

Protein Kinase C-Dependent Phosphorylation Regulates the Cell Cycle-Inhibitory Function of the p73 Carboxy Terminus Transactivation Domain^{∇†}

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The transcription factor p73, a member of the p53 family of proteins, is involved in the regulation of cell cycle progression and apoptosis. However, the regulatory mechanisms controlling the distinct roles for p73 in these two processes have remained unclear. Here, we report that p73 is able to induce cell cycle arrest independently of its amino-terminal transactivation domain, whereas this domain is crucial for p73 proapoptotic functions. We also characterized a second transactivation domain in the carboxy terminus of p73 within amino acid residues 381 to 399. This carboxy terminus transactivation domain was found to preferentially regulate genes involved in cell cycle progression. Moreover, its activity is regulated throughout the cell cycle and modified by protein kinase C-dependent phosphorylation at serine residue 388. Our results suggest that this novel post-translational modification within the p73 carboxy terminus transactivation domain is involved in the context-specific guidance of p73 toward the selective induction of cell cycle arrest.

The p73 protein was discovered in 1997 and, together with p53 and p63, constitutes the p53 family of transcription factors (17). All three family members show significant sequence homology, especially with regard to the amino-terminal transactivation (TA) domain, the central DNA binding domain (DBD), and the carboxy-terminal oligomerization domain (38).

Similar to p53, p73 has the ability to induce cell cycle arrest and/or apoptosis (7). In addition, both p73 and p63 have been shown to play critical roles during development and to regulate cell differentiation (3). Although transcription-independent functions have been described, most p73 effects are believed to be mediated through the modulation of expression of specific target gene. These include some obvious candidates to mediate either cell cycle arrest or apoptosis, such as the *p21*, *mdm2*, *bax*, *cyclin G*, *PIG3*, and *CD95* genes (12, 13, 27, 29).

Several groups reported previously that the function of p73 in cell cycle arrest or apoptosis induction is regulated by interactions with other proteins. Indeed, the transcriptional coactivator Yes-associated protein has been shown to interact with p73, increasing its activity at the *mdm2*, *bax*, and *p53AIP1* promoters and thereby enhancing p73 proapoptotic activity (2, 33, 34). However, no effect was seen at the *p21* promoter (33). The interaction of p73 with the prolyl isomerase Pin1 increases p73 stability, its activity on *p21* and

bax promoters, and proapoptotic effects upon DNA damage (18).

In addition to protein interactions, the ability of p53 family members to transactivate specific genes can be affected by posttranslational modifications, including phosphorylation, acetylation, and sumoylation (16, 23, 30). Posttranslational phosphorylation and/or acetylation of p53 generally results in p53 stabilization and an increase in sequence-specific DNA binding and transcriptional activity (5, 21). The protein kinase C δ (PKC δ) catalytic fragment, generated during the apoptotic response to DNA damage, phosphorylates p73 at serine 289 (30). The tyrosine kinase c-Abl, activated in response to DNA-damaging agents, phosphorylates p73 at tyrosine residue 99 (40). These two modifications markedly enhance p73-mediated apoptosis (1, 30, 40). It has also been reported that p300 acetylates p73 at lysines 321, 327, and 331. Interestingly, it has been proposed that the acetylation of p73 by p300 potentiates the apoptotic function of p73 by enhancing its ability to selectively activate the transcription of proapoptotic target genes (8). Thus, posttranscriptional modification by acetylation could be a key event in the decision of p73 to promote apoptosis instead of cell cycle arrest (20).

Despite the increasing number of identified p73 downstream target genes and the identification of several p73 posttranslational modifications, it is still unclear how p73 may preferentially affect the expression of cell cycle-regulatory genes and, therefore, promote cell cycle arrest rather than apoptosis (8, 26). In this study, we have identified a TA domain within the carboxy terminus of p73 that preferentially regulates genes involved in cell cycle progression. This TA domain is located within amino acid residues 381 to 399 of p73 and possesses cell type-specific activity. Moreover, the activity of the carboxy-terminal TA domain was found to be modified by PKC-dependent phosphorylation and differentially regulated throughout the cell cycle.

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MATERIALS AND METHODS

Cell culture, transfection, and treatments. The human small-cell lung carcinoma (SCLC) cell line NCI-H82 (ATCC HTB-175), the human embryonic kidney 293 cell line (ATCC CRL-1573), the human neuroblastoma SH-SY5Y cell line (ATCC CRL-2266), the human cervical carcinoma HeLa S9 cell line (NCCC), and the rat pheochromocytoma PC12 cell line (ATCC CRL-1721) were used in this study. Cells were cultured at 37°C and 5% CO₂ in RPMI 1640 medium (H82 and PC12), Dulbecco's modified Eagle's medium (MEM) (HEK-293), MEM mixed with F-12 nutrient mixture (Ham) at ratio of 1:1 (SH-SY5Y), or MEM mixed with 1× MEM nonessential amino acids and 1 mM MEM sodium pyruvate (HeLa), all supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml). Twenty-four hours after seeding into culture dishes with fresh medium, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, cells were treated with 5 μM VP16. The cell density was kept at levels allowing exponential growth.

Reagents and antibodies. VP16 was obtained from Bristol-Myers, and PKC inhibitor PKC-412 was obtained from Novartis. Hoechst 33342 dye was obtained from Molecular Probes, Geneticin was obtained from Gibco, propidium iodide (PI) was obtained from Sigma, and RNase A was obtained from Boehringer. For Western blot and immunofluorescence experiments, primary mouse monoclonal anti-p73α/β (Ab-4) antibody (NeoMarkers), rabbit polyclonal anti-p73 (Ab-6) antibody (NeoMarkers), rabbit polyclonal anti-Bax antibody (BD Pharmingen), mouse monoclonal anti-Bax (6A7) antibody (BD Pharmingen), mouse monoclonal anti-hemagglutinin (12CA5) antibody (Roche), rabbit polyclonal anti-Mdm2 (N-20) antibody (Santa Cruz Biotechnology, Inc.), or rabbit polyclonal anti-phosphoserine antibody (Zymed Laboratories) was used. Secondary Alexa Fluor 488- and 594-conjugated goat anti-immunoglobulin G (IgG) (Molecular Probes) was used in immunofluorescence and fluorescence-activated cell sorter (FACS) analysis experiments. For Western blot experiments, rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (anti-G3PDH) antibody (Trevigen) and horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Pierce) were used. For bromodeoxyuridine (BrdU) incorporation studies, BrdU and fluorescein isothiocyanate-conjugated mouse anti-BrdU monoclonal antibody (BD Pharmingen) were used. Pre-designed and tested ON-TARGETplus siRNA SMARTpool against human PKCα (GenBank accession number NM_002737) and human PKCβ2 (accession number NM_002738) were obtained from Dharmacon. Nocodazole and hydroxyurea were obtained from Roche.

Plasmids. Human p73 isoforms (α, β, ΔNα, γ, δ, and ε) and p73 TA domain-inactive mutants (having an amino acid substitution at position 156) were kind gifts from G. Melino and were described previously (10, 11). To generate the naturally occurring mutants P405R and P425L, having amino acid substitutions at either position 405 or 425, either full-length p73α p73β or their NH₂-TA-mutated counterparts were used. To generate p73Δ380 and TAmut p73Δ380, full-length p73β and TAmut p73β were used, and a stop codon was introduced at amino acid 381. To generate PKC phosphorylation site mutant TAmut p73β S388A, a base pair substitution was made at amino acid 388. Mutagenesis was performed according to the manufacturer's protocol (QuikChange site-directed mutagenesis kit; Stratagene) using primers P405R (5'-CAC CTA CAG CCC CGG TCC TAC GGG C-3' [forward] and 5'-G CCC GTA GGA CCG GGG CTG TAG GTG-3' [reverse]), P425L (5'-ATG AAC AAG CTG CTC TCC GTC AAC C-3' [forward] and 5'-G GTT GAC GGA GAG CAG CTT GTT CAT-3' [reverse]), 380stop (5'-CTG ATG GAG TTG TAG CCG CAG CCA CTG-3' [forward] and 5'-CAG TGG CTG CGG CTA CAA CTC CAT CAG-3' [reverse]), and S388A (5'-G CCA CTG GTG GAC GCC TAT CGG CAG CA-3' [forward] and 5'-TG CTG CCG ATA GGC GTC CAC CAG TGG C-3' [reverse]). Gal4DBD-p73/N (amino acids 1 to 112) and Gal4DBD-p73/C (amino acids 380 to 513) were kind gifts from K. Somasundaram and were described previously (9). Plasmids containing the *PIG3* promoter-luciferase construct (*PIG3-luc*) (containing a 1.4-kb promoter fragment [positions -861 to +546] encompassing the canonical p53 binding site between positions -330 and -309) (28), the *CD95* promoter-luciferase construct (*CD95-luc*) (containing bases -1435 to +236 of the human FAS/CD95 gene) (37), p63α, and p63γ were kind gifts from T. Soussi. T. Perlmann kindly provided us with plasmids encoding β-galactosidase and MH100 Gal4RE-luc. The promoter-luciferase constructs *mdm2-luc* (containing the promoter and upstream promoter sequence of human *mdm2*, including the two p53-responsive elements) (41), *bax-luc* (containing the fragment at positions -687 to -318 of the human *bax* promoter, including one p53-responsive element) (14, 22), *p21-luc* (containing 2.4 kb of the human *p21* promoter and upstream sequence, including one p53-responsive element) (14), and *cyclin G-luc* (1.48 kb of the *cyclin G* gene) (14) were kind gifts from M. Oren. All promoters were cloned into the pGL reporter vector (Promega), except for

the *CD95* promoter, which was cloned into a pGV-B vector (Wako PicaGene). Both vectors bear a simian virus 40 promoter upstream of the luciferase gene. Enhanced green fluorescent protein (EGFP) plasmid was obtained from Clontech, and pCMV-DsRed-Express (pDsRed) was obtained from BD Biosciences. Plasmid encoding wild-type p53 was kindly provided by B. Vogelstein. PKC and dominant negative PKC (DN-PKC) expression plasmids were kind gifts from Jae-Won Soh. The protein levels of p73, p63, and p53 variants after expression of the various cDNAs were examined by immunoblotting. There was no significant difference in the levels of protein (25) (see Fig. S3 in the supplemental material).

