The p53 Isoform Δp53 Lacks Intrinsic Transcriptional Activity and Reveals the Critical Role of Nuclear Import in Dominant-Negative Activity

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Abstract

The transcription factor p53 is one of the most frequently mutated tumor suppressors. Recent progress has unraveled several novel isoforms of p53. Intriguingly, one of the p53 isoform, Δp53, which lacks part of the DNA binding domain, was reported to be transcriptionally active toward some p53 target genes and is critical for the intra–S phase checkpoint. Here, we show that, in contrast to full-length p53, ectopically expressed Δp53 neither transactivated the promoters of $p21^{CIP1/WAF1}$ or murine double minute-2 (MDM2) nor repressed the cyclin B1 promoter in unstressed H1299 cells. Due to the deletion of a nuclear localization signal, Δp53 was not imported into the nucleus. Engineering of nuclear localization signals to Δp53 restored nuclear accumulation. However, the nuclear-targeting Δp53 remained inactive, indicating that the lack of intrinsic activity of Δp53 was not simply due to subcellular localization but to its incomplete DNA binding domain. Similar to p53, Δp53 was subjected to MDM2-mediated ubiquitination/proteolysis. The cytoplasmic localization of Δp53 correlated with the instability of the protein because forcing Δp53 into the nucleus increased its stability. Although Δp53 could form a complex with p53 and stimulated the cytoplasmic retention of p53, it was not a robust inhibitor of p53. Targeting Δp53 into the nucleus enhanced the dominant-negative activity of Δp53. These observations underscore the critical role of subcellular localization in the dominant-negative action of p53. [Cancer Res 2007;67(5):1959–69]

Introduction

Loss of the p53 tumor suppressor function is one of the most common steps in tumorigenesis. Germ line mutations of *p53* (*TP53*) are present in cancer-prone families with Li-Fraumeni syndrome (1), and somatic mutations are found in more than half of all cancer cases (2).

The *p53* gene encodes a protein with a central DNA binding domain, flanked by an NH_2-terminal transactivation domain and a COOH-terminal tetramerization domain (3). The active form of p53 is a tetramer of four identical subunits, consisting of a dimer of a dimer (4). Consistent with its tetrameric state, p53 binds DNA sites that contain four repeats of the pentamer sequence motif 5′-Pu-Pu-Pu-C-A/T-3′ (Pu is purine). The majority of the mutations in p53 are missense point mutations clustered in the DNA binding domain (5). The structure of the DNA binding domain consists of a large β-sandwich that acts as a scaffold for three loop-based elements that contact the DNA (6). Importantly, the residues most frequently mutated in cancers are all at or near the protein-DNA interface, and over two thirds of the missense mutations are within the DNA binding loops (7).

Many studies have detailed the role of p53 as a transcription factor. A myriad of genes are transactivated by p53 and many of which are believed to be underlie the antiproliferative functions of p53 (8), including genes whose products are critical for cell cycle arrest ($p21^{CIP1/WAF1}$, 14-3-3σ, and GADD45) and apoptosis (BAX, NOXA, and PUMA). Given the critical role of p53 in controlling cell proliferation, it is not surprising that its levels and activities are tightly regulated. Under normal conditions, murine double minute-2 (MDM2; also one of the transcriptional targets of p53) binds to the transactivation domain of p53 and abrogates p53-mediated transcription. MDM2 also shuttles p53 out of the nucleus and targets p53 for ubiquitin-mediated proteolysis, keeping p53 at a low level under unstressed conditions (9). Other ubiquitin ligases including MDMX (10), PIRH2 (11), and COP1 (12) also seem to contribute to p53 ubiquitination. On DNA damage or other stresses, p53 is phosphorylated by ataxia telangiectasia mutated (ATM)/ATM and Rad3-related (ATR) at Ser^{15} (13) and CHK1/CHK2 at Ser^{20} (14, 15). Phosphorylation of these residues (as well as other NH_2-terminal residues by various kinases) disrupts the p53-MDM2 interaction and promotes p53 accumulation. Besides ubiquitination and phosphorylation, p53 is also regulated by other posttranslational modifications, including acetylation by CREB binding protein/p300 at multiple COOH-terminal lysine residues, neddylation, and sumoylation (16).

Two *p53*-related genes, *p63* (*TP63*) and *p73* (*TP73*), encode proteins that share high sequence homology with p53, particularly at the DNA binding domain. This enables p63 and p73 to also transactivate p53-responsive genes, causing cell cycle arrest and apoptosis (17). A notable feature of *p63* and *p73* is that both genes express a large number of isoforms (17). Human *p63* encodes at least six different isoforms: three are derived from alternative splicing of the COOH terminus (TAp63α, TAp63β, and TAp63γ) and three are transcribed from an alternative promoter located in the intron 3, producing proteins lacking the NH_2-terminal transactivation domain (ΔNp63α, ΔNp63β, and ΔNp63γ; ref. 18). Human *p73* expresses at least seven alternatively spliced COOH-terminal isoforms (α, β, γ, δ, ε, ζ, and η) and at least four alternatively spliced NH_2-terminal isoforms initiated at different ATG. Like *p63*, *p73* can be transcribed from an alternative promoter located in the intron 3 (ΔNp73). Both ΔNp63 and ΔNp73 can bind DNA through p53-responsive element and can exert dominant-negative effects over p53, p63, and p73 activities either by competing for DNA binding sites or by direct protein-protein interaction (19).

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Until recently, the prevailing view was that the structure of *p53* gene is much simpler than that of *p63* and *p73*. Recent progress, however, has unraveled that *p53* also encodes several isoforms. These include p53β (also called p53i9) and p53γ, which are produced from alternative splicing from intron 9 and lack the COOH-terminal tetramerization domain (20, 21). In addition, NH_2-terminally truncated isoforms (Δ40p53, Δ40p53β, and Δ40p53 γ) are derived from alternative splicing of intron 2 or by alternative initiation of translation (22, 23). Another type of NH_2-terminally deleted isoforms (Δ133p53, Δ133p53β, and Δ133p53γ) is transcribed from an internal promoter located in intron 4 (24). Similar to ΔNp63 and ΔNp73, both Δ40p53 (23) and Δ133p53 (24) have dominant-negative effect on wild-type p53 transcriptional activity and p53-mediated apoptosis. Furthermore, Δ40p53 can modify the subcellular localization of p53 and prevent p53 degradation by MDM2 (23).

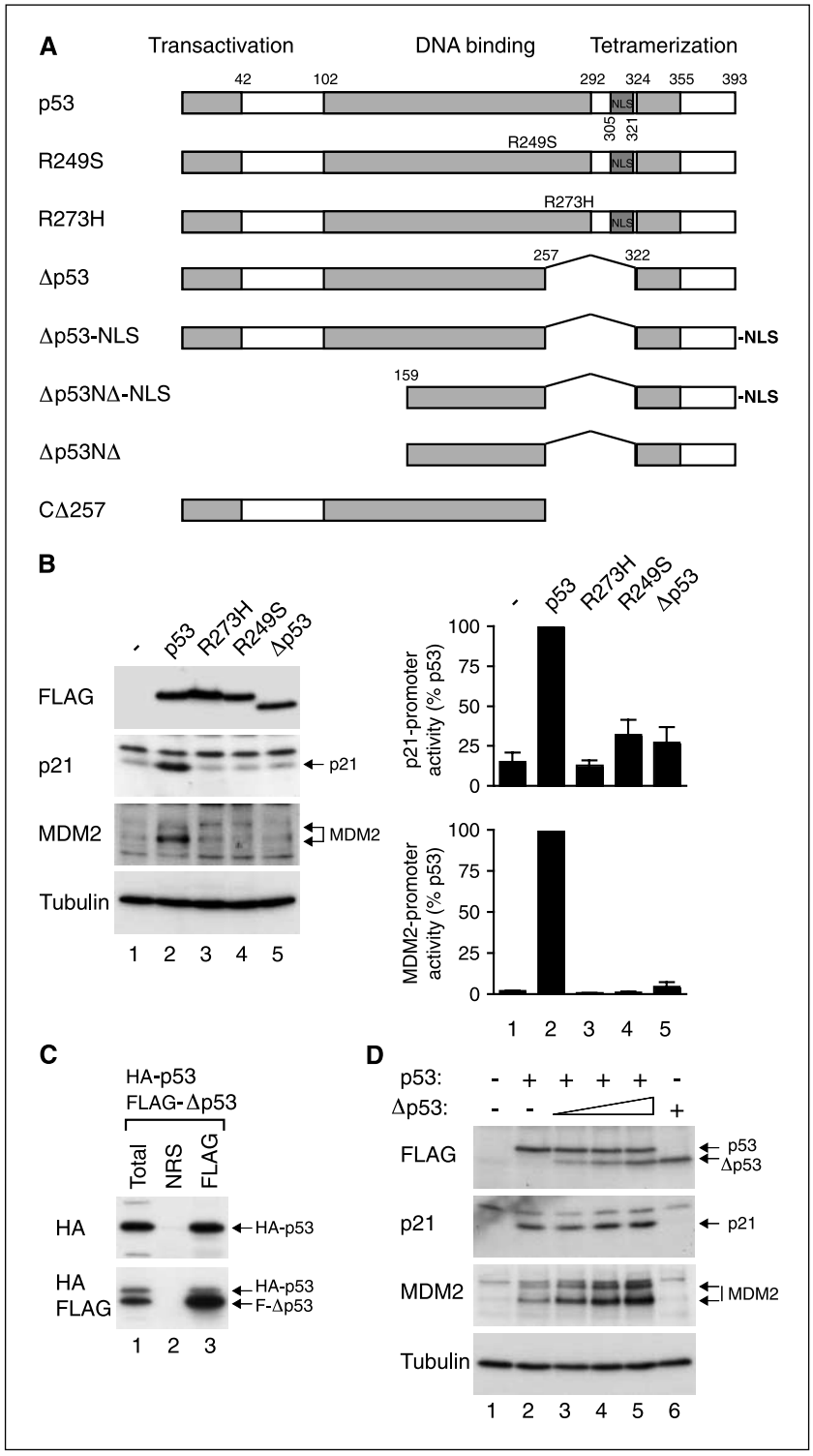

Figure 1. Δp53 interacts with p53 but transactivates p53-responsive promoters ineffectively. *A,* schematic diagram of the p53 constructs used in this study. The positions of the various functional elements of human p53 are shown to scale. All constructs, except Δp53-NLS, are FLAG tagged at the NH_2 terminus. Also used in this study were untagged p53 and Δp53-NLS, HA-tagged p53, and V5-tagged p53 and Δp53. *B,* Δp53 does not activate $p21^{CIP1/WAF1}$ and MDM2. H1299 cells were transfected with control vector or plasmids expressing p53, Δp53, R249S, or R273H mutants as indicated. Cell extracts were prepared at 24 h after transfection and the abundance of $p21^{CIP1/WAF1}$ and MDM2 was detected by immunoblotting (*left*). The recombinant p53 and Δp53 were detected by immunoblotting for the FLAG tag. Equal loading of lysates was confirmed by immunoblotting for tubulin. Cells were also cotransfected with plasmids expressing luciferase reporters under the control of $p21^{CIP1/WAF1}$ promoter or MDM2 promoter and a *Renilla* luciferase-expressing construct. The luciferase activity was measured, normalized with the *Renilla* luciferase activity to correct for variations in transfection efficiency between samples, and plotted as a percentage of p53 (*right*). *Columns,* mean of three independent experiments; *bars,* SD. *C,* Δp53 can bind to wild-type p53. HA-tagged p53 was coexpressed with FLAG-Δp53 in H1299 cells. Cell extracts were prepared and 100 μg were subjected to immunoprecipitation with either control normal rabbit serum (*NRS*) or FLAG antiserum. The immunoprecipitates were immunoblotted for HA. The blot was then probed for FLAG to verify the immunoprecipitation. Total cell lysates (10 μg) were applied to indicate the input. *D,* expression of p53 targets is not attenuated by Δp53. Constant amount of FLAG-p53 and increasing amount of FLAG-Δp53 were expressed in H1299 cells as indicated. At 24 h after transfection, cell extracts were prepared and the abundance of $p21^{CIP1/WAF1}$ and MDM2 was detected by immunoblotting. The expression of FLAG-tagged p53 and Δp53 was confirmed by immunoblotting for FLAG. Tubulin analysis was included to assess protein loading and transfer.

Recently, Rohaly et al. (25) discovered that a novel p53 isoform, denoted as Δp53, is generated by alternative splicing between exon 7 and exon 9. Sixty-six residues in the DNA binding domain of p53 are absent in Δp53 (Δ257–322). Paradoxically, Δp53 was reported to be transcriptionally active toward *CIP1/WAF1* and *14-3-3σ*, but not *MDM2, BAX*, and *PIG3*. It was also reported that Δp53 is expressed in several cell lines and is an essential element of the ATR-mediated intra–S phase checkpoint.

The presence of activity from Δp53 is somewhat intriguing as the isoform contains an incomplete DNA binding domain (see Fig. 1*A*). As Δp53 still retains the tetramerization domain, it is more likely that Δp53 can form tetramers with wild-type p53 and acts in a dominant-negative manner. Extensive data from studies in cell culture suggest that many missense mutant p53 can inhibit the transactivation of target genes. Mutated p53 present within a tetramer is thought to abolish the DNA binding capacity of the entire complex. This has the important implication that a heterozygous mutation in *p53* could result in the functional inactivation of cellular *p53*. We have previously shown that DNA binding–defective p53 mutants can impair the transcriptional activity of p53, albeit rather ineffectively: at least three mutants are required to inactivate a tetramer (26). In marked contrast, NH_2-terminally truncated p53 is a very potent inhibitor of p53: one NΔ subunit per tetramer is sufficient to abolish the transcriptional activity.

In this study, we explored whether Δp53, like the other DNA binding mutants of p53, has the potential to interact with p53 and act in a dominant-negative manner. We found that, in contrast to full-length p53, Δp53 did not transactivate the promoters of $p21^{CIP1/WAF1}$ or MDM2. Furthermore, Δp53 was ineffective in impairing the activity of p53. Significantly, our data revealed that Δp53 was not imported into the nucleus. The cytoplasmic localization of Δp53 correlated with the short half-life of the protein through ubiquitin-mediated proteolysis. Finally, whereas forcing Δp53 into the nucleus did not activate the transcriptional activity of Δp53 per se, this enhanced the dominant-negative activity of Δp53. These observations underscore the critical role of nuclear localization in the dominant-negative action of p53.

Materials and Methods

Materials. All reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

DNA constructs. Human p53 in pRcCMV (27), MDM2 in pCMV (28), pLINX (29), luciferase reporters under the control of $p21^{CIP1/WAF1}$ promoter (30) or MDM2 promoter (27), and hemagglutinin (HA)-ubiquitin in pUHD-P2 (31) were obtained from sources as previously described. Constructs for HA-p53, FLAG-p53, p53 (R249S), and p53 (R273H) were as previously described (26). Cyclin B1 promoter-luciferase reporter was a generous gift from Dr. Denise Galloway (Fred Hutchinson Cancer Center, Seattle, WA). FLAG-p53 in pUHD-P1 (26) was amplified by PCR with a vector forward primer and 5′-CTTCTAGAGTGATGATGGTGAGGATGGGCCT-3′; the PCR product was cut with *Nhe*I-*Xba*I and ligated into pUHD-P1 (32) to generate FLAG-p53(CΔ257) in pUHD-P1. FLAG-p53 in pUHD-P1 was amplified by PCR with 5′-CCTCTAGATGGAGAATATTTCACCC-3′ and a vector reverse primer; the PCR product was cut with *Xba*I-*Bam*HI and ligated into FLAG-p53(CΔ257) in pUHD-P1 to create FLAG-Δp53. This construct was then amplified by PCR with a vector forward primer and 5′-TTTCTCGAG-TAAGTCTGAGTCAGGCCCTT-3′ (p53-*Xho*I reverse primer); the PCR product was cut with *Nco*I-*Xho*I and ligated into pCMV/myc/nuc (Invitrogen, Carlsbad, CA) to create Δp53(NΔ159)-nuclear localization signal (NLS)-myc. The *Nco*I-*Bam*HI (the *Bam*HI site was introduced with a primer at the myc tag) fragment was then put into pUHD-P1 to generate FLAG-Δp53(NΔ159)-NLS-myc in pUHD-P1. The *Nco*I-*Nco*I fragment from p53 cDNA was ligated into *Nco*I-cut Δp53(NΔ159)-NLS-myc in pCMV/myc/nuc to create Δp53-NLS-myc in pCMV/myc/nuc. This construct and full-length p53 were amplified by PCR with 5′-CGAATTCCATGGAGGAGCCG-CAGT-3′ (p53-*Eco*RI forward primer) and p53-*Xho*I reverse primer; the PCR products were cut with *Eco*RI-*Xho*I and ligated into pcDNA6/V5-HisA (Invitrogen) to create Δp53-V5-His and p53-V5-His in pcDNA6/V5-HisA, respectively. FLAG-Δp53(NΔ159) was obtained by removing the *Nco*I-*Nco*I fragment from FLAG-Δp53 in pUHD-P1.

Cell culture. H1299 cells (non–small-cell lung carcinoma; ref. 33) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (Invitrogen) in a humidified incubator at 37°C in 5% CO_2. Cycloheximide (10 μg/mL), doxycycline (1 μg/mL), and G418 (1 mg/mL) were used at the indicated concentrations. UV radiation was delivered with UVB (290–320 nm) erythemal tubes (Philips, Eindhoven, the Netherlands). The UV dose was calibrated with a UV meter from InternationalLight (Peabody, MA). The medium and the lid of the plate were removed before the cells were irradiated. Transfection was carried out with a calcium phosphate precipitation method (34). The amount of total DNA transfected was adjusted to the same level with blank vectors. H1299 cells were transfected with pLINX (a plasmid expressing the tTA transactivator; ref. 35) and grown in medium containing G418. After ~2 weeks of selection, single colonies were isolated and tested for inducible gene expression using doxycycline. Cell-free extracts were prepared as previously described (36). The protein concentration of cell lysates was measured with the bicinchoninic acid protein assay system (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Transactivation assays. The transcriptional activity of p53 was assayed by transfecting cells with a promoter-luciferase (firefly) reporter construct and a *Renilla reniformis* luciferase construct. The activities of the two luciferases were analyzed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The activity of the firefly luciferase was normalized with that of the *Renilla* luciferase.

