c-Jun Regulates the Stability and Activity of the p53 Homologue, p73*

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Chemotherapeutic drugs and stress signals activate p73, the structural and functional homologue of p53, both by transcriptional activation and post-translational modifications. However, cisplatin, a DNA damage-inducing chemotherapeutic agent, is thought to regulate p73 only by affecting its stability through mechanisms involving the MLH-1/c-Abl signaling cascade. Here we show that c-Jun, a component of the AP-1 family of transcription factors, contributes to p73 induction by cisplatin. c-Jun−/− cells are defective in p73 induction, and ectopic c-Jun expression augments p73 levels. c-Jun-mediated accumulation of p73 requires the transactivation activity of c-Jun and occurs in a c-Abl- and Mdm2-independent manner. c-Jun expression increases p73 half-life by preventing it from proteasome-mediated degradation, resulting in the potentiation of p73-mediated transcriptional activity. Moreover, mouse fibroblasts lacking c-Jun are resistant to cisplatin-induced apoptosis, and reintroduction of c-Jun restores p73 activation and sensitivity to cisplatin. Furthermore, p73-mediated apoptosis is abrogated in c-Jun−/− cells. Together, these findings demonstrate a possible role for c-Jun in regulating p73 function and highlight the importance of the cooperativity between transcription factors in potentiating apoptosis.

Many stress signals and chemotherapeutic agents activate the c-Jun NH2-terminal kinase signaling pathway leading to the activation of c-Jun, a major component of the mammalian Jun protein family that includes JunB and JunD (1). c-Jun, together with the Fos proteins, form the set of dimers belonging to the AP-1 family of DNA-binding proteins (1). c-Jun is a central component of all AP-1 complexes, which act often as transcriptional activators (2). c-Jun is required for a variety of biological processes that influence oncogenic transformation including cell differentiation, proliferation, and apoptosis (3). Its expression is elevated in response to many stimuli, including 12-O-tetradecanoylphorbol-13-acetate, growth factors, cytokines, chemotherapeutic drugs, UV irradiation, and other stress stimuli (4). Fibroblasts lacking c-Jun have severe proliferation defects and are resistant to several forms of stress-induced apoptosis (5–8). It has been shown that Fas ligand is a transcriptional target of c-Jun, contributing to some forms of p73-mediated cell death (8, 9). However, the detailed mechanisms by which c-Jun regulates apoptosis is not well understood.

Many chemotherapeutic agents that activate c-Jun also often induce both p53-dependent and -independent cell death (10, 11). These agents have been shown to activate p73, a structural and functional homologue of p53 (12, 13). p73 exists as several distinct isoforms because of extensive splicing at the COOH-terminal domain, resulting in at least six splice variants (p73α–ϕ) (14–16). In addition, the use of an alternative promoter in the intron 3 of the p73 gene leads to the expression of a p73 protein that lacks the NH2-terminal transactivation domain (ΔNp73) (17). The full-length p73 isoforms have been shown to have tumor-suppressive properties because ectopic overexpression in cell lines often leads to p53-independent apoptosis and suppression of colony formation (15, 18). The c-Abl tyrosine kinase appears to play a central role in DNA damage-induced activation of p73 and consequent cell death (12, 13). Upon DNA damage, activated c-Abl has been shown to bind to the PXXP motif of p73 and phosphorylate on tyrosine residue 99 and to a lesser extent, on residues 121 and 240 (19, 20). p73 was not activated in c-Abl−/− cells, and hence, these cells were found to be resistant to DNA damage-induced cell death (12). Moreover, we have recently shown that multiple stress signals activate p73, resulting in cell death, and that p73−/− cells are resistant to apoptosis induced by these stress signals (21). Hence, activation of p73 appears to contribute to another line of defense against stress signals, in the absence of p53.