Immunofluorescence and laser-scanning confocal microscopy. One day after transfection with p73 plasmids, H82 cells were harvested, and cytospins were prepared. Cells were fixed in 4% paraformaldehyde (PFA) and blocked/permeabilized in phosphate-buffered saline (PBS) with 10 mM HEPES, 0.3% Triton X-100, and 3% bovine serum albumin. Slides were incubated with primary mouse anti-p73α/β antibody or primary rabbit anti-p73 antibody at room temperature for 4 h, followed by secondary Alexa Fluor 488-conjugated antibody (room temperature for 1 h). Subsequently, slides were incubated with primary rabbit anti-Bax antibody, primary mouse anti-Bax (6A7) antibody, or primary rabbit anti-Mdm2 antibody (4°C overnight) and then incubated with secondary Alexa Fluor 594-conjugated antibody (room temperature for 1 h). Nuclei were counterstained with Hoechst dye (1 μg/ml), and slides were mounted in Vectashield H-1000 (Vector Laboratories, Inc.) and analyzed under a Zeiss 510 Meta confocal laser scanning microscope equipped with an inverted Zeiss Axiovert 200m microscope. Mix dyes were acquired by sequential multiple-channel fluorescence scanning to avoid bleedthrough. Three independent experiments were made, and average values are shown. Error bars represent standard deviations (SD).

Apoptosis detection. H82 cells were cotransfected with plasmids encoding EGFP and p73 at a ratio of 1:10. One day after transfections, cells were treated with 5 μM VP16. At 24 h posttreatment, cells were stained with Hoechst dye and scored in a fluorescence microscope as the percentage of EGFP-expressing cells with condensed nuclei.

Flow cytometric analysis of BrdU incorporation. H82 cells were cotransfected with pCMV-DsRed-Express and p73 plasmids (ratio of 1:10). Forty-eight hours after transfection, cells were incubated with 10 μM BrdU for 15 min, harvested, and prepared according to a protocol for BrdU incorporation (BD Pharmingen). Samples were analyzed using a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences). For each sample, 10,000 cells sorted for red fluorescence were assessed for BrdU incorporation.

Cell cycle analysis. PC12 cells were cotransfected with EGFP and p73 expression plasmids (ratio of 1:5). Twenty-four hours posttransfection, cells were fixed in 1% PFA and subsequently frozen overnight in 95% ethanol. Thirty minutes prior to analysis, cells were resuspended in PBS with 50 μg/ml PI and 5 μg/ml RNase A. Samples were analyzed using a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences). In each sample, 10,000 cells sorted for green fluorescence were assayed.

Flow cytometric analysis of mdm2 expression. One day after cotransfection with pDsRed and p73 plasmids (ratio of 1:5), H82 cells were harvested, and cells were fixed in 1% PFA and blocked/permeabilized in PBS with 10 mM HEPES, 0.3% Triton X-100, and 3% bovine serum albumin. Cells were incubated with primary rabbit anti-mdm2 antibody at room temperature for 1 h, followed by secondary Alexa Fluor 488-conjugated antibody (room temperature for 30 min). Subsequently, cells were resuspended in PBS, and samples were analyzed using a FACSCalibur flow cytometer using Cell Quest software (BD Biosciences). For each sample, 10,000 cells sorted for red fluorescence were assessed for intensity of *mdm2* staining. Kolmogorov-Smirnov (K-S) statistics (CellQuest Software) were generated to compare the fluorescence intensity distributions between mock-transfected control and p73-transfected cells.

Reporter gene assays. Transfections were performed in 24-well plates with Lipofectamine 2000 according to the manufacturer's protocol. For promoter-luciferase constructs, each well was transfected with 100 ng of reporter plasmid (*mdm2*, *bax*, *p21*, *cyclin G*, *PIG3*, or *CD95*), 300 ng of p73 expression vector, and 100 ng of pCMV-β-gal reference plasmid containing a bacterial β-galactosidase gene. For Gal4-RE-luciferase constructs, each well was transfected with 100 ng reporter plasmid (MH100 Gal4RE-luc), 200 ng p73 expression vector, and 100 ng pCMX-β-gal reference plasmid. Where indicated, a total of 600 ng of the different PKC or DN-PKC expression vectors or 100 nM PKCα or PKCβ2 small interfering RNA (siRNA) was added to each transfection mixture. The same amount of DNA was added to each well. For PKC-412 treatment, 1 μM PKC-412 was added to cells 6 h after transfection. For VP16 treatment, a final concentration of 5 μM VP16 was added to cells 24 h after transfection and incubated for 6 h before harvesting. Cells were harvested 24 h after transfection and lysed, and extracts were assayed for luciferase and β-galactosidase activities in a microplate

luminometer/photometer reader (Orion microplate luminometer; Berthold Detection Systems). Values shown are representative of at least three independent experiments made in triplicate, with error bars representing SD.

Growth rate analysis. PC12 cells were transfected with mock or p73 vectors and treated with 500 μ g/ml Geneticin for 48 h. Transfected/surviving cells were counted and reseeded. Cell counts were then performed at 3, 6, and 9 days, as indicated, using a hemacytometer and/or a CellCoulter apparatus (BD).

Cell cycle synchronization. Nocodazole (50 ng/ml) or hydroxyurea (2 mM) was added to H82 cells 24 h after transfection, and cells were incubated for 12 or 15 h, respectively. Cells were washed to remove the nocodazole or hydroxyurea and left in culture for the indicated time periods. Cells were subsequently assayed for cell cycle phase using PI or BrdU staining and FACS analysis (as described above). For gene reporter assays, H82 cells were transfected with *mdm2*-luc, β -gal, and mock plasmid or TA-mutated p73 β . Twenty-four hours after transfections, nocodazole or hydroxyurea was added to cells and incubated for 12 or 15 h, respectively. Cells were washed and left in culture for the indicated time periods before being subjected to gene reporter analysis, as described above.

Immunoprecipitation and immunoblotting. Twenty-four hours after transfection with plasmids encoding p73 β , TAmut p73 β , and TAmut p73 β S388A, H82 cells were lysed in NP-40 buffer (150 mM sodium chloride, 1% NP-40, 50 mM Tris-HCl [pH 8]). Samples were precleared with protein G-Sepharose beads and then incubated with p73 antibody overnight. Protein G-Sepharose beads were added, and samples were incubated for 1 h. Beads were washed in radioimmunoprecipitation assay lysis buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl [pH 8]), resuspended in Laemmli loading buffer, and boiled for 3 min. Samples were resolved on 10% sodium dodecyl sulfate-polyacrylamide gels and blotted onto nitrocellulose membranes (Amersham Biosciences). Membranes were then probed with rabbit antiphosphoserine antibody and mouse antihemagglutinin antibody. Primary antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (Pierce). After repeated washing in Tris-buffered saline, bands were visualized by enhanced chemiluminescence (ECL Plus) according to the manufacturer's instructions (Amersham Biosciences) and analyzed using phosphorimaging (Image Reader LAS-1000 Pro V2.6; Fujifilm).

Chromatin immunoprecipitation. H82 cells were transfected with plasmids encoding p73 β , TAmut p73 β , and TAmut p73 β S388A. Chromatin immunoprecipitations were carried out using a ChIP assay kit (Upstate, Millipore) according to the manufacturer's protocol and p73 antibody (Ab-4). PCRs were performed using primers for the p21 promoter (forward primer 5'-GTG GCT CTG ATT GGC TTT CTG-3' and reverse primer 5'-CTG AAA ACA GGC AGC CCA AG-3'), the distal p21 promoter (2.8 kb upstream of the p53-responsive element) (forward primer 5'-GGA GTC CTG TTT GCT TCT GG-3' and reverse primer 5'-CTT TGG CCA CAC TGA GGA AT-3'), the *bax* promoter (forward primer 5'-TAA TCC CAG CGC TTT GGA AG-3' and reverse primer 5'-TGC AGA GAC CTG GAT CTA GCA-3'), the distal *bax* promoter (~2.5 kb upstream of the p53-responsive element) (forward primer 5'-GAC CTT GCT TTG CTC TAA GCT ATC-3' and reverse primer 5'-GAG CCT GTC TCA AAA AGA AAA AAG-3'), the *mdm2* promoter (forward primer 5'-GGT TGA CTC AGC TTT TCC TCT TG-3' and reverse primer 5'-GGA AAA TGC ATG GTT TAA ATA GCC-3'), and the distal *mdm2* promoter (~2.5 kb upstream of the p53-responsive element) (forward primer 5'-TGA ATC TAC TCT TGG TGG TCC-3' and reverse primer 5'-AAG GAA ATT TGG GCT TTC GAC-3'). Quantification of DNA band intensity was made using ImageJ software according to the supplier's instructions.

