Differential Regulation of c-Jun Protein Plays an Instrumental Role in Chemoresistance of Cancer Cells*

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Background: The mechanism underlying intrinsic tumor resistance to the widely used drug cisplatin remains elusive.

Results: Cisplatin induced c-Jun down-regulation via cleavage and ubiquitylation-dependent degradation of c-Jun in CDDP-sensitive cancer cells and c-Jun up-regulation via activation of JNK2 in CDDP-resistant cancer cells.

Conclusion: c-Jun plays an instrumental role in chemoresistance of cancer cells.

Significance: c-Jun is a molecular target for improving cancer therapy.

The chemotherapeutic drug cisplatin (cis-diamminedichloroplatinum(II) (CDDP)) is widely used in the treatment of human cancers. However, the mechanism underlying intrinsic tumor resistance to CDDP remains elusive. Here, we demonstrate that treatment with CDDP resulted in down-regulation of c-Jun expression via caspase-9-dependent cleavage of c-Jun at Asp-65 and MEKK1-mediated ubiquitylation and degradation of c-Jun in CDDP-sensitive cancer cells. In contrast, activation of JNK2 (but not JNK1) phosphorylated and up-regulated the expression of c-Jun in CDDP-resistant cancer cells. Activated c-Jun bound to the promoter regions of the MDR1 gene and promoted the expression of MDR1. Expression of a cleavage-resistant c-Jun mutant (D65A) suppressed CDDP-induced apoptosis of CDDP-sensitive cells, whereas depletion of JNK2, c-Jun, or MDR1 in CDDP-resistant cancer cells promoted apoptosis upon CDDP treatment. In addition, mammary gland tumors induced by polyomavirus middle T antigen in JNK2−/− mice were more sensitive to CDDP compared with those in JNK2+/+ mice. These findings highlight the instrumental role of c-Jun in the resistance of tumors to treatment with CDDP and indicate that c-Jun is a molecular target for improving cancer therapy.

The proto-oncogene c-jun is the cellular homolog of v-jun, the transforming oncogene in the genome of avian sarcoma virus 17 (1). Jun family proteins are major components of AP-1 (activating protein 1) transcription factors (2–4) that recognize either 12-O-tetradecanoylphorbol-13-acetate response elements (5′-TGA/GC/TCA-3′) or cAMP response elements (5′-TGACGTCG-3′) (5). c-Jun is activated by various physiologic and pathologic stimuli, including growth factors, oncoproteins, proinflammatory cytokines, and chemotherapeutic drugs (6–9). c-Jun activation is instrumental in cellular growth and differentiation, apoptosis, cell transformation, tissue morphogenesis, and inflammatory responses (10). Mouse embryos lacking c-Jun expression exhibit impaired hepatogenesis, altered fetal liver erythropoiesis, and generalized edema and die at middle-to-late gestation (11, 12).

The activity of individual AP-1 components can be regulated at multiple levels, including transcription; interaction with other proteins; and post-translational modifications, such as phosphorylation, ubiquitylation, and sumoylation (13, 14). c-Jun can bind to AP-1-binding sites in the c-jun enhancer to enhance c-Jun expression by a positive autoregulatory loop (1). c-Jun activity can be post-translationally regulated by the MAPK signaling pathway, which is composed of a cascade of three kinases: an MAPK, an MAPK kinase, and an MAPK kinase kinase (MEKK) (6). In response to stress stimuli, c-Jun is activated by JNK-dependent phosphorylation at Ser-63 and Ser-73 in the transactivation domain near its N terminus (1). c-Jun expression can be down-regulated by ubiquitin (Ub)2/proteasome-mediated degradation via distinct E3 Ub ligases or sumoylation, depending on the cell and tissue type involved and the cellular regulatory influences on the cells (13–21). In response to osmotic stress, c-Jun expression is down-regulated by histone deacetylase 3-dependent histone deacetylation at c-jun promoter regions (22).