Although p73 is able to induce apoptosis when overexpressed, p73-mediated transactivation of many p53-responsive promoters, including the apoptotic targets, is less efficient compared with p53-mediated transactivation (22). This raises the possibility that expression of p73 alone might not be sufficient to execute cell death. In this regard, it is noteworthy that p73 null mice do not develop spontaneous tumors as do their p53 null counterparts (23), reinforcing the idea that p73 might be a weaker tumor suppressor of the two, and it might require the cooperation of other apoptosis activators.

c-Jun has been shown to negatively regulate transcription of both p53 and its target, the cyclin-dependent kinase inhibitor, p21, thereby antagonizing p53-mediated growth suppression (6, 24). Hitherto, it is not evident whether c-Jun has any role in regulating p73 function. We report here that c-Jun contributes to the increase in p73 half-life by preventing it from proteasome-mediated degradation, thus resulting in p73 induction.
and potentiation of p73-mediated cell death. These findings demonstrate a critical role for c-Jun in regulating p73 function.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—COS7, H1299, and all mouse fibroblasts were cultured as described (6, 21, 25). cjun−/− cells were infected with c-Jun retroviral particles derived from a pBabe-c-Jun construct as described (6) and selected on puromycin for stable infected. These c-jun−/+ and c-jun−/− cells were used as controls with the c-jun−/− cells. Transfections were performed by using LipofectAMINE reagent as per the manufacturer’s instructions. The total amounts of transfected DNA were equalized with appropriate amounts of pCDNA3 vector in all cases. pCDNA3-based expression plasmids for p73α, p73β, ΔNp73α, and ΔNp73β have been described (15). Point mutations of p73α and p73β were generated using the site-directed mutagenesis kit as per the manufacturer’s instructions (Stratagene), and c-Jun mutants were generated by standard molecular biological techniques. Transfected cells were harvested 48 h post-transfection for analysis of proteins and RNA. The cells were treated with cisplatin with the indicated amounts and harvested for protein and cell survival analysis.

**Immunoblot and Immunoprecipitation Analysis**—The cells lysates were prepared in lysis buffer containing 0.5% Nonidet P-40 as described (26). The proteins were separated on SDS-polyacrylamide gels and Western blotted with anti-p73α (ER15; Oncogene), anti-p73β (GC15; Oncogene), anti-c-Jun (H79, Santa Cruz; G56–206, Pharmingen), anti-Actin (Sigma), and anti-Egfp (Clontech) antibodies. The specificity of the p73 antibodies are described by Lin et al. (21). Generally, 150 μg of lysate was used from transfected cells to monitor steady-state levels of proteins. In the following cases, 300 μg of total cell extracts were used: Figs. 1C and 2B.

The half-life of p73 was determined by starving cells in methionine/cysteine-free minimum essential medium for 1 h, followed by labeling in minimum essential medium with 300 μCi ml−1 of [35S]methionine (ICN) for 2 h. After washing away the [35S]methionine with 1× phosphate-buffered saline, the cells were chased with normal Dulbecco’s modified Eagle’s medium for different lengths of time. The lysates were prepared as described earlier. 500 μg of lysate was immunoprecipitated overnight with anti-p73β antibody and separated on a 12% SDS-PAGE and analyzed as described (26). The amount of [35S] in p73 was quantified by phosphorimaging.

**RNA Analysis**—Total RNA was prepared from cells using the TRI Reagent (Sigma) as per the manufacturer’s instructions. Reverse transcriptase-PCR and Northern blot analysis were performed using total RNA as described (21, 25). Full-length p73 PCR was performed using a forward 5’-TCTGGAACCAGACAGCACCT-3’ primer and a reverse 5’-GTTGCTGACTGCTGGAAAGT-3’ primer under the following conditions, as described (21).

**Luciferase Assays**—Transient transfections of H1299 and the indicated mouse fibroblasts were generally performed with 10 ng of expression plasmids for p73β and c-Jun, the mdm2-luc plasmid, or the PG13-luc plasmid (0.5 μg) together with 0.5 μg plasmid encoding the β-galactosidase gene for evaluating the transfection efficiency. The cells were collected 48 h post-transfection. Luciferase assays were performed as described (27).