Statistical analysis. Statistical analyses were performed using a two-tailed, paired Student's *t* test and one-way analysis of variance (ANOVA), where *P* values of <0.01 and *P* values of <0.05 were considered to be significant.

RESULTS

The amino terminus TA domain of p73 is not required for TA of cell cycle-regulatory genes in H82 cells. The decision between cell cycle arrest and cell death might be due to different abilities of the expressed p73 isoforms to regulate these cell fates. In order to characterize the structural requirements of different p73 isoforms for their functions in the regulation of cell cycle progression and/or cell death, a set of p73 α and p73 β derivatives was tested on *bax* and *mdm2* promoters in luciferase gene reporter assays. Human SCLC H82 cells were transiently transfected with expression vectors for p73 α , p73 β ,

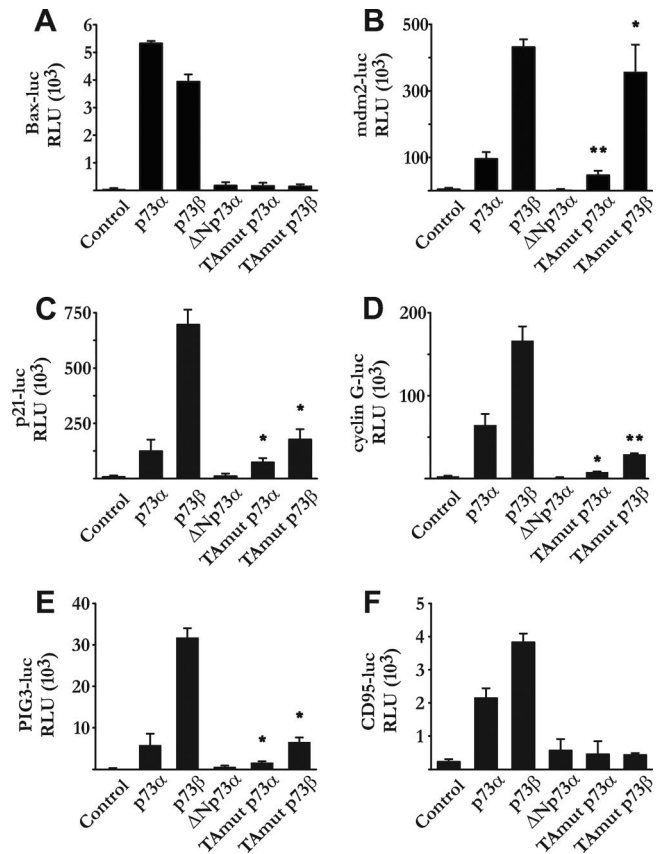


FIG. 1. The amino terminus TA domain of p73 is not required for transactivation of cell cycle-regulatory genes in H82 cells. H82 cells were cotransfected with promoter-luciferase vectors (*mdm2* [A], *bax* [B], *p21* [C], *cyclin G* [D], *PIG3* [E], and *CD95* [F]) and an empty vector or p73 expression vectors (p73 α , p73 β , Δ Np73 α , TAmut p73 α , or TAmut p73 β), as indicated. Cells were harvested after 24 h, and cell extracts were assayed for luciferase and β -galactosidase activities. Relative luciferase units (RLU) were compared after normalization to β -galactosidase activities. All promoters were cloned into pGL vectors, except CD95, which was cloned into the pGV-B vector. All vectors bear a simian virus 40 promoter upstream of the luciferase gene. Values shown are representatives of at least three independent experiments made in triplicate, with error bars representing SD. *, *P* < 0.05; **, *P* < 0.01 (Student's *t* test with ANOVA).

Δ Np73 α , TAmut p73 α , or TAmut p73 β (constructs carrying a point mutation in their NH₂ termini) (see Fig. S1 in the supplemental material), together with *mdm2* or *bax* promoter luciferase vectors. Interestingly, whereas TAmut p73 α and TAmut p73 β were inactive on the *bax* promoter (Fig. 1A), they retained significant transcriptional activity on the *mdm2* promoter (Fig. 1B).

To investigate whether the amino terminus TA domain (NH₂-TA)-independent transcriptional activity of p73 α and p73 β could have promoter specificity, luciferase gene reporter assays were performed on the *p21*, *cyclin G*, *PIG3*, and *CD95* promoters. The transcriptional activity of both NH₂-TA-mutated constructs was observed in *p21*, *cyclin G*, and *PIG3* reporter assays (Fig. 1C, D, and E), whereas none of the NH₂-TA domain-mutated constructs were active in gene reporter assays using the *CD95* promoter (Fig. 1F). The function of these genes in the regulation of either cell cycle or apoptosis

is well known. The role of *PIG3* is the least investigated; however, it appears that *PIG3* levels increase during p53-mediated growth arrest, and so far, the activation of *PIG3* has not been shown to be sufficient to cause apoptosis (13). Thus, the NH₂-TA-mutated constructs did not transactivate the promoters of the apoptosis-related genes *bax* and *CD95*. In contrast, both constructs were active on the promoters of the cell cycle-related genes *mdm2*, *p21*, and *PIG3*. Together, these results argue for an NH₂-TA-independent activity that is involved in the specific regulation of cell cycle-related genes.

Amino terminus TA domain-independent activity of p73 is cell type specific. Many functions of p73 depend on the cellular context. To investigate whether the NH₂-TA-independent transcriptional activity of p73 is cell type specific, four additional cell lines were tested: human neuroblastoma SH-SY5Y, rat pheochromocytoma PC12, human embryonic kidney HEK-293, and human cervical carcinoma HeLa cells. Cells were transiently transfected with expression vectors encoding p73 α , p73 β , Δ Np73 α , TAMut p73 α , or TAMut p73 β , together with *mdm2* or *bax* promoter-luciferase vectors. These experiments demonstrated that TAMut p73 α and TAMut p73 β are transcriptionally inactive on both promoters in HEK-293 and HeLa cells (Fig. 2A to D). However, both NH₂-TA-mutated p73 constructs were found to be active on the *mdm2* promoter in SH-SY5Y and PC12 cells (Fig. 2E and G). It is worth noting that these two cell lines share a neuroendocrine origin, as do H82 cells. Similarly to the observation in H82 cells, no effect of the NH₂-TA-mutated constructs was found in those cells on the *bax* promoter (Fig. 2F and H). These results demonstrated the existence of a cell type-specific NH₂-TA-independent transcriptional activity of p73 on the *mdm2* promoter.

Amino terminus TA domain-mutated p73 is able to induce expression of endogenous Mdm2. Our next aim was to uncover whether the NH₂-TA-independent activity of p73 could have effects on the expression of endogenous protein. Subsequently, H82 cells were transfected with plasmids expressing p73 α , p73 β , Δ Np73 α , TAMut p73 α , or TAMut p73 β and subjected to immunofluorescence staining using antibodies against p73 and Mdm2. Cells transfected with NH₂-TA-mutated p73 constructs exhibited an increased level of expression of endogenous Mdm2 protein, compared to that of mock-transfected control cells. The Δ Np73 α -transfected cells did not display an increase in endogenous Mdm2 protein levels, whereas cells transfected with either p73 α or p73 β did (Fig. 3A). Furthermore, the fluorescence staining intensity for Mdm2 in the images was quantified using the image analysis software from the confocal microscope. Results showed that cells transfected with either p73 α , p73 β , or NH₂-TA-mutated p73 displayed a higher level of endogenous Mdm2 protein than did control cells (Fig. 3B). Moreover, cells transfected with plasmids expressing p73 α , p73 β , Δ Np73 α , TAMut p73 α , or TAMut p73 β were subjected to immunofluorescence staining using antibodies against p73 and Bax (data not shown). Using the confocal image analysis module, Bax protein staining intensity in p73-expressing cells was compared to the fluorescence intensity in the nonexpressing cells. The p73 α - and p73 β -transfected cells, but not cells transfected with the NH₂-TA-mutated p73 constructs, displayed an increase in endogenous Bax protein expression (Fig. 3C). It is worth noting that the induction of endogenous Bax protein expression by p73 α and p73 β was not sufficient to

