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DNA-Binding and Transactivation Activities Are Essential for TAp63 Protein Degradation

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The p53-related p63 gene encodes six isoforms with differing N and C termini. TAp63 isoforms possess a transactivation domain at the N terminus and are able to transactivate a set of genes, including some targets downstream of p53. Accumulating evidence indicates that TAp63 plays an important role in regulation of cell proliferation, differentiation, and apoptosis, whereas transactivation-inert ΔNp63 functions to inhibit p63 and other p53 family members. Mutations in the p63 gene that abolish p63 DNA-binding and transactivation activities cause human diseases, including ectrodactyly ectodermal dysplasia and facial clefting (EEC) syndrome. In this study, we show that mutant p63 proteins with a single amino acid substitution found in EEC syndrome are DNA binding deficient, transactivation inert, and highly stable. We demonstrate that TAp63 protein expression is tightly controlled by its specific DNA-binding and transactivation activities and that p63 is degraded in a proteasome-dependent, MDM2-independent pathway. In addition, the N-terminal transactivation domain of p63 is indispensable for its protein degradation. Furthermore, the wild-type TAp63γ can act in *trans* to promote degradation of mutant TAp63γ defective in DNA binding, and the TA domain deletion mutant of TAp63γ inhibits transactivation activity and stabilizes the wild-type TAp63 protein. Taken together, these data suggest a feedback loop for p63 regulation, analogous to the p53-MDM2 feedback loop.

The p63 gene is a recently discovered member of the p53 gene family (38). Unlike p53, p63 has six different isoforms. The transactivation (TA) isoforms, which resemble p53, are generated by the use of an upstream promoter and consist of an acidic N-terminal transactivation domain, a central DNA-binding domain, and a C-terminal oligomerization domain (25, 39). The primary amino acid sequences in the DNA-binding domains of p63 and p53 share over 60% identity, whereas in the TA domain they share 25% identity. TAp63 isoforms are capable of transactivating a set of target genes, some of which overlap with targets downstream of p53, including Bax, MDM2, and p21 (39). The ΔN isoforms, produced from an intronic promoter, contain the same DNA-binding and oligomerization domains as the TA isoforms but lack the transactivation domain. The ΔN isoforms also contain a region of 26 amino acids (aa) at the very N-terminal end of the protein (TA2) in which an activation function was recently identified (7). In addition, the ΔN isoforms are capable of forming protein complexes with p53 family proteins to inhibit the function of p53 family members (39). Furthermore, both the TAp63 and ΔNp63 isoforms can undergo alternative splicing to yield three different C-terminal tails (TAp63α, -β, and -γ isoforms and ΔNp63α, -β, and -γ isoforms). Among these isoforms, TAp63γ is the most transactivation-active isoform of p63 (39). In the C-terminal extension of the α-isoforms, there is a sterile alpha motif implicated in protein-protein interactions and thought to be important for mammalian development (32).

Despite their structural homology, the p53 family members have distinctive biological functions. While p53 is a key gatekeeper for genomic stability by regulating cell cycle, DNA damage repair, and apoptosis, p73 and p63 are critical during development and differentiation. In particular, p63 appears to be essential in epithelial and limb development as demonstrated by the mouse models (33). Several dominant human syndromes involving limb development and ectodermal dysplasia have been mapped to the p63 gene, including ectrodactyly, ectodermal dysplasia and cleft lip/palate (EEC) syndrome; nonsyndromic split hand/foot malformation (SHFM); ankyloblepharon, ectodermal dysplasia, clefting (AEC) syndrome; acro-dermato-ungual-lacrimal-tooth (ADULT) syndrome; and limb-mammary syndrome (LMS) (2, 4). Most mutations in the p63 gene identified in EEC patients so far result in amino acid substitutions that are predicted to abolish the DNA-binding capacity of p63. In contrast to p53, the p63 gene is rarely mutated in cancer (12, 21, 25). However, overproduction of ΔNp63 isoforms has been reported in squamous cell carcinoma (10) and in many other types of epithelial tumors (6, 24, 26, 27).

The p53 protein is usually labile in normal cells but is dramatically stabilized upon a variety of cellular stresses. The key negative regulator of p53 is the MDM2 protein, which functions as an E3 ubiquitin ligase for p53 to promote its protein degradation (9, 15). In addition, MDM2 physically binds to the p53 N-terminal transactivation domain, thereby directly inhibiting p53 transactivation activity (5). Importantly, MDM2 is a bona fide target gene downstream of p53. Thus, activation of

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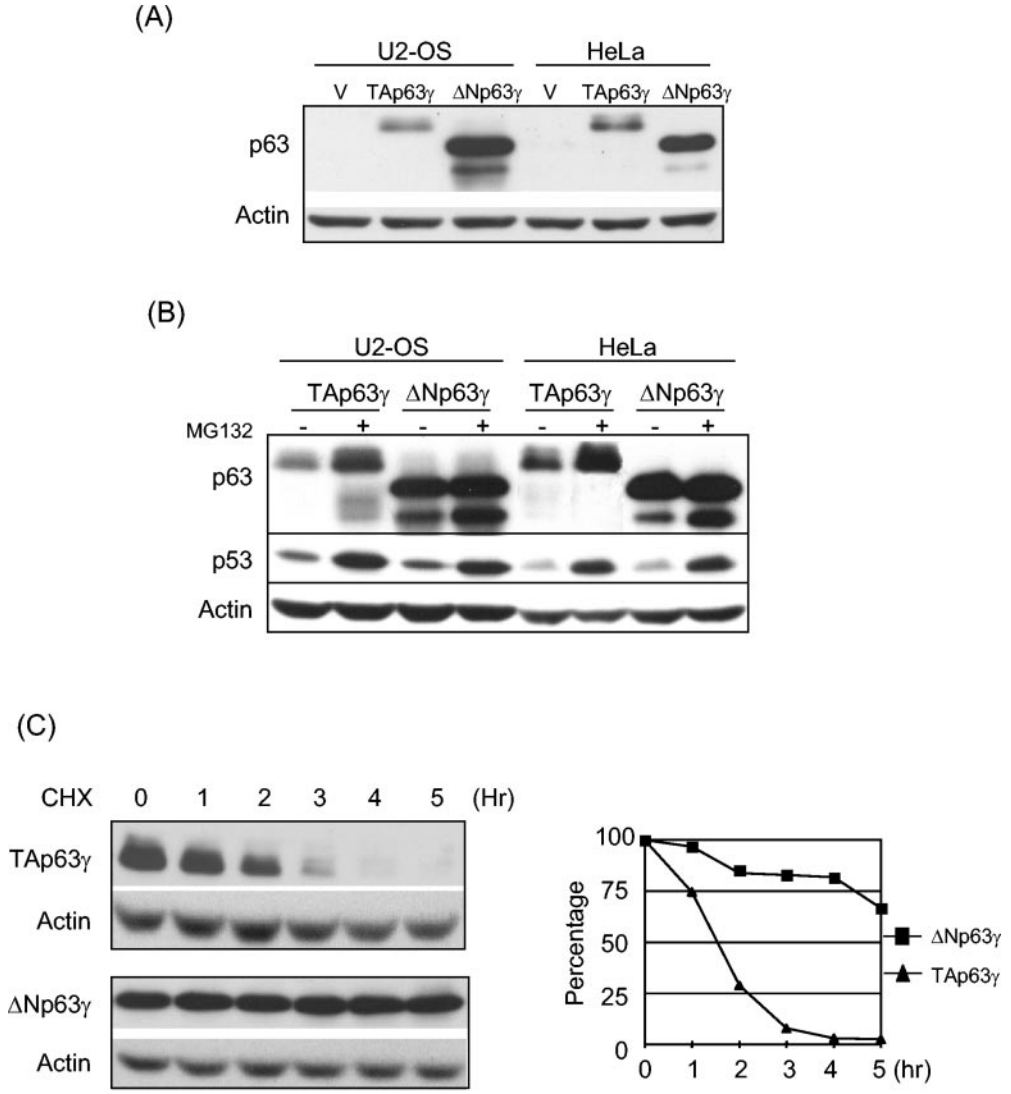


