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Inhibition of Stress-Inducible Kinase Pathways by Tumorigenic Mutant p53

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More than 50% of human cancers contain p53 gene mutations and as a result accumulate altered forms of the full-length p53 protein. Although certain tumor types expressing mutant p53 protein have a poor prognostic process, the precise role of mutant p53 protein in highly malignant tumor cells is not well defined. Some p53 mutants, but not wild-type p53, are shown here to interact with Daxx, a Fas-binding protein that activates stress-inducible kinase pathways. Interaction of Daxx with p53 is highly dependent upon the specific mutation of p53. Tumorigenic mutants of p53 bind to Daxx and inhibit Daxx-dependent activation of the apoptosis signal-regulating kinase 1 stress-inducible kinases and Jun NH_2-terminal kinase. Mutant p53 forms complexes with Daxx in cells, and consequently, mutant p53 is able to rescue cells from Daxx-dependent inhibition of proliferation. Thus, the accumulation of mutant p53 in tumor cells may contribute to tumorigenesis by inhibiting stress-inducible kinase pathways.

The tumor suppressor gene p53 is one of the most frequently mutated genes in a wide variety of human cancers (61), indicating that the p53 protein plays a critical role in growth control and tumorigenesis. Deletion of the gene and loss of wild-type p53 function by viral or cellular oncoproteins clearly contribute to tumorigenesis. The wild-type p53 protein functions as a tetrameric transcription factor, and different forms of stress activate signal-transduction pathways that culminate in posttranslational modification to stabilize and activate p53. This accumulation of p53 protein activates the transcription of genes that are involved in various activities, including cell cycle inhibition and apoptosis—depending on the cellular context, the extent of damage, and other unknown parameters. The majority of p53 mutations apparently result in a loss of normal function, since no mutant p53 so far isolated functions as a transcription activator, as does wild-type p53. Missense mutations in p53 may also play a role in malignant transformation by generating a dominant negative form that inhibits the function of wild-type p53 (17). In such a case, expression of a dominant negative mutant p53 would result in a phenotype that is indistinguishable from that seen in p53-null cells. Such mutations have been identified by genetic analysis, and they contribute to the tumorigenic phenotype (12, 16, 51).

In principle, missense mutations could also contribute to tumorigenesis by creating gain-of-function forms. Such a gain-of-function mutation of p53 can be distinguished from a dominant negative mutation because it results in a novel phenotype that is not observed in the p53-null cell. Individuals with Li-Fraumeni syndrome (LFS), who carry a congenital mutation in one p53 allele, frequently develop additional mutations necessary for malignant transformation. The cancer rates observed in LFS families are consistent with the abolition of a rate-limiting step rather than with one less step in a multistep carcinogenic process (3, 52). Most human sporadic cancer cells that carry a missense mutation to one p53 allele show a deletion of the other wild-type p53 allele and continue to produce only a mutant form of p53 protein. This situation is quite different from that for most other tumor suppressor genes, where deletions of both alleles are common. These results support the notion that a gain-of-function by mutant p53 contributes to the development of human cancer.

The expression of a mutant p53 gene in a p53-null cell enhances malignant transformation in cultured cells (60) and affects tumor progression (8, 10, 21, 26, 45, 49). A role for mutant p53 in generating aneuploidy in human cells has also been suggested (14), and an accumulation of aneuploid cells has been found in fibroblasts from LFS patients (3). Moreover, the expression of mutant p53 proteins in human colon carcinoma cells results in a tendency for the increase in ploidy during growth in culture (1) or in response to radiation or treatment with doxorubicin hydrochloride (Adriamycin; Pharmacia & Upjohn) (57). Mutant p53 also disrupts the spindle checkpoint control in fibroblasts from LFS patients (14). Specific transcriptional activation of certain growth-promoting genes by mutant p53 has been reported. Mutant p53 does not activate the same gene promoters as does wild-type p53 but instead activates transcription of the genes for epidermal growth factor receptor, multiple-drug resistance 1, proliferating cell nuclear antigen, and c-myc (13, 32). Recently, the mouse mutant p53 172RH, which corresponds to the 175RH hot-spot mutation in human tumors, has been shown to convey

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high metastatic potential to tumors in experiments using a transgenic mouse system (36). Since the mutant p53 protein may contribute to cell survival under conditions where apoptotic programs are reduced by the expression of mutant protein, we aimed to identify a potential linkage of mutant p53 protein for the modulation of apoptotic pathways. We found that tumorigenic mutant, but not wild-type, p53 proteins interact with the nuclear protein Daxx and inhibit both Daxx-dependent apoptosis signal-regulating kinase 1 (ASK1) and Jun NH_2-terminal kinase (JNK) activation. Daxx was originally identified as a Fas-binding protein that enhances Fas-dependent apoptosis by activating stress-inducible kinase pathways (65).

Various environmental factors, including UV and γ irradiation, chemotherapy drugs, osmotic shock, heat, nitric oxide (NO), lipopolysaccharide, arsenic oxide, and inflammatory cytokines such as tumor necrosis factor alpha or interleukin 1, induce the cellular activation of stress-inducible kinase pathways. Activated kinases, such as JNK, appear to regulate gene expression and/or other biochemical functions in cells exposed to environmental stress. JNKs phosphorylate transcription factors, such as c-Jun, ATF-2, and Elk-1, and strongly augment their transcriptional activity (15, 29, 59). Activation of JNK has been shown to induce apoptosis in some cells. For example, overexpression of MEKK, a JNK kinase kinase, has a lethal effect on fibroblasts (27, 31). In JNK2 knockout mice or mice expressing a dominant negative form of JNK, immature thymocytes are resistant to apoptosis induced by administration of anti-CD3 antibody (46). Disruption of the JNK3 gene in mice induces neuronal cells to become resistant to the excitotoxic glutamate receptor agonist, kainic acid, in the hippocampus (64). Fibroblasts with simultaneous targeted disruptions of JNK1 and JNK2 genes are protected against UV-stimulated apoptosis (55). In addition, the tumor suppressor p53, which is essential for radiation-induced apoptosis (37), is activated by JNK1 in vivo (40). These data suggest that the JNK cascade may participate in apoptosis.

Daxx directly interacts with ASK1 (4), a mitogen-activated protein kinase kinase kinase (24, 53, 63), which in turn activates the JNK- and p38-dependent cascades. Mutant p53 inhibits the Daxx-ASK1 cascade most likely as a result of a novel gain-of-function phenotype. We hypothesize that mutant p53 supports cell survival by inhibiting the stress-inducible kinase activation and that it contributes to both cellular transformation and resistance to cytotoxic agents.

MATERIALS AND METHODS

Identification of Daxx cDNA. A yeast two-hybrid screening was performed as described previously (56) by using as bait a COOH-terminal region of p53 containing a nuclear localization sequence, an oligomerization domain, and a regulatory basic amino acid region. The positive clones were identified by two criteria. The first was growth capability on nutrient-restricted agar plates: five essential components, leucine, lysine, histidine, tryptophan, and uracil, were omitted. In this system, histidine is provided only when a protein from the cDNA library has an affinity for the bait protein. The expression of the HIS3-coding sequence was driven by a minimal GAL1 promoter fused to multimerized LexA binding sites. The second criterion was the expression of β-galactosidase. Because the *lacZ* gene is driven by the same promoter as HIS3, the yeast clones that carry a positive cDNA clone should express β-galactosidase. After confirmation with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining for β-galactosidase activity, we isolated positive plasmids from the yeast colonies and transferred them to *Escherichia coli* strain HB101, which carries a mutation of the LueB gene and can therefore grow on an agar plate without leucine only when it contains a plasmid that carries the Leu2 gene. Isolated plasmid DNAs were then transferred back to the yeast, which carries bait plasmid, to confirm the presence of β-galactosidase activities (data not shown).

DNA cloning and protein production in vitro and in bacteria. Full-length human Daxx cDNA was isolated by reverse transcriptase PCR, and the sequence was verified. cDNA was cloned into a pcDNA3.1 vector (Invitrogen) and subjected to in vitro transcription-translation by using the TNT T7 coupled reticulocyte lysate system (Promega). A different portion of the COOH-terminal region of p53 was cloned into a pGEX-2T expression vector (Pharmacia); glutathione *S*-transferase (GST) fusion proteins were produced in BL21(DE3) pLysS bacteria (Novogen) and purified by a glutathione-Sepharose column as described previously (20).

Cell culture and cell lines. Saos2, H1299, HT-29, and 293 cells were maintained at 5% CO_2 in Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 10% fetal calf serum (FCS). HeLa cells were maintained at 5% CO_2 in minimum essential medium supplemented with 10% FCS. The transfections were performed by using FuGENE 6 (Roche) as recommended by the manufacturer.

Saos2-derived cell lines expressing 143VA, 175RH, 248RW, 273RH, or 281DG mutant p53 genes were established. In brief, Saos2 cells were transfected with a mutant p53-expressing plasmid followed by G418 (400 μg/ml) selection. Two weeks after selection by the drug, colonies were isolated and tested for the expression of mutant p53. Saos2/175RH cells were then further transfected with a FLAG-Daxx-expressing plasmid followed by hygromycin selection (100 μg/ml) for 2 weeks to establish cells in which both the 175RH mutant p53 and FLAG-Daxx were expressed.