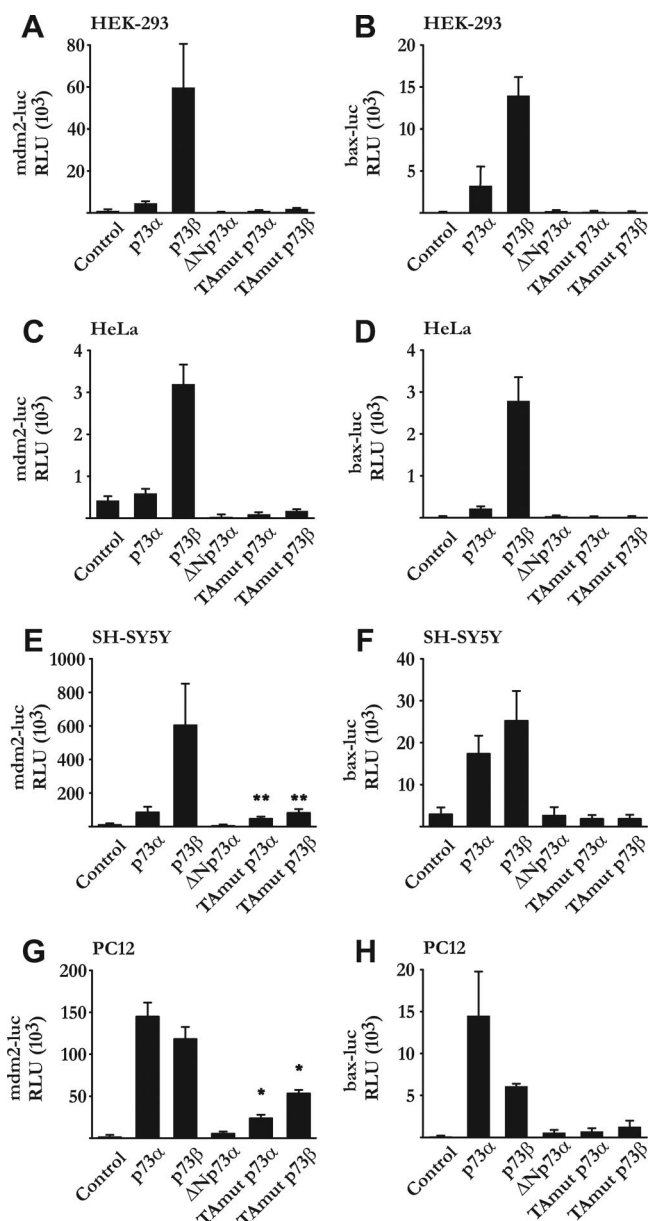


FIG. 2. Amino terminus TA domain-independent activity of p73 is cell type specific. HEK-293 (A and B), HeLa (C and D), SH-SY5Y (E and F), and PC12 (G and H) cells were cotransfected with *mdm2* (A, C, E, and G) or *bax* (B, D, F, and H) promoter-luciferase reporter gene plasmids and the indicated p73 expression vectors. Experiments were performed and results are presented as described in the legend to Fig. 1. RLU, relative luciferase units.

cause apoptosis and did not correlate with the conformational activation status of Bax (data not shown). Furthermore, H82 cells were cotransfected using pDsRed expression plasmid and p73 isoforms (ratio of 1:5) and subjected to immunofluorescence staining using antibody against Mdm2. pDsRed-expressing cells were sorted, and the fluorescence intensity of Mdm2 was analyzed using FACS. Cells transfected with plasmids expressing p73 α , p73 β , TAMut p73 α , or TAMut p73 β displayed a higher fluorescence intensity in Mdm2 staining than did mock-transfected control cells (Fig. 3D). Results are displayed

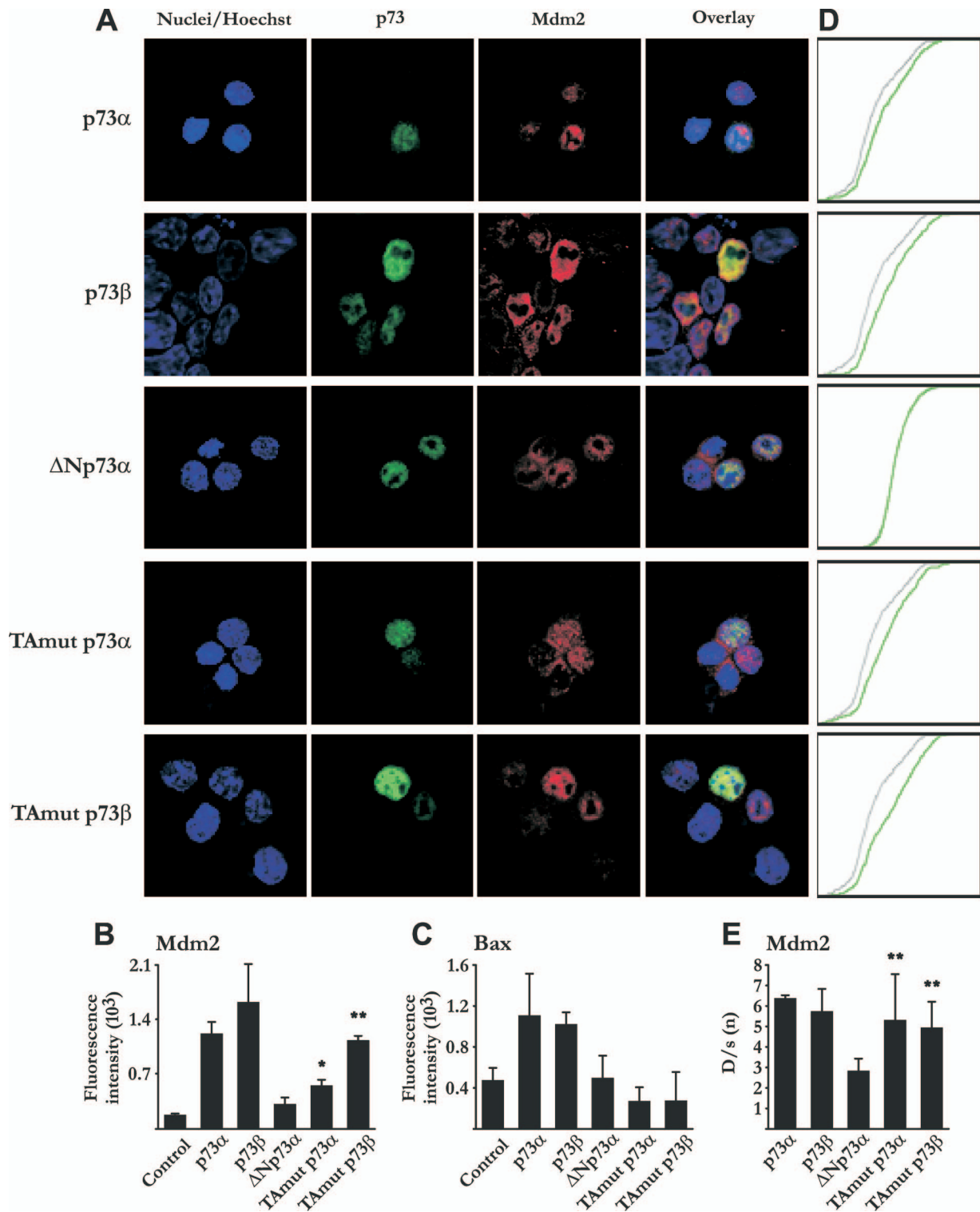


FIG. 3. Amino terminus TA domain-mutated p73 is able to induce expression of endogenous mdm2. H2 cells were transfected with different p73 expression vectors and stained with anti-p73 and anti-Mdm2 (A and B) or anti-Bax (C) antibodies. (A) An increased level of Mdm2 protein expression can be observed in cells expressing p73 α , p73 β , TAmut p73 α , and TAmut p73 β . Data are representative of three independent experiments. Using confocal image analysis software, the intensities of Mdm2 (B) and Bax (C) staining in p73-expressing cells were measured and compared to the intensity of protein staining in non-p73-expressing control cells. Values are means \pm SD. *, $P < 0.05$; **, $P < 0.01$ (Student's t test with ANOVA). H2 cells were cotransfected with pDsRed and p73 plasmids and stained with anti-mdm2 antibody (D and E). (D) Using FACS analysis and K-S statistics, an increase in mdm2 intensity can be observed upon expression of p73 α , p73 β , TAmut p73 α , and TAmut p73 β (dark gray line), compared to fluorescence intensity in mock-transfected control cells (light gray line). Data are representatives of at least three independent experiments. (E) Quantification of K-S statistics of at least three independent experiments. Values are means \pm SD. *, $P < 0.05$; **, $P < 0.01$ (Student's t test with ANOVA).

as K-S statistics, i.e., comparison of fluorescence intensity distributions between mock-transfected control cells and p73-transfected cells. The K-S statistics were quantified and showed that cells transfected with either p73 α , p73 β , or NH₂-TA-mutated p73 displayed a significant increase in the fluorescence intensity of Mdm2 compared to control cells (Fig. 3E). Altogether, these results show that the NH₂-TA domain is crucial for the transactivation of the proapoptotic gene *bax*. On the other hand, the transactivation of *mdm2* is independent of a functional NH₂-TA domain. Furthermore, these data suggested to us the possible existence of a TA domain within the carboxy terminus of p73.