**Apoptosis Assays**—Cisplatin-treated cells were analyzed for cell death by staining with Annexin-V fluorescent isothiocyanate as per the manufacturer’s instruction and analyzed by flow cytometry as described (25).

**Cell death analysis of transfected cells** (1.0 μg of p73 in the presence or absence of 1.0 μg of c-Jun expression plasmids, together with 0.1 μg of Egfp encoding plasmid) was performed by sub-2N analysis of DNA...
Fig. 2. Ectopic c-Jun expression increases the steady-state levels of p73. A, c-jun−/− and c-jun−/− + c-jun mouse embryonic fibroblasts were transfected with plasmids encoding p73β (1.0 μg) and egfp (0.3 μg). In each case, total amount of transfected DNA were equalized with pCDNA3 vector. The cells were harvested 48 h after transfection and subjected to immunoblot analysis as indicated. B, COS7 cells were transfected transiently with the transactivation proficient or deficient p73α or p73β isoforms (1.0 μg) without or with c-jun (1.0 μg) or the pCDNA3 empty vector as indicated. In each case, total amount of transfected DNA were brought up to 2.3 μg with pCDNA3 vector. The cells were harvested 48 h after transfection and subjected to Western blot analysis with both p73α- and p73β-specific antibody (ER-15 and GC15, respectively) and c-Jun-specific antibody. The asterisks indicate the detection of endogenous p73β in c-jun transfected cells. C, COS7 cells were transfected with the indicated plasmids encoding p73α, egfp, and c-jun at the indicated concentrations and were analyzed as described in B. D, COS7 cells were transfected with the indicated point mutants of the p73α or p73β without or with c-jun (1.0 μg) and were analyzed as described above. E, levels of p73β and glyceraldehyde-3-phosphate dehydrogenase (gapdh) RNA were determined in the above cells as in B.
c-Jun Regulates p73 Stability

Fig. 3. The transactivation domain of c-Jun is required for p73 accumulation. A, schematic diagram indicating the functional domains of c-Jun. B, increasing amounts of the various c-jun plasmids (0, 0.2, 0.4, 0.6, and 0.8 μg) were co-transfected with 0.2 μg of p73β expression plasmid, and the steady-state levels of p73β were determined as described above.

content by staining ethanol-fixed cells with propidium iodide. DNA content of Egfp-positive cells were determined by flow cytometry. Visual inspection of the Egfp-positive cells was performed by conventional fluorescence microscopy.

RESULTS

c-Jun Is Required for Cisplatin-induced p73 Accumulation—We have recently shown that multiple stress signals activate p73, both through the induction of p73 mRNA and through the prolongation of p73 half-life (21). Among them, the chemotherapeutic drug cisplatin, which has been shown by several groups to induce p73 protein (12), was not able to induce p73 mRNA (Fig. 1A and Ref. 12). This was in contrast to Taxol, another chemotherapeutic drug that was able to activate p73 mRNA (Fig. 1A). Cisplatin treatment leads to activation of a plethora of signaling modules, including the c-Jun NH2-terminal kinase signaling cascade that regulates c-Jun (29).

Because c-jun−/− cells are resistant to cisplatin-induced cell death (29), we examined whether c-Jun is a critical determinant of cisplatin-induced p73 activation. Treatment of wild-type mouse fibroblasts with cisplatin resulted in a time-dependent increase in the levels of c-Jun protein, indicating that c-Jun is activated by cisplatin (Fig. 1B). Immunoblot analysis using a p73β-specific antibody that is able to recognize endogenous human, mouse, and simian p73 revealed that exposure to cisplatin for 24 h induced expression of p73 in c-jun−/− mouse embryonic fibroblasts stably re-expressing a single copy of c-jun by retroviral infection (c-jun−/− + c-jun cells) but not in cells lacking c-Jun, demonstrating that c-Jun can potentiate p73 expression upon cisplatin treatment (Fig. 1C). Although basal p53 levels were elevated in c-jun−/− cells as previously reported and re-expression of c-Jun led to a decrease in p53 basal levels (6), p53 was induced in both the cell types by cisplatin (Fig. 1C). Together, the results indicate that c-Jun contributes to the accumulation of endogenous p73 protein.