FIG. 1. The transactivation activity of p63 correlates with its protein stability. (A) TAp63γ and ΔNp63γ were transiently expressed in U2-OS and HeLa cells. Cell lysates were subjected to Western blot analysis for p63 and actin. V, vector. (B) U2-OS or HeLa cells transfected with TAp63γ and ΔNp63γ expression plasmids were treated with proteasome inhibitor MG132 (+). Cell lysates were subjected to Western blot analysis. The endogenous p53 protein levels were also analyzed. (C) U2-OS cells transiently transfected with TAp63γ or ΔNp63γ were treated with cycloheximide (CHX) for the indicated times (in hours). Equal amounts of total proteins were subjected to Western blot analysis for p63. Quantitation of the p63 protein bands was performed using densitometry scanning and presented as a percentage of the remaining p63 protein level.

p53 up-regulates its own inhibitor (MDM2). This feedback loop ensures that p53 protein levels are maintained at low levels in normal cells.

Here we show that, like p53, the protein stability of the transactivation form of p63, TAp63γ, is also tightly regulated by its DNA-binding and transactivation activities. The N-terminal TA domain is required for its protein degradation independent of MDM2. Our data suggest that TAp63γ is regulated through a feedback mechanism similar to the p53-MDM2 feedback loop.

MATERIALS AND METHODS

Cell culture and drug treatment. U2-OS, HeLa, and Saos-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin G/streptomycin sulfate at 37°C in a humidified 5% CO_2 incubator. Wild-type or $p53^{-/-}$ $MDM2^{-/-}$ mouse embryonic fibroblasts (MEF) were maintained in DMEM supplemented with 15% FBS. U2-OS-Tet-TAp63γ and U2-OS-Tet-Luciferase cells were maintained in DMEM supplemented with 10% FBS, 2 μg/ml doxycycline (BD Biosciences–Pharmingen), and 200 μg/ml hygromycin (BD Biosciences–Pharmingen).

Plasmid construction and transfection. pcDNA-myc-TAp63γ and pcDNA-myc-ΔNp63γ were described previously (39). A BamHI/XhoI or BamHI/XbaI PCR fragment containing TAp63γ was subcloned into pcDNA-HA or pTRE-tight (BD Biosciences–Pharmingen) to generate pcDNA-HA-TAp63γ or pTRE-tight-TAp63γ. The pcDNA-myc-TAp63γ and pcDNA-HA-TAp63γ plasmids were used to generate deletion mutants or point mutants by site-directed mutagenesis according to the manufacturer's instructions (Stratagene). The TA domain swapping mutant of TAp63γ, HA-p53TA-p63γ, was generated from pcDNA-HA-TAp63γ and pcDNA-HA-p53 (40) using overlapping extension as described previously (11). All constructs were confirmed by DNA sequencing.

U2-OS and HeLa cells were transfected with FuGENE 6 (Roche). Saos-2 cells were transfected with CalPhos transfection kit (BD Biosciences–Pharmingen). MEF were transfected with Lipofectamine 2000 (Invitrogen). To examine

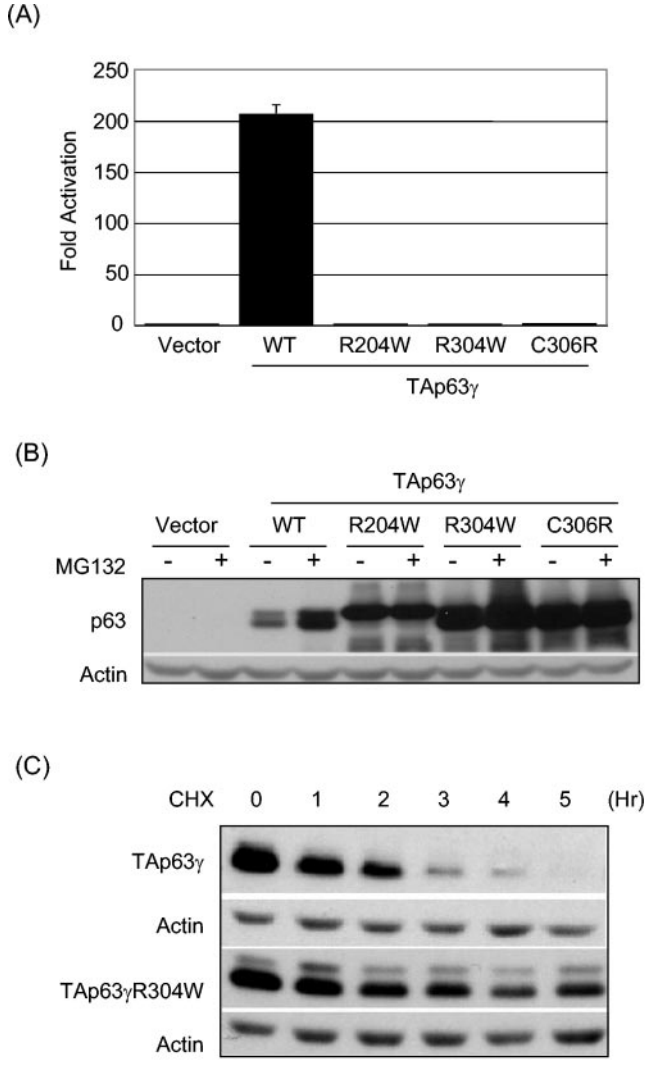


FIG. 2. The DNA-binding activity of p63 is essential for its protein degradation. (A) Saos-2 cells were transfected with either wild-type (WT) TAp63γ or a mutant TAp63γ defective in DNA binding (R204W, R304W, or C306R) in the presence of Bax-Luciferase reporter and β-galactosidase plasmids. Cell lysates were subjected to luciferase and β-galactosidase assays. The luciferase activity was normalized to β-galactosidase activity and reported as an increase in activation (mean ± standard deviation [error bar]). (B) U2-OS cells transfected with an indicated plasmid were treated with MG132 (+). Cell lysates were subjected to Western blot analysis. (C) U2-OS cells transfected with TAp63γ or TAp63γ(R304W) were treated with cycloheximide (CHX) for the indicated times (in hours). Cell lysates were subjected to Western blot analysis for p63 and actin.