Region between amino acid residues 381 and 399 defines a TA domain within the p73 carboxy terminus. In 1999, Takada et al. reported that the carboxy-terminal domain of p73 has TA abilities (35). To further investigate the presence of a potential TA domain in the carboxy terminus of p73 in our experimental system, we took advantage of GAL4 constructs, which represent a widely used in vitro detection approach to identify domain-specific activity in transcription. The transcriptional activity of the p73 domains containing amino acid residues 1 to 112 (p73/N) or 380 to 513 (p73/C) (see Fig. S1 in the supplemental material) was investigated. In all cell lines tested, the fusion construct GAL4-p73/N was active in transcription (Fig. 4A to E). In contrast, the GAL4-p73/C fusion constructs were active in transcription only in H82, SH-SY5Y, and PC12 cells (Fig. 4A to C). These results further strengthen the idea of a cell type-specific TA domain within the carboxy terminus of p73.

It is notable that although mutations in the p73 gene are rare, two missense mutations (P405R and P425L) located within the carboxy terminus have been described for tumors of the lung and in neuroblastoma (24). However, the effect of these naturally occurring point mutations on the transcriptional activity of p73 is still controversial. Indeed, the two mutations were reported to cause a significant reduction in the TA ability of the p73 carboxy-terminal domain (35). However, another group reported that the same mutations have no major effect on the transcriptional ability of the full-length proteins (24). Therefore, we investigated if the two mutations could have an effect on the carboxy terminus TA domain in our model system. Using the *mdm2* promoter-luciferase reporter gene assay, the activities of single (P405R or P425L) or double (TAmut and P405R or P425L) p73 mutants were investigated (see Fig. S1 in the supplemental material). None of the mutations alone (P405R or P425L) or in combination with NH₂-TA mutation had any significant effects on the transcriptional activity of p73 α or p73 β on the *mdm2* promoter (see Fig. S2 in the supplemental material). The observation that the two mutations P405R and P425L did not affect the activity of the carboxy-terminal TA domain led us to further investigate the carboxy-terminal amino acid residues needed for its transcriptional activity. Transcriptional activities of the p73 isoforms α , β , γ , δ , and ϵ (see Fig. S1 in the supplemental material), as well as a series of NH₂-TA p73 mutants, were studied using *mdm2*-luciferase reporter gene assay. In line with data from previous reports, p73 ϵ did not have any activity in transcription, whereas both p73 γ and p73 δ were active on the *mdm2* promoter (10, 36) (Fig. 4F). The NH₂-TA-mutated p73 α , p73 β , and p73 δ were transcriptionally active on the *mdm2* promoter, whereas TAmut p73 γ did not have any transcriptional activity. The only

common amino acids between the active carboxy-terminal region were residues 380 to 513 (GAL4 system) (Fig. 4A to C), and the shortest NH₂-TA domain-mutated p73 isoform (p73 δ) (Fig. 4F) found to be active in transcription were amino acids 380 to 399. Therefore, this suggested to us that amino acids 380 to 399 of p73 include the carboxy-terminal TA domain. Consequently, a set of experiments was performed to investigate the exact location of the carboxy-terminal TA domain. Carboxy-terminal p73 δ deletion mutants were made by the insertion of a stop codon at amino acid residue 381 (see Fig. S1 in the supplemental material). Whereas p73 δ Δ 380 and NH₂-TA-mutated p73 δ constructs were transcriptionally active, TAmut p73 δ Δ 380 was transcriptionally inactive at the *mdm2* promoter (Fig. 4G). These results provide further evidences that the carboxy-terminal TA domain is located in the region encompassing amino acid residues 381 to 399.

The carboxy-terminal TA domain does not enhance VP16-induced apoptosis. Our results demonstrated that the NH₂-TA-mutated p73 α and p73 β were able to activate the promoters of the cell cycle-regulatory genes *mdm2*, *p21*, *cyclin G*, and *PIG3* but not the promoters of the apoptosis-related genes *bax* and *CD95*. Therefore, we decided to investigate whether the induction of cell damage using the topoisomerase II inhibitor etoposide (VP16) could affect the transcriptional activity of the carboxy-terminal TA domain. For this purpose, H82 cells were transfected with the *mdm2*-luc reporter and p73 β , TAmut p73 β , p73 δ , or TAmut p73 δ ; treated with VP16 for 6 h; and analyzed for transcriptional activity. As shown in Fig. 5A, VP16 treatment had no significant effect on the transcriptional activity of the carboxy-terminal TA domain (Fig. 5A). We have previously reported that full-length p73 α repressed VP16-induced cell death in SCLC cells, whereas p73 β promoted drug-induced cell death (25). To investigate whether NH₂-TA-mutated p73 can affect the outcome of VP16-induced apoptosis, H82 cells were transfected with different p73 constructs along with an EGFP reporter plasmid and then treated with VP16 for 24 h. Similar to the repressive effect of p73 α and Δ Np73 α on drug-induced cell death, the NH₂-TA-mutated p73 α and p73 β did not increase VP16-induced cell death but, rather, repressed it (Fig. 5B). Altogether, these results confirm that the p73 NH₂-TA is required for the ability of p73 to enhance drug-induced apoptosis and furthermore that the transcriptional induction of *mdm2* by the carboxy-terminal TA domain is not affected upon VP16 treatment.

The carboxy-terminal TA domain regulates cell cycle progression. The selective regulation of cell cycle-related genes by the carboxy-terminal TA domain could be linked to the ability of p73 to control cell cycle progression. If the carboxy-terminal TA domain indeed controls cell cycle progression, one would expect its transcriptional activity to be regulated throughout the cell cycle. To test this possibility, H82 cells were transfected with the *mdm2*-luc reporter and TAmut p73 δ or the corresponding empty vector, synchronized using nocodazole, and analyzed using a luciferase gene reporter assay (Fig. 6A and B). Nocodazole treatment arrests cells at the G₂/M boundary of the cell cycle. After release from the nocodazole block, the proportion of cells in different phases of the cell cycle was monitored by FACS analysis at different time points using DNA content analysis upon PI staining (Fig. 6A). This revealed that the transcriptional activity of the carboxy-terminal

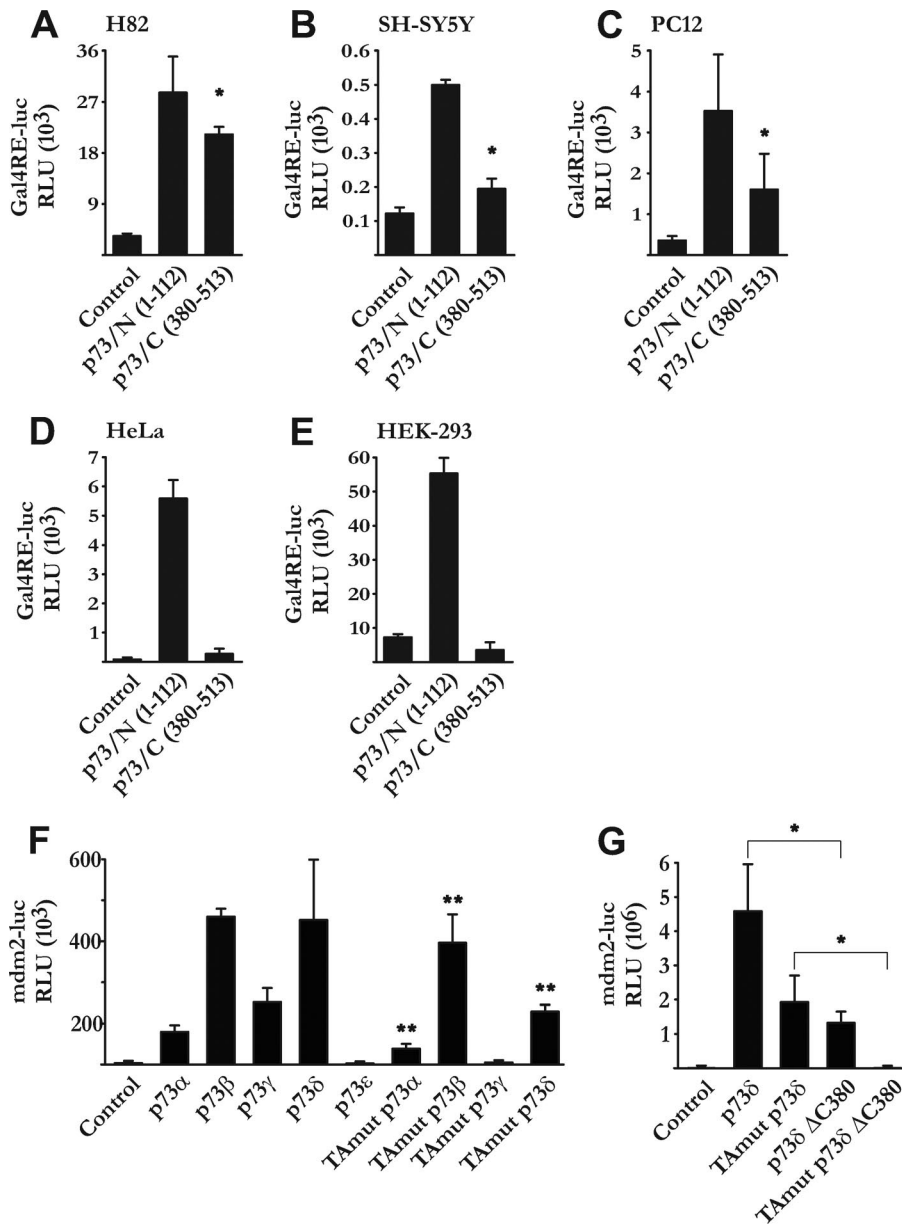


FIG. 4. Region between amino acid residues 381 and 399 defines a TA domain within the p73 carboxy terminus. H82 (A), SH-SY5Y (B), PC12 (C), HeLa (D), and HEK-293 (E) cells were cotransfected with MH100 Gal4RE-luc (containing a GAL4-responsive element) and p73/N or p73/C expression vectors. (F and G) The carboxy-terminal TA domain of p73 is located between amino acid residues 381 and 399. H82 cells were cotransfected with the *mdm2*-luc reporter and p73 isoforms α , β , γ , δ , and ϵ and their NH₂-TA domain-mutated counterparts (F) or with p73 δ , TAmut p73 δ , p73 δ Δ C380, or TAmut p73 δ Δ C380 (G). Experimental procedures were performed as described in the legend to Fig. 1. RLU, relative luciferase units.