Cisplatin (cis-diamminedichloroplatinum(II) (CDDP)) is one of the most potent anticancer agents, displaying significant

*The abbreviations used are: Ub, ubiquitin; CDDP, cis-diamminedichloroplatinum(II) (cisplatin); PyV MT, polyomavirus middle T.
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clonal antibodies against actin, JNK (FL), and MDR1 (multi-drug resistance1) and monoclonal antibodies against phosphor-
ning antibodies as described previously (27). The same experiments were performed at least three times.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture Conditions**—MEKK1+/+, MEKK1−/−, caspase-8+/+, caspase-8−/−, caspase-9+/+, caspase-9−/−, caspase-7−/−, caspase-3−/−, JNK1/2−/−, JNK1/2−/−, JNK1−/−, and JNK2−/− mouse fibroblasts; PC14 and A549 lung cancer cells; LN18, U87, U251, and U343 brain tumor cells; and 293T human embryonic kidney cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone, Logan, UT).

**Cell Treatment with CDDP**—Quiescent cells that were grown in medium containing 0.5% serum for 16 h were treated with 50 µg/ml CDDP.

**Transfection**—Cells were plated at a density of 4 × 105/60-mm dish for 18 h before transfection. Transfection was performed as described previously (22).

**Materials**—SP600125, the FAS-Fc fusion protein, the caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone, and MG132 were obtained from Calbiochem. The caspase inhibitor benzyloxycarbonyl-VAD-fluoromethyl ketone was expressed in bacteria were purified as described previously (27). The same experiments were performed at least three times.

**In Vitro Caspase Cleavage Assay**—As described previously (29), 500 ng of purified His-WT c-Jun, or His-c-Jun(D65A) protein was incubated with 2 units of caspases at 37 °C for 4 h in a reaction solution containing 50 mM NaCl, 50 mM HEPES, 10 mM EDTA, 5% glycerol, and 0.1% CHAPS (pH 7.2).

**DNA Constructs and Mutagenesis**—PCR-amplified human c-jun and truncated c-jun cDNAs (amino acids 66–331) were cloned into pFLAG and pcDNA6/V5-HisB vectors between BamHI and NotI. c-jun cDNA was cloned into a pHis8 vector between BamHI and HindIII. pHis8/c-Jun(D65A), pcDNA6/V5-HisB/c-Jun(D59A), and pcDNA6/V5-HisB/c-Jun(D65A) were constructed using a Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA).

pGIPZ c-Jun shRNA was generated with AACCTGAAAG-CTCAGAAGTC. pGIPZ MDR1 shRNA was generated with GCCTAGCAAATTTTGTGTTTTG. pGIPZ MDR1 shRNA was generated with AAGTTCCAGCCTCAGAC. pGIPZ control shRNA was generated with the control oligonucleotide GTCTAACCACGGAGGTCTT.

**Immunoprecipitation and Immunoblot Analysis**—Extraction of proteins from cultured cells using a modified buffer was followed by immunoprecipitation and immunoblotting with corresponding antibodies as described previously (27). The same experiments were performed at least three times.

**Purification of Recombinant Proteins**—His-WT c-Jun and His-c-Jun(D65A) expressed in bacteria were purified as described previously (28).

**RT-PCR**—Total RNA was extracted using an RNApure high-purity total RNA rapid extraction kit (Signalway Biotechnol). cDNA was prepared using oligonucleotides (dT), random primers, and a Thermo reverse transcription Kit (Signalway Biotechnol). PCR analysis was performed using 2 × SYBR real-time PCR premix (Signalway Biotechnol) under the following conditions: 5 min at 95 °C followed by 40 cycles at 95 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min using an ABI PRISM 7700 sequence detection system (Applied Biosystems).

The following primer pairs were used for quantitative real-time PCR: MDR1, 5′-GAGATAGGGCTGTTTGTGATG-3′ (forward) and 5′-CTGCTCCAGCTTTGATACA-3′ (reverse); GSTP1, 5′-GCTGGAAGGAGGAGGTGTG-3′ (forward) and 5′-GGACACGCAGGCTTCAA-3′ (reverse); and MRPI, 5′-ACACTGAATGGCATCACCCT-3′ (forward) and 5′-GCCGT-GTATCCAGGACCTG-3′ (reverse).

**In Vitro Caspase Cleavage Assay**—As described previously (29), 500 ng of purified His-WT c-Jun, or His-c-Jun(D65A) protein was incubated with 2 units of caspases at 37 °C for 4 h in a reaction solution containing 50 mM NaCl, 50 mM HEPES, 10 mM EDTA, 5% glycerol, and 0.1% CHAPS (pH 7.2).

**Cell Apoptosis Assay**—Plated cells (5 × 105) were left untreated or treated with CDDP for 12 h and then collected and processed for annexin V-FITC/propidium iodide staining. The cells were then washed with phosphate-buffered saline, and 105 cells (in 100 µl buffer) were stained with 5 µl of annexin V-FITC and 5 µg/ml propidium iodide in annexin-binding buffer containing 10 mM HEPES (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2 at ambient temperature. After staining for 15 min, 400 µl of annexin-binding buffer was added to the cell samples, which were immediately analyzed using a FACS Calibur flow cytometer (BD Biosciences). The data are presented as the means ± S.D. from three independent experiments.

**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 then examined under a Zeiss laser scanning microscope with a 60-Å oil immersion objective. The AxioVision software program (Zeiss) was used to deconvolve Z-series images.
**RESULTS**

**CDDP Induces Caspase-9-dependent c-Jun Cleavage at Asp-65**—We treated PC14 and A549 human non-small cell lung cancer cells with CDDP for different time periods. Fig. 1A shows that transient up-regulation of c-Jun phosphorylation and expression followed by their down-regulation occurred in PC14 cells, whereas persistent up-regulation of c-Jun phosphorylation and expression occurred in A549 cells. In addition, we detected a cleaved c-Jun fragment in PC14 cells, but not in A549 cells. We also observed c-Jun cleavage and reduced c-Jun phosphorylation and expression in LN18 and U87 human glioblastoma cells (Fig. 1B). Intriguingly, tumor cells with up-regulated c-Jun expression were more resistant to apoptosis compared with those with down-regulated c-Jun expression upon CDDP treatment (Fig. 1C), suggesting a role for c-Jun expression in CDDP resistance of tumors. To determine whether activation of caspases is involved in c-Jun cleavage, we pretreated PC14 cells with benzylxoycarbonyl-VAD-fluoromethyl ketone, a general caspase inhibitor. This treatment blocked c-Jun cleavage (Fig. 1D), indicating that caspase activity is required for CDDP-induced c-Jun cleavage.

To identify the caspase that cleaves c-Jun, we performed an in vitro caspase cleavage reaction by mixing purified recombinant c-Jun with purified recombinant caspase-3, -4, -6, -7, -8, and -9. Fig. 1E shows that only caspase-9 cleaved c-Jun. In line with this result, pretreatment of PC14 cells with a caspase-9-specific inhibitor (Fig. 1F) and deficiency of caspase-9, but not caspase-7 or caspase-3 (Fig. 1G), significantly blocked CDDP-induced c-Jun cleavage. These results indicate that caspase-9 cleaves c-Jun in vitro and in vivo.

We detected the truncated c-Jun fragment, which was ~8 kDa less compared with the full-length protein, using immunoblotting with an antibody against the C terminus of c-Jun (Fig. 1A), suggesting that the c-Jun cleavage site is located close to the N terminus. Analyses of the c-Jun sequence revealed that Asp-59 and Asp-65 were potential caspase cleavage residues (35), which are ~8 kDa from the N terminus. Expression of V5-tagged WT c-Jun and the c-Jun(D59A) and c-Jun(D65A) mutants, in which aspartic acid was mutated to alanine, showed that the c-Jun(D65A) mutant, but not WT c-Jun or the c-Jun(D59A) mutant, was resistant to cleavage upon CDDP treatment (Fig. 1H). Incubation of caspase-9 with purified recombinant WT c-Jun and the c-Jun(D65A) mutant showed that c-Jun(D65A), but not its WT counterpart, was resistant to cleavage by caspase-9 (Fig. 1I). These results indicate that caspase-9 cleaves c-Jun at Asp-65.