Ubiquitination assays. *In vivo* ubiquitination assays were done as previously described (31). Briefly, constructs expressing FLAG-tagged proteins were cotransfected with HA-ubiquitin in pUHD-P2. The cells were treated with 50 μmol/L of LLnL for 6 h before harvested. Cell extracts prepared from the transfected cells were immunoprecipitated with either normal rabbit serum or FLAG antiserum. The presence of HA-ubiquitin–conjugated proteins in the immunoprecipitates was detected by immunoblotting with the anti-HA monoclonal antibody 12CA5.

Fractionation. After harvest and washing with PBS, the cells were resuspended in 600 μL of buffer [10 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, and 1 mmol/L DTT] supplemented with protease inhibitors (2 μg/mL aprotinin, 15 μg/mL benzamidine, 1 μg/mL, leupeptin, 10 μg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/mL soybean trypsin inhibitor) and incubated at 4°C for 10 min. The cells were then homogenized with 10 strokes in a tight pestle Wheaton Dounce homogenizer (Millville, NJ). The lysates were centrifuged at 240 × *g* for 5 min and the supernatant was collected (cytoplasmic fraction). The pellets were then washed thrice with buffer [10 mmol/L HEPES (pH 8), 50 mmol/L NaCl, 25% glycerol, and 0.1 mmol/L EDTA], centrifuged for 5 min, and resuspended in 30 μL of buffer [10 mmol/L HEPES (pH 8), 350 mmol/L NaCl, 25% glycerol, and 0.1 mmol/L EDTA]. After incubation at 4°C for 10 min, the lysates were centrifuged at 13,000 rpm in a microfuge for 30 min and the supernatant was collected (nuclear fraction). The protein concentrations in the cytoplasmic and nuclear fractions were then determined. The quality of the fractionation was assessed by immunoblotting with histone H3 and tubulin.

Antibodies and immunologic methods. Immunoblotting and immunoprecipitation were done as described (36). The intensities of signals on immunoblots were quantified with ImageJ software (NIH) using appropriate serial dilution of the samples as calibration. Indirect immunofluorescence microscopy was done as previously described (37). TRITC- and FITC-conjugated secondary antibodies were from DAKO (Glostrup, Denmark).

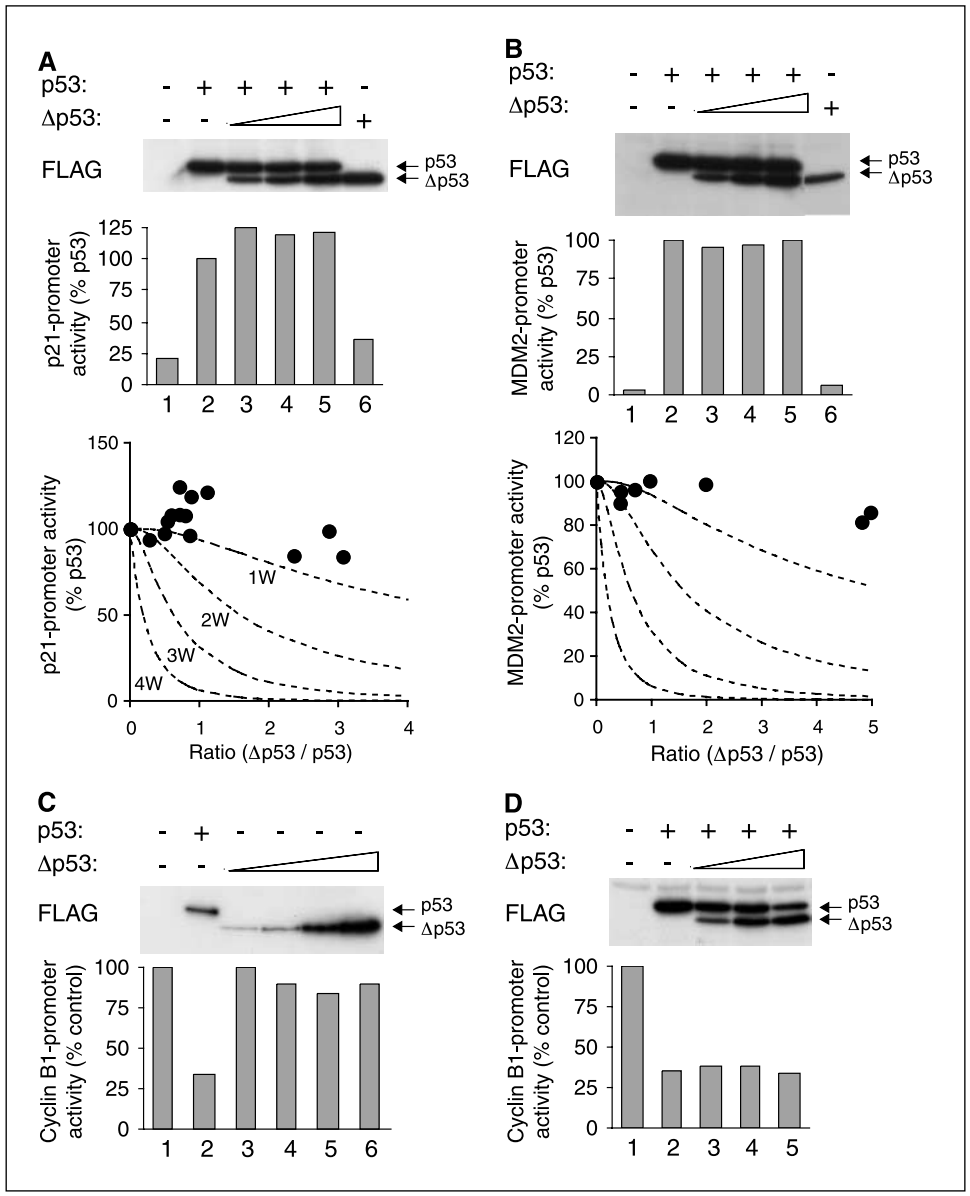

Figure 2. Δp53 can form complexes with p53 but does not affect the transcriptional activity of p53. *A,* transactivation of $p21^{CIP1/WAF1}$ promoter by p53 is not affected by Δp53. Cells were transfected with plasmids expressing a $p21^{CIP1/WAF1}$ promoter-luciferase reporter and *Renilla* luciferase. Constant amount of FLAG-p53 and varying amounts of FLAG-Δp53 were transfected as indicated (*top*). Cell extracts were prepared and the luciferase activities were determined. The transcriptional activity was expressed as a percentage of p53 alone (*lane 2*). The expressions of p53 and Δp53 were detected together by immunoblotting for FLAG. Data from several experiments were pooled to construct the inhibition curve of Δp53 on p53 activity (*bottom*). *Dotted lines,* theoretical inhibition curves as previously described (26). The various curves are based on the assumption that tetramers are only active with the number of wild-type p53 ranging from four to one. *4W*, 4; *3W,* ≥3; *2W,* ≥2; *1W,* ≥1. *B,* transactivation of MDM2 promoter by p53 is not impaired by Δp53. Experiments were done as in (*A*) except that an MDM2 promoter-luciferase reporter was used. *C,* Δp53 does not suppress cyclin B1 promoter. Cells were transfected with plasmids expressing a cyclin B1 promoter-luciferase reporter and *Renilla* luciferase. FLAG-tagged p53 or Δp53 was coexpressed as indicated. The cells were harvested at 24 h after transfection and cell lysates were prepared. The luciferase activities were determined, normalized with the *Renilla* luciferase activity, and plotted as a percentage of control. The expression of FLAG-tagged p53 and Δp53 was confirmed by immunoblotting. *D,* Δp53 does not impair the suppression of the cyclin B1 promoter by p53. Experiments were done as in (*A*) except that a cyclin B1 promoter-luciferase reporter was used.

Rabbit polyclonal antibodies against FLAG tag (29) and monoclonal antibodies against FLAG tag (M2; ref. 31), HA tag (12CA5; ef. 29), and tubulin (YL1/2; ref. 38) were obtained from sources as previously described. Monoclonal antibodies against MDM2 (2A10; Calbiochem, San Diego, CA), myc tag (9E10; DAKO), V5 tag (R960-25; Invitrogen), and p53 (DO1; Santa Cruz Biotechnology, Santa Cruz, CA) and polyclonal antibodies against $p21^{CIP1/WAF1}$ (sc-397; Santa Cruz Biotechnology) were obtained from the indicated sources.

Results

Δp53 is not active as a transcriptional factor for *CIP1/WAF1* and *MDM2*. Given that Δp53 lacks a portion of the DNA binding domain (Fig. 1*A*), we first compared the transcriptional activity of Δp53 with those of other DNA binding–defective mutants of p53. The various constructs of wild-type and mutant p53 used in this study are shown in Fig. 1*A*. Compared with the control reaction, ectopic expression of wild-type p53 in H1299 cells (a p53-null cell line) induced the p53-responsive gene products $p21^{CIP1/WAF1}$ and MDM2 (or HDM2; Fig. 1*B*). As expected, neither R273H nor R249S (both are missense "hotspot" mutants found in a variety of tumors) stimulated the expression of the same p53-responsive products. We found that even when expressed to the similar levels as p53, Δp53 did not activate the endogenous $p21^{CIP1/WAF1}$ or MDM2.

To further validate that Δp53 did not possess transcriptional activity, luciferase reporters under the control of $p21^{CIP1/WAF1}$ or MDM2 promoters were coexpressed with Δp53 (Fig. 1*B*). The luciferase activities were normalized with the *Renilla* luciferase activity from a cotransfected plasmid to correct for transfection efficiency. As expected, both $p21^{CIP1/WAF1}$ and MDM2 promoters were robustly transactivated by wild-type p53. In contrast, neither the DNA binding–defective mutants (R273H and R249S) nor Δp53 significantly transactivated the promoters. These data indicate that ectopically expressed Δp53 does not display intrinsic transcriptional activity toward endogenous or cotransfected $p21^{CIP1/WAF1}$ and MDM2 promoters.

Δp53 can bind to p53 but does not inhibit the transcriptional activity of p53. Because Δp53 did not possess transcriptional activity, it is conceivable that it can act in a dominant-negative manner by virtue of its tetramerization with full-length p53. To test this hypothesis, we first examined if Δp53 could indeed form a complex with p53. FLAG-tagged Δp53 was coexpressed with HA-tagged p53 and was immunoprecipitated using a FLAG antiserum. Figure 1*C* shows that HA-p53 was coimmunoprecipitated with FLAG-Δp53 but not with the control serum, confirming that Δp53 could form a complex with p53.

To determine if the activity of p53 could be altered by Δp53, a constant amount of p53-expressing plasmids was cotransfected with increasing amount of Δp53-expressing plasmids (Fig. 1*D*). As both p53 and Δp53 were FLAG tagged and of different sizes, their relative levels could be assessed by immunoblotting for FLAG. In agreement with the above data, p53 but not Δp53 alone induced the expression of $p21^{CIP1/WAF1}$ and MDM2. Unexpectedly, Δp53 did not suppress the expression of $p21^{CIP1/WAF1}$ and MDM2 induced by p53. We instead observed a slight increase in MDM2 expression when Δp53 was coexpressed with p53.

To validate that Δp53 was inadequate in reducing the activity of p53, a $p21^{CIP1/WAF1}$ promoter-luciferase reporter was cotransfected with p53 and Δp53. Figure 2*A* shows that the $p21^{CIP1/WAF1}$ promoter was activated by p53, but it was not hindered in the presence of Δp53. Because both wild-type p53 and Δp53 were detected together with the same monoclonal antibody on the same blot, their relative level could be quantified by densitometry with the appropriate serial dilution standards. Given that this approach depended only on the relative expression between p53 and Δp53, data from several independent experiments could be

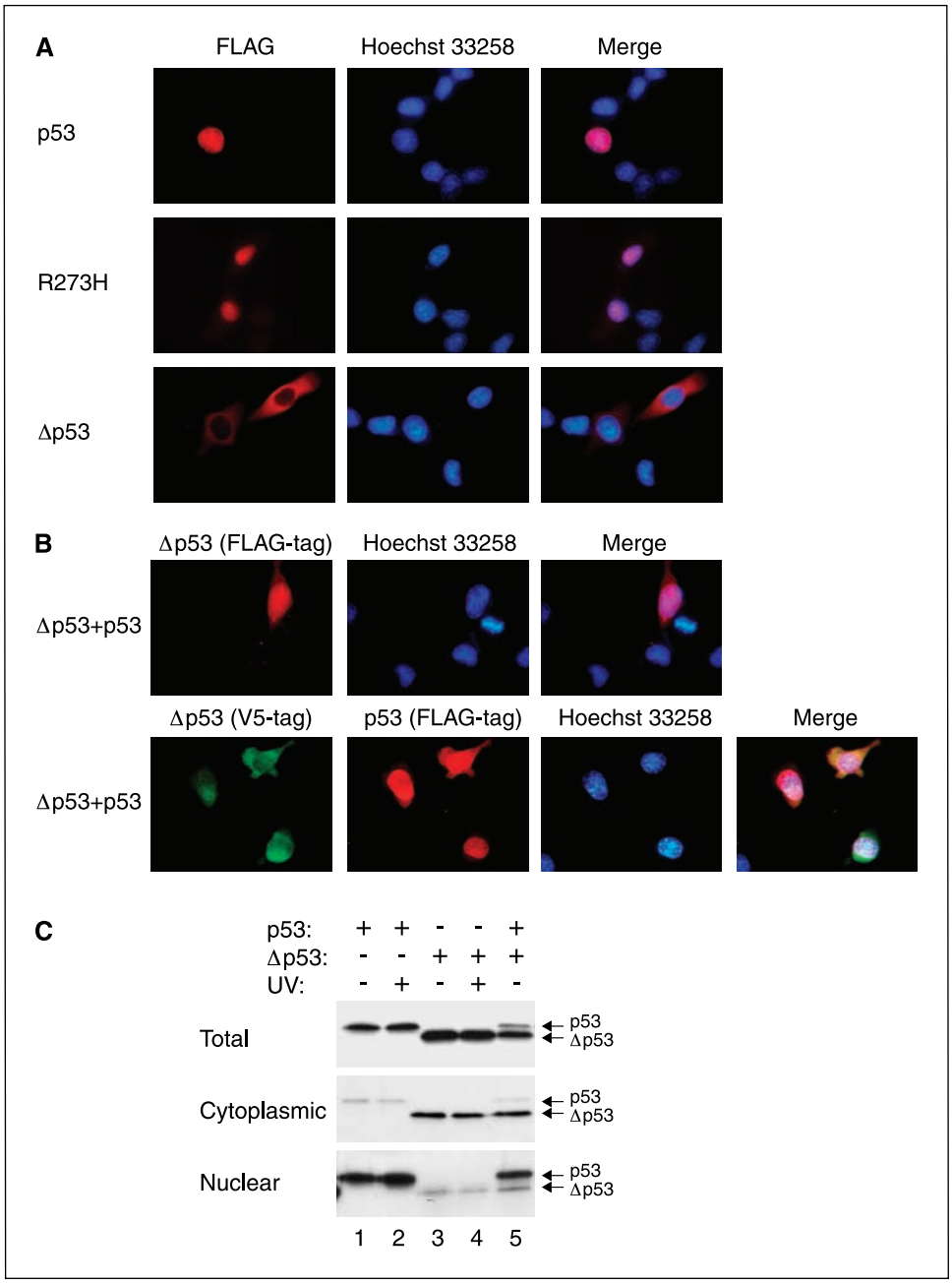

Figure 3. Δp53 is localized to the cytoplasm and can influence the localization of full-length p53. *A,* Δp53 is localized to the cytoplasm. H1299 cells were transfected with plasmids expressing FLAG-tagged p53, R273H, or Δp53 as indicated. At 24 h after transfection, the cells were fixed and the FLAG-tagged proteins were detected by immunostaining with a monoclonal antibody against FLAG, followed by a TRITC-conjugated antimouse immunoglobulin G (IgG) secondary antibody (*red*). Nuclei were counterstained with Hoechst 33258 (*blue*). *Right,* merged images. *B,* Δp53 and p53 mutually affect each other's subcellular localization. Cells were cotransfected with plasmids expressing untagged p53 and FLAG-tagged Δp53 (*top*) or FLAG-tagged p53 and V5-tagged Δp53 (*bottom*). At 24 h after transfection, the cells were fixed and subjected to immunostaining. FLAG-Δp53 was detected with a monoclonal antibody against FLAG, followed by a TRITC-conjugated antimouse IgG secondary antibody (*red*). FLAG-p53 was detected with a polyclonal antibody against FLAG, followed by a TRITC-conjugated antirabbit IgG secondary antibody (*red*). V5-Δp53 was detected with a monoclonal antibody against V5, followed by a FITC-conjugated antimouse IgG secondary antibody (*green*). Nuclei were counterstained with Hoechst 33258 (*blue*). *Right,* merged images. *C,* confirmation of the cytoplasmic localization Δp53 by subcellular fractionation. H1299 cells were transfected with plasmids expressing FLAG-tagged p53 and Δp53 as indicated. At 24 h after transfection, the cells were either mock treated or irradiated with 50 J/m^2 UVB. After incubation for 6 h, the cells were harvested and subjected to subcellular fractionation. The abundance of p53 and Δp53 in total lysates, cytoplasmic fractions, and nuclear fractions was detected by immunoblotting for the FLAG tag.