TA domain seems to peak just before the cells enter the S phase of the cell cycle (18 h [Fig. 6A] and 15 h [B]). To further confirm the increase of transcriptional activity of the carboxy-terminal TA domain during entry into S phase, H82 cells were synchronized using hydroxyurea treatment, which arrests cells at the G₁/S boundary of the cell cycle (Fig. 6C and D). Upon release from the hydroxyurea block, accumulation of the cells in S phase was monitored by FACS analysis of BrdU incorporation and DNA content upon PI staining (Fig. 6C and data not shown). In line with the above-described results, maximum transcriptional activity of the carboxy-terminal TA domain was

observed at 3 h after washing, just before the cell population showed the maximal number of BrdU-positive cells (3 h [Fig. 6D] and 6 h [Fig. 6C] after wash). Thus, the transcriptional activity of the carboxy-terminal TA domain appears to be regulated throughout the cell cycle. Consequently, we decided to investigate the possible effect of different p73 constructs on cell cycle progression. H82 cells were cotransfected with the pDS-Red expression vector and various p73 constructs, pulsed with BrdU, and analyzed for BrdU incorporation. Both p73 α and p73 β expressions prevented cell cycle progression, as depicted by a decrease of the cell population in S phase compared to

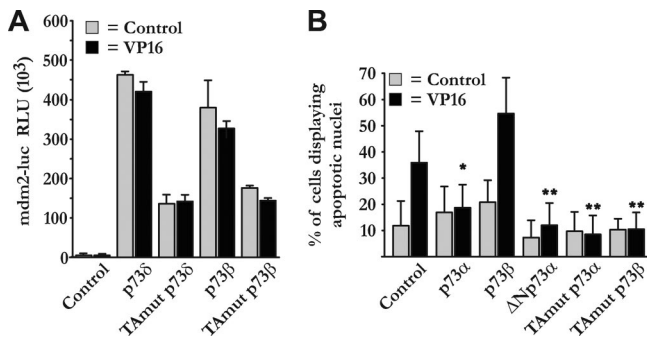


FIG. 5. The carboxy-terminal TA domain does not enhance VP16-induced apoptosis. (A) VP16-treatment does not affect the transcriptional activity of the carboxy-terminal TA domain. H82 cells were cotransfected with *mdm2-luc* reporter, p73 β , p73 δ , and their NH₂-TA domain-mutated counterparts and treated with VP16 for 6 h. Experimental procedures were performed as described in the legend to Fig. 1. RLU, relative luciferase units. (B) NH₂-TA-mutated p73 does not enhance DNA damage-induced apoptosis. H82 cells were cotransfected with EGFP- and p73-expressing plasmids (ratio 1:10), treated with VP16 for 24 h, and stained with Hoechst dye. Results are expressed as a percentage of EGFP-positive cells displaying apoptotic nuclei.

control cells (Fig. 6E). Importantly, similar effects could also be seen in cells expressing NH₂-TA-mutated p73 α and p73 β . p73 α and p73 β were previously reported to induce cell cycle arrest when overexpressed (42). To investigate if NH₂-TA-mutated p73 α and p73 β can also promote cell cycle arrest, PC12 cells were cotransfected with EGFP and p73 constructs and stained with PI. Subsequently, EGFP-expressing cells were sorted and analyzed for the distribution of cells throughout the cell cycle. The overexpression of both isoforms p73 α and p73 β induced cell cycle arrest at G₁ phase compared to mock-transfected control cells (Fig. 6F). Importantly, both NH₂-TA-mutated constructs also led to G₁ arrest. Finally, the effect of p73 expression on PC12 cell growth was also examined. The expression of p73 α , p73 β , and both NH₂-TA domain-mutated constructs exhibited significant repressive effects on the growth of PC12 cells (Fig. 6G). The transcriptional activity of the carboxy-terminal TA domain appears to be regulated throughout the cell cycle, and the NH₂-TA domain seems to play a negligible role in the inhibition of cell death, in the induction of cell cycle arrest, and in the repression of cell growth.

The carboxy-terminal TA domain is regulated by PKC phosphorylation. The finding of a sequence consisting of less than 20 amino acids that is able to regulate transcription from specific genes inspired us to investigate whether this region could have homology to TA domains in other proteins. Subsequent PROSITE searches revealed that this sequence contains a putative PKC phosphorylation site at amino residue serine 388. This site appeared to be conserved between p73 and its family member p63. Further BLAST searches also showed the whole carboxy terminus TA domain to be significantly conserved between the two proteins (Fig. 7A). To test if PKC-dependent phosphorylation could have any effects on the transcriptional activity of p73, H82 cells were transfected with plasmids encoding p73 α , p73 β , and their NH₂-TA domain-mutated counterparts and treated with the specific PKC inhibitor PKC-412. Treatment with PKC-412 drastically lowered the activities of

all p73 constructs tested on the *mdm2* promoter (Fig. 7B). The PKC inhibitor also reduced the transcriptional activity of p63 γ on the *mdm2* promoter (Fig. 7C) but had no effect on p53 transcriptional activity (Fig. 7D). PKC412 is a potent inhibitor of the classical PKC isoforms α , β 1, β 2, and γ (19). We therefore investigated the effect of each individual classical PKC isoform on the activity of the p73 carboxy terminus TA domain. In fact, PKC α and PKC β 2 expressions significantly enhanced the transcriptional activity of the p73 carboxy terminus (Fig. 7E). To further confirm the involvement of p73 phosphorylation by PKC α and PKC β 2 in enhancing p73 carboxy terminus activity in TA, cells were transfected with DN-PKC α , DN-PKC β 2, or a combination of both of them. Indeed, both DN-PKC α and DN-PKC β 2, alone or in combination, reduced the level of TA by p73/C (Fig. 7F). In addition, cells were transfected with siRNAs targeting either human PKC α or human PKC β 2 (Fig. 7G). Similar to the results shown in Fig. 7F, both siRNAs reduced the level of transactivation by p73/C (Fig. 7H). Hence, the transcriptional activity of the p73 carboxy-terminal TA domain seems to depend on its phosphorylation by PKC.

Modification of p73 serine 388 affects promoter binding and transcriptional activity of p73. In an attempt to investigate the importance of p73 phosphorylation at serine 388, this site was point mutated in TAmut p73 β (see Fig. S1 in the supplemental material). Mutation at serine residue 388 decreased the overall p73 serine phosphorylation, as shown by p73 immunoprecipitation followed by phosphorimaging (Fig. 8A). Moreover, this mutation significantly reduced the recruitment of NH₂-TA domain-mutated p73 β to the *mdm2* promoter but had no effect on binding to the *bax* promoter (Fig. 8B). Having identified a PKC phosphorylation site at serine 388 of p73 affecting the recruitment of p73 to the *mdm2* promoter, we decided to define the influence of this site on the transcriptional activity of the carboxy terminus TA domain of p73. The suppression of the phosphorylation site at serine 388 resulted in a substantial decrease in the TA ability of the p73 carboxy terminus TA domain on the *mdm2* promoter (about one-third of the activity detected with NH₂-TA domain-mutated p73 β) (Fig. 8C). These results reveal the important role for the PKC-dependent phosphorylation of p73 Ser388 in the transactivation ability of the carboxy terminus TA domain.

DISCUSSION

Despite the increasing number of p73 downstream target genes being identified, and the identification of several p73 posttranslational modifications, little is still known regarding how p73 may preferentially affect the expression of cell cycle-regulatory genes and thereby favor cell cycle arrest.