To determine whether CDDP treatment induces recruitment of WT c-Jun to the c-jun promoter for c-jun transcription and to determine the effect of cleavage of c-Jun on its ability to bind to its promoter, we performed ChIP analyses by immunoprecipitation of FLAG-tagged WT c-Jun and a truncated c-Jun mutant (amino acids 66–331). As expected, treatment of PC14 cells with CDDP for 4 h induced the binding of WT c-Jun to the c-jun promoter (Fig. 1J), which contributed to temperate up-regulation of c-jun expression. However, c-Jun(66–331), with a deletion of Ser-63, which is the residue phosphorylated by JNK and important for c-jun transcription (1), failed to bind to the c-jun promoter upon treatment with CDDP. These results suggest that caspase-9-dependent c-Jun cleavage down-regulates the transcriptional activity of c-Jun, reducing its own expression.

**FAS Ligand-dependent Caspase Activation Plays a Role in CDDP-induced c-Jun Cleavage**—In response to stress and DNA damage stimulation, such as osmotic stress and ultraviolet radiation, cells can secrete FAS ligand (22, 29). FAS ligand binds to the FAS (also called APO-1 or CD95) death receptor, leading to trimerization of FAS, activation of caspase-8, and subsequent release of cytochrome c from mitochondria and activation of caspase-9 (45–47). To determine the possible involvement of FAS in CDDP-induced c-Jun cleavage, serum-free media were collected after CDDP treatment of PC14 and A549 cells. Immunoblot analysis with an anti-FAS ligand antibody showed increased secretion of FAS ligand from PC14 cells upon pro-
longed CDDP treatment (Fig. 2A, middle panel), whereas a lack of detectable actin in these media indicated that they were not contaminated with cell fragments. In contrast, no FAS ligand secretion was detected in A549 cells (Fig. 2A, right panel). Incubation of PC14 cells with a neutralizing FAS-Fc fusion protein that binds to FAS ligand reduced CDDP-induced c-Jun cleavage (Fig. 2B), indicating that secreted FAS ligand contributes to caspase-9-induced c-Jun cleavage. In agreement with these results, deficiency of caspase-8 inhibited CDDP-induced caspase-9 activation, as indicated by generation of a cleaved
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C-Jun Plays an Instrumental Role in CDDP Resistance of Tumors—CDDP treatment results in enhanced binding of FLAG-tagged full-length c-Jun, but not the c-Jun(66–331) mutant, to the MDR1 promoter region (Fig. 5C), suggesting that WT c-Jun binds to the MDR1 promoter and regulates MDR1 transcription. Consistent with these findings, CDDP treatment induced up-regulation of MDR1 expression in CDDP-resistant A549 cells, but not in CDDP-sensitive PC14 cells (Fig. 5C). In addition, c-Jun depletion blocked CDDP-induced up-regulation of MDR1 expression (Fig. 5D). These results indicate that CDDP promotes the binding of c-Jun to the MDR1 gene and increases MDR1 expression.

C-Jun Regulates Chemoresistance—CDDP resistance can result from increased expression of drug transporters, leading to reduced intracellular CDDP accumulation and enhanced drug detoxification in tumor cells. Given that c-Jun is phosphorylated and activated upon CDDP treatment in CDDP-resistant cells, we examined whether up-regulation of c-Jun expression is related to regulation of the expression of several efflux transporter proteins, including ATP-binding cassette transporter family members MDR1 (P-glycoprotein) and MRPI (multidrug resistance-associated protein 1), the copper transporters ATP7A and ATP7B, and GST (23–26). Real-time PCR analysis showed that CDDP treatment increased MDR1 mRNA expression in A549 cells, but not mRNA expression of MRPI and GSTPI (GST Pi 1) (Fig. 5A) and also ATP7A and ATP7B (data not shown), which can be inhibited by c-Jun depletion. In line with a previous report showing that AP-1 is able to bind to the MDR1 promoter (36), ChIP assay with an anti-FLAG antibody showed that CDDP treatment resulted in enhanced binding of FLAG-tagged full-length c-Jun, but not the c-Jun(66–331) mutant, to the MDR1 promoter region (Fig. 5B), suggesting that WT c-Jun binds to the MDR1 promoter and regulates MDR1 transcription.