c-Jun Expression Increases Steady-state p73 Levels—Because p73 induction appears to be dependent on c-Jun, we further examined whether p73 expression was altered by the absence of c-Jun. Immunoblot analysis revealed that the levels of transiently transfected p73β expression was much reduced in c-jun−/− fibroblasts as compared with the c-jun−/− + c-jun cells (Fig. 2A, compare lanes 2–5). Transient expression of c-Jun together with p73 in c-jun−/− fibroblasts resulted in higher levels of p73 expression (Fig. 2A, compare lanes 2 and 3). In addition, expression of c-Jun with p73 in c-jun−/− + c-jun cells led to the highest levels of p73 expression (Fig. 2A, lane 6), indicating that the presence of c-Jun affected the levels of ectopically expressed p73. In contrast, the status of c-Jun did not affect the levels of green fluorescent protein (Egfp), which was used to normalize the transfection efficiency (Fig. 2A).

We assessed the effect of c-Jun overexpression on steady-state p73 levels in an attempt to investigate the mechanism by which c-Jun regulates p73 expression. Monkey COS7 cells were transiently transfected with the expression plasmids for c-Jun either alone or together with p73α or p73β, and p73 levels were determined by Western blotting 48 h later. Ectopic expression of c-Jun alone led to the accumulation of endogenous p73β, as indicated by the asterisk, as compared with cells transfected with “empty” vector (Fig. 2B, compare lanes 1 and 2). Moreover, co-expression of equal amounts (1.0 μg) of c-Jun with p73α or p73β resulted in a significant increase in the steady-state levels of p73 compared with expression of p73 alone (Fig. 2B, compare lanes 3 and 7 for p73α and compare lanes 4 and 8 for p73β). This accumulation was seen in several other human cell lines (H1299, MCF-7, and 293 cells; data not shown). Using various amounts of both p73α and c-Jun expression plasmids, we observed that c-Jun overexpression led a dose-dependent accumulation of p73α (Fig. 2C) and p73β (data not shown). In contrast, expression of c-Jun did not affect the levels of Egfp, which was used to normalize the transfection efficiency (Fig. 2C).

We next investigated the domains of p73 that are required for c-Jun-mediated increase in steady-state levels. Transfection of the expression constructs for ΔNp73α and ΔNp73β, the anti-apoptotic proteins that lack the NH2-terminal transactivation domain, with c-Jun expression construct did not lead to in-
c-Jun prolongs p73 half-life. A, wild-type fibroblasts were treated with 25 μM of MG132 for 6 h, and the cell extracts were analyzed by Western blotting for the indicated proteins. B, induction of p73β accumulation by MG132. COS7 cells transfected with the indicated plasmids (1.0 μg each) as described for Fig. 2A, were treated 45 h after transfection with the proteasomal inhibitor MG132, where indicated (+), to a final concentration of 25 μM. After an additional 3 h, the cells were harvested and analyzed as described in Fig. 3. C, half-life determination. COS7 cells were transfected with p73β and c-jun plasmids or p73β alone and pulse-labeled with [35S]Met/Cys followed by a 4-h chase. p73 expression was detected by immunoprecipitation with anti-p73β antibody followed by autoradiography. The lower panel shows the quantification of the remaining p73 using phosphorimaging. D, half-life of p73 in was also determined in wild-type and c-jun−/− cells after 25 μM cisplatin treatment as described for C.
creased expression of these proteins, as compared with their full-length counterparts (Fig. 2B, compare lanes 5 and 9, 6 and 10, 3 and 7, and 4 and 8), suggesting that the NH2 terminus of p73 is required for c-Jun-mediated p73 accumulation. However, co-expression with c-Jun led to the accumulation of two p73 mutants that have been shown to be defective in c-Abl-mediated p73 stability, namely the 338-p73 (which is defective in c-Abl binding) and the 99-p73 (which cannot be phosphorylated by c-Abl) (Fig. 2D), indicating that c-Jun-mediated p73 accumulation is probably independent of the pathway by which the tyrosine kinase c-Abl stabilizes p73.