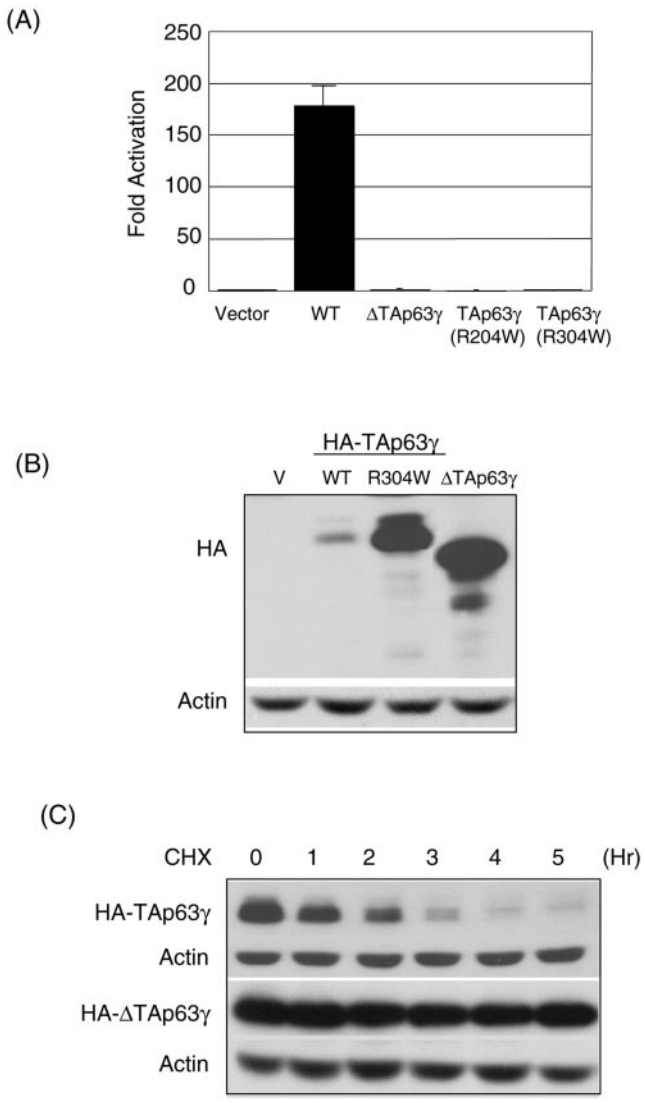


FIG. 3. The transactivation activity of p63 is critical for its protein degradation. (A) Saos-2 cells transfected with wild-type (WT) TAp63γ or a mutant TAp63γ, either lacking the TA domain (ΔTAp63γ) or defective in DNA binding (R204W and R304W) in the presence of Bax-Luciferase reporter and β-galactosidase plasmids. The luciferase activity was normalized to β-galactosidase activity and reported as an increase in activation (mean ± standard deviation [error bar]). (B) Protein expression was examined by Western blot analysis. V, vector. (C) U2-OS cells transfected with TAp63γ or ΔTAp63γ were treated with cycloheximide (CHX) for the indicated times (in hours). Cell lysates were subjected to Western blot analysis.

TAp63γ protein stability, U2-OS or HeLa cells were transfected with 500 ng of pcDNA-TAp63γ or cotransfected with 500 ng of pcDNA-TAp63γ and 1.0 μg of either pcDNA-TAp63γ(R304W), pcDNA-ΔTAp63γ, or pcDNA-TAp63γ(FWL-A). Sixteen hours posttransfection, cells were treated with cycloheximide (Sigma) at a final concentration of 50 μg/ml. Cells were collected at time intervals. For MG132 treatment, cells transfected for 30 h were treated with MG132 (20 μM) (Sigma) for 5 h. To generate U2-OS-Tet-TAp63γ and U2-OS-Tet-Luc cells, U2-OS-Tet_{off} cells (gift from Qiang Yu, Boston University School of Medicine) were cotransfected with 5 μg of pTRE-tight-TAp63γ or pTRE-tight-Luciferase (BD Biosciences–Pharmingen) and 0.5 μg of pTK-Hyg. Twelve hours posttransfection, cells were selected with doxycycline (2 μg/ml) and hygromycin (400 μg/ml) for 4 weeks. Single colonies were selected. All clonal cell lines were tested for induction of TAp63γ or luciferase in the absence of doxycycline by Western blot analysis or luciferase activity assay. A stable U2-OS-TetTAp63γ cell line (clone 2) was chosen for further analysis for its effective repression and induction by doxycycline administration or withdrawal.

Luciferase reporter assay and Western blot analysis. For luciferase report assay, Saos-2 cells grown in six-well tissue culture dishes at 80% confluence were transfected with 100 ng of either wild-type or mutant pcDNA-TAp63γ in the presence of 2 μg of Bax-Luciferase (Bax-Luc) and 100 ng of pCMV-β-galactosidase plasmids. To examine the dominant-negative effect of p63 mutants, Saos-2 cells were transfected with 100 ng of pcDNA-myc-TAp63γ, 400 ng of either pcDNA-HA-ΔTAp63γ or pcDNA-HA-TAp63γ(R304W) in the presence of 2 μg of Bax-Luc and 100 ng of pCMV-β-galactosidase. Thirty-six hours posttransfection, cells were harvested in 1× reporter lysis buffer (BD Biosciences–Pharmingen) and subjected to β-galactosidase assay and luciferase activity assay (BD

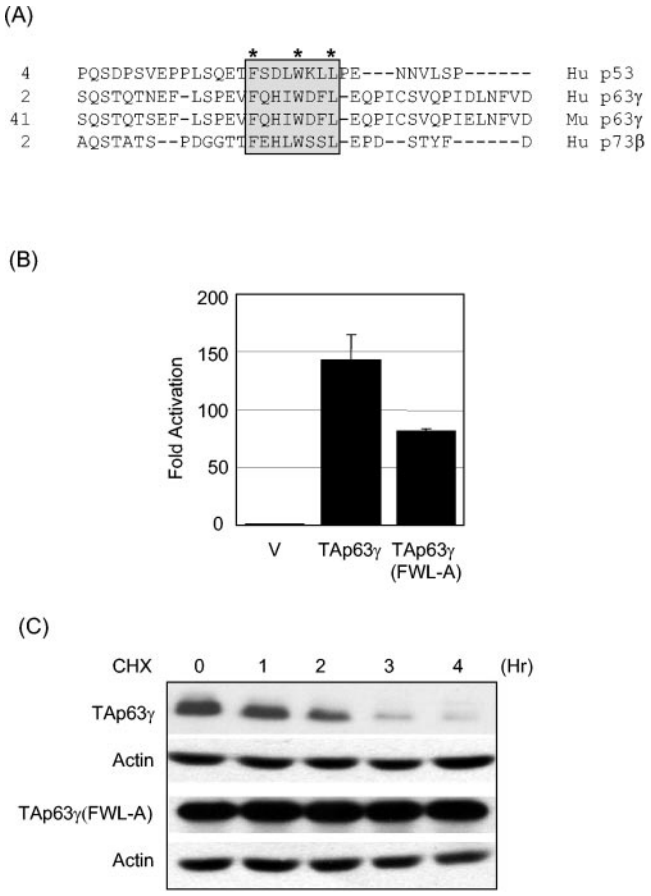


FIG. 4. The mutant TAp63γ(FWL-A) bearing three point mutations at the FWL motif in the TA domain retains partial transactivation activity but is highly stable. (A) The FWL motif (indicated by asterisks) is evolutionarily conserved among p53 family members. Gaps introduced to maximize alignment are indicated by dashes. Hu, human; Mu, murine. (B) Saos-2 cells were transfected with wild-type or mutant TAp63γ(FWL-A) in the presence of Bax-Luciferase and β-galactosidase constructs. The luciferase activity was normalized to β-galactosidase activity and reported as an increase in activation (mean ± standard deviation [error bar]). V, vector. (C) U2-OS cells transfected with TAp63γ or TAp63γ(FWL-A) were treated with cycloheximide (CHX) for the indicated times (in hours). Cell lysates were subjected to Western blot analysis for p63 and actin.