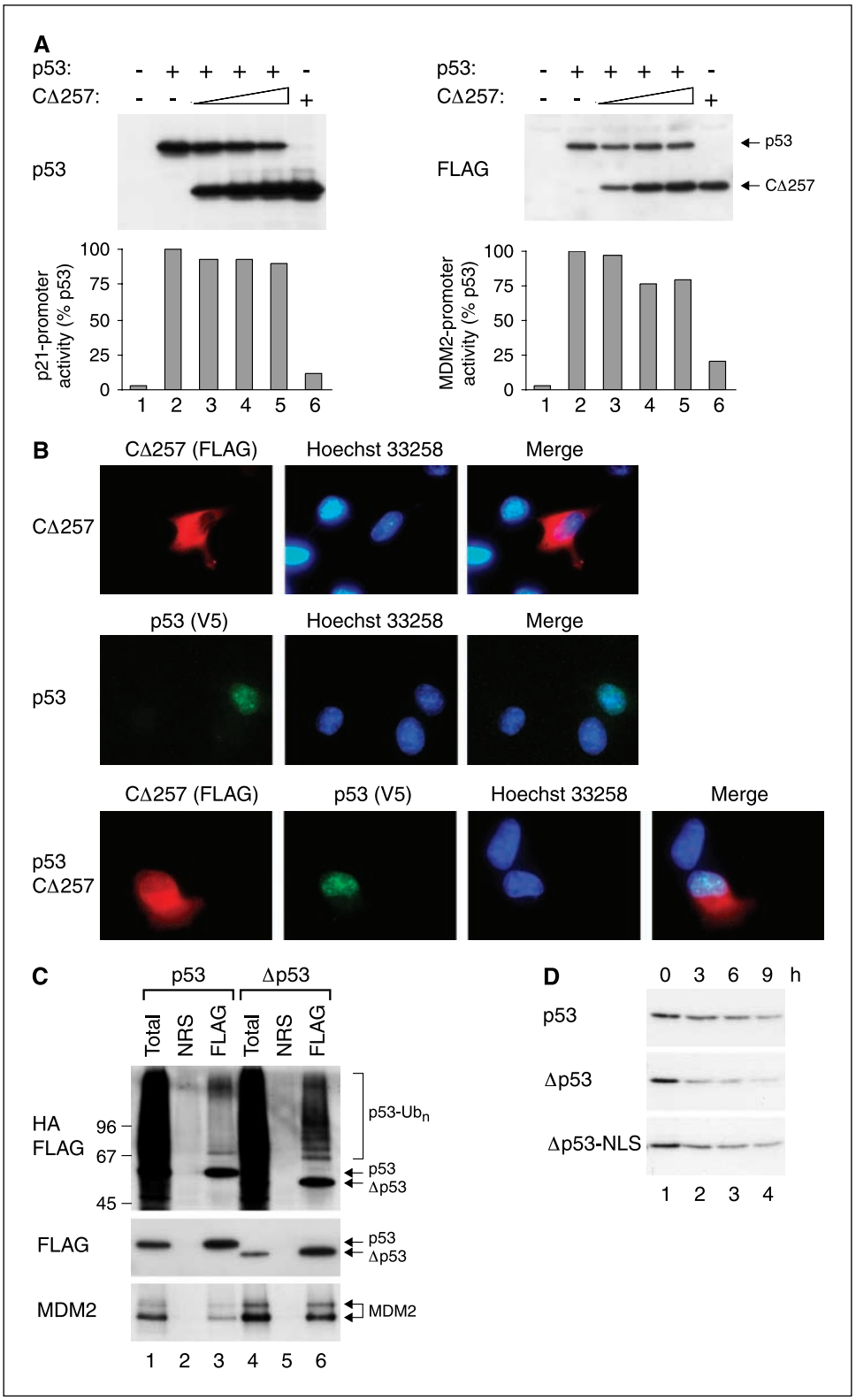

Figure 4. Δp53 is targeted to ubiquitination-dependent degradation, and Δp53 lacking the COOH-terminal tetramerization domain does not affect the activity or localization of p53. *A,* CΔ257 does not inhibit the transactivation of $p21^{CIP1/WAF1}$ promoter by p53. Cells were transfected with plasmids expressing *Renilla* luciferase and $p21^{CIP1/WAF1}$ promoter (*left*) or MDM2 promoter (*right*) luciferase reporters. FLAG-tagged p53 and CΔ257 were expressed as indicated. Cell extracts were prepared and luciferase activities were determined (*bottom*). The transcriptional activity was expressed as a percentage of p53 alone (*lane 2*). The expression of FLAG-tagged p53 and CΔ257 was confirmed by immunoblotting. *B,* CΔ257 is localized to the cytoplasm and does not affect the nuclear localization of p53. H1299 cells were transfected with plasmids expressing FLAG-tagged CΔ257 and V5-p53. At 24 h after transfection, the cells were fixed and subjected to immunostaining. V5-p53 was detected with a monoclonal antibody against V5, followed by a FITC-conjugated antimouse IgG secondary antibody (*green*). FLAG-CΔ257 was detected with a polyclonal antibody against FLAG, followed by a TRITC-conjugated antirabbit IgG secondary antibody (*red*). Nuclei were counterstained with Hoechst 33258 (*blue*). *Right,* merged images. *C,* Δp53 is targeted to MDM2-dependent ubiquitination. FLAG-tagged p53 or Δp53 was coexpressed with MDM2 and HA-ubiquitin (*Ub*) in H1299 cells. The cells were treated with the proteasome inhibitor LLnL for 6 h before harvest to stabilize the ubiquitinated products. Cell extracts were prepared and subjected to immunoprecipitation with either control normal rabbit serum or FLAG antiserum. The immunoprecipitates were immunoblotted with antibodies against HA, FLAG, and MDM2 as indicated. The positions of unmodified and polyubiquitinated p53 are indicated. *Left,* positions of molecular size standards (in kilodaltons). *D,* Δp53 is less stable than wild-type p53. FLAG-tagged p53, Δp53, or Δp53-NLS was coexpressed with MDM2 in H1299 cells. At 48 h after transfection, doxycycline and cycloheximide were added and cell extracts were prepared at the indicated time points. The stability of the FLAG-tagged proteins was examined by immunoblotting.

pooled. Figure 2*A* summarizes the experiments that examined the effects of Δp53 on p53.

The dominant-negative activity of p53 mutants depends on the number of mutant molecules needed to be present in a tetramer to inhibit the activity of whole tetramer. We have previously made theoretical predictions of the inhibition characteristics of p53 DNA binding–defective mutants when the concentration of mutant is increased relative to the wild-type (26). The various inhibition curves, based on the assumption that tetramers are only active with the number of wild-type subunit required for full activity ranging

from four (4W) to only one (1W), are also plotted in Fig. 2*A*. Our data revealed that Δp53 did not inhibit p53 even when Δp53 was expressed at a concentration much higher than p53. Similarly, we found that Δp53 was equally ineffective in suppressing the activity of p53 on the MDM2 promoter (Fig. 2*B*).

Apart from transcription activation, p53 also represses the transcription of several genes like *cyclin B1* (39). When expressed on its own, Δp53 did not significantly reduce the expression from a cyclin B1-promoter reporter construct (Fig. 2*C*). As a control, wild-type p53 was able to suppress the cyclin B1 promoter (*lane 2*). Furthermore, coexpression of Δp53 did not affect the p53-mediated repression of cyclin B1 promoter (Fig. 2*D*). Taken together, these data indicate that although Δp53 can form a complex with p53, it is ineffective in inhibiting the activity of p53.

Δp53 lacks the major NLS and is mainly localized to the cytoplasm. Nuclear localization of p53 is mediated by a NLS situating between the DNA binding domain and the tetramerization domain (40). This NLS (residues 305–321; see Fig. 1*A*) is notably absent in Δp53. To examine the subcellular localization of Δp53, epitope-tagged p53 or Δp53 was expressed in H1299 cells and their localization was detected by indirect immunofluorescence microscopy (Fig. 3*A*). As expected, both p53 and R273H mutant were predominantly localized to the nucleus. In marked contrast, Δp53 was excluded from the nucleus and accumulated in the cytoplasm.

Because Δp53 and p53 were individually localized to distinct compartments but could form a complex when coexpressed, this prompted us to explore the localization of Δp53 and p53 when they were coexpressed. When Δp53 (FLAG tagged) was coexpressed with full-length p53 (untagged), many cells displayed a prominent nuclear staining of Δp53. Representational images are shown in Fig. 3*B*. To detect both p53 and Δp53 simultaneously in the same cells, Δp53 and p53 were engineered to fuse with V5 and FLAG tags, respectively. Similar to FLAG-Δp53, V5-Δp53 was exclusively localized to the cytoplasm when expressed on its own (data not

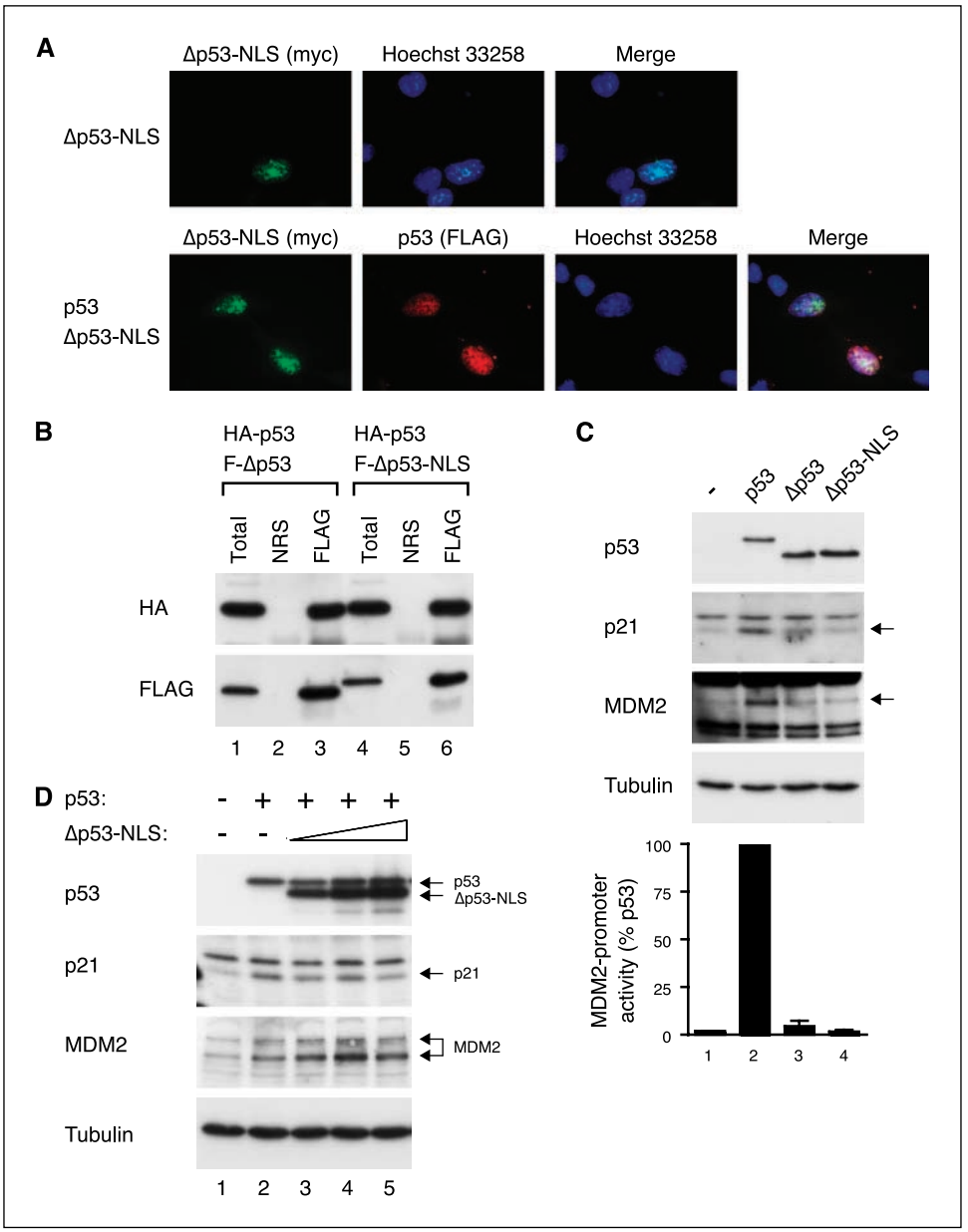

Figure 5. NLS-containing Δp53 does not possess transcriptional activity and is a weak dominant-negative protein. *A,* Δp53-NLS does not affect the localization of p53. Cells were transfected with plasmids expressing FLAG-tagged p53 and myc-tagged Δp53-NLS. At 24 h after transfection, the cells were fixed and subjected to immunostaining. FLAG-p53 was detected with a polyclonal antibody against FLAG, followed by a TRITC-conjugated antirabbit IgG secondary antibody (*red*). Δp53-NLS was detected with a monoclonal antibody against myc, followed by a FITC-conjugated antimouse IgG secondary antibody (*green*). Nuclei were counterstained with Hoechst 33258 (*blue*). *Right,* merged images. *B,* Δp53-NLS can form a complex with p53. HA-tagged p53 was coexpressed with FLAG-Δp53 or FLAG-Δp53-NLS. Cell extracts were prepared and 100 μg were subjected to immunoprecipitation with either control normal rabbit serum or FLAG antiserum as indicated. The immunoprecipitates were immunoblotted for HA and FLAG. Total cell lysates (10 μg) were applied to indicate the input. *C,* MDM2 and $p21^{CIP1/WAF1}$ are not activated by Δp53-NLS. An MDM2 promoter-luciferase reporter and a *Renilla* luciferase construct were coexpressed with p53, Δp53, or Δp53-NLS as indicated. Cell extracts were prepared at 24 h after transfection and the abundance of endogenous $p21^{CIP1/WAF1}$, MDM2, and recombinant p53 proteins was detected by immunoblotting (*top*). Equal loading of lysates was confirmed by immunoblotting for tubulin. The luciferase activity was determined, normalized with the *Renilla* luciferase activity to correct for variations in transfection efficiency between samples, and plotted as a percentage of p53 (*bottom*). *Columns,* mean of three independent experiments; *bars,* SD. *D,* effects of Δp53-NLS on the activity of p53. Constant amount of FLAG-p53 and increasing amount of Δp53-NLS were expressed in H1299 cells as indicated. At 24 h after transfection, cell extracts were prepared and the abundance of $p21^{CIP1/WAF1}$ and MDM2 was detected by immunoblotting. The expression of p53 and Δp53-NLS was confirmed by immunoblotting. Tubulin analysis was included to assess protein loading and transfer.