In this study, we report the characterization of a TA domain in the carboxy terminus of p73. In fact, in spite of a functional inactivation of the amino-terminal TA domain, the p73 isoforms α and β remained active on a set of target genes tested, i.e., *mdm2*, *p21*, *cyclin G*, and *PIG3*. Furthermore, we successfully mapped the location of the carboxy-terminal TA domain to amino acid residues 380 to 399. Previously, it was reported that both p73 and p63 carry potential TA abilities within their carboxy termini. GAL4 systems proved the potential carboxy-terminal TA domain to be located within exons 11 and 12 of

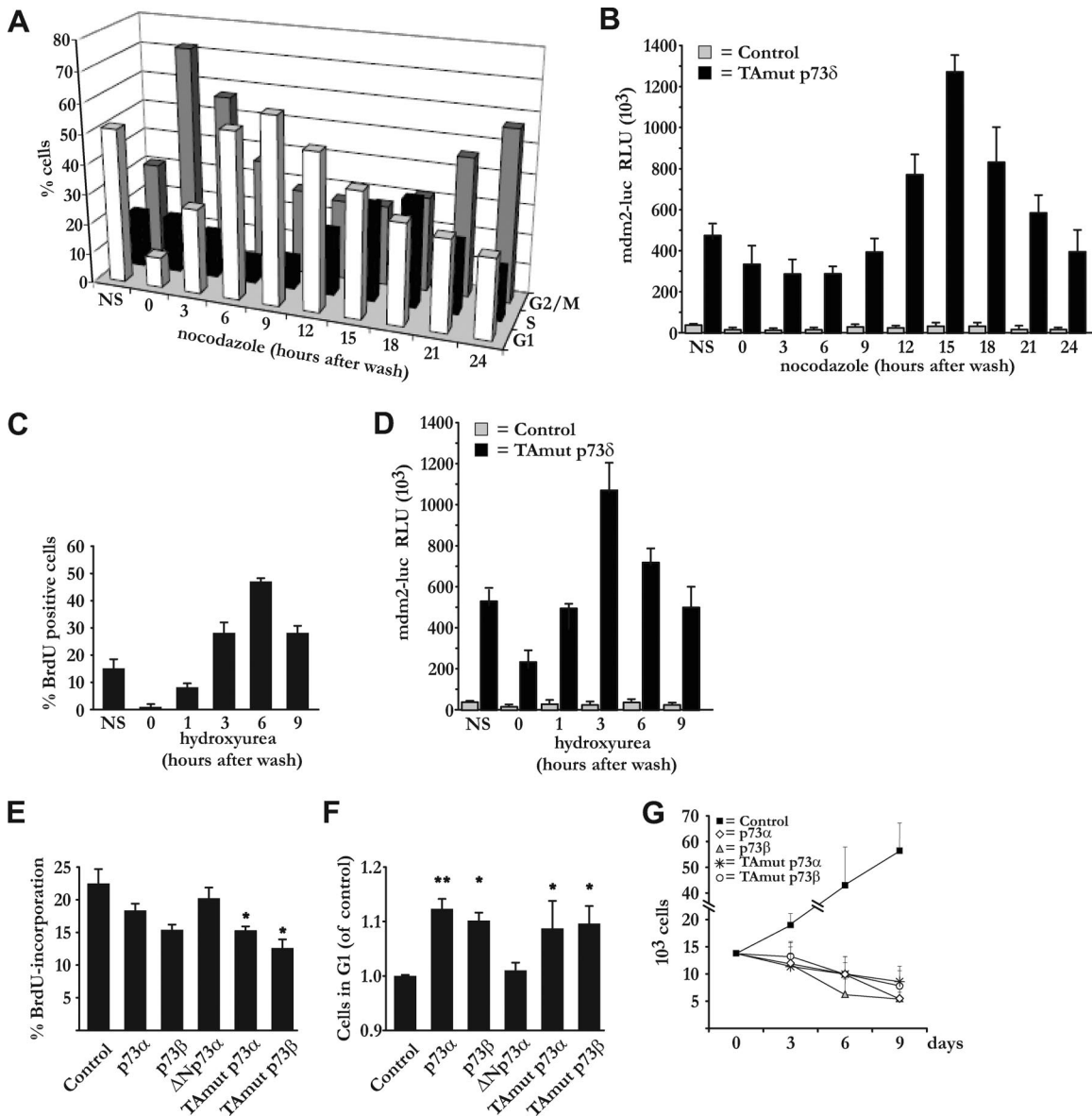


FIG. 6. The carboxy-terminal TA domain regulates cell cycle progression. (A) Nocodazole treatment arrests cells at the G_2/M boundary. H82 cells were mock transfected, treated with nocodazole for 12 h, washed, and analyzed using PI staining and FACS analysis for amounts of cells in the different phases of the cell cycle at different time points postwashing. (B) Transcriptional activity of the carboxy-terminal TA domain is dependent on the cell cycle phase. H82 cells were cotransfected with the *mdm2-luc* reporter and mock plasmid or NH_2 -TA-mutated p73 δ , treated, and analyzed as described above (A). (C) Hydroxyurea treatment arrests cells at the G_1/S boundary. H82 cells were mock transfected, treated with hydroxyurea for 15 h, washed, and analyzed using BrdU and FACS analysis for amounts of cells in S phase at different time points postwashing. (D) Transcriptional activity of the carboxy-terminal TA domain is dependent on the cell cycle phase. H82 cells were cotransfected with *mdm2-luc* reporter and mock plasmid or NH_2 -TA-mutated p73 δ , treated, and analyzed as described above (C). NS, nonsynchronized cells; RLU, relative luciferase units. (E) NH_2 -TA-mutated p73 can cause cell cycle arrest. H82 cells were cotransfected with pCMV-DsRed and p73 expression plasmids. Forty-eight hours posttransfection, cells were pulsed with 10 μ M BrdU and subjected to immunofluorescence staining prior to FACS analysis. Analysis was performed using 10,000 pCMV-DsRed-positive cells. (F) Overexpression of NH_2 -TA-mutated p73 causes G_1 -phase cell cycle arrest. PC12 cells were cotransfected with EGFP and p73 expression plasmids. Twenty-four hours posttransfection, cells were fixed and stained with PI. Analysis was performed on 10,000 EGFP-positive cells. Values are expressed as control of cells in G_1 phase. (G) Expression of NH_2 -TA-mutated p73 affects cell growth. PC12 cells were transfected with mock control plasmid or p73 plasmids, and transfected cells were selected using Geneticin. Cells were seeded and counted at 0, 3, 6, and 9 days as indicated. Counts of all cells transfected with p73 constructs differ significantly from those of mock-transfected control cells at days 3, 6, and 9 (p73 α , 3**, 6*, and 9*; p73 β , 3*, 6**, and 9*; TAmut p73 α , 3*, 6*, and 9*; and TAmut p73 β , 3*, 6*, and 9*). All graphs display a representative of at least three independent experiments. Values are means \pm SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test with ANOVA).

p63. In addition, the potential p73 carboxy-terminal TA domain was located in the corresponding sequence of p73, consisting of amino acid residues 380 to 499 (15, 35). Interestingly, the identified regions in p63 and p73 are enriched in proline

residues, a pattern frequently found in activation domains of many other transcription factors and known to play an important role in the regulation of RNA polymerase II activity (35).