C-Jun Is Required for CDDP-induced Up-regulation of MDR1 Expression—CDDP resistance can result from increased expression of drug transporters, leading to reduced intracellular CDDP accumulation and enhanced drug detoxification in tumor cells. Given that c-Jun is phosphorylated and activated upon CDDP treatment in CDDP-resistant cells, we examined whether up-regulation of c-Jun expression is related to regulation of the expression of several efflux transporter proteins, including ATP-binding cassette transporter family members MDR1 (P-glycoprotein) and MRPI (multidrug resistance-associated protein 1), the copper transporters ATP7A and ATP7B, and GST (23–26). Real-time PCR analysis showed that CDDP treatment increased MDR1 mRNA expression in A549 cells, but not mRNA expression of MRPI and GSTPI (GST Pi 1) (Fig. 5A) and also ATP7A and ATP7B (data not shown), which can be inhibited by c-Jun depletion. In line with a previous report showing that AP-1 is able to bind to the MDR1 promoter (36), ChIP assay with an anti-FLAG antibody showed that CDDP treatment resulted in enhanced binding of FLAG-tagged full-length c-Jun, but not the c-Jun(66–331) mutant, to the MDR1 promoter region (Fig. 5B), suggesting that WT c-Jun binds to the MDR1 promoter and regulates MDR1 transcription. Consistent with these findings, CDDP treatment induced up-regulation of MDR1 expression in CDDP-resistant A549 cells, but not in CDDP-sensitive PC14 cells (Fig. 5C). In addition, c-Jun depletion blocked CDDP-induced up-regulation of MDR1 expression (Fig. 5D). These results indicate that CDDP promotes the binding of c-Jun to the MDR1 gene and increases MDR1 expression.

JNK1 (but Not JNK2) Is Instrumental in CDDP-induced Up-regulation of c-Jun Expression—JNK1 and JNK2 are the primary kinases that phosphorylate and activate c-Jun (1). We observed that pretreating A549 cells with the JNK inhibitor SP600125 blocked CDDP-induced c-Jun phosphorylation and up-regulation of c-Jun expression (Fig. 4A), suggesting that JNK1/2 activity is required for CDDP-induced c-Jun expression. Consistent with these results, JNK1/2 deficiency blocked CDDP-induced c-Jun phosphorylation and up-regulation of c-Jun expression in response to CDDP treatment (Fig. 4B, left panel). To determine whether JNK1 or JNK2 is the primary regulator of c-Jun regulation upon treatment with CDDP, we treated JNK1−/− and JNK2−/− cells with CDDP and observed that JNK2 (but not JNK1) deficiency largely abrogated CDDP-induced c-Jun phosphorylation and up-regulation of c-Jun expression (Fig. 4B, right panel). In line with these observations, JNK2 depletion induced by expression of a shRNA against JNK2 mRNA in A549 cells significantly inhibited c-Jun phosphorylation and expression up-regulation upon CDDP treatment (Fig. 4C). These results indicate that JNK2 (but not JNK1) is primarily responsible for CDDP-induced up-regulation of c-Jun expression.

C-Jun Phosphorylation and Up-regulation of c-Jun Expression upon CDDP Treatment—CDDP-resistant PC14 cells overexpressed cleavage-resistant FLAG-c-Jun(D65A) in PC14, U87, and LN18 cells. The cells transfected with a vector expressing c-Jun(D65A) had increased resistance to CDDP-in
duced apoptosis in contrast with the untransfected cells, which had condensed and fragmented nuclei (Fig. 6A). In addition, depletion of JNK2 (Fig. 4C), c-Jun (Fig. 5D), or MDR1 in A549 cells significantly promoted CDDP-induced apoptosis as measured using annexin V-FITC/propidium iodide staining of the cells (Fig. 6B).

To determine the effect of regulation of c-Jun on the efficacy of tumor treatment with CDDP, we intraductally injected a len-
tivirus expressing PyV MT antigen into the mammary glands of JNK2+/+ and JNK2−/− mice and administered CDDP to the mice via intraperitoneal injection for 2 weeks. We noticed that tumors grew at a faster rate in JNK2−/− mice than in JNK2+/+ mice (Fig. 6C), which is in line with a previous observation (37). Notably, CDDP treatment inhibited tumor growth in JNK2−/− mice much more significantly than in JNK2+/+ mice (Fig. 6D).

In addition, immunohistochemical staining of tumor tissues showed that the c-Jun protein level in mammary gland tumor cells in JNK2+/+ mice was higher before CDDP treatment than that in JNK2−/− mice (Fig. 6E). CDDP treatment induced c-Jun up-regulation in tumor tissue in JNK2+/+ mice, but not in JNK2−/− mice. These results strongly suggest that JNK2-dependent up-regulation of c-Jun expression plays an instrumental role in tumor resistance to CDDP.

**DISCUSSION**

CDDP is a DNA-damaging antitumor agent widely used for the treatment of various human cancers. A number of mechanisms contribute to tumor cell resistance to CDDP (23). We have shown that c-Jun expression was down-regulated by post-translational modifications, including FAS ligand secretion-induced and caspase-9-dependent cleavage of c-Jun and MEKK1-mediated ubiquitylation and degradation of c-Jun in CDDP-sensitive cells. In contrast, c-Jun expression was persistently up-regulated in a JNK2-dependent manner in CDDP-resistant cells, which led to MDR1 expression. Importantly, depletion of c-Jun or blockage of up-regulation of c-Jun expression by JNK2 deficiency sensitized tumor cells to CDDP. These results highlight the essential role of c-Jun expression in the protection of tumor cells against CDDP-induced apoptosis and intrinsic tumor resistance to CDDP.

c-Jun was rapidly phosphorylated and activated in CDDP-sensitive cells, subsequently contributing to the initial and transient up-regulation of c-Jun expression. However, this up-regulation of c-Jun was counteracted by caspase-9-dependent c-Jun cleavage and MEKK1-dependent c-Jun ubiquitylation and degradation, leading to down-regulation of c-Jun expression. In contrast, CDDP-resistant cells, which did not have secreted FAS ligand for activation of caspase-9 or MEKK1-regulated c-Jun ubiquitylation (data not shown), exhibited maintenance of high levels of c-Jun phosphorylation and up-regulation in response to CDDP treatment. Inhibition of JNK2 activity, which disrupted the autoregulation of c-Jun, blocked CDDP-enhanced c-Jun expression and promoted cell apoptosis. A previous study of liver-specific inactivation of c-Jun demonstrated that c-Jun is required for chemically induced hepatocellular carcinoma development in mice (38). Our findings, which are in line with previously reported results (37), show that JNK2 deficiency does not inhibit PyV MT antigen-induced tumor growth, suggesting that the role of c-Jun in tumorigenesis is context-dependent and can differ according to oncogenesis signaling.

C-Jun is a transcription factor with a protective role in the cellular response to DNA damage (39). c-Jun-deficient cells undergo premature senescence due to spontaneous DNA damage (40), suggesting a role for c-Jun in DNA repair. JNK1 and JNK2, which are protein kinases upstream of c-Jun, are activated in response to CDDP treatment (41, 42). Biochemical studies established that both JNK1 and JNK2 function as posi-

