T3 cells with pMT35-His-c-Jun, the cells were treated with sorbitol for 12 h. Cells expressing His-c-Jun (red) were detected with an anti-His antibody. Nuclei were stained with Hoechst 33342 (blue). The arrows point to cells expressing His-c-Jun. (C) Surviving cells transfected with or without pMT35-His-c-Jun and treated with sorbitol were counted under a fluorescent deconvolutional microscope. The relative levels of cell survival were normalized to the levels of untreated cells. Data are means plus standard deviations for three independent experiments.

mutation escapes recognition by FBW7 (43). Enhanced c-Jun degradation in response to osmotic stress is different from human De-etiolated 1 (hDET1)-promoted ubiquitination and degradation of c-Jun, which occurs in nonstimulated cells by assembling a multisubunit Ub ligase containing DNA damage binding protein 1 (DDB1), cullin 4A (CUL4A), regulator of cullins 1 (ROC1), and constitutively photomorphogenic 1 (COP1) (44). Our results are also distinct from the finding that Itch, a HECT domain-containing E3, promotes c-Jun and JunB ubiquitination in a T-cell system (17). In this system, c-Jun ubiquitination is independent of c-Jun S63/73 phosphorylation but depends on the activation of JNK. Activated JNK1 phosphorylates Itch at S199, S232, and T222, which disrupts an inhibitory interaction between the WW domain of Itch and its catalytic HECT domain and induces a conformational change that greatly enhances Itch E3 ligase activity (16).

JNK1 and JNK2 differentially regulate c-Jun phosphorylation and stability, which are enhanced in JNK2$^{-/-}$ fibroblasts, whereas the absence of JNK1 has the opposite effect (37). In our experiments with fibroblasts, both WT MEKK1 and the E3 ligase-defective MEKK1 C433/478A mutant were able to activate JNK1/2; however, ectopic expression of the MEKK1 C433/478A mutant, but not WT MEKK1, significantly increased the level of c-Jun protein. Furthermore, the D1369A kinase-dead MEKK1 mutant, which did not activate JNK, was still able to downregulate c-Jun expression. These results suggest that neither JNK1 activation nor JNK2 activation is required for sorbitol-induced c-Jun degradation. Therefore, c-Jun ubiquitination is likely mediated by multiple E3 ligases depending on the cell and tissue type involved and the cellular regulatory influences that the cells are receiving. In addition to c-Jun, which is a substrate of MEKK1 E3 ligase, MEKK1 also regulates the stability of another AP-1 family member, Fra-2, by inducing Fra-2 ubiquitination and degradation (10).

c-Jun was downregulated in MEKK1$^{-/-}$ cells in response to osmotic stress. Nevertheless, MEKK1 deficiency blocked sorbitol-induced c-Jun downregulation in the presence of a de novo protein synthesis inhibitor. These results indicate that MEKK1 plays an important role in the posttranslational regulation of c-Jun and that osmotic stress-induced c-Jun downregulation results from the combined effects of MEKK1-mediated ubiquitination and a potential transcriptional repression. Indeed, osmotic stimulation results in transcriptional repression of c-*jun* by regulating histone deacetylase 3 (our unpublished data).