Furthermore, the increased levels of p73 in the presence of c-Jun was not due to increased p73 transcription, because co-expression of c-Jun with p73β did not affect the levels of p73 RNA (Fig. 2E), further suggesting that c-Jun-mediated p73 accumulation occurs at a post-transcriptional level. Together, the results demonstrate that the overexpression of c-Jun can lead to a substantial increase in the amount of p73 in human and monkey cells, which appears not to be dependent on the binding and phosphorylation of p73 by c-Abl.

The Transactivation Domain of c-Jun Is Required for p73 Accumulation—To determine the mechanism by which c-Jun contributes to p73 accumulation, we determined whether c-Jun could be co-immunoprecipitated with p73. Several strategies to evaluate protein-protein interactions failed to reveal reciprocal binding between c-Jun and p73 (data not shown). We therefore
determined the critical domains of c-Jun that were required for increasing p73 steady-state levels. To this end, the following expression plasmids were generated: transactivation domain deficient c-Jun (TAM67) (30), deletion mutant of c-Jun that does not have the COOH-terminal DNA-binding and leucine zipper domains that are required for DNA-binding and oligomerization with other transcription factors, respectively (Δ195–331), and a mutant that only expresses the COOH terminus of c-Jun containing the DNA-binding domain and the leucine zipper domains (Δ1–244) (Fig. 3A). Co-expression of p73β with increasing amounts of these mutants, unlike full-length c-Jun, did not cause an increase in the steady-state levels of p73β (Fig. 3B). The transactivation and DNA-binding domains together appear to be critical for c-Jun-mediated p73 accumulation, because c-Jun mutants expressing the DNA-binding domain without the transactivation domain (TAM-67) or the Δ195–331 mutant that lacks the DNA-binding domain but retains the transactivation domain were unable to increase p73 levels (Fig. 3B). Expression of the DNA-binding and leucine zipper domains of c-Jun (1–244) alone was also not sufficient to increase p73 levels (Fig. 3B). The data together suggest that the transactivation property of c-Jun is required for p73 accumulation.

**c-Jun Prolongs p73 Half-life**—Intracellular p73 levels were shown to be regulated by c-Abl at the level of protein stability (12). To assess whether the effect of c-Jun is mediated through interference with the proteasomal degradation of p73, we first evaluated the effect of the proteasomal inhibitor MG132 on endogenous p73. Treatment of wild-type fibroblasts with MG132 resulted in the increase of endogenous p73, indicating that p73 is indeed degraded through the proteasomal pathway (Fig. 4D). To further confirm that p73 stability is dependent on c-Jun expression upon cisplatin treatment, the half-life of p73 was determined in wild-type and c-jun−/− cells. Pulse-chase experiments indicated that p73 has a half-life of approximately 1 h in wild-type fibroblasts (Fig. 4D). In contrast, p73 was less stable in c-jun−/− cells with a much shorter half-life, and almost all the p73 protein had disappeared by 1 h (Fig. 4D). Together, these data suggest that c-Jun regulates the accumulation of p73 by controlling protein stability.

**p73 Transcriptional Activity Is Potentiated by c-Jun**—To assess whether c-Jun-mediated p73 stability affects the transactivation function of p73, reporter assays were performed in H1299 cells with constructs containing the luciferase gene driven by the p53/p73 responsive mdm2 promoter (mdm2luc) or the multimerized consensus p53 binding site (PG13). Co-transfection of the mdm2luc with a vector expressing c-Jun had only a slight effect on luciferase activity (Fig. 5A, left panel). By contrast, co-transfection of the reporters with 10 ng of the p73β expression vector resulted in the activation of luciferase activity, which was potentiated by co-expression with c-Jun. The elevated level of p73 activity in the presence of c-Jun was comparable with the activity of higher amounts of p73β (100 ng) (Fig. 5A, left panel). Similar results were obtained using the PG13-luc construct (Fig. 5A, right panel). RNA analysis indicated that co-expression of c-Jun with p73β potentiated the expression of endogenous p53AIP-1 (Fig. 5B), an apoptotic target gene that is induced by p73 (13), further demonstrating that expression of c-Jun leads to enhanced p73 activity. Furthermore, analysis of transfected p73 activity was assessed in mouse fibroblasts lacking p53 alone or those lacking both c-Jun and p53. Expression of p73β resulted in activation of luciferase activity in p53−/− fibroblasts, which was compromised in the absence of c-Jun (Fig. 5C, right panel), indicating that endogenous c-Jun contributes to the transcriptional activity of p73.