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**FIGURE 5. c-Jun is required for CDDP-induced up-regulation of MDR1 expression.** A, A549 cells with or without c-Jun shRNA expression were treated with or without CDDP (50 μg/ml) for 6 h. The levels of MDR1, MRP1, and GSTP1 mRNA expression were determined by quantitative real-time PCR. B, 293T cells transiently expressing FLAG-WT c-Jun or FLAG-c-Jun(66–331) were treated with or without CDDP (50 μg/ml) for 4 h. ChiP analysis with an anti-FLAG antibody for immunoprecipitation (IP) was carried out with primers for the human MDR1 promoter. Normal mouse IgG was used as a control. C, the indicated cells were treated with or without CDDP (50 μg/ml) for 24 h. WB, Western blot. D, A549 cells with or without c-Jun shRNA expression were treated with or without CDDP (50 μg/ml) for 24 h.
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FIGURE 6. c-Jun plays an instrumental role in CDDP resistance of tumors. A, the indicated cells, which transiently expressed a FLAG-c-Jun(D65A) construct, were treated with CDDP (50 μg/ml) for 24 h. The cells were then immunostained with an anti-FLAG antibody. Nuclei were stained with Hoechst 33342 (blue). The white arrows point to fragmented nuclei from untransfected cells (left panel). One hundred untransfected and transfected cells were analyzed for cell survival rates. The data are presented as the means ± S.D. from three independent experiments (right panel). B, A549 cells stably expressing c-Jun shRNA, JNK2 shRNA, or MDR1 shRNA were treated with CDDP (50 μg/ml) for 24 h. The cells were then stained with annexin V-FITC and propidium iodide and analyzed using a FACS Calibur flow cytometer. The data are presented as the means ± S.D. from three independent experiments. The immunoblot analyses were performed with the indicated antibodies. C, lentiviruses expressing PyV MT antigen (20 μl in a concentration of 3.4 × 10^8 transduction units/ml) were injected into each nipple of JNK2^+/+ and JNK2^-/- mice. Two weeks after the injections, the mice were killed. Tumor sizes were measured and calculated. The data are presented as the means ± S.D. for 12 tumors in each group. D, lentiviruses expressing PyV MT antigen (20 μl in a concentration of 3.4 × 10^8 transduction units/ml) were injected into each nipple of JNK2^+/+ and JNK2^-/- mice. Two weeks after the injections, the mice were administered treatment with or without intraperitoneal injection of CDDP (2 mg/kg) for 2 consecutive weeks. The control and CDDP-treated groups of mice were killed and dissected, and their tumor sizes (on days 0 and 14 of treatment) were measured and calculated. The data are presented as the means ± S.D. from three independent experiments. The immunoblot analyses were performed with the indicated antibodies. E, immunohistochemistry staining with an anti-c-Jun antibody was performed with tumor samples obtained from JNK2^+/+ and JNK2^-/- mice at day 14 compared with untreated JNK2^+/+ mice. Two weeks after the injections, the mice were killed and dissected, and their tumor sizes (on days 0 and 14 of treatment) were measured and calculated. The data are presented as the means ± S.D. for 12 tumors in each group. Relative tumor size change reflects the combinational value of both tumor progression and the effect of CDDP on tumor progression; we set the fold increase in tumor size of untreated mice as 100% and compared it with that of treated mice. Expression of c-Jun in stress response and apoptosis.

tive regulators of c-Jun phosphorylation and expression. Deficiencies in JNK1 and JNK2 differentially affect the growth of mouse embryonic fibroblasts (43), suggesting that JNK1 and JNK2 do not have the same cellular functions. A study of transgenic mice harboring a Jnk2 mutation that expands the ATP pocket so that it can be sensitized to inhibition by the general protein kinase inhibitor PPI demonstrated that JNK2 is important for up-regulation of c-Jun expression and is required for cell survival (44). In line with these observations, we found that deficiency of JNK2 (but not JNK1) largely abrogated CDDP-induced c-Jun phosphorylation and up-regulation of c-Jun expression and sensitized mammary gland tumors to CDDP. These results indicate a critical role for JNK2 in regulation of c-Jun expression and CDDP resistance of tumors, highlighting the potential for regulation of c-Jun expression as an approach to enhancing cancer treatment with CDDP.

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