Biosciences–Pharmingen). Luciferase activity was normalized to β-galactosidase activity and presented as the mean ± standard deviation of three independent experiments performed in triplicate.

For Western blot analysis, cells were lysed in EBC buffer (50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% Nonidet P-40, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml leupeptin, 2 μg/ml aprotinin, 50 mM NaF, and 0.5 mM Na_3VO_4). Equal amounts of total protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Antibodies used were specific for p63 (4A4; Santa Cruz), hemagglutinin (HA) (Y-11; Santa Cruz), p53 (DO-1; Santa Cruz), Myc (9E10; Santa Cruz), β-galactosidase (23781; Promega), and actin (C-11; Santa Cruz).

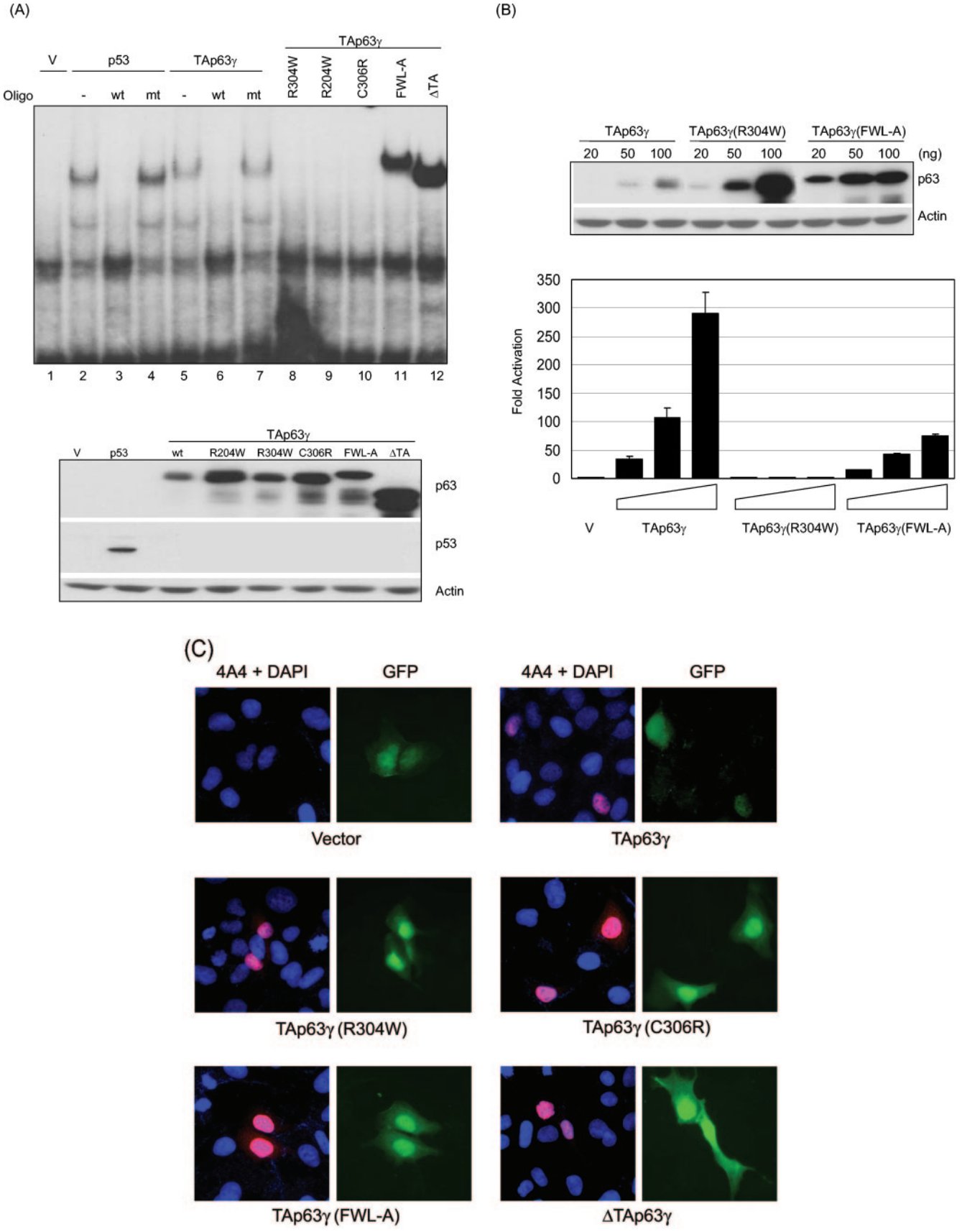
Electrophoresis mobility shift assay (EMSA). H1299 cells were transfected with 3 μg of p53 or a TAp63γ construct. Twenty-four hours posttransfection, nuclear extracts were prepared. Briefly, cells were collected and lysed in hypotonic buffer (10 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 0.5 mM PMSF, 0.2% Nonidet P-40), followed by centrifugation at 4,000 rpm for 10 min at 4°C. The pellets were then lysed in hypertonic buffer (20 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol), followed by centrifugation at 14,000 rpm for 20 min at 4°C. The reaction mixture (total volume of 25 μl) containing 20 μg nuclear extracts in binding buffer [10 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 0.25 μg/μl bovine serum albumin, and 0.1 μg/μl of poly(dI-dC)] was incubated at room temperature for 20 min. The competition assay was performed by the addition of 50-fold excess of unlabeled wild-type oligonucleotide (sc-2579; Santa Cruz Biotechnology) or mutant oligonucleotide (sc-2580; Santa Cruz Biotechnology) into the reaction mixture and incubation for 20 min prior to the addition of ^{32}P-labeled probe. DNA-protein complexes were resolved by electrophoresis on 4% polyacrylamide gels in Tris-glycine buffer and revealed by autoradiography. Thirty nanograms of oligonucleotide containing the p53-binding consensus site (sc-2579; Santa Cruz Biotechnology) was end labeled in the presence of 25 μCi of [$\gamma^{32}P$]ATP (NEG002H; New England Nuclear) and 10 U of T_4 polynucleotide kinase (M0201; New England BioLabs) for 30 min at 37°C.

Immunofluorescence staining. U2-OS cells in six-well plates were cotransfected with either 500 ng of pcDNA vector or pcDNA-TAp63γ, or 200 ng of pcDNA-TAp63(R304W), pcDNA-TAp63(C306R), pcDNA-TAp63(FWL-A), or pcDNA-TAp63(ΔTA) in the presence of 50 ng of pEG-GFP using FuGENE 6. Twelve hours posttransfection, cells were trypsinized and 5×10^4 cells were plated onto Lab-Tek II chamber slides (154461; Nalge Nunc International). Twenty-four hours later, cells were fixed in 4% paraformaldehyde (in phosphate-buffered saline, pH 7.4), permeabilized with 0.2% Triton X-100, blocked in phosphate-buffered saline containing 1% bovine serum albumin, and then immunostained with p63 antibody (sc-8431; Santa Cruz Biotechnology) followed by a Cy3-conjugated goat anti-mouse immunoglobulin G antibody (115-165-146; Jackson ImmunoResearch Laboratory). The cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (300 nM) prior to mounting using ProLong Gold Antifade (P36930; Molecular Probes). Staining was visualized using the following excitation/emission wavelengths: 358/461 nm for DAPI, 595/615 nm for Cy3, and 494/518 nm for green fluorescent protein (GFP). Fluorescent images were captured on a Zeiss Axiovert 200M microscope with Axiovision v 4.3 program.