Together, the data indicate the c-Jun potentiates p73 activity.

**Mdm2 Is Not Required for c-Jun-mediated p73 Stability**—Because the transcriptional activity of c-Jun is required for p73 accumulation and because no direct reciprocal interactions were observed between c-Jun and p73, we investigated whether Mdm2, a transcriptional target of c-Jun (32) that has been shown to stabilize p73 (33), is the intermediate through which c-Jun stabilizes p73. Transfection of p73β in p53−/− and p53−/−
FIG. 7. c-Jun potentiates cisplatin and p73-mediated apoptosis. A, c-abl<sup>−/−</sup>, c-jun<sup>−/−</sup>, and c-jun<sup>−/−</sup> + c-jun mouse embryonic fibroblasts were treated with the indicated concentrations of cisplatin for 24 h, and the extent of programmed cell death was determined by staining cells with annexin-V fluorescein isothiocyanate and subsequent flow cytometric analysis (left panel). p53<sup>−/−</sup>, c-jun<sup>−/−</sup> p53<sup>−/−</sup>, and c-jun<sup>−/−</sup> p53<sup>−/−</sup> cells were...
mammalian fibroblasts revealed that p73 expression was reduced in p53<sup>−/−</sup> mdm2<sup>−/−</sup> cells (Fig. 6, compare lanes 5 and 6), confirming previous reports that Mdm2 potentiated p73 stability (33). However, p73 levels were higher when co-expressed with c-Jun in both cell types (Fig. 6, lanes 7 and 8). Although the levels of p73 was slightly lower in p53<sup>−/−</sup> mdm2<sup>−/−</sup> cells compared with p53<sup>−/−</sup> cells, it was much more than when p73 was transfected alone (compare lanes 6 and 8), indicating that the absence of Mdm2 does not affect c-Jun-potentiated p73 stabilization. Together, the data excludes Mdm2 as a possible transcriptional target of c-Jun that is required for p73 stability.

**p73-mediated Apoptosis Is Potentiated by c-Jun**—We finally evaluated whether c-Jun affected p73-potentiated cell death. Analysis of cisplatin-induced cell death of c-jun<sup>−/−</sup> or c-jun<sup>−/−</sup> + c-jun cells with 4 μm and 20 μm of cisplatin revealed a reduction in cell death of c-jun<sup>−/−</sup> cells compared with c-jun<sup>−/−</sup> + c-jun cells when assessed 24 h post-treatment (number of viable c-jun<sup>−/−</sup> cells, 72% [4 μm] and 64% [20 μm]; c-jun<sup>−/−</sup> + c-jun cells, 50% [4 μm] and 24% [20 μm]) (Fig. 7A, left panel). Similarly, c-Abi deficient fibroblasts were also resistant to apoptosis (Fig. 7A, left panel), as previously reported (12). p53 is also a mediator of cisplatin-induced cell death (10). Therefore, to eliminate the possibility of c-Jun acting via p53 to induce cisplatin-potentiated cell death, we determined the sensitivity of fibroblasts lacking both p53 and c-Jun (p53<sup>−/−</sup> c-jun<sup>−/−</sup> cells) or p53<sup>−/−</sup> cells to cisplatin. p53<sup>−/−</sup> c-jun<sup>−/−</sup> cells were found to be more resistant than p53<sup>−/−</sup> cells (number of viable p53<sup>−/−</sup> c-jun<sup>−/−</sup> cells, 80% [4 μm] and 55% [20 μm]; p53<sup>−/−</sup> cells, 63% [4 μm] and 38% [20 μm]), although p53<sup>−/−</sup> cells were also partially resistant compared with the control p53<sup>−/−</sup> c-jun<sup>−/−</sup> cells (Fig. 7A, right panel). These data, together with the earlier findings that p73 is not activated by cisplatin in c-jun<sup>−/−</sup> cells, suggest a critical role for c-Jun in p73-potentiated, p53-independent cell death.