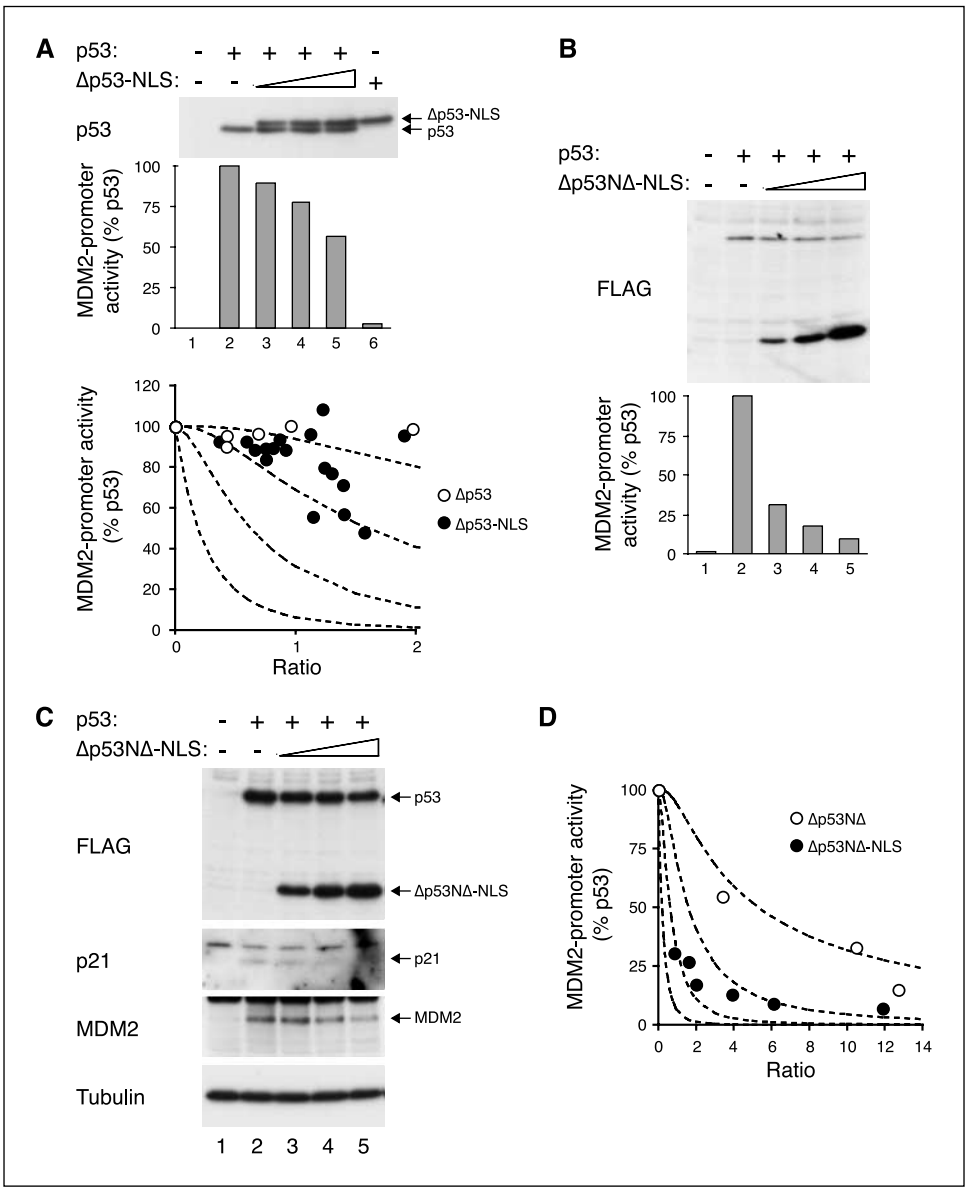

Figure 6. The dominant-negative activity of Δp53 lacking the NH_2-terminal region is increased by nuclear localization. *A,* Δp53-NLS only slightly reduces the transcriptional activity of p53. H1299 cells were transfected with plasmids expressing a MDM2 promoter-luciferase reporter, p53, and FLAG-tagged Δp53-NLS as indicated. Cell extracts were prepared and the luciferase activities were determined. The transcriptional activity was expressed as a percentage of p53 alone (*lane 2*). The expression of p53 and Δp53-NLS was confirmed by immunoblotting. Data from several experiments were pooled to construct the inhibition curve of FLAG-Δp53 (○) or FLAG-Δp53-NLS (●) on p53 activity (*bottom*). The transcriptional activity was plotted against the ratio of Δp53/p53 or Δp53-NLS/p53 as described in Fig. 2*A*. *B,* Δp53NΔ-NLS strongly inhibits the transcriptional activity of p53. H1299 cells were transfected with plasmids expressing a MDM2 promoter-luciferase reporter, FLAG-p53, and FLAG-Δp53NΔ-NLS as indicated. Cell extracts were prepared and the luciferase activities were determined (*bottom*). The transcriptional activity was expressed as a percentage of p53 alone (*lane 2*). The expression of p53 and Δp53NΔ-NLS was confirmed by immunoblotting. *C,* Δp53NΔ-NLS decreases the transactivation of $p21^{CIP1/WAF1}$ and MDM2 by p53. Constant amount of FLAG-p53 and increasing amount of FLAG-Δp53NΔ-NLS were expressed in H1299 cells as indicated. At 24 h after transfection, cell extracts were prepared, and the abundance of $p21^{CIP1/WAF1}$ and MDM2 was detected by immunoblotting. The expression of p53 and Δp53NΔ-NLS was confirmed by immunoblotting for FLAG. Tubulin analysis was included to assess protein loading and transfer. *D,* Δp53NΔ-NLS is a more potent inhibitor of p53 than Δp53NΔ. H1299 cells were transfected with plasmids expressing a MDM2 promoter-luciferase reporter and different ratios of plasmids expressing FLAG-tagged p53 and Δp53NΔ (○) or Δp53NΔ-NLS (●). The transcriptional activity was plotted against the ratio of Δp53NΔ/p53 or Δp53NΔ-NLS/p53 as described in Fig. 2*A*.

shown), but a portion was redistributed to the nucleus when coexpressed with FLAG-p53 (Fig. 3*B*). Conversely, Δp53 caused a slight increase of FLAG-p53 in the cytoplasm. The effect of Δp53 on p53 was not as profound as the converse, as many cells still retained a predominantly nuclear staining of p53.

The cytoplasmic localization of Δp53 was further confirmed by subcellular fractionation. Figure 3*C* shows that, in marked contrast to p53, Δp53 was predominantly present in the cytoplasmic fractions. There was a slight increase of Δp53 in the nucleus when it was coexpressed with p53. In addition, we found that the localization of Δp53 was not altered after DNA damage induced by UVB.

As a further control, we constructed a COOH-terminally deleted mutant (CΔ257) that removed the tetramerization domain from Δp53 (Fig. 1*A*). As expected, CΔ257 did not possess transcriptional activity on $p21^{CIP1/WAF1}$ promoter or MDM2 promoter (Fig. 4*A*). Moreover, CΔ257 did not diminish the transactivation of $p21^{CIP1/WAF1}$/MDM2 promoters by wild-type p53. Significantly, CΔ257 (which was exclusively cytoplasmic) did not affect the localization of p53, or vice versa (Fig. 4*B*).

Collectively, these results indicate that, unlike full-length p53, Δp53 is not imported into the nucleus. This may explain, in part, why Δp53 is not active as a transcription factor and is not an inhibitor of p53.

Δp53 is subjected to ubiquitination and is less stable than wild-type p53. A major pathway of p53 proteolysis involves MDM2-mediated ubiquitination. MDM2 binds to the NH_2-terminal region of p53, shuttles p53 to the cytoplasm, and targets p53 for ubiquitination. Ubiquitinated p53 is then degraded by the proteasome complex. Ubiquitination occurs at lysine residues at the COOH-terminal region and at the NH_2-terminal half of the DNA binding domain (ref. 41 and references therein). Given that Δp53 still retains the NH_2-terminal MDM2 binding site, as well as the potential ubiquitin-acceptor sites, we next investigated if Δp53 is subjected to ubiquitination. Cell-free extracts were prepared from cells expressing FLAG-tagged p53 or Δp53 together with MDM2 and

HA-ubiquitin. The FLAG-tagged proteins were immunoprecipitated and the ubiquitinated proteins were detected by immunoblotting for HA (Fig. 4*C*). As expected, high molecular size products representing ubiquitinated proteins could be detected in the FLAG-p53 immunoprecipitates but not in the control serum immunoprecipitates. Likewise, high molecular size products containing HA-ubiquitin were readily detected in the immunoprecipitates of FLAG-Δp53, indicating that Δp53 was ubiquitinated. Finally, MDM2 bound specifically with p53 and Δp53 but not with the control serum immunoprecipitates.

Given that, unlike p53, Δp53 is already in the cytoplasm and does not require a nuclear exporting step for degradation, it is conceivable that Δp53 is degraded more efficiently than p53. To test this hypothesis, cells expressing p53 or Δp53 were treated with doxycycline (the promoters of these constructs were under the negative control of doxycycline) and cycloheximide. As we have previously shown with the same assay (41), ectopically expressed p53 was degraded relatively slowly (Fig. 4*D*). In contrast, a similar expression level of Δp53 was degraded quicker than p53.

Taken together, these data indicate that, similar to full-length p53, Δp53 can bind MDM2 and be targeted for ubiquitination. Moreover, Δp53 exhibits a shorter half-life than p53, probably due, in part, to its cytoplasmic localization.

Forcing Δp53 into the nucleus potentiates its dominant-negative activity. To test if the relatively short half-life of Δp53 was due to its cytoplasmic localization, three NLS (as well as a myc tag) were added to the COOH terminus of Δp53 (Fig. 1*A*). As expected, reinstating NLS to Δp53 restored the nuclear localization (Fig. 5*A*). In contrast to Δp53, Δp53-NLS was degraded at a similar rate as wild-type p53 (Fig. 4*D*), suggesting that the instability of Δp53 may be due, in part, to its cytoplasmic localization.

It is possible that the relatively weak dominant-negative action of Δp53 on p53 was also due to its subcellular localization. To test this hypothesis, we examined if Δp53-NLS could modulate the transcriptional activity of p53. One of our concerns was that the addition of the three NLS and myc tag at the COOH terminus might affect the function of the nearby tetramerization domain. Immunoprecipitation revealed that, similar to Δp53, Δp53-NLS was able to form a complex with p53 (Fig. 5*B*), validating that the extra NLS targeted Δp53 to the nucleus without affecting oligomerization. We next examined the subcellular localization of p53 and Δp53-NLS by immunostaining. In marked contrast to Δp53, Δp53-NLS did not affect the nuclear localization of p53 (Fig. 5*A*).

Interestingly, although Δp53-NLS was localized to the nucleus, it was unable to induce the expression of $p21^{CIP1/WAF1}$ or MDM2 (Fig. 5*C*). This was further validated by the lack of transactivation activity of Δp53-NLS on a cotransfected MDM2 promoter (Fig. 5*C*). These data unequivocally show that the lack of intrinsic transcriptional activity of Δp53 was not simply due to deficiency of NLS but was likely to be due to the incomplete DNA binding domain.

Because nuclear-targeting Δp53 did not possess transcriptional activity, it is possible that it could act in a dominant-negative fashion. To test this hypothesis, Δp53-NLS was coexpressed with wild-type p53 and the transcriptional activity was measured. Figures 5*D* and 6*A* show that the activities of p53 were only marginally reduced by Δp53-NLS. The effect was slightly more prominent at high doses of Δp53-NLS. Consistent with the effects of Δp53 (Fig. 1*D*), endogenous MDM2 expression was actually stimulated by lower doses of Δp53-NLS (Fig. 5*D*). The inhibitory activity of Δp53-NLS seemed to be slightly stronger than that of Δp53 (Fig. 6*A*) and is comparable to other DNA binding–defective mutants (3). To obtain more definite evidence of the importance of nuclear localization in the dominant-negative action of p53, we also removed the entire NH_2-terminal region in the Δp53 backbone (NΔ159). The basis of this is that NH_2-terminally truncated versions of p53 are more powerful inhibitors of p53 functions than DNA binding–defective mutants (3). Figure 6*B* shows that the transcriptional activity of p53 was effectively attenuated by Δp53NΔ-NLS. Moreover, the expression of endogenous $p21^{CIP1/WAF1}$ and MDM2 was also down-regulated by Δp53NΔ-NLS (Fig. 6*C*). Significantly, Δp53NΔ-NLS inhibited p53 function better than Δp53NΔ (without NLS), indicating a critical role of nuclear localization in the dominant-negative function of p53 (Fig. 6*D*).