Anchorage-independent cell growth assay. An anchorage-independent cell growth assay was performed as described previously (8). In brief, Saos2-derived cells expressing mutant p53 were resuspended in 0.3% Noble agar in Dulbecco's modified Eagle's medium supplemented with 10% FCS at a density of 2×10^4 cells per well in six-well plates and were plated onto a medium containing solidified 0.5% Noble agar in the bottom layer. Cultures were fed weekly, and colonies containing more than 30 cells were counted after 3 weeks.

GST protein binding assay. GST pull-down experiments were preformed as described previously (20). In short, in vitro-translated full-length human Daxx and deletion mutant Daxx Δ1, Δ2, Δ3, and Δ5, labeled with [^{35}S]methionine were incubated with GST or a GST-p53 fusion protein on glutathione-Sepharose beads, and the bound fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) followed by autofluorography.

Immunoprecipitation. Saos2 or H1299 cells were transfected with the plasmid DNA expressing FLAG-Daxx and the mutant p53-expressing plasmid. Transfected cells were harvested 48 h after transfection. Cells were lysed in E1A buffer (50 mM HEPES [pH 7.4], 1% NP-40, 250 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol [DTT], 50 mM NaF, 1 mM sodium orthovanadate, 5 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 0.5 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, 0.7 μg of pepstatin A/ml, and 10% glycerol). Lysates were subjected to immunoprecipitation with the DO-1 anti-p53 antibody or the M2 anti-FLAG antibody, and the precipitated material was analyzed by immunoblotting with the M5 anti-FLAG antibody or the DO-1 antibody, respectively.

To identify mutant p53-Daxx complexes in HT-29 cells, cell lysates were immunoprecipitated with PAb421 antibody, and the precipitated materials were analyzed by Western blotting using anti-Daxx polyclonal antibody (M-112; Santa Cruz Biotechnology). Arsenic trioxide (5 μM) treatment was performed for 4 h. For cells constitutively expressing both mutant p53 and FLAG-Daxx, 5 μM arsenic trioxide treatment was performed for 4 h or stimulation with the CH-11 anti-Fas antibody (300 ng/ml; Upstate Biotechnology) was performed for 40 min. Cell lysates (160 μg of protein) were subjected to immunoprecipitation with control immunoglobulin G2a (IgG2a) or with the PAb421 anti-p53 antibody (0.5 μg) followed by immunoblotting with the M5 anti-FLAG antibody. Total Daxx proteins in cells were detected by immunoblotting with anti-Daxx polyclonal antibody (M-112; Santa Cruz Biotechnology).

IP-kinase assay. For the immunoprecipitation kinase (IP-kinase) assay, pCFLAG-JNK (0.5 μg) was cotransfected with pcDaxx (0.75 μg) and a mutant p53-expressing plasmid DNA (0.75 μg) in the indicated combinations into HeLa cells. Four hours after transfection, cells were serum starved for 20 h and then harvested and lysed in lysis buffer (25 mM HEPES [pH 7.5], 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 50 mM NaF, 0.5 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, 0.7 μg of pepstatin A/ml, and 10% glycerol). FLAG-JNK1 was immunoprecipitated from cleared lysates with the M2 anti-FLAG antibody and used in in vitro kinase reactions in a buffer containing 25 mM HEPES (pH 7.4), 25 mM β-glycerophosphate, 25 mM $MgCl_2$, 0.1 mM sodium

orthovanadate, 0.5 mM DTT, 1 μCi of [γ-^{32}P]ATP, 20 μM ATP, and 1 μg of GST-c-Jun(1-79).

ASK1 kinase activity was measured in an IP-kinase assay using the 12CA5 antihemagglutinin (anti-HA) antibody. 293 cells were cotransfected with pcDNA3-ASK1-HA (0.5 μg), pCFLAG-Daxx (0.75 μg), and p53 (wild-type, 143VA, 175RH, 273RH) expression plasmids (0.75 μg). Kinase assays were performed by sequential phosphorylation of GST-MKK6 and GST-SAPK/p38γ to amplify the activities as described before (24). In brief, 24 h after transfection, cells were lysed in the lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EGTA, 3 mM DTT, 1% Triton X-100, 12 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM NaF, 0.5 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, 0.7 μg of pepstatin A/ml, and 10% glycerol) and subjected to immunoprecipitation with an anti-HA antibody for 2 h at 4°C. The beads were washed twice with the lysis buffer and once with the kinase reaction buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 0.1 mM sodium orthovanadate) and then incubated with 0.2 μg of GST-MKK6 for 10 min at 30°C in a final volume of 25 μl of the kinase reaction buffer supplemented with 100 μM ATP. Next, the activated complex was incubated with 0.3 μCi of [γ-^{32}P]ATP and 1 μg of GST-SAPK/p38γ in the same reaction (final volume, 35 μl) for 10 min at room temperature. The reactions were terminated by addition of 10 μl of 5× SDS sample buffer. Samples were heated at 95°C for 3 min and analyzed by SDS-PAGE. The phosphorylated GST-SAPK/p38γ was measured by PhosphorImager (Bio-Rad) after resolution by SDS-PAGE. The expression levels of ASK1-HA, FLAG-Daxx, and mutant p53 were identified by immunoblotting.

Cell survival assay. Fas-dependent apoptosis enhanced by Daxx was measured by cotransfecting pCMVβ (0.25 μg), pcDNA-Fas (0.25 μg), and mutant p53 (0.375 μg) into HeLa cells together with Daxx or Daxx deletion mutant expression plasmids (0.375 μg). Twenty-four hours after transfection, transfected cells were identified by X-Gal staining. The percentage of apoptotic cells was determined by dividing the number of blue cells with apoptotic morphology by the total number of blue cells. The specific apoptosis was determined as the percentage of blue cells with apoptotic morphology minus the percentage of blue cells with apoptotic morphology in pcDNA3-transfected cells. At least 200 cells from 10 random fields were counted in each experiment. Statistical analyses between two groups were performed with an unpaired Student *t* test, and probability (*P*) values were calculated.

Clonogenic cell survivals were measured as follows. Saos2 cells (2×10^5 cells) carrying an empty vector (pcDNA3.1/Neo) or a mutant p53 expression plasmid (mutants 143VA, 175RH, or 273RH) were transfected with a Daxx expression plasmid (pcDaxx/Hyg), an empty vector (pcDNA/Hyg) as a positive control, or a negative control vector (pCMVβ) that does not carry a hygromycin resistance gene. Drug-resistant cells were selected with hygromycin (800 μg/ml) for 2 weeks, colonies were stained with 0.05% crystal violet in a 20% ethanol–0.37% formaldehyde solution, and surviving colonies containing more than 50 cells were counted.

RESULTS

Identification of a p53 binding protein as a potential signal mediator. To identify potential partners of mutant p53 in the cell signaling pathways, we employed the yeast two-hybrid screening (56) using the COOH-terminal region of wild-type p53 as bait. This region, shared between wild-type and a variety of common mutant p53s, contains a nuclear localization sequence, an oligomerization domain, and a regulatory basic amino acid region (see Fig. 2). Sequence analysis of the positive clones revealed that one of the clones carries part of the human Daxx cDNA (see Fig. 2). Thus, it is likely that the COOH-terminal region of p53 and the COOH-terminal half of Daxx form complexes in yeast cells. Daxx was initially identified as a Fas-binding protein; however, the majority of the protein has been found in the nucleus (5, 65).

Identification of the regions responsible for the Daxx-p53 interaction. To characterize the domain within the COOH-terminal region of p53 responsible for its binding with Daxx, we performed an in vitro binding assay using GST-p53 (9, 20) and Daxx translated in vitro (see Fig. 2). In vitro-translated full-length human Daxx protein labeled with [^{35}S]methionine

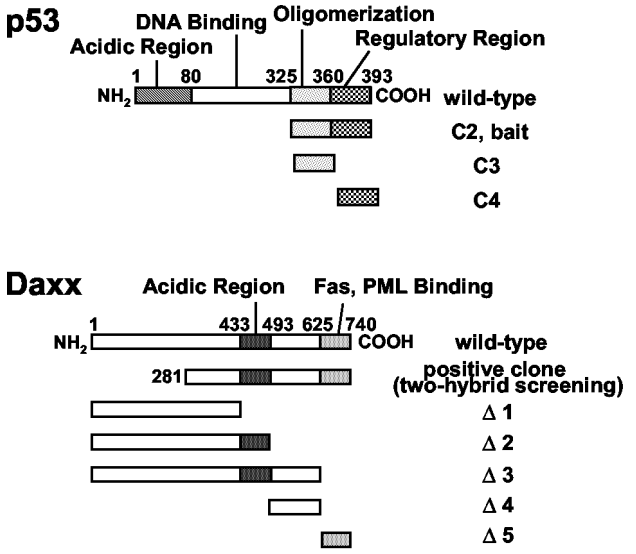

FIG. 1. Schematic diagram of p53 and Daxx mutants. Amino acid numbers of the domain boundaries and descriptions of domains are indicated. p53C2 is the portion used as a bait in the yeast two-hybrid screen. The cDNA fragment encoding amino acids 281 through 740 of human Daxx (positive clone) is the original fragment obtained in the yeast two-hybrid screen. The COOH terminus of p53 (C4, regulatory domain) is the Daxx-binding domain. The acidic domain (amino acids 433 to 493) and the COOH terminus Δ5 (amino acids 625 to 740) of Daxx are p53-binding domains.

was incubated with a series of truncated forms of p53 fused to GST protein on glutathione-Sepharose beads, and the bound fraction was analyzed by SDS-PAGE followed by autofluorography (20). Figure 2A shows the proteins used in this assay visualized with Coomassie brilliant blue stain. Human Daxx was found to bind specifically to the COOH-terminal region of p53 (C2), identical to that used in the yeast two-hybrid screen as bait (see Fig. 2B, lane 3). These results confirm the result in the two-hybrid screening (Fig. 1). Within the C2 portion, human Daxx protein was found to bind to the COOH-terminal end of p53, which contains a regulatory domain for the binding of p53 to specific DNA sequences (Fig. 2B, lane 5). We next identified the Daxx domain responsible for its interaction with p53. We created Daxx deletion mutant constructs, as shown in Fig. 1, and proteins were produced by in vitro transcription-translation in the presence of [^{35}S]methionine. The retention of these Daxx deletion mutants on the glutathione beads with the COOH-terminal end of p53 (GSTP53C4) were then measured. The deletion mutant Δ1, which contains the amino acids up to position 433, lost the ability to bind to GSTP53C4 (Fig. 2C, lanes 7 and 8), but Δ2, which contains the amino acids up to position 493, was retained on the beads (Fig. 2C, lanes 9 and 10). These results indicate that the p53-binding domain of Daxx is located between amino acids 434 and 493, the acidic region of Daxx. It is noteworthy that the COOH-terminal end of Daxx that was reportedly responsible for the interaction with Fas or the promyelocytic leukemia protein (PML) (54, 65) bound specifically to GSTP53C4 (Fig. 2D, lanes 2 and 3). These results show that the region containing amino acids 434 to 493 and possibly the COOH-terminal region of the Daxx

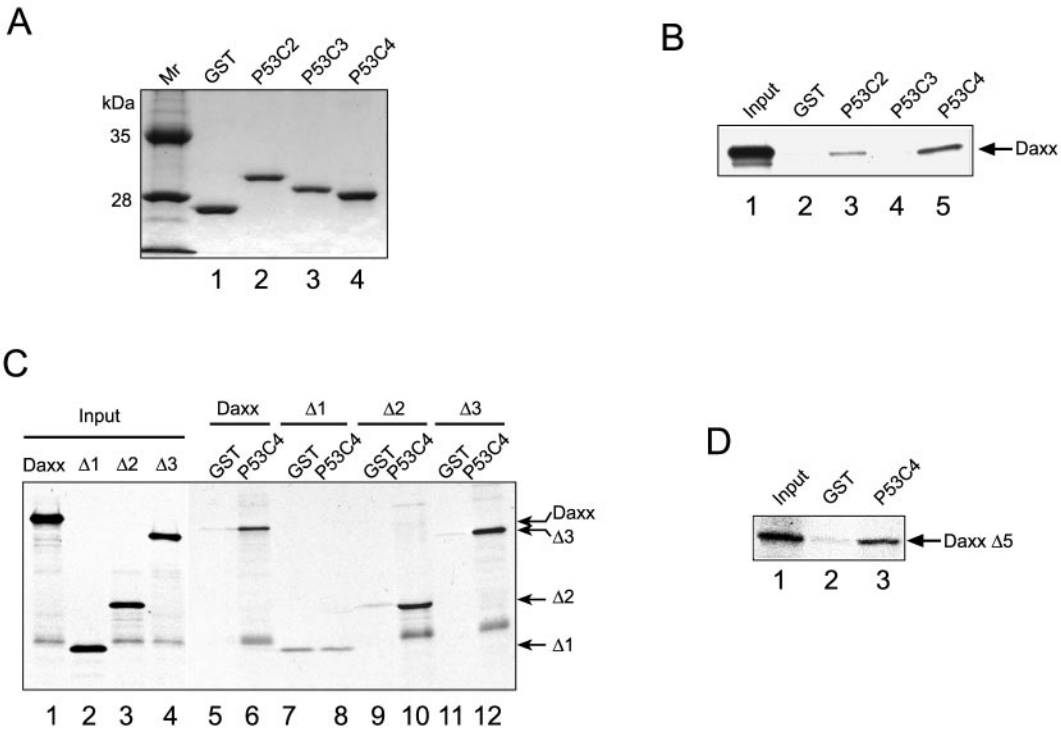

FIG. 2. Interaction of p53 and Daxx in vitro. (A) Coomassie brilliant blue staining of purified GST-p53 fusion proteins. GST (lane 1), GST-p53C2 (lane 2), GST-p53C3 (lane 3), and GST-p53C4 (lane 4) were produced in bacteria and purified as described previously. (B) Daxx binds to the COOH terminus of p53. In vitro-translated full-length human Daxx labeled with [^{35}S]methionine (lane 1) was incubated with GST (lane 2) or GST-p53 fusion proteins (lanes 3 to 5) on glutathione-Sepharose beads as indicated, and the bound fraction was analyzed by SDS–10% PAGE followed by autofluorography. (C) The acidic domain of Daxx binds to the COOH terminus of p53. Full-length Daxx and deletion mutants Daxx Δ1, Δ2, and Δ3 were translated in vitro in the presence of [^{35}S]methionine (lanes 1 to 4) and incubated with GST (lanes 5, 7, 9, and 11) or the GST-p53C4 fusion protein (lanes 6, 8, 10, and 12) on glutathione-Sepharose beads. Bound fractions were analyzed as described for panel B (lanes 5 to 12). Arrowheads indicate the positions of Daxx proteins. (D) The COOH terminus of Daxx binds to p53. A deletion mutant, Daxx Δ5 (codons 625 to 740), was translated in vitro (Daxx Δ5, lane 1) with [^{35}S]methionine and incubated with GST (lane 2) or the GST-p53C4 fusion protein (lane 3) on glutathione-Sepharose beads, and the bound fraction was analyzed as described for panel B.

protein are involved in its interaction with the COOH-terminal region of p53.

Daxx interacts with tumorigenic mutant p53 but not with wild-type p53. We next examined the interaction of p53 with Daxx in the cell by coimmunoprecipitations. Wild-type p53 expression plasmid or 175RH mutant p53 expression plasmid was cotransfected with FLAG-Daxx expression plasmids into human osteosarcoma Saos2 cells ($p53^{-/-}$), and then 48 h later the cells were lysed in E1A buffer (250 mM NaCl). Cell lysates were subjected to immunoprecipitation with DO-1 anti-p53 monoclonal antibody. Immunoprecipitated materials were separated by SDS-PAGE and analyzed by Western blotting (IP-Western blotting) with M5 anti-FLAG monoclonal antibody. Daxx did not immunoprecipitate with wild-type p53 but precipitated with mutant p53 (Fig. 3A). We then tested the abilities of various mutant p53s to bind to Daxx by IP-Western blotting using DO-1 antibody, which reacts equally well with all mutant p53s used. We tested five mutants, 175RH, 248RW, 273RH, 281DG, and 143VA (a temperature-sensitive mutant used at a nonpermissive temperature), and found that there were significant differences in the ability of different mutant proteins to coprecipitate Daxx (Fig. 3B, IP: p53 and WB: FLAG-Daxx). The 175RH and 273RH mutants precipitated the highest amount of Daxx (Fig. 3B, lanes 3 and 5), while 248RW and 281DG precipitated moderate amounts (Fig. 3B, lanes 4 and 6). The 143VA mutant precipitates the lowest amount of Daxx (Fig. 3, lane 2). The amounts of FLAG-Daxx and mutant p53 proteins were almost equal in each transfection, suggesting that these differences in affinities were due to an inherent difference between the mutant p53 proteins. The difference of the abilities for mutant p53 to interact with Daxx was also observed by reciprocal immunoprecipitation with M2 anti-FLAG antibody followed by Western blotting with DO-1 (Fig. 3B, IP:FLAG-Daxx and WB:p53).