In an attempt to further investigate the role of c-Jun in p73-potentiated cell death, we analyzed whether p73-potentiated cell death is dependent on the presence of c-Jun. p73β was transiently expressed in jun<sup>−/−</sup> + c-jun mouse fibroblasts or those lacking either c-Jun or c-Abi, and the fate of transfected cells were followed by including an appropriate green fluorescent protein expression plasmid in the transfections (see “Experimental Procedures”). Analysis of cells under fluorescence microscopy indicated that unlike c-jun<sup>−/−</sup> + c-jun fibroblasts and similar to c-abl<sup>−/−</sup> cells, c-jun deficient cells were relatively resistant to p73β-mediated cell death, as ascertained by their morphology (number of dead c-jun<sup>−/−</sup> cells, 7%; c-abl<sup>−/−</sup> cells, 5%; wild-type cells, 20%) (Fig. 7B). However, co-transfection of c-Jun with p73β resulted in appreciable amounts of cell death of c-jun<sup>−/−</sup> cells, similar in extent to that found with c-jun<sup>−/−</sup> + c-jun cells (c-jun<sup>−/−</sup> cells, 37%; c-jun<sup>−/−</sup> + c-jun cells, 43%) (Fig. 7C).

Additional experiments were performed to determine the role of c-Jun in p73-potentiated cell death in the absence of p53. Ectopic expression of p73β alone or in combination with c-Jun expression plasmids in p53<sup>−/−</sup> cells and subsequent analysis by sub-2N DNA content, which represents the amount of cells undergoing apoptosis, by flow cytometry indicated that c-Jun potentiated p73-induced apoptosis in p53<sup>−/−</sup> cells (percentage of dead cells: Vector, 3.9%; p73β, 12.1%; c-jun + p73β, 32.5%) (Fig. 7D). Moreover, c-jun<sup>−/−</sup> p53<sup>−/−</sup> cells were found to be markedly resistant to apoptosis induced by ectopic expression of p73β compared with p53<sup>−/−</sup> cells (p53<sup>−/−</sup> cells, 12.1% dead cells versus c-jun<sup>−/−</sup> p53<sup>−/−</sup> cells, 0.7% dead cells) (Fig. 7D). Together, these experiments indicate a role for c-Jun in p73-mediated apoptosis.

We finally investigated whether the absence of p73 would result in resistance to cisplatin-mediated cell death. Wild-type fibroblasts treated with cisplatin underwent massive apoptosis as determined Annexin-V staining (Fig. 7E). However, treatment of p73<sup>−/−</sup> cells resulted in a dramatic reduction in the apoptotic rates (percentage of Annexin-V<sup>+</sup> cells in wild type versus p73<sup>−/−</sup>: cisplatin, 24% versus 10%) (Fig. 7E), indicating that the presence of p73 is required for cisplatin-mediated cell death.

**DISCUSSION**

The data presented in this study demonstrate that c-Jun contributes to efficient p73 induction by cisplatin as well as p73-mediated cell death. The apoptosis inducing full-length p73, but not the anti-apoptotic ΔNp73, is stabilized in the presence of c-Jun, which appears to interfere with the normal proteasomal degradation of p73 leading to its accumulation. Thus, activation and overproduction of c-Jun may qualify as another signal that enhances the cellular p73 response.