RESULTS

p63 protein instability correlates with its transactivation activity. It is well documented that the predominant species of p63 in epithelial cells and cancer cells are ΔN isoforms, which lack the transactivation domain at the N terminus, whereas the levels of TA isoforms are very low under physiological conditions (10, 20, 38). We asked whether this phenomenon is reminiscent of p53, whose mutants defective in transactivation activity unavoidably become stabilized. Thus, we examined the protein expression levels of transactivation-potent TAp63γ and transactivation-inert ΔNp63γ in U2-OS or HeLa cells by transient transfection. As shown in Fig. 1A, the protein levels of TAp63γ were significantly less than those of ΔNp63γ in both cell lines. Treatment with proteasome inhibitor MG132 led to stabilization of endogenous p53 proteins, as expected, and to a dramatic increase of TAp63γ protein levels. ΔNp63γ protein was also stabilized, albeit to a much lesser extent (Fig. 1B). These data suggest that TAp63γ is degraded through a proteasome-dependent pathway and that the differences in the protein levels of ectopically expressed TAp63γ and ΔNp63γ are likely due to different protein turnover rates. Thus, we performed cycloheximide treatment to determine the protein half-lives of TAp63γ and ΔNp63γ in U2-OS cells. As shown in Fig. 1C, TAp63γ exhibited a much shorter half-life (~90 min) than ΔNp63γ did (>5 hours). These data revealed a correlation between the transactivation activity and protein stability of p63 isoforms.

The specific DNA-binding and transactivation activities of TAp63γ are essential for its protein degradation. Mutations in the p63 gene have been linked to several human genetic diseases, including EEC syndrome and SHFM (33). In EEC syndrome and SHFM, the germ line mutations in the p63 gene are



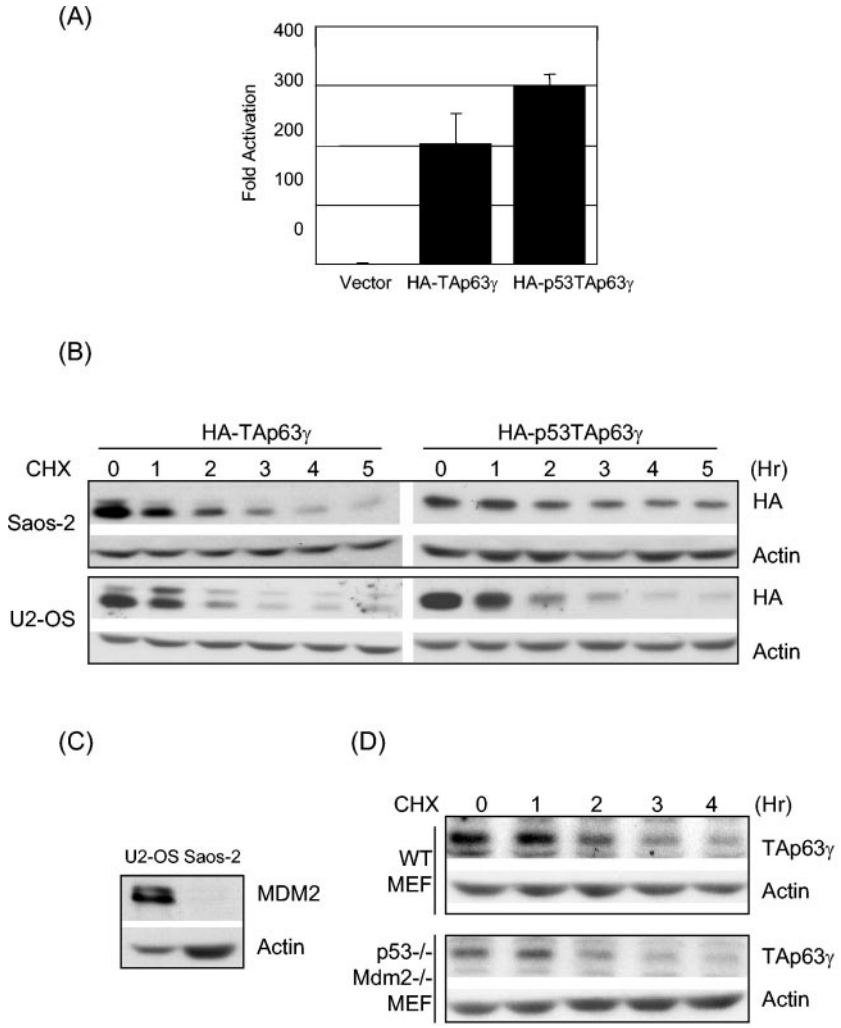


FIG. 6. The TA domain of p63 is essential for its protein instability independent of MDM2. (A) Saos-2 cells were transfected with wild-type TAp63γ or a chimeric construct (HA-p53TAp63γ) in the presence of Bax-Luciferase and β-galactosidase constructs. The luciferase activity was normalized to β-galactosidase activity and reported as an increase in activation (mean ± standard deviation [error bar]). (B) Saos-2 ($p53^-$) or U2-OS ($p53^+$) cells transfected with wild-type HA-TAp63γ or HA-p53TA-p63γ were treated by cycloheximide (CHX) for the indicated times (in hours). Cell lysates were subjected to Western blot analysis using antibodies specific for HA tag or actin. (C) Cell lysates (50 μg of total protein) from U2-OS or Saos-2 cells were subjected to Western blot analysis for MDM2 or actin. (D) Wild-type (WT) or $p53^{-/-}$ $MDM2^{-/-}$ MEF were transfected with TAp63γ, followed by cycloheximide (CHX) treatment for the indicated times (in hours). Cell lysates were subjected to Western blot analysis using antibody specific for p63.

frequently located in the central DNA-binding domain (4, 34, 36). Strikingly, these missense mutations correspond very well with the somatic mutational hot spots in the p53 gene, which inactivate p53 DNA binding and p53 growth suppression function. We generated three point mutations in the DNA-binding domains of TAp63γ, TAp63γ(R204W), TAp63γ(R304W), and TAp63γ(C306R). These mutations are found in EEC syndrome and are predicted to abolish the DNA-binding activity

FIG. 5. The mutant TAp63γ proteins defective in DNA binding or in transactivation are nuclear and stable. (A) H1299 cells were transfected with an indicated p53 or TAp63γ expression plasmid. Equal amounts of nuclear extracts were subjected to EMSA using a ^{32}P-labeled p53 oligonucleotide (Oligo) (top panel). Cold competition was performed using either the wild-type (wt) p53 oligonucleotide (lanes 3 and 6) or a mutant (mt) p53 oligonucleotide (lanes 4 and 7). Expression of ectopically expressed p53 or p63 proteins was shown by Western blot analysis for p53, p63, and actin (bottom panel). V, vector. (B) Dose effects of wild-type or mutant TAp63γ on transactivation activity. Saos-2 cells were transfected with the indicated amounts of wild-type or mutant TAp63γ [TAp63γ(FWL-A) or TAp63γ(R304W)] in the presence of Bax-Luciferase reporter and pCMV-β-galactosidase plasmids. Equal amounts of total proteins (20 μg) were subjected to Western blot analysis for p63 and actin (top panel) or to assays measuring luciferase and β-galactosidase activities. The luciferase activity was normalized to β-galactosidase activity and reported as an increase in activation (*n*-fold) (mean ± standard deviation [error bar]) (bottom panel). V, vector. (C) U2-OS cells were cotransfected with wild-type or mutant TAp63γ and GFP expression plasmids. Thirty-six hours posttransfection, cells were fixed and subjected to immunohistochemical staining with p63-specific antibody (4A4). Nuclei were costained with DAPI. Coexpressed GFP is shown (magnification of ×400).

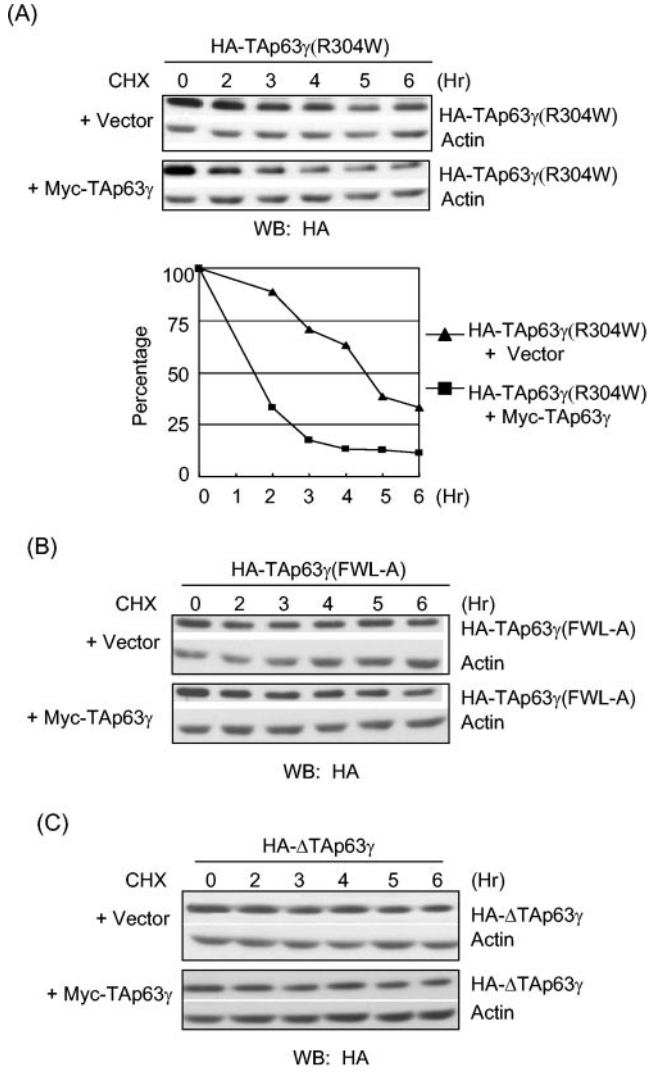


FIG. 7. TAp63γ can act in *trans* to promote degradation of the p63 DNA-binding mutant, but not the p63 TA domain mutants. U2-OS cells were cotransfected with Myc-tagged wild-type TAp63γ and a HA-tagged TAp63γ mutant, HA-TAp63γ(R304W) (A), HA-TAp63γ(FWL-A) (B), or HA-ΔTAp63γ (C). Cells were then treated with cycloheximide (CHX) for the indicated times (in hours). Cell lysates were subjected to Western blot (WB) analysis using antibodies specific for HA tag or actin. Quantitative analysis of the data in panel A was performed.

of p63 (4). Indeed, all three mutant proteins exhibited no DNA-binding activity (see Fig. 5A) and, as expected, no detectable transcriptional activity (Fig. 2A), yet they were expressed at much higher levels than wild-type TAp63γ (Fig. 2B). Inhibition of proteasomes led to a dramatic increase in protein stability of wild-type TAp63γ but not the p63 mutant proteins defective in DNA binding (Fig. 2B). Indeed, the mutant proteins exhibited significantly prolonged protein half-lives (Fig. 2C and data not shown). Thus, these data indicate that the specific DNA-binding activity of TAp63γ is essential for its protein degradation.

To further demonstrate the link between the transactivation activity of TAp63γ and its protein degradation, we generated an N-terminal deletion mutant of TAp63γ, ΔTAp63γ, which lacks the entire TA domain (aa 1 to 69). Although ΔTAp63γ possessed a strong DNA-binding activity (see Fig. 5A) and localized in nuclei (see Fig. 5C), it exhibited no transactivation activity (Fig. 3A) and was highly stable (Fig. 3B and C). Therefore, these data indicate that the transcriptional activity of TAp63γ is critical for its protein instability.

The FWL motif in the TA domain is essential for p63 protein degradation. The p53-MDM2 feedback regulation is a well-established mechanism for the control of p53 protein levels. Three hydrophobic amino acids within the p53 N terminus, F19 W23 L26, which are buried in a cleft within the interface between p53 and MDM2, are critical for the MDM2-mediated degradation of p53 protein (16). Despite a poor homology (25%) among the p53 family members in the N-terminal transactivation domain, the FWL motif is well conserved (Fig. 4A). Therefore, we examined whether the FWL motif is also critical for p63 degradation. As shown in Fig. 4, the triple point mutant, TAp63γ(FWL-A) in which these three amino acid residues (F16, W20, and L23) are replaced by alanine, retained strong DNA-binding activity (Fig. 5A) and partial transactivation activity in a dose-dependent manner (Fig. 4B and 5B), yet the protein was highly stable (Fig. 4C). These data suggest either that the partial transactivation activity is insufficient to promote p63 protein degradation or that the FWL motif is critical for p63 protein turnover. To address this question, we generated a HA-tagged chimeric protein HA-p53TA-p63γ in which the TA domain (aa 1 to 64) of TAp63γ is replaced with the TA domain (aa 1 to 45) of p53. As shown in Fig. 6, p53TA-p63γ was fully competent in transactivation (Fig. 6A). Strikingly, it was stable in Saos-2 cells (Fig. 6B), indicating that the transactivation activity alone is not sufficient for protein degradation. However, in contrast to remarkable protein stability in Saos-2 cells, p53TA-p63γ protein was rapidly turned over in U2-OS cells (Fig. 6B). Since U2-OS cells contain wild-type p53 and express much higher levels of MDM2 in comparison to the p53 null Saos-2 cells (Fig. 6C), it is likely that degradation of the p53TA-p63γ chimeric protein is dependent on MDM2, which binds to the TA domain of p53. Of note, the FWL motif is important for p53 interaction with MDM2 (16). Thus, the conserved FWL motif in the TA domain of TAp63γ may serve as a binding site for MDM2, which might in turn promote p63 degradation. However, the ectopically expressed TAp63γ exhibited similar protein half-lives in wild-type MEF and $p53^{-/-}$ $MDM2^{-/-}$ MEF (Fig. 6D), indicating that degradation of TAp63γ does not require MDM2. Thus, the FWL motif in the TA domain of TAp63γ is critical for its protein degradation in an MDM2-independent manner.

Wild-type TAp63γ can act in *trans* to promote degradation of the p63 DNA-binding mutant, but not the p63 TA domain mutants. Since both the transcriptional activity and the FWL motif of TAp63γ are crucial for its protein instability, it is very possible that there may be a feedback control mechanism for regulation of p63 protein levels, analogous to the p53-MDM2 feedback loop. This hypothesis would predict that TAp63γ transactivates expression of an unidentified target gene whose product interacts with the FWL motif in the TA domain to promote TAp63γ protein degradation via a proteasome-dependent pathway. Accordingly, the wild-type TAp63γ should be able to act in *trans* to promote the degradation of mutant

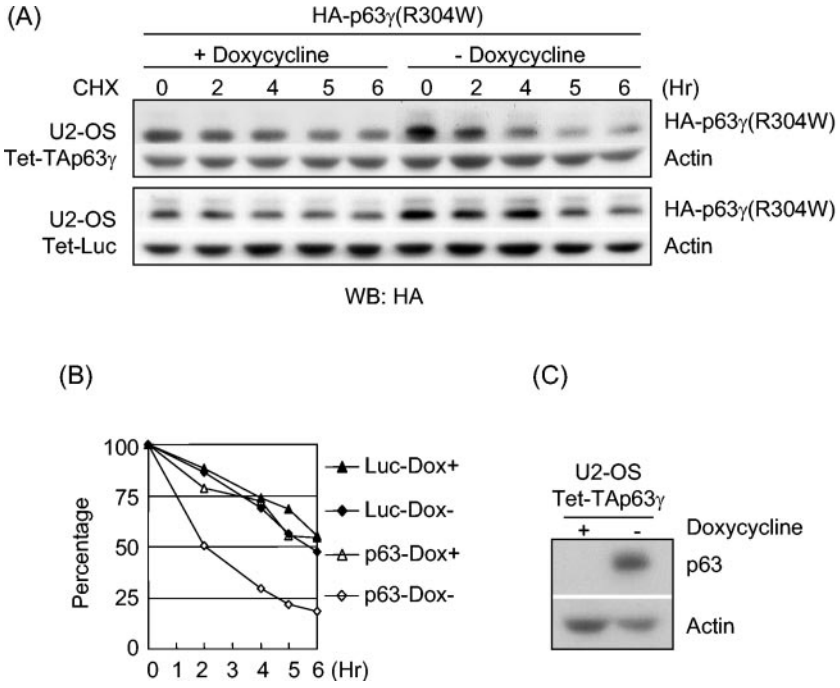


FIG. 8. Induction of TAp63γ promotes degradation of p63 DNA-binding mutant. Doxycycline-repressible stable U2-OS cells, U2-OS-Tet-TAp63γ and U2-OS-Tet-Luc, were transfected with HA-TAp63γ(R304W). Transfected cells were grown in the presence (+) or absence (−) of doxycycline for 24 h, followed by cycloheximide (CHX) treatment for the indicated times (in hours). Cell lysates were subjected to Western blot (WB) analysis using antibodies specific for HA tag or actin (A). The data in panel A were subjected to quantitative analysis and presented as a percentage of remaining protein levels (B). Induction of TAp63γ expression in U2-OS-Tet-TAp63γ stable cells was assessed by Western blot analysis for p63 (C).

p63 protein defective in DNA binding, but not the mutant p63 lacking the TA domain or the mutant p63 with a defective FWL motif. Indeed, in cotransfection experiments, the protein half-life of HA-tagged TAp63γ(R304W) was decreased by 50% in the presence of Myc-tagged wild-type TAp63γ, while the half-life of HA-tagged ΔTAp63γ or TAp63γ(FWL-A) was not affected by the presence of wild-type TAp63γ (Fig. 7). A similar phenomenon was observed using an inducible U2-OS-Tet-TAp63γ stable cell line. Upon withdrawal of doxycycline, expression of TAp63γ was markedly induced (Fig. 8C). Induction of TAp63γ, but not induction of luciferase in the control cell line, led to a significant decrease in the protein half-life (~2 h) of HA-TAp63γ(R304W) (Fig. 8A and B).

It has been shown that nontransactivation ΔNp63 isoforms are predominantly expressed in cancer cells and in epithelial progenitor cells (10, 20, 38). We therefore investigated the influence of transactivation-inert p63 mutant ΔTAp63γ on the wild-type TAp63γ protein stability. As shown in Fig. 9, coexpression of ΔTAp63γ led to a marked suppression of transactivation activity and significant stabilization of TAp63γ protein. Interestingly, coexpression of DNA-binding-defective TAp63γ (R304W) exhibited a much lower inhibitory effect on the TAp63γ transactivation activity and no significant effect on the stability of TAp63γ protein (Fig. 9). Thus, nontransactivation ΔTAp63γ can effectively function in a dominant-negative fashion in suppression of transactivation activity of wild-type TAp63γ and modulate the stability of transactivation p63 isoforms.

DISCUSSION

Although the regulation of p53 protein stability has been extensively studied, how p63 protein stability is regulated is largely unknown (19). Here we demonstrate that the transactivation-potent species of p63, TAp63γ, is tightly regulated likely through a feedback mechanism, analogous to the p53-Mdm2 feedback loop (Fig. 10). We show that both DNA-binding and transactivation activities are essential for TAp63γ instability and, furthermore, that the unique features of the TAp63γ TA domain play an important role in protein degradation. Moreover, we show that the wild-type TAp63γ can act in *trans* to induce the degradation of the p63 DNA-binding mutant and that the p63 deletion mutant lacking the TA domain can stabilize the TAp63γ protein through its dominant-negative effect.

p53 is frequently mutated in human cancers with a high frequency of point mutations, referred to as hot spots, in the DNA-binding domain. These mutant p53 proteins are usually stable, since they are incapable of inducing the expression of MDM2 (22). Strikingly, many of the naturally occurring p63 mutations, especially those found in human EEC syndrome, correspond very well with hot spot mutations found in the p53 gene (2, 4). For instance, the missense mutations creating R204W, R304W, and C306R substitutions in the p63 DNA-binding domain correspond to R175, R273, and C275 of p53, respectively. These DNA-binding-defective TAp63γ proteins are highly stable, thus supporting the notion that specific

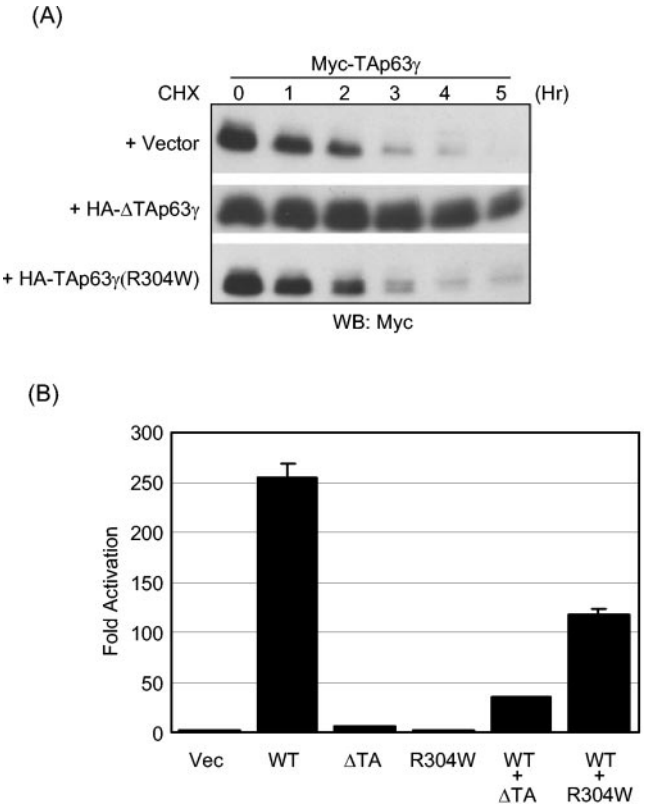


FIG. 9. ΔTAp63γ exhibits dominant-negative effects on the transactivation activity and protein stability of wild-type TAp63γ. (A) U2-OS cells were cotransfected with Myc-TAp63γ and HA-ΔTAp63γ (A) or HA-TAp63γ(R304W). Cells were then treated with cycloheximide (CHX) for the indicated times (in hours). Cell lysates were subjected to Western blot (WB) analysis using an monoclonal antibody specific for Myc. (B) Saos-2 cells were cotransfected with the indicated plasmids in the presence of Bax-Luciferase and β-galactosidase plasmids. The luciferase activity was normalized to β-galactosidase activity and reported as an increase in activation (mean ± standard deviation [error bar]). Vec, vector; WT, wild-type TAp63γ; ΔTA, HA-ΔTAp63γ; R304W, HA-TAp63γ(R304W).