It has been demonstrated that p53 mutants increase malignant transformation of the Saos2 cell to various degrees depending on the type of p53 mutant (8). We determined the ability of each mutant p53 to induce anchorage-independent growth of Saos2 cells in soft agar. Saos2 cell lines that stably express each of the aforementioned five mutant p53s were established, and the plating efficiencies in agar were determined by counting colonies (>30 cells). Interestingly, we observed gain-of-function activities of mutant p53s similar to those previously described for Saos2 cells, which induced anchorage-independent growth (8). Our results for vector, 143VA, 175RH, 248RW, 273RH, and 281DG were −, −, ++, −, ++, and +, respectively, and Dittmer et al. reported respective results of −, −, ++, +, ++, and +, where the sym-

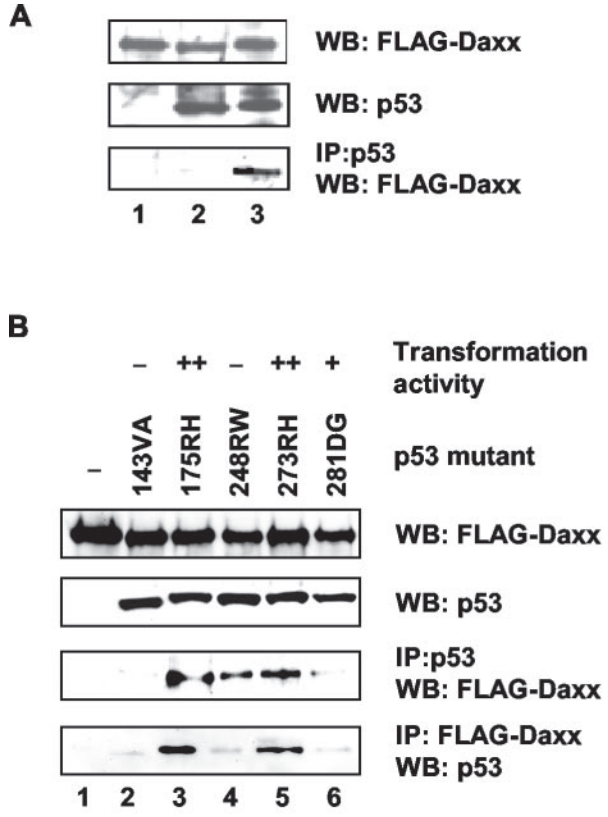

FIG. 3. Daxx interacts with tumorigenic mutant p53 but not with wild-type p53. (A) Mutant p53 but not wild-type p53 interacts with Daxx. Saos2 cells were transfected with empty vector (lane 1), wild-type p53 (lane 2), or 175RH mutant p53 (lane 3) expression plasmid together with FLAG-Daxx expression plasmid. Two days posttransfection, cells were lysed in the E1A buffer and the expression level of FLAG-Daxx and wild-type or mutant p53 was analyzed by Western blotting (WB) using M5 anti-FLAG antibody or DO-1 anti-p53 (both wild-type and mutant forms) antibody, respectively. Immunoprecipitations (IP) were performed by using DO-1 anti-p53 antibody, and precipitated materials were analyzed by Western blotting using M5 anti-FLAG antibody (WB). (B) Daxx binds to various p53 mutants. H1299 cells ($p53^{-/-}$) were transfected with the plasmids expressing FLAG-Daxx and mutant p53 (143VA, 175RH, 248RW, 273RH, and 281DG). Cell lysates were subjected to immunoprecipitation with DO-1 or M2 anti-FLAG antibody, and the precipitated material was analyzed by Western blotting with the M5 anti-FLAG antibody or DO-1, respectively. The summary of transformation activities of mutant p53 to induce anchorage-independent growth of cells is shown (top). The expressions of FLAG-Daxx and mutant p53 (143VA [lane 2], 175RH [lane 3], 248RW [lane 4], 273RH [lane 5], and 281DG [lane 6]) in cotransfected cells are shown.

bols for transformation activities are the following: −, 0%; +, ≤0.05%; ++, ≥0.1%. The potential of mutant p53s to induce anchorage-independent growth of Saos2 cells was found to correlate with Daxx binding ability. These results suggest that the interaction of mutant p53 with Daxx would play a role in the malignant transformation process.

Daxx interacts with mutant p53 under physiological conditions. We next examined the interaction between endogenous 273RH mutant p53 and endogenous Daxx in HT-29 cells by IP-Western blotting. Cell lysates were immunoprecipitated with PAb421 anti-p53 monoclonal antibody, and the precipitated material was analyzed by Western blotting with anti-Daxx polyclonal antibody. As shown in Fig. 4A, endogenous Daxx was specifically coimmunoprecipitated with 273RH mutant p53 when cells were treated with arsenic trioxide (As_2O_3), which enhances Daxx function in apoptosis (Fig. 4A, lanes 2 and 3), whereas the specific interaction was not detected without the treatment (Fig. 4A, lanes 4 and 5). Approximately 10% of the total cellular Daxx was coprecipitated with 273RH mutant p53 under the experimental conditions described in Materials and Methods. The interaction between mutant p53 and Daxx was further studied using a Saos2-derived cell line ectopically expressing both FLAG-Daxx and the 175RH mutant p53. The expression levels of FLAG-Daxx and endogenous Daxx were determined by Western blotting. Although FLAG-Daxx protein is expressed in this cell line, the total level of Daxx expression is almost identical to that of HeLa or parental Saos2 cells, indicating that the expression of FLAG-Daxx in these cells is at the physiological level (Fig. 4B, lanes 2 to 4). The expression level of 175RH mutant p53 in this cell line was determined by Western blotting and compared with the level of endogenous mutant p53 (273RH) expressed in HT-29. The expression level of mutant p53 in the Saos2-derived cells was much lower than that of HT-29, indicating that the expression level of 175RH mutant p53 is within the physiological range (Fig. 4C). Cell lysates of Saos2-derived cells were immunoprecipitated with PAb421 anti-p53 monoclonal antibody, and the precipitated material was analyzed by Western blotting with M5 anti-FLAG antibody. The FLAG-Daxx protein was specifically coimmunoprecipitated with the 175RH mutant p53 from the cell lysate (Fig. 4E, lanes 1 and 2). Furthermore, the interaction between mutant p53 and Daxx was significantly induced by treating cells with anti-Fas monoclonal antibody, which activates the Fas-dependent apoptosis pathway, or As_2O_3 (Fig. 4E, compare lane 2 with lanes 3 and 4). Since the amount of FLAG-Daxx and mutant p53 proteins expressed in cells are constant throughout the treatment (Fig. 4D), the treatment of cells with anti-Fas monoclonal antibody or As_2O_3 drastically enhances the affinity between 175RH mutant p53 and Daxx in cells. These results indicate that mutant p53 interacts with Daxx under physiological conditions, that the interaction is not a cell line-dependent phenomenon, and that the induction of complex formation by stresses, such as exposure to As_2O_3, is not a cell type-specific phenomenon. These results suggest that the expression of mutant p53 may modify Daxx-dependent stress-signaling pathways under physiological conditions.

Tumorigenic mutant p53 inhibits the Daxx-dependent stress-inducible kinase pathway. To determine the effect of mutant p53 on the Daxx signaling pathway, we measured ASK1 activities induced by Daxx overexpression. ASK1 is a mitogen-activated protein kinase kinase kinase that interacts with Daxx and activates the JNK and p38 pathways (4, 24). Thus, we examined the effect of mutant p53 on Daxx-dependent activation of ASK1. 293 cells were transfected with FLAG-JNK, ASK1, and p53 expression plasmids in the combinations indicated (Fig. 5). The ASK1 activity measured by a sequential IP kinase assay using GST-MKK6 and GST-p38γ as substrates was induced by overexpression of Daxx as expected (Fig. 5, lane 2). The strong Daxx-interacting 175RH and 273RH mutants strongly inhibited the activation of ASK1 induced by

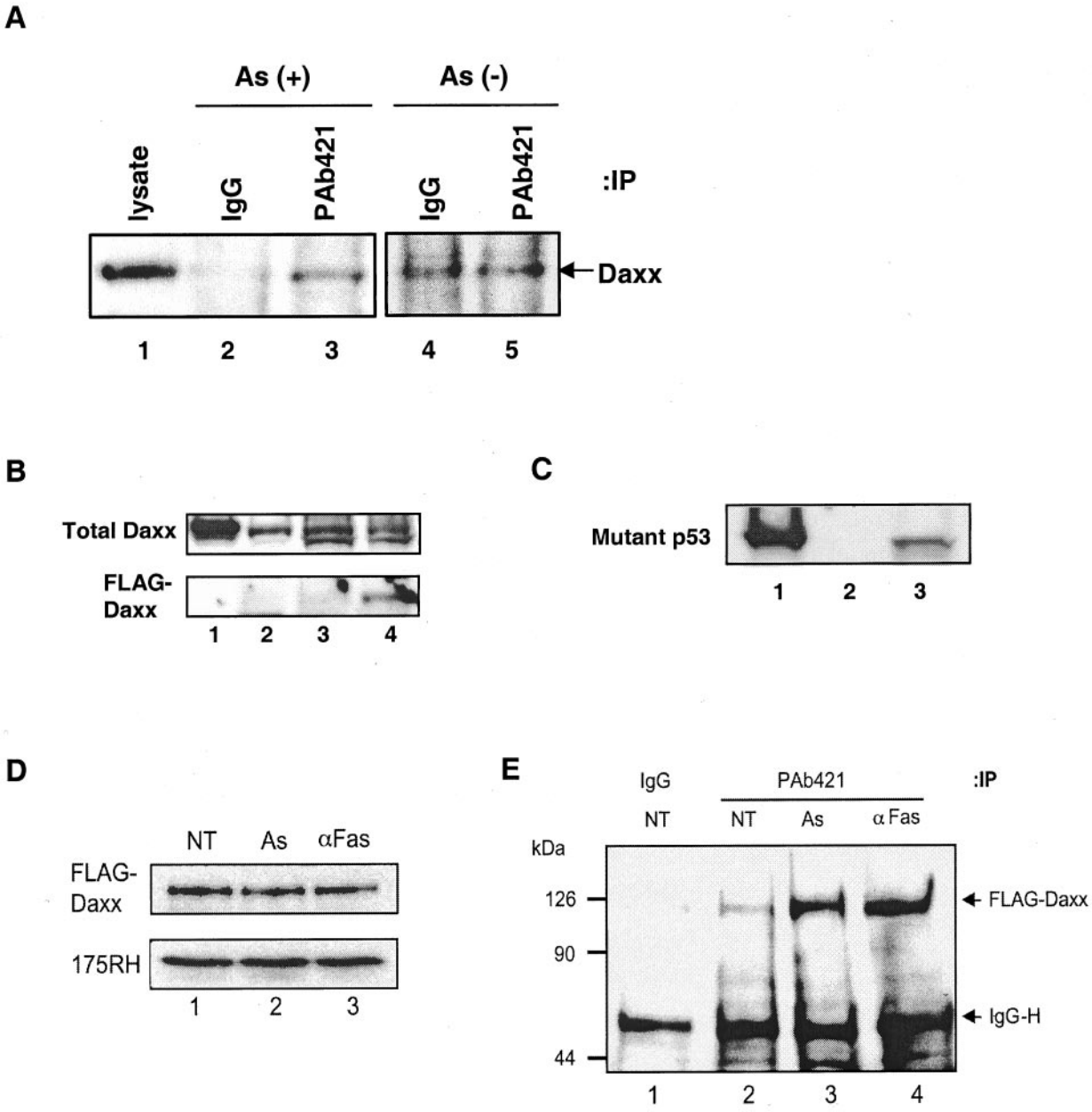

FIG. 4. Daxx interacts with mutant p53 under physiological conditions. (A) Mutant p53 interacts with Daxx in tumor cells exposed to arsenic trioxide. Cell lysates prepared from HT-29 cells originally expressing 273RH mutant p53 were subjected to immunoprecipitation with normal mouse IgG2a (lanes 2 and 4) or Pab421 anti-p53 antibody (lanes 3 and 5), and precipitated materials were analyzed by Western blotting with anti-Daxx antibody. HT-29 cells were treated with (lanes 2 and 3) or without (lanes 4 and 5) 5 μM arsenic trioxide for 4 h. Lanes 4 and 5 were exposed three times longer. Lane 1 shows the amount of Daxx in cell lysate (10 μg). (B) Expression of FLAG-Daxx in a Saos2-derived cell line. A cell line expressing both the 175RH mutant of p53 and FLAG-Daxx was established. Total Daxx expression was determined by anti-Daxx polyclonal antibody, and FLAG-Daxx expression was determined by M5 anti-FLAG antibody. Lane 1, Saos2 cells transfected with Daxx expression plasmid (10 μg of protein; positive control); lane 2, HeLa cells (30 μg of protein); lane 3, parental Saos2 cells (30 μg of protein); lane 4, established Saos2-derived cell line. (C) Expression of mutant p53 in established cell lines. Cell lysates (30 μg) were analyzed for the expression level of mutant p53 by Western blotting. Lane 1, HT-29 cells (expressing endogenous 273RH mutant p53; positive control); lane 2, Saos2 (p53-null, parental cells); lane 3, Saos2-derived established cells (expressing 175RH mutant p53). (D) Expression of mutant p53 and FLAG-Daxx in Saos2-derived cells during Fas cross-linking and arsenic trioxide treatment. Arsenic trioxide treatment (5 μM) was performed for 4 h, and stimulation of Fas with CH-11 anti-Fas antibody (300 ng/ml) was performed for 40 min. Lane 1, no treatment (NT); lane 2, arsenic trioxide treatment (AS); lane 3, Fas-cross-linking (αFas). (E) Fas-cross-linking and arsenic trioxide treatment induced the formation of complexes between Daxx and mutant p53 in cells. Cell lysate (160 μg) was subjected to immunoprecipitation with control IgG2a or the PAb421 anti-p53 antibody (0.5 μg) followed by Western blotting with the M5 anti-FLAG antibody. Lane 1, immunoprecipitation with control IgG; lane 2, no treatment (NT); lane 3, arsenic trioxide treatment (As); lane 4, Fas cross-linking (αFas).

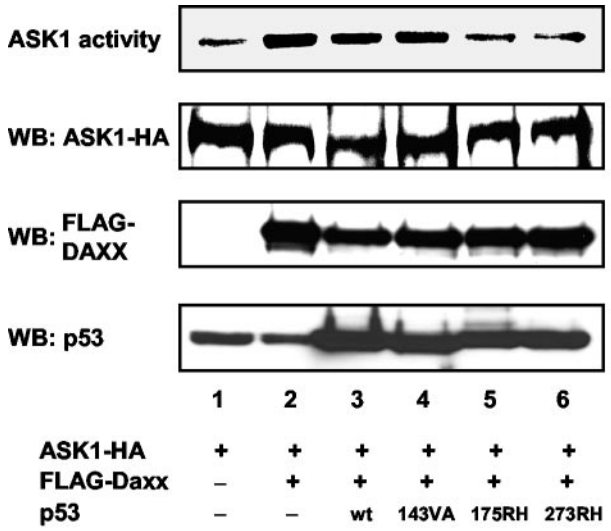

FIG. 5. Mutant p53 but not wild-type p53 inhibits Daxx-dependent ASK1 activation. 293 cells were cotransfected with pcDNA3-ASK1-HA (0.5 μg), pCFLAG-Daxx (0.75 μg), and a p53 (wild-type, 143VA, 175RH, or 273RH) expression plasmid (0.75 μg) in the indicated combinations. ASK1 kinase activity was measured in an IP kinase assay as described in Materials and Methods. The expression levels of ASK1-HA, FLAG-Daxx, and p53 were identified by Western blotting.

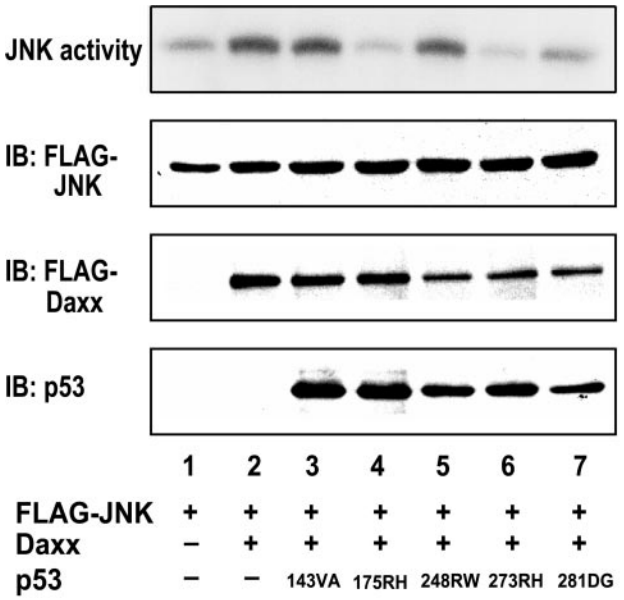

FIG. 6. Mutant p53 inhibits Daxx-induced JNK activation. pCFLAG-JNK (0.5 μg) was cotransfected with pcDaxx (0.75 μg) and a mutant p53-expressing plasmid (0.75 μg) in the indicated combinations into HeLa cells. Four hours after transfection, cells were serum starved for 20 h and then harvested and lysed in the lysis buffer. IP kinase assays were performed as described in Materials and Methods. The cleared lysates were subjected to Western blotting to measure the expression levels of FLAG-JNK, FLAG-Daxx, and mutant p53 proteins.

Daxx (Fig. 5, lanes 5 and 6). In contrast, the wild-type and the weak Daxx-interacting mutant p53, 143VA, showed weak inhibition of ASK1 activity (Fig. 5, lanes 3 and 4). The results of these experiments demonstrated that the strong Daxx-binding mutant p53 inhibits the Daxx ability to activate ASK1.

It has been shown that overexpression of Daxx activates ASK1 and the JNK pathway to enhance Fas-dependent apoptosis (65). Therefore, we next determined the effect of mutant p53 on the Daxx-dependent JNK activation. HeLa cells were transfected with a Daxx expression plasmid followed by an IP kinase assay using GST-c-Jun(1-79) as a substrate. The cleared lysates were also subjected to Western blotting to measure the expression levels of FLAG-JNK, Daxx, and mutant p53. Daxx-induced JNK activity was strongly inhibited by the expression of 175RH or 273RH mutant p53 (Fig. 6, lanes 4 and 6) and was weakly inhibited by 143VA or 248RW mutant p53 (Fig. 6, lanes 3 and 5). The magnitude of inhibition of JNK activity correlated with the strength of the interaction of mutant p53 with Daxx (Fig. 3B), suggesting that the complex formed between Daxx and mutant p53 caused the inhibition of the Daxx-dependent activation of JNK.

The effect of mutant p53 on the ASK1-dependent activation of JNK. We next examined the effect of mutant p53 on ASK1-dependent activation of JNK. HeLa cells were transfected with FLAG-JNK, ASK1, and mutant p53 expression plasmids in the combinations as indicated (Fig. 7). FLAG-JNK was immunoprecipitated from cell lysates, and in vitro kinase assays were performed as described in Materials and Methods. JNK activity was induced by the expression of ASK1 as expected (Fig. 7, lane 2). In contrast to the Daxx-dependent activation of JNK, both the weak Daxx-binding 143VA and the strong Daxx-binding 175RH mutant p53 weakly inhibited the activation of JNK induced by ASK1 (Fig. 7, lanes 3 and 4). Another strong Daxx-binding 273RH mutant p53 did not show an inhibitory effect (Fig. 7, lane 5). These results indicate that mutant p53s could have a weak inhibitory effect on the ASK1-dependent activation of the JNK pathway. However, the ability of each mutant p53 to inhibit the ASK1-dependent activation of JNK does not correlate with the strength of the interaction with

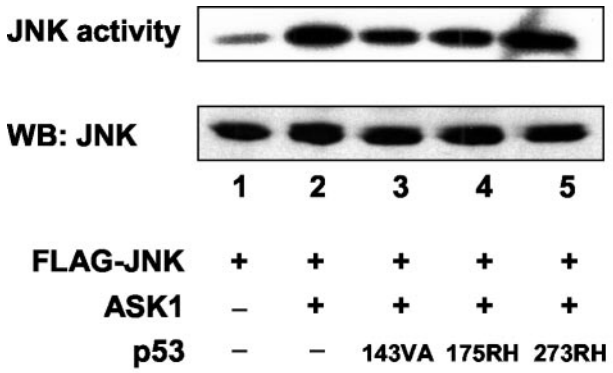

FIG. 7. The effect of mutant p53 on the ASK1-dependent JNK activation. FLAG-JNK expression plasmid (0.5 μg) was cotransfected with ASK1 expression plasmid (0.75 μg) and mutant p53-expressing plasmid (0.75 μg) in the indicated combinations into HeLa cells. Four hours after transfection, cells were serum starved for 20 h, and then they were harvested and lysed in the lysis buffer. The activity of JNK was determined by IP kinase assay as described in the legend for Fig. 6. The cell lysates were subjected to Western blotting to determine the expression of FLAG-JNK protein.

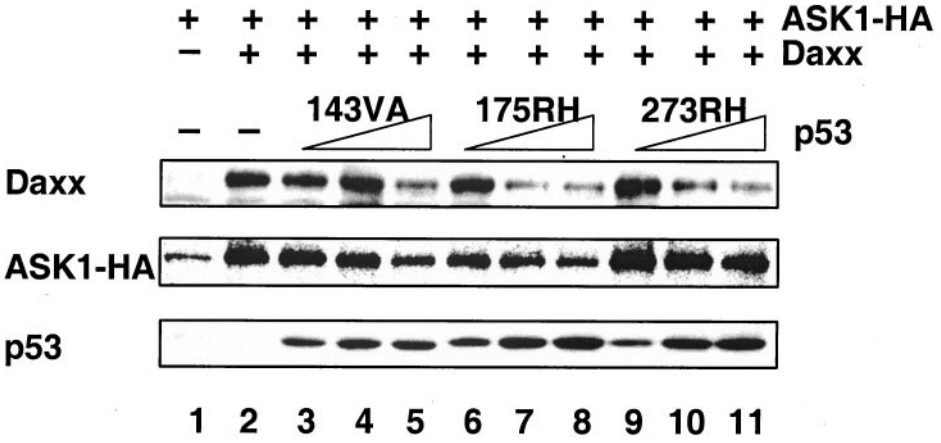

FIG. 8. Mutant p53 reduces the amount of Daxx in cells. Expression plasmids for Daxx (0.75 μg) and ASK1-HA (0.5 μg) were transfected into HeLa cells together with an increasing amount of mutant p53 expression plasmid (0.25, 0.5, and 0.75 μg). Lanes 3 to 5, 143VA; lanes 6 to 8, 175RH; lanes 9 to 11, 273RH. Cells were harvested 2 days posttransfection, and the expression levels of Daxx, ASK1-HA, and mutant p53 were determined by Western blotting using anti-Daxx, anti-HA, and anti-p53 antibodies, respectively.

Daxx. Therefore, mutant p53 likely inhibits the Daxx-dependent JNK activation pathway by inhibiting mainly the Daxx-dependent activation of ASK1.

Mutant p53 reduces the amount of Daxx in the cell. The expression of p53 mutants slightly reduced the amount of FLAG-Daxx as shown in Fig. 5, although p53 mutants do not squelch transcription factors from promoters as does wild-type p53 (20, 47). To understand how mutant p53 inhibits Daxx-dependent activation of ASK1, we examined the effect of mutant p53 on the expression of Daxx in the cell. The levels of Daxx and ASK1-HA in HeLa cells were measured by Western blotting after cotransfection of Daxx and ASK1-HA expression plasmids with an increasing amount of mutant p53 expression plasmid (Fig. 8). The expression level of Daxx was decreased by the expression of all three mutant p53s tested in dose-dependent manners, whereas the level of ASK1-HA was minimally affected. Furthermore, the expressions of the strong Daxx-interacting mutant p53s (175RH, 273RH) showed a stronger effect on the expression of Daxx (Fig. 8, lanes 6 to 8 and 9 to 11) than a weak Daxx-interacting mutant p53 (143VA; Fig. 8, lanes 3 to 5). These results suggest that mutant p53s inhibit the Daxx-dependent activation of ASK1 by reducing the level of Daxx in cells through their interaction.

Mutant p53 inhibits the function of Daxx to enhance Fas-dependent apoptosis. Daxx enhances Fas-dependent apoptosis of HeLa cells by activating the JNK pathway (65). Therefore, we examined whether the interaction of mutant p53 with Daxx inhibits this effect. Fas-dependent apoptosis of HeLa cells was enhanced by the cotransfection of Daxx expression plasmid as expected (Fig. 9, lane 3). The Daxx deletion construct Δ3 (Fig. 1), which contains Δ4, a domain activating ASK1 constitutively (Fig. 9, lane 7), also enhanced Fas-dependent apoptosis (Fig. 9, lane 5). The effect of Daxx or Daxx deletion mutant Δ3 on enhancing Fas-dependent apoptosis was inhibited by the expression of strong Daxx-binding 175RH mutant p53 (Fig. 9, compare lanes 3 and 4 and lanes 5 and 6 [$P = 0.025$ and 0.038, respectively]). By contrast, 175RH mutant p53 failed to inhibit enhanced apoptosis by Daxx deletion mutant Δ4, which lacks the acidic domain responsible for the interaction with mutant

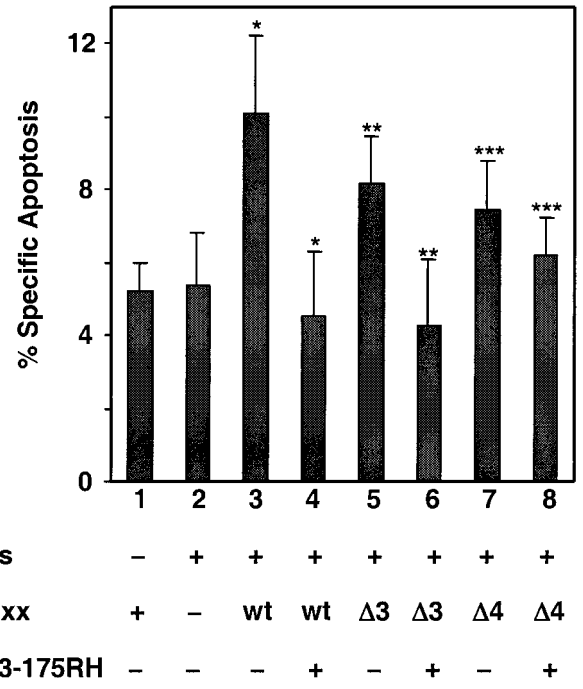

FIG. 9. Mutant p53 inhibits the function of Daxx to enhance apoptosis induced by Fas. Fas-dependent apoptosis enhanced by Daxx was measured by cotransfecting pCMVβ (0.25 μg) and pcDNA-Fas (0.25 μg) into HeLa cells together with Daxx or Daxx deletion mutant (Δ3 or Δ4) expression plasmids (0.375 μg). The inhibitory effect of 175RH mutant p53 on the function of Daxx in apoptosis was determined by cotransfection with mutant p53 expression plasmid (0.375 μg) as indicated (lanes 4, 6, and 8). Twenty-four hours after transfection, transfected cells were identified by X-Gal staining and apoptotic cells were counted. Specific apoptosis was calculated as the percentage of enhanced apoptosis over the expression of vector-transfected cells as described in Materials and Methods. Probability values (P) are as follows: *, $P = 0.025$; **, $P = 0.038$; ***, $P = 0.273$.