Although the activity of transcription factors are generally regulated by many proteins that are components of signaling cascades, the data presented here provides a paradigm for the regulation of the activity of one transcription factor by another. It is thus plausible that the regulation of p73 activity by c-Jun occurs downstream of the signaling cascades that activate both c-Jun and p73. Such a cross-talk between two transcription factors could conceivably enhance the physiological outcome. In this respect, it should be noted that p73 transcriptional activity was found to be dependent on c-Jun and was enhanced by its expression. Moreover, both c-Jun and p73 are inducers of apoptosis and p73-mediated apoptosis appear to depend on the presence of endogenous c-Jun, because c-Jun null cells were refractory to apoptosis induced by p73 overexpression and to cisplatin treatment. Thus, it is likely that induction of p73 in response to c-Jun overexpression may augment apoptosis, thereby providing a safeguard against uncontrolled proliferation. In this regard, c-Jun appears to regulate p73 differently from p53, because the latter was shown to be negatively regulated by c-Jun (6). In addition, c-Jun does not stabilize ΔNp73, the anti-apoptotic member of the p73 family (17), indicating that c-Jun cooperates with full-length p73 to bring about efficient apoptosis of cells. Our findings imply that cells undergoing an increase in c-Jun levels, resulting for example from stress-induced signals, may respond by up-regulating p73. This would restrain subsequent proliferation, providing a p73-mediated tumor suppressor function. Induction of p73 will also provide a safeguard against uncontrolled proliferation in the absence of p53, as is the case in many cancers. In this respect, we have recently shown that multiple stress signals that acti-
c-Jun can also induce the accumulation of endogenous p73β in the absence of p53 (21).

What might be the mechanism underlying this effect of c-Jun on p73? In the absence of evidence for a direct physical reciprocal association between p73 and c-Jun (data not shown), the mechanism is most likely indirect. One possibility is that a rate-limiting component of the ubiquitin-proteasome pathway is shared between p73 and c-Jun, which like p73 is also a target for proteolytic degradation by this pathway (34). The ubiquitanation of mammalian c-Jun has been shown to be mediated through its interaction with the c-Jun NH2-terminal kinase (34). Thus, it would be of interest to examine whether c-Jun might be a shared component in the degradation pathway for a common E3 ubiquitin ligase.

Alternatively, c-Jun may reprogram the cell in a way that compromises the normal rapid degradation of p73. The latter may be brought about by c-Jun regulating other factors that are required for the stability of p73. In this respect, it is noteworthy that the transactivation function of c-Jun is required for p73 stabilization. Our data indicate that Mdm2, a transcriptional target of c-Jun (32) that stabilizes p73 (33) is not required for p73 stabilization. Our data indicate that Mdm2, a transcriptional target of c-Jun (32) that stabilizes p73 (33) is not required for p73-mediated p73 stability. Thus, it is conceivable that c-Jun activates other targets that are required for regulating p73 stability. Moreover, it is evident that the NH2-terminal region of p73 is essential for c-Jun-mediated p73 accumulation. Many proteins, including p300/cAMP-responsive element-binding protein-binding protein, c-Myc, and MM1, a c-Myc-binding protein, and Yes-associated protein have been shown to interact with p73 (35–37). In addition, mismatch repair protein PMS2, cyclin G, and c-Abl have been shown to regulate p73 stability (12, 38, 39). It is thus also possible that c-Jun might be a shared component in the degradation pathway for a common E3 ubiquitin ligase.

It is of interest to note that constitutive activation of c-Jun can also lead to increased cellular proliferation in different cell types. Hence, it is likely that the effect of c-Jun on p73 turnover may also be very different in different cell types.

Collectively, our data support the existence of a signaling pathway that involves both p73 and c-Jun as mediators of apoptosis. The results presented here provide evidence that c-Jun, a known mediator of apoptosis, collaborates with the tumor suppressor p73 in inducing p53-independent cell death. Because p73 is thought to be only activated by a subset of agents that activate p53 (23) and because p73 only weakly activates the death-inducing target genes in comparison with p53 (22), it is conceivable that concerted effort with other death activators is necessary for the potentiation of the death-inducing function of p73. Thus, modulators of pathways involving p73 and c-Jun may be able to increase the response of a variety of p53-defective cancer cells that do not have mutations in the p73 gene to therapeutical agents.