It appears that several human tumor-derived p53 mutants

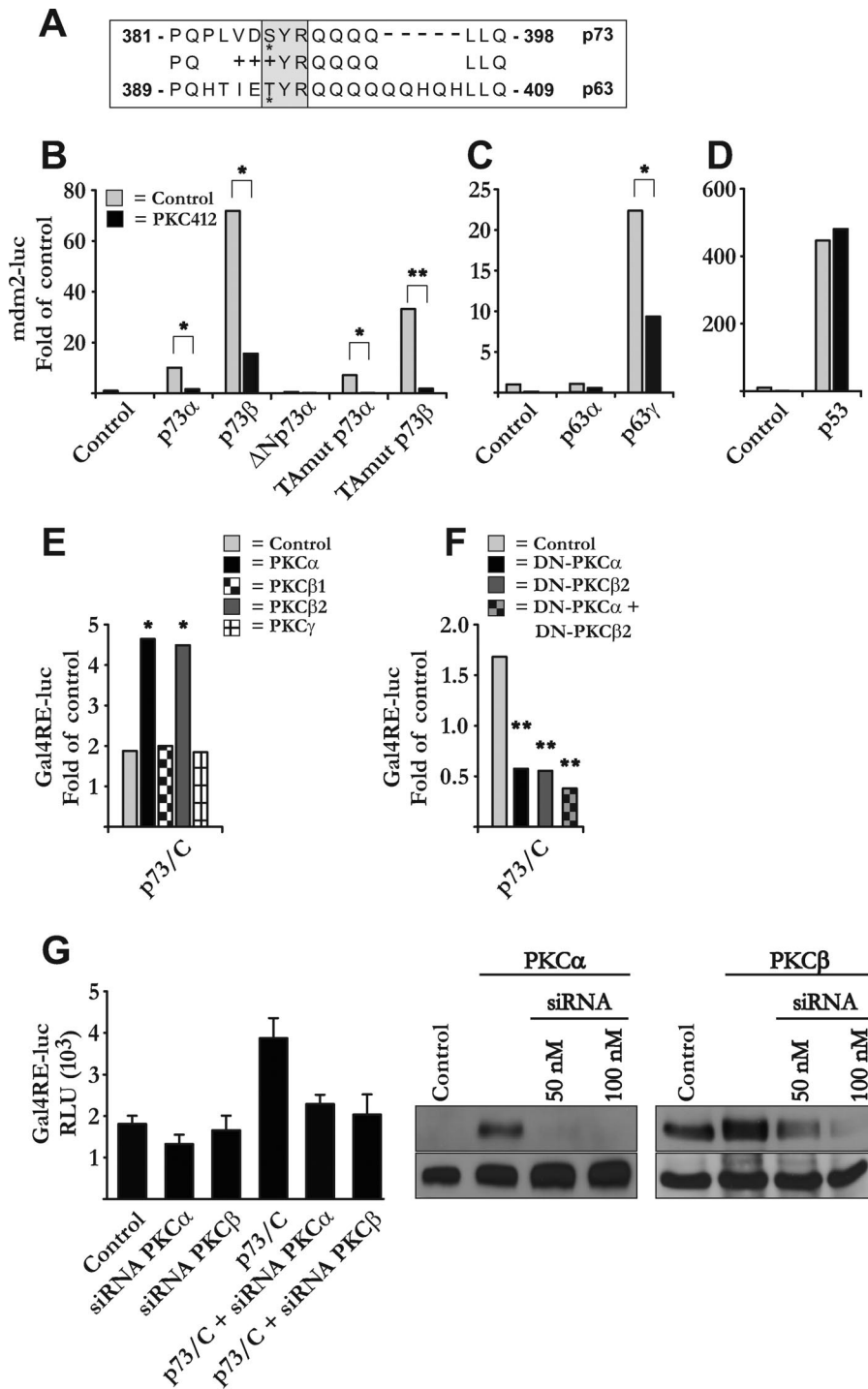


FIG. 7. The carboxy-terminal TA domain is regulated by PKC. (A) The carboxy-terminal TA domain and its internal PKC phosphorylation site are conserved between p73 and p63. A BLAST search was performed using amino acids 380 to 399 of p73. A further PROSITE search was performed on the same sequence as well as with the corresponding sequence in p63 found in BLAST. (B to D) The carboxy-terminal TA domain is regulated by PKC. H82 cells were transfected with the *mdm2* promoter-luciferase reporter gene and p73, p63, or p53 expression plasmids. At 6 h posttransfection, 100 nM phorbol myristate acetate or 1 μ M PKC-412 was added, and cells were incubated for a further 18 h. Experiments were performed as described in the legend of Fig. 1. Graphs display control of empty vector-transfected cells without treatment. (E to G) PKC α and PKC β 2 affect the p73 carboxy-terminal ability in TA. (E and F) H82 cells were transfected with GalRE-luc, p73/C, and different PKC and DN-PKC isoform expression vectors. Experiments were performed as described in the legend of Fig. 1. (G) H82 cells were transfected with 100 ng siRNA against PKC α or PKC β 2. Endogenous as well as overexpressed PKC α and PKC β 2 were sufficiently downregulated. H82 cells were transfected with GalRE-luc, p73/C, and siRNA against PKC α or PKC β 2. Experiments were performed as described in the legend to Fig. 1.

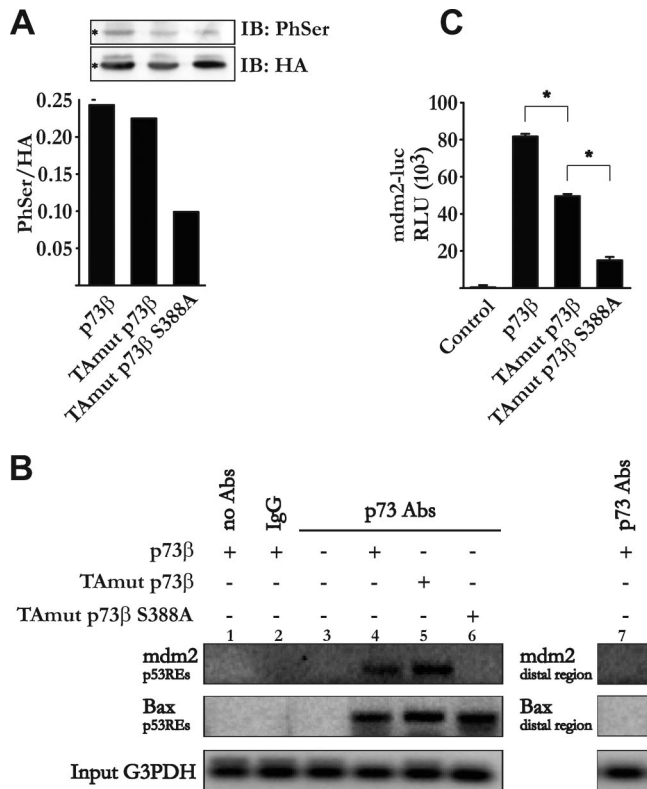


FIG. 8. Modification of p73 serine 388 affects promoter binding and transcriptional activity of p73. (A) p73 serine residue 388 is a potential PKC phosphorylation site. H82 cells were transfected with p73 β , TAMut p73 β , and TAMut p73 β S388A expression vectors. Samples were immunoprecipitated using p73 antibody, and the serine phosphorylation (PhSer) status was analyzed using Western blotting and phosphorimaging. IB, immunoblot. (B) p73 binding to the *mdm2* promoter is reduced by the p73 S388A mutation. H82 cells were transfected with p73 β , TAMut p73 β , and TAMut p73 β S388A expression vectors. Samples were immunoprecipitated with p73 antibody (Ab), and binding to promoters was detected using PCR. (C) Mutation of the PKC phosphorylation site at Ser388 renders TAMut p73 β inactive in transcription. H82 cells were transfected with the *mdm2*-luc reporter and p73-expressing plasmids. Experimental procedures are described in the legend to Fig. 1A. Quantification of the DNA band intensity (B) was done using ImageJ software and displays the intensity of bands in arbitrary units. All graphs display a representative of at least three independent experiments. Values are means \pm SD. *, $P < 0.05$; **, $P < 0.01$ (Student's *t* test with ANOVA). RLU, relative luciferase units.

have lost their ability to either transactivate proapoptotic genes (14, 31) or modulate genes involved in the regulation of cell cycle progression (32). The likely basis for p53 mutants that retain their ability to cause cell cycle arrest, but not apoptosis, is due to a decreased affinity for DNA. In fact, these mutants remain active only on promoters with high-affinity p53 binding sites, like in the *p21* promoter (6). Here, we report that the characterized p73 carboxy terminus TA domain selectively transactivates promoters of specific target genes known to be involved in cell cycle regulation but not promoters of apoptosis-related proteins. We found that the carboxy-terminal TA domain alone is sufficient to induce cell cycle arrest and repress cell growth and that its activity is regulated differentially throughout the cell cycle. In addition, we confirmed that the

p73 amino-terminal TA domain is crucial when it comes to enhancing drug-induced cell death (25).

Posttranslational phosphorylation has been reported to affect the activity of p73 and p53. More specifically, PKC has been shown to phosphorylate p73 serine 289 and p53 serine 378. In p73, phosphorylation at serine 289 increases the proapoptotic activity of the protein (30), whereas in p53, PKC-dependent phosphorylation at serine 378 enhances p53 sequence-specific DNA binding (21). Furthermore, phosphorylation of p73 at Tyr99 by c-Abl appeared to enhance p73 acetylation by p300 and thereby drive the protein toward the selective induction of proapoptotic target genes (8).

Here, we have identified a putative PKC phosphorylation site at serine 388 located within the characterized carboxy terminus TA domain. PKC isoforms have been suggested to play a central role in various cellular processes like apoptosis, differentiation, and cell cycle progression. The observation that different PKC isoforms are specifically expressed in certain cell types suggests that PKC could have some cell type-specific effect (4). Furthermore, we show that the selective PKC inhibitor PKC-412, and/or a mutation at serine 388, can markedly reduce the ability of p73 to transactivate the *mdm2* promoter. In addition, coexpression with PKC isoform α or β 2 enhances the ability of p73 to transactivate the *mdm2* promoter, whereas the coexpression of either DN-PKC α or -PKC β 2, or siRNA against PKC α or PKC β 2, represses this activity. Interestingly, the identified PKC phosphorylation site is present in p63, located within the putative carboxy-terminal TA domain (15). Whereas PKC-412 exerted a repressive effect on the transcriptional activity of p63, it had no effect on the transcriptional activity of p53.

In vivo studies have revealed that both p63- and p73-deficient mice suffer from severe developmental defects, but they show no increased susceptibility to spontaneous tumorigenesis. In contrast, only a small percentage of p53 knockout mice show developmental defects; rather, these mice are particularly prone to developing tumors (39). Connecting our study to reports from in vivo studies of knockout mice, one might therefore speculate that the characterized carboxy-terminal TA domain, or the lack of it, contributes to the phenotypes observed in p73- and p63-deficient mice.

p73 has been described as a multifunctional protein. The protein's various functions might depend on different posttranslational modifications. In fact, it was previously shown that DNA damage-dependent acetylation of p73 potentiates its apoptotic function. Here, we show that the PKC-dependent phosphorylation of the carboxy-terminal TA domain promotes the selective induction of cell cycle-related genes. These findings indicate that specific posttranslational modifications of different domains of p73 are regulatory events underlying the mechanisms to promote either cell cycle arrest or apoptosis.

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