Both JNK and c-Jun may have pro- and antiapoptotic functions that depend on the cell or tissue type and the specific apoptotic stimuli involved (21, 25, 35). Tumor necrosis factor alpha (TNF-α) induces sustained JNK activation by inhibition of JNK-inactivating phosphatases (23). Activated JNK enhances Itch E3 ligase activity, which ubiquitinates and degrades antiapoptotic protein c-FLIP, an inhibitor of caspase 8, and promotes TNF-α-induced apoptosis (9). JNK phosphorylates the proapoptotic BH3-only subgroup of Bcl2-related proteins, Bim and Bmf, and induces Bax/Bak-dependent apoptosis (26). However, the JNK antiapoptotic function was also exemplified by a report showing that interleukin 3 withdrawal-induced apoptosis is suppressed by expression of a constitutively active JNK, which phosphorylates BAD at threonine 201, thereby inhibiting BAD association with the antiapoptotic molecule BCL-X_L (48). Both WT MEKK1 and the C433/478A MEKK1 mutant can activate JNK. Nevertheless, MEKK1$^{-/-}$ cells reconstituted for expression of the C433/478A MEKK1 mutant, which have a higher level of c-Jun expression than MEKK1$^{-/-}$ cells reconstituted for expression of WT MEKK1, showed increased resistance to sorbitol-induced apoptosis. These data imply that c-Jun, whose stability is regulated by MEKK1 but not JNK, plays a role in cells against apoptosis induced by osmotic stress. The fact that overexpression of c-Jun significantly blocked sorbitol-induced apoptosis provides additional evidence of the antiapoptotic functions of c-Jun. These results are consonant with the fact that c-Jun$^{-/-}$ fibroblasts are much less capable than c-Jun$^{+/+}$ cells of escaping UV-induced apoptosis (45). These results are also consistent with the massive level of apoptosis of hepatoblasts and erythroblasts that results from c-Jun deficiency in the developing mouse liver in vivo (14). Although more studies are needed to characterize the antiapoptotic functions of c-Jun, it has been shown that c-Jun suppresses p53 transcription by directly binding to a variant AP-1 site in the p53 promoter (38). Additionally, c-Jun protects hepatocytes from apopto-

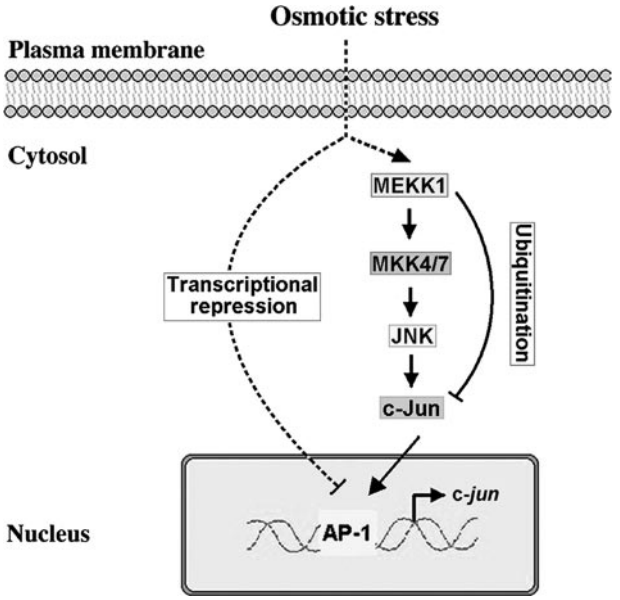

FIG. 6. Mechanism of osmotic stress-induced downregulation of c-Jun. c-Jun is phosphorylated and activated by the MEKK1-MEK4/7-JNK kinase cascade at an early stage of osmotic stress. Prolonged osmotic stress downregulates c-Jun protein through MEKK-1-mediated ubiquitination and degradation and transcriptional repression. Downregulation of c-Jun functions as a negative feedback mechanism for regulating cell survival pathways during persistent exposure to stress stimuli.

sis by antagonizing p53 activity in liver-specific c-Jun conditional mutant mice (13).

In summary, our results reveal a novel mechanism for the regulation of signal transduction in response to extracellular stimuli and provide a model for the dynamic regulation of c-Jun expression during stress responses in living cells (Fig. 6). c-Jun is phosphorylated and activated by the MEKK1-MKK4/7-JNK1/2 kinase cascade in response to brief treatment with sorbitol. Activated c-Jun counteracts extracellular stress by transcriptionally suppressing the expression of proapoptotic proteins or by activating the expression of antiapoptotic proteins, which allows cells to recover from transient stress stimuli. When exposure to the stress is prolonged, c-Jun expression is downregulated, which involves MEKK1-mediated ubiquitination and degradation of c-Jun. The depletion of c-Jun, which downregulates the activation of c-Jun during an early stage of exposure to stress, promotes osmotic stimulation-induced apoptosis. Our proposed model for the dynamic regulation of cell survival molecules, which are activated at an early stage of stress for counteracting apoptosis and downregulated at a late stage for promoting apoptosis, may represent an important general cellular mechanism.

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