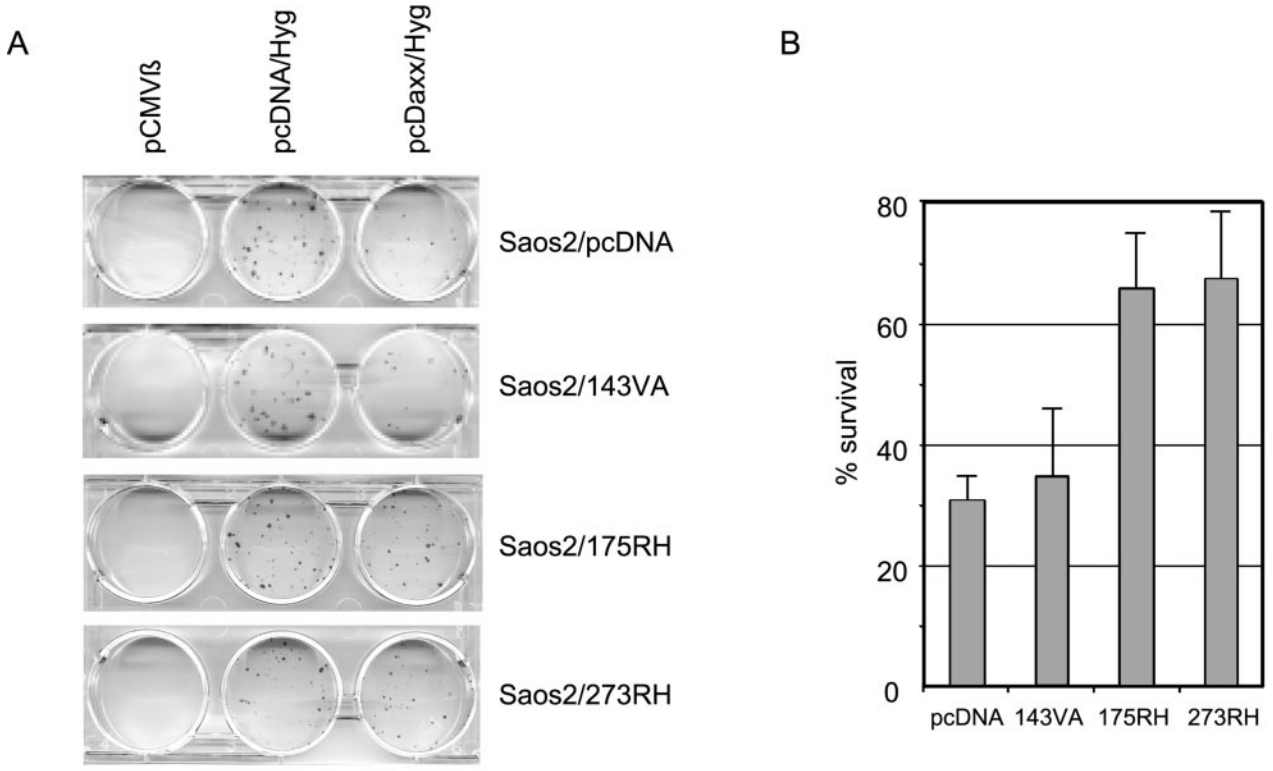

FIG. 10. Mutant p53 rescued cells from growth inhibition caused by Daxx. (A) Mutant p53 increased cell survival from Daxx-dependent growth inhibition. Three stable Saos2 cell lines expressing 143VA, 175RH, or 273RH mutant p53 and one control cell line (Saos2/pcDNA) were tested for the Daxx-dependent growth inhibition by clonogenic survival assay. Cells were transfected with Daxx expression plasmid (pcDaxx/Hyg), an empty vector (pcDNA/Hyg), or a negative control plasmid (pCMVβ) that does not carry a hygromycin resistance gene. Drug-resistant cells were selected with hygromycin (800 μg/ml) for 2 weeks, and colonies were stained with 0.05% crystal violet in a 20% ethanol–0.37% formaldehyde solution. (B) Quantitative analysis of surviving colonies. Stained colonies (>50 cells) were counted, and the survival ratio was calculated by dividing the number of stained colonies by the numbers of colonies from pcDNA/Hyg-transfected cells. The averages of survival ratios are shown with standard deviations from three independent experiments.

p53 (Fig. 9, compare lanes 7 and 8 [$P = 0.273$]). Thus, mutant p53 appears to inhibit the function of Daxx to enhance Fas-dependent apoptosis through a direct interaction with Daxx.

Tumorigenic mutant p53 relieves cells from Daxx-dependent growth inhibition. To further establish the biological significance of the interaction of mutant p53 and Daxx, we examined the effect of mutant p53 on the Daxx-dependent inhibition of cell growth. The ability of mutant p53 to rescue cells from such growth inhibition was assayed by measuring clonogenic survival in mutant p53-expressing cell lines (Fig. 10). Four cell lines were established by transfecting Saos2 cells with an empty vector (pcDNA3.1/Neo) or a mutant p53 expression plasmid (143VA, 175RH, or 273RH) followed by selection with G418. The expression of mutant p53 in each cell line was confirmed by Western blotting. The cells were then transfected with an empty vector (pcDNA3.1/Hyg) or with a Daxx expression plasmid (pcDaxx/Hyg). Drug-resistant cells were selected by hygromycin treatment for 2 weeks, and surviving colonies were stained for counting (Fig. 10A). In each cell line, the survival was normalized to cells receiving pcDNA3.1/Hyg. Transfections with pCMVβ, which does not carry a hygromycin-resistant gene, were included to demonstrate that the four Saos2 cell lines were sensitive to hygromycin selection (zero survival). We found that the transfection of a Daxx expression plasmid into control cells (Saos2/pcDNA) reduced colony formation to 30% of pcDNA/Hyg-transfected cells. The constitutive expression of strong Daxx-binding p53 mutants 175RH and 273RH significantly increased the survival of cells, while the weak Daxx-binding mutant p53, 143VA, had little effect (Fig. 10B). Thus, mutant p53 inhibits the function of Daxx and therefore increases cell survival.

DISCUSSION

More than 50% of all human cancer cells carry a mutation in the p53 tumor suppressor gene that produces a point mutant of the p53 protein. Since mutant p53s are generally inert in the activation of the MDM2 gene, which encodes a ubiquitin ligase for p53 to induce its degradation, mutant p53 often accumulates in tumor cells (11, 19). Accumulated mutant p53 may be beneficial for deregulated cell growth, and it may acquire an unidentified growth-promoting function, termed gain-of-function. Indeed, some mutant p53s have been shown to be tumorigenic (8, 10, 21, 26, 45, 49); however, the molecular mechanisms of this function are still largely unknown.

We report here that the mutant p53 protein interacts with Daxx, suggesting a possible physiological role for the mutant p53 in altering the cellular signaling process when cells are

exposed to stresses. Daxx was first identified as a Fas Death-domain binding protein. Fas is a cell surface receptor that induces apoptosis upon oligomerization (42). Fas can activate two independent signaling pathways. One such well-characterized pathway involves the adapter protein FADD, which recruits procaspase-8 and activates a protease cascade leading to apoptosis (2, 41, 42). The second pathway is mediated by Daxx, which can enhance Fas-induced apoptosis by activating the JNK cascade, culminating in the phosphorylation and activation of transcription factors, such as c-Jun (30, 38, 65). We found that tumorigenic mutant p53s bound to Daxx whereas wild-type p53 did not. Interestingly, the binding ability of each mutant p53 to Daxx did not correlate with a conformational status of its DNA-binding domain. Two p53 mutants, 143VA and 175RH, undergo a conformational change to a mutant form in their DNA-binding domain, evident from their interactions with the heat shock cognate 70 (HSC70) or PAb240, a monoclonal antibody specific to mutant forms of p53 (33). However, 143VA's activity in inducing malignant transformation in Saos2 cells is weaker than that of 175RH (Fig. 3B) (8). Daxx distinguishes these two p53 mutants and interacts preferentially with 175RH. The p53 mutants 248RW and 273RH have lost an amino acid that is involved in the direct contact with DNA (6) and do not have a detectable conformational change in their DNA-binding domain as judged by their inability to bind HSC70 or PAb240. Again, Daxx distinguishes these two p53 mutants from the wild-type p53 and preferentially binds to these mutant p53s. Because both 143VA and 175RH mutant p53s localize in the nucleus, the potential difference of localization of mutant p53 is not the reason for the weak interaction between 143VA mutant p53 and Daxx. The molecular mechanism by which Daxx recognizes and distinguishes these p53 mutants from wild-type p53 although they share the identical COOH-terminal domain remains to be solved. One amino acid mutation within the central DNA binding domain may induce conformation change, and since the regulatory COOH terminus is expected to interact with the central DNA binding domain (23), the conformation change within the central domain could affect the availability of the COOH-terminal domain to interact with Daxx.