Taken together, these data show that Δp53 remains inactive even when nuclear localization is restored. Targeting Δp53 to the nucleus does enhance its dominant-negative activity, thus explaining why Δp53 is not a robust dominant-negative protein.

Discussion

It is remarkable that whereas *p53* is one of the most investigated human genes, it has only recently been recognized that it has the potential to encode a large number of isoforms. Whereas the existence of Δp53 has been subjected to some debates (17), the mechanistic insights drawn from the study of novel forms of this critical tumor suppressor can be revealing. Similar to DNA binding–defective mutants like R249S and R273H, Δp53 did not display intrinsic transcriptional activity. We found that Δp53 activated neither endogenous $p21^{CIP1/WAF1}$ nor MDM2 (Fig. 1*B*). Likewise, cotransfected $p21^{CIP1/WAF1}$ or MDM2 promoters were not transactivated by Δp53 (Fig. 1*B*). In addition, unlike wild-type p53, Δp53 failed to repress the cyclin B1 promoter. Hence, it is paradoxical that Δp53 was reported to display activity, in particular after DNA damage during S phase (25). The molecular mechanism underlying this activity remains to be elucidated. It is conceivable that posttranslational modifications triggered during S phase or after DNA damage may contribute to the activation of Δp53. Here, we mainly compared the intrinsic transcriptional activities between transiently transfected p53 and Δp53 without additional stress. However, we were also not able to detect significant $p21^{CIP1/WAF1}$ transcriptional activity[1] or a change in subcellular localization (Fig. 3*C*) with transfected Δp53 after UV irradiation (cells were irradiated with 50 J/m^2 UVB and harvested after 6 h). As Rohaly et al. (25) used stable H1299 cell lines that conditionally expressed Δp53, a possibility is that additional mutations in the cell lines may contribute to the activity of Δp53.

Two factors may account for the inactivity of Δp53. First, the COOH-terminal 35 residues of the DNA binding domain are absent in Δp53. As mutation of single residues in this region (e.g., R273H) is sufficient to disrupt the transcriptional activity of p53, it is a fair postulation that Δ257–322 may be detrimental to the structure of the DNA binding domain. Another factor that may contribute to the inactivity of Δp53 is that it is not imported into the nucleus. Nuclear localization of p53 is mediated by a major NLS (absent in Δp53) and two minor NLS at the COOH-terminal region (40). Indeed, we found that ectopically expressed Δp53 was excluded from the nucleus (Fig. 3*A* and *C*). Addition of leptomycin B, a CRM1 inhibitor, did not affect the localization of Δp53.[1] These

[1] Our unpublished data.

observations indicate that the cytoplasmic localization of Δp53 is due to a defect in nuclear import and not due to a more active nuclear export in comparison with p53.

To see if the inactivity of Δp53 can be explained entirely by its cytoplasmic localization, we constructed a version of Δp53 containing three NLS from the SV40 large T antigen. However, although Δp53-NLS was correctly localized to the nucleus, it did not activate $p21^{CIP1/WAF1}$ or MDM2 (Fig. 5*C*). These results indicate that the lack of an effective NLS in Δp53 is not the main reason for the absence of transcriptional activity and underscore the critical role of the DNA binding domain.

The localization of Δp53 to the cytoplasm does seem to contribute to the relative instability of the protein. We showed that, similar to full-length p53, Δp53 was ubiquitinated in the presence of MDM2 (Fig. 4*C*). This is not too surprising as Δp53 still retains the NH_2-terminal MDM2 binding site. Indeed, we found that MDM2 was coimmunoprecipitated with Δp53 (Fig. 4*C*). Furthermore, the potential ubiquitin-acceptor sites are retained in Δp53. Although a cluster of six lysine residues found in p53 are absent in Δp53, we have shown that these lysine residues are not critical ubiquitination sites (41). Instead, both the NH_2-terminal and COOH-terminal clusters of ubiquitination acceptor sites are still present in Δp53. Moreover, there is a high degree of flexibility in the sites of ubiquitination, so the sequence missing in Δp53 is unlikely to impair the overall ubiquitination of the protein.

Although Δp53 seemed to be more efficiently ubiquitinated than full-length p53 (Fig. 4*C*), the ubiquitination assays were not quantitative and we were not able to unequivocally conclude that Δp53 is more susceptible to ubiquitination than p53. Analysis of the stability of the proteins revealed that Δp53 was less stable than full-length p53 (Fig. 4*D*). We attribute this difference of the half-lives mainly to the subcellular localization of Δp53 and p53. Although p53 can be ubiquitinated by MDM2 inside the nucleus (42), one major pathway of p53 degradation is through the export of the p53-MDM2 complexes to the cytoplasm before p53 is delivered to the ubiquitin/proteasome pathway. As Δp53 is already in the cytoplasm, it is possible that it can be degraded by the ubiquitin/proteasome pathway more efficiently. In support of this, we found that Δp53-NLS, which was imported into the nucleus, was more stable than Δp53 (Fig. 4*D*). Because ubiquitination itself also contributes to the efficient export of p53 to the cytoplasm (43, 44), it could be hypothesized that the two events, ubiquitination and export, simply act reciprocally on each other for Δp53. We think that this is unlikely because treatment with leptomycin B did not increase the nuclear localization of Δp53,[1] suggesting that Δp53 was never imported into the nucleus in the first place.

Another consequence of the cytoplasmic localization of Δp53 is the lack of dominant-negative activity. We found that the transcriptional activities of p53 (including the activation of $p21^{CIP1/WAF1}$ and MDM2 promoters as well as the repression of the cyclin B1 promoter) were not significantly affected by Δp53 (Fig. 2). It is interesting that the activation of MDM2 by p53 was actually increased by Δp53 (Figs. 1*D* and 5*D*). This is also consistent with the increase of p53 activity on the MDM2 promoter in the presence of the R273H mutant (26). Because the presence of one or two molecules of Δp53 (or R273H) within a tetramer may not be inhibitory, the addition of Δp53 (up to certain level) may in fact increase the abundance of active tetramers.

Although Δp53 did not strongly inactivate p53, the localization of p53 was nevertheless altered because some p53 staining could be detected in the cytoplasm (Fig. 3*B*). We postulate that this was due to the complex formation between Δp53 and p53, rendering a portion of p53 to be imported into the nucleus less efficiently. This impeded nuclear accumulation was apparently not sufficient to reduce the activity of p53, possibly because the majority of p53 was still imported into the nucleus. It is not too surprising as nuclear import is an active process and does not depend on tetramerization (COOH-terminally truncated p53 lacking the tetramerization domain is still imported; ref. 26).

Conversely, a portion of Δp53 was imported into the nucleus when it was coexpressed with p53 (Fig. 3*B* and *C*). This was presumably again due to the interaction between p53 and Δp53, with Δp53 piggybacked into the nucleus. Indeed, some proteins without NLS are imported into the nucleus by a similar principle. For example, cyclin D1-CDK4 complexes are targeted to the nucleus by binding to NLS-containing CDK inhibitors (45, 46). This increase in nuclear Δp53 was insufficient to inhibit p53, presumably because of the relatively low levels of Δp53 in the nucleus. Another reason is that even when Δp53 is imported into the nucleus, several copies per tetramer are probably required to abolish the transcriptional activity. This was verified by the experiments involving Δp53-NLS (Fig. 6*A*). Although Δp53-NLS was localized to the nucleus and could bind p53, it displayed an inhibitory profile similar to the theoretical prediction that at least three subunits are required to inhibit the tetramer. This is similar to the activity displayed by other DNA binding–defective mutants like R273H and R249S (26).

To obtain a clearer indication of the effect of nuclear localization on Δp53, we accentuated the dominant-negative effect of Δp53 by removing its NH_2-terminal region. In marked contrast to DNA binding–defective mutants, NΔ mutants are powerful inhibitors of p53 function and about one mutant per tetramer is sufficient to abolish the transcriptional activity (26). Consistent with this, we found that Δp53NΔ-NLS was a more robust inhibitor of p53 than Δp53-NLS (Figs. 6*B* and *C*). Furthermore, Δp53NΔ-NLS inhibited p53 more efficiently than Δp53NΔ (Fig. 6*D*), indicating that the nuclear localization is critical for the dominant-negative function of p53.

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The p53 Isoform Δp53 Lacks Intrinsic Transcriptional Activity and Reveals the Critical Role of Nuclear Import in Dominant-Negative Activity

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