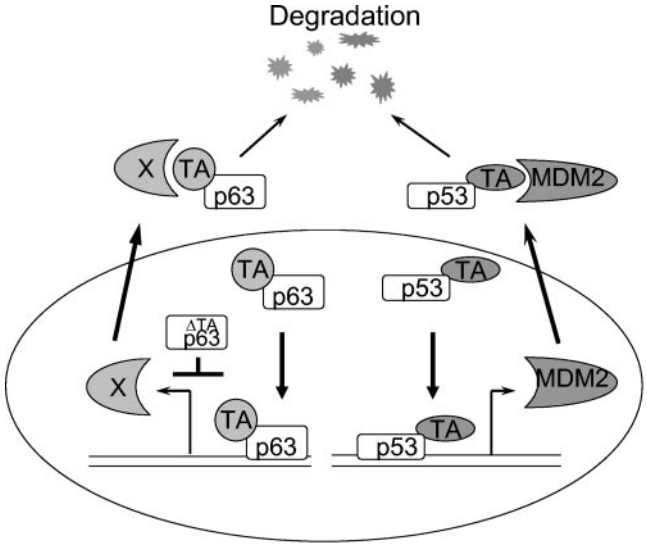


FIG. 10. Feedback model for TAp63γ regulation. p53 activates the expression of MDM2 which in turn binds to the N-terminal transactivation domain of p53 to promote proteasome-dependent degradation. Similarly, TAp63γ binds to a subset of specific DNA promoters and activates transcription of downstream target genes, one of which encodes protein X. Protein X interacts with the TA domain of TAp63γ and promotes its protein degradation via proteasome-dependent pathway. Since TAp63γ contains a unique transactivation domain that is distinct from p53, MDM2 is unable to promote degradation of TAp63γ. ΔTAp63γ can bind DNA but is unable to transactivate, thereby acting as a dominant-negative mutant.

DNA-binding activity, while being a prerequisite for TAp63γ-mediated transcription, is critical for its protein degradation. Alternatively, it is possible that interaction of TAp63γ with DNA is required for its protein degradation in a manner similar to some transcription factors, such as estrogen receptor, Myc, and VP16, whose DNA-binding activity is critical for protein degradation (13, 28, 29, 35). However, our data reveal that wild-type TAp63γ can promote the degradation of a DNA-binding-defective mutant, suggesting that TAp63γ interaction with DNA is not a prerequisite for its protein degradation. Notably, high levels of wild-type p53 or MDM2 have been shown to promote the degradation of mutant p53 proteins defective in DNA binding (1, 22).

The N-terminal domain of TAp63γ is indispensable for its protein degradation. The ΔTAp63γ mutant, which lacks the entire transactivation domain and thus is completely inert in transactivation, is highly stable. Replacement of the TA domain of TAp63γ with the p53 TA domain in the chimeric protein p53TA-p63γ results in increased protein stability in cells expressing low levels of MDM2, despite its high transactivation activity, indicating that transcription activity is not sufficient for p63 protein degradation. In addition, the TAp63γ(FWL-A) mutant bearing the point mutations in the F16-W20-L23 motif also leads to protein stability. Of note, not only are both ΔTAp63γ and TAp63γ(FWL-A) highly stable but both are also resistant to the degradation induced by wild-type TAp63γ. These data indicate that the TA domain is required for the degradation of TAp63γ protein, probably by serving as an interaction domain for a p63 transcription target protein that can induce the degradation of TAp63γ. Interestingly, a recent study reported that the F16-W20-L23 motif functions as a protein-protein interaction site with which the transactivation-inhibitory domain on the C terminus of TAp63α is able to interact, resulting in stabilization of the TAp63α protein (31). Importantly, these three hydrophobic amino acids (F19-W23-L26) within the p53 N terminus are critical for the MDM2-mediated degradation of p53 protein (16). Despite the poor homology between the p53 family members, those three amino acids are well conserved in p53, p63, and p73; this may mean that MDM2 might also be able to bind the FWL motif and promote p63 protein degradation. However, our data indicate that MDM2 is not required for p63 degradation, as evidenced by the observation that p63 protein can be degraded equally well in $p53^{-/-}$ $MDM2^{-/-}$ MEF and wild-type MEF. These data are consistent with the observations indicating that overexpression of MDM2 does not lead to p63 protein instability (3, 18).

The notion that p63 is regulated by a feedback loop is further supported by the observation that the ΔTAp63γ mutant can significantly inhibit the transactivation activity of the wild-

type TAp63γ and lead to its protein stabilization. It is possible that excess expression of ΔTAp63γ may occupy the promoter regions to which the transactivation p63 normally binds and thereby block TAp63-mediated transcription, which may in turn suppress the expression of proteins involved in p63 protein degradation (Fig. 10). Interestingly, a recent study showed that the transactivation activity of p73 is also critical for p73 protein degradation (37). It is plausible that cells may have developed similar feedback regulation mechanisms during evolution in regulation of p53 family protein expression despite their distinct biological functions.

It has been established that certain unstable transcription factors contain the degron sequences in the transactivation domains so that transcription can be coupled to proteolysis (17, 23, 30). Our data demonstrated that TAp63γ degradation is also tightly coupled to its transcriptional activity, which may partially explain the low levels of expression of transactivation-active p63 isoforms in most tissues and cell lines. Recent studies have demonstrated that among the p63 proteins TAp63 isoforms are the first to be expressed during embryogenesis and are required for commitment to an epithelial stratification program. ΔNp63 is the predominant isoform expressed in the basal cells of many epithelial tissues (14, 20). Since TAp63 isoforms seem to inhibit terminal differentiation, their activities must be counterbalanced by various mechanisms, such as transcription-coupled degradation of TAp63 and expression of ΔNp63 in neutralizing TAp63, to allow cells to respond to signals required for the maturation of embryonic epidermis. It is possible that uncontrolled expression of transactivation-potent p63 isoforms leads to adverse effects on cells, since TAp63γ has been shown as an inducer for apoptosis (8, 39).

Interestingly, the p63 mutations in the DNA-binding domain observed in several dominant human syndromes are highly stable. It is conceivable that these mutations may regulate the function of p53 family proteins to disrupt normal cell proliferation and development.

Although our data indicate the TAp63 protein stability is tightly controlled by its transcriptional activity, the precise molecular mechanism, however, remains to be elucidated.

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