Arsenic trioxide is clinically used on patients with refractory acute promyelocytic leukemia and is known to accentuate the localization of PML protein to the PML oncogenic domain. The antileukemic effects of arsenic trioxide may be directly mediated by its ability to induce the relocalization and degradation of PML, as well as the degradation of PML-RARα fusion protein (48, 66), the specific product of the t(15;17) of acute PML (7, 28). PML plays a critical role in apoptosis induced by Fas, tumor necrosis factor alpha, or interferons (58), suggesting that apoptotic signals initiated at the membrane receptors are transferred to the nucleus. Recently, arsenic trioxide was also shown to enhance Daxx function to promote Fas-dependent apoptosis (54) although the phosphorylation of JNK was not observed in this experimental system, suggesting that arsenic trioxide has an effect on enhancing Daxx-dependent stress responses. Stress-inducible complex formation between mutant p53 and Daxx has been demonstrated in the tumor cell line expressing endogenous mutant p53 and ectopically expressing mutant p53 (Fig. 4), which strongly suggests that certain stresses are able to enhance complex formation in cells in which wild-type p53 has been abolished. These results also suggest that the interaction as well as the induction of complex formation by stresses is not a cell type-specific event. Arsenic treatment induces posttranslational modification of PML with SUMO-1, a ubiquitin-like protein, to recruit Daxx into the PML oncogenic domain (25). Thus, posttranslational modifications and/or recruitment of additional protein(s) to the mutant p53 and/or Daxx could mediate the induction of the Daxx-mutant p53 complex formation by arsenic trioxide (Fig. 4A and E). Immunoprecipitated Daxx from cells treated with arsenic trioxide was analyzed by Western blotting with anti-SUMO-1 antibody, and this showed that Daxx underwent SUMO-1 modification under our experimental conditions (data not shown). Daxx has been shown to translocate from the nucleus to the cytoplasm upon Fas activation in the presence of z-VAD-fmk (5). The molecular mechanism by which Fas signal or arsenic treatment stimulates formation of mutant p53-Daxx complexes remains to be addressed.

Several proteins have been reported to interact with Daxx, including Fas, ASK1, DNA methyltransferase I, PML, Ets1, Pax3 and Pax5, histone deacetylase, HSP27, and possibly with a centromere-associated protein, CENP-C (18, 34, 35, 39, 44), suggesting that Daxx has multiple functions. Targeted disruption of the Daxx gene in the mouse results in an early embryonic lethal phenotype with increased apoptosis directly or indirectly, indicating an important developmental function of this protein (39). Little is known about the embryonic expression pattern of the characterized death-signaling complexes, and the function of Daxx during embryogenesis is unknown; thus, further studies are needed to clarify the molecular mechanism of inducing apoptosis of cells that lack the wild-type function of Daxx.

Daxx-dependent ASK1 activation was effectively inhibited by the strong Daxx-binding mutant p53 (Fig. 5). One possible explanation for this effect is that mutant p53 reduces the cellular level of Daxx (Fig. 8). Since the magnitude of the effect of p53 mutants to decrease Daxx seems to correlate with their abilities to interact with Daxx, the effect is likely through the direct interaction of mutant p53 and Daxx. The mechanism of reducing the amount of Daxx by mutant p53 is still unclear. However, since the ectopically expressed Daxx was driven by the cytomegalovirus (CMV) promoter, which is the same as the other promoters in the system, it is unlikely that the effect of mutant p53 reducing Daxx is on a transcriptional level. Moreover, it seems that it is not due to the masking effect of the epitope for the antibody by posttranslational modifications of Daxx induced by mutant p53, because ectopically expressed FLAG-tagged Daxx was also reduced by mutant p53 detected by anti-FLAG monoclonal antibody (Fig. 5 and data not shown). Therefore, it is likely that mutant p53 destabilizes Daxx, possibly inducing its degradation. Mutant p53 seems to inhibit the activation of Daxx-dependent stress response by two ways. Mutant p53 may reduce the amount of Daxx if the stress also activates ASK1; however, if the stress does not activate ASK1 and therefore the reduction of the amount of Daxx by mutant p53 is not significant, mutant p53 binds to Daxx and inhibits ASK1-dependent JNK activation as shown in Fig. 6.

Activation of the Daxx-dependent signaling pathway showed a moderate level of enhancement (about 10%) of Fas-depen-

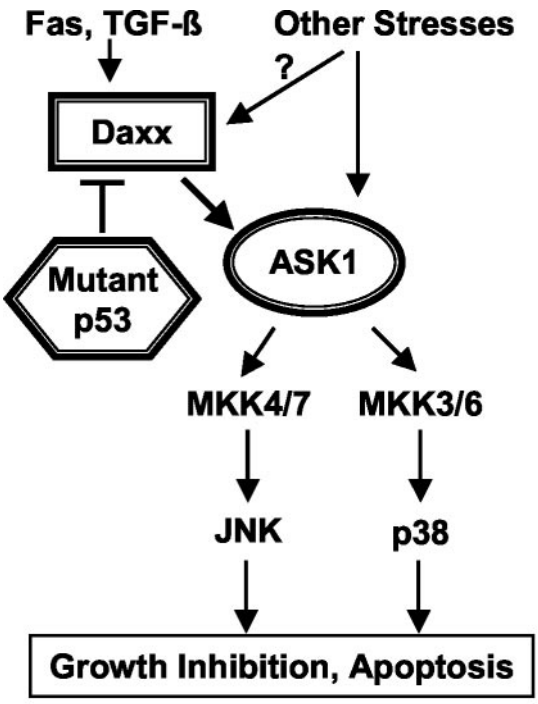

FIG. 11. Mutant p53 supports unregulated cell growth by inhibiting Daxx-dependent stress-response. Tumorigenic mutant p53 binds to Daxx and inhibits ASK1-mediated stress-inducible kinase pathways. This inhibition may relieve tumor cells from growth inhibition or apoptosis induced by the activation of stress-inducible kinases.

dent apoptosis (Fig. 9) as previously reported (65). It is not clear whether both the acidic domain and the Fas interaction domain of Daxx are necessary for the interaction with mutant p53. Fas-dependent apoptosis promoted by Δ3 Daxx, which lacks the Fas-binding domain but contains the acidic domain, was inhibited by 175RH mutant p53 (Fig. 9), whereas the apoptosis promoted by Δ4 Daxx, which does not have either the Fas-binding domain or the acidic domain, was not inhibited by 175RH mutant p53. Therefore, it is likely that mutant p53 does not bind to Δ4 Daxx and thus does not inhibit Daxx-dependent promotion of apoptosis. These results suggest that the interaction of mutant p53 through the acidic domain of Daxx is critical, as shown by in vitro experiments (Fig. 2), but the Fas-binding domain is dispensable to bind to 175RH mutant p53 under our experimental conditions. It is possible that the Fas interaction domain plays a role in determining the strength of the interaction of mutant p53 and Daxx.

The growth-inhibiting effect of Daxx was clearly observed by the clonogenic survival assay (Fig. 10), suggesting that activation of the Daxx-dependent signaling pathway has indeed a negative effect on cell growth and may induce nonapoptotic cell death, such as necrosis. Some mutants of p53 (175RH and 273RH) that have high Daxx-binding ability neutralize the growth repression function of Daxx, whereas a mutant p53 with a weak Daxx-binding activity (143VA) failed to rescue cells from Daxx-dependent growth repression. These results are consistent with the observations that tumorigenic mutant p53 interacts with Daxx and strong Daxx-interacting mutants of p53 interfere with Daxx-mediated stress-inducible kinase activations.

The biological significance of Daxx-dependent activation of the JNK pathway in Fas-dependent apoptosis may vary among cell lines. Daxx is also involved in mediating JNK activation by tumor growth factor β (TGF-β), and therefore it is directly associated with the TGF-β apoptotic signaling pathway (43). The activation of stress response kinases during apoptosis is well documented (22, 24, 27, 50, 62). The effect of JNK activation on the regulation of the apoptotic pathway appears to be cell type dependent as well as affected by other conditions to which cells are exposed. Recently, phosphorylated HSP27 dimer was shown to inhibit Daxx-mediated JNK activation and Fas-induced FADD-independent apoptosis (5). HSP27 prevents JNK activation by inhibiting Daxx from the interaction with ASK1, which is in clear contrast to the mechanism of the inhibition by mutant p53, where the expression of Daxx protein is reduced.

We have thus identified a novel gain-of-function of tumorigenic mutant p53 that inhibits stress-inducible kinase pathways and, consequently, relieves cells from the growth inhibition caused by Daxx. As mutant p53 frequently accumulates in large amounts in human tumor cells, our results suggest that the accumulation of mutant p53 in cells may establish acquired resistance during its malignant transformation to microenvironmental stresses, at least in part by inhibiting Daxx function to activate stress-inducible kinase pathways (Fig. 11). Moreover, ionizing radiation and chemotherapy drugs also activate stress-inducible kinase pathways. Therefore, these data may identify the Daxx-mutant p53 protein interaction as a potential drug target that can sensitize highly malignant tumor cells to selected cancer treatments.

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