The p73 gene, a member of the p53 family, encodes several variants through differential splicing and use of alternative promoters. At the NH2 terminus, two different promoters generate the full-length and the ΔN isoforms, with or without the transactivating domain. At the COOH terminus, seven isoforms generated through alternative splicing have been cloned. Previous studies have demonstrated that ΔNp73 isoforms exert a dominant-negative effect on p73 by blocking their transactivation activity and hence the ability to induce apoptosis. Considerable efforts are made to identify the functional diversity of the COOH-terminal p73 variants. In this study, we found that p73α inhibited drug-induced apoptosis in small cell lung carcinoma cells, whereas p73β promoted it. p73α prevented Bax activation, mitochondrial dysfunction, and caspase activation. In addition, p73α was also able to reduce apoptosis induced by the BH3-only protein PUMA (p53 up-regulated modulator of apoptosis). Furthermore, we discovered that p73α is able to inhibit the pro-apoptotic effect of p73β, demonstrating the existence of equilibrium between these two p73 isoforms. In conclusion, the reported overexpression of p73α in certain tumor types, and our findings that p73α exerts anti-apoptotic functions, indicate a potential oncogenic activity for p73.

p73 is a transcription factor, which has been shown to elicit cell-cycle arrest, apoptosis, neuronal, epidermal, myogenic, and oligodendrogliocyte differentiation (1–5). The p73 gene along with p53 and p63 constitute the p53 family (6). p73 can activate the transcription of p53-responsive genes and inhibit cell growth in a p53-like manner by inducing apoptosis. It can substitute for p53 when the latter is deleted or mutated. p73 has a high sequence homology with p53 within the amino-terminal transactivation domain (TA), the sequence specific DNA-binding domain (DBD), and the oligomerization domain (OD). In fact, like p53, p73 induces cell growth arrest (1, 6), activates the transcription of some endogenous p53 target genes such as p21Waf1, mdm2, bax, cyclin G, GADD4, IGF-BP3, and 14-3-3σ (7), and induces apoptosis irrespective of p53 status (1, 6). However, despite similarities with p53, it is clear that p73 presents significant differences in terms of regulation and function. In contrast to p53-deficient mice, the p73-null mice show no increased susceptibility to spontaneous tumorigenesis (8). On the other hand, they exhibit profound developmental defects, including dysgenesis in the brain (9).

In contrast to the human p53 gene, which is found to encode one protein, the human p73 gene gives rise to 14 isoforms by both alternative splicing and by the use of alternative promoters (p73α-η) and ΔNp73(α-η) (7). The isoforms generated by two promoters are called p73 and ΔNp73, the former contains a TA domain and the latter has a shorter amino terminus without the TA domain. In addition, alternative splicing generates at least seven transcripts with different carboxyl terminal mini (α, β, γ, δ, ε, ζ, and η). p73α is the longest form of the p73 proteins, and contains in its carboxyl-terminal extension a sterile α motif (SAM) domain. p73β is a smaller polypeptide, missing the extreme carboxy-terminal region and most of the SAM domain present in p73α (6, 10). Of all the p73 isoforms, p73β seems to be the most potent in transactivating and growth suppression. p73α, p73β, and p73η are less active than p73β, whereas p73γ and p73ε are ineffective in this respect (11, 12).

Whereas p73 isoforms function as transcription factors that might induce cell cycle arrest, differentiation, and apoptosis, the ΔNp73 isoforms that lack the TA domain are incapable of activating transcription and do not induce growth arrest or cell death. However, the ΔNp73 variants have a very important regulatory role, as they exert a dominant-negative effect on p53 and p73 by blocking their transactivation activity, and hence their ability to induce apoptosis (13). This scenario suggests that the p73 gene exemplifies the “two genes in one” idea with products that play opposite roles. Their impact on cell proliferation, differentiation, and cell death might therefore depend on the balance between “pro” p73 isoforms and the “anti” ΔNp73 isoforms. p73α, which is most abundantly expressed in many tissues and cells among the alternatively spliced forms of p73, has an additional long carboxyl-terminal tail that might explain the distinct function of p53 and p73α or other p73 splicing variants. The SAM domain is found in p73-related proteins, and in several unrelated proteins that are involved in the regulation of development (10). In some signaling proteins, the SAM domain is involved in protein-protein interactions. However, it is not clear whether the SAM domain in p73α has similar activity.

A current idea is that the diverse functions of p73 in controlling cell proliferation, differentiation, and cell death, might be because of the different ability of its isoforms to regulate these processes. In the attempt of delineating the functions of the different p73 isoforms, we found that, in small cell lung carcinoma cells (SCLC), p73α inhibits drug-induced apoptotic cell death. On the contrary, p73β enhances that process. The observed inhibitory effect is dependent on the carboxyl terminus region of p73α containing the SAM domain and required the
p73α Inhibits Apoptosis in SCLC Cells

DNA binding domain. The inhibition of cell death occurs upstream of caspase activation and mitochondrial events, such as cytochrome c release, suggesting that p73α acts at the initiation phase of apoptosis and has a rather broad range inhibitory effect preventing the caspase- and mitochondria-dependent cell death pathways. Furthermore, we observed that p73α is able to inhibit the pro-apoptotic effect of p73β and, inversely, that p73β could counteract the anti-apoptotic function of p73α, demonstrating an equilibrium between these two isoforms. In addition to the previously described antagonistic effect of ΔNp73 on the p73 isoforms, our finding of the existence of pro- and anti-apoptotic members within the p73 variants reveal the complexity of the outcome of p73 gene expression.

MATERIALS AND METHODS

Reagents—Epitope (VP16) and cisplatin were both from Bristol-Myers. Staurosporine (STS) and MitoTracker Red CM-H2XRos were purchased from Sigma and Molecular Probes, respectively.

Plasmids—Human p73 isoforms, p73α transcription inactive mutants, and the Δ84p73β mutant were kind gifts from Dr. G. Melino and have been described (3, 11). The p73α DBD mutant having amino acid substitutions at positions 268 and 300 on the DNA binding domain was a generous gift from Dr. S. Ded (14). Carboxyl-terminus-deleted p73α mutants, p73αΔC565, p73αΔC505, p73αΔC424, and ΔODP73α were kind gifts from Dr. M. Hiji (15). Plasmid encoding PUMA and the (−336/+157) PUMA-luc reporter plasmid were a kind gift from Dr. B. Vogelstein (16). Enhanced green fluorescent protein (EGFP) plasmid was from Clontech. Protein expression level and subcellular localization of each p73 variant used in these studies was examined. No significant difference in their expression at the protein level was noticed. In addition, their localization was found to be mainly nuclear (Figs. 2 and 4, and data not shown).

Cell Culture, Transfection, and Treatments—The human small cell lung carcinoma cell lines, NCI-H82 (ATCC HTB-175) and U1285, human non-small cell lung carcinoma cell line, H1299 (ATCC CRL-5803), and human osteosarcoma cell line, Saos-2 (ATCC HTB-85), were used in this study. Cells were cultured at 37 °C, 5% CO2 in RPMI 1640 medium (NCI-H82 and U1285) or Dulbecco’s modified Eagle’s medium (NCI-H82 and Saos-2), both 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 setting in culture dishes with fresh medium, cells were transfected. Transfections were performed in 24-well plates with Lipofectamine PLUS for Saos-2 and H1299 cells or with Lipofectamine 2000 for H82 and U1285 cells, according to the manufacturer’s protocol. Twenty-four hours after transfection cells were treated with 5 μM VP16, 1 μM STS, or 40 μM cisplatin. The cell density was kept at levels allowing exponential growth.

Western Blot Analysis—Laemmli’s loading buffer (100 μl/106 cells) was added to harvested cells and samples were boiled for 3 min. Thirty μl of protein extracts were resolved on a 12% SDS-polyacrylamide gel and transblotted onto nitrocellulose membranes (Amersham Biosciences). Membranes were then probed with a monoclonal antibody anti-p73α/β (Ab-4; NeoMarker) or monoclonal antibody anti-HA (Roche Diagnostics). Immuno blot with anti-glyceraldehyde-3-phosphate dehydrogenase rabbit polyclonal antibody (Trevena) was used for standardization of protein loading. Primary antibody binding was detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgGs (Pierce). After repeated washing in phosphate-buffered saline bands were visualized by enhanced chemiluminescence (ECL Plus) following the manufacturer’s instructions (Amersham Biosciences).

Immunofluorescence and Confocal Microscopy—At the indicated time points post-treatment, 15 × 10⁴ H82 cells were harvested, and cytospins were prepared. Slides were stored at −20 °C and thawed for 1 h prior to staining. The following antibodies were used in these studies: monoclonal anti-p73α/β (Ab-4; clones ER-13 + ER-15 + GC-15, NeoMarker), polyclonal anti-p73α/β (Chemicon), polyclonal anti-AIF (Santa Cruz Biotechnology), polyclonal anti-active Bax (clone 6A7, BD Pharmingen), polyclonal anti-cleaved (Asp⁷³⁷) caspase-3 (Cell Signaling), monoclonal anti-cytochrome c (clone 6H2-B4; BD Pharmingen), polyclonal anti-cleaved PARP (Cell Signaling), and polyclonal anti-PUMA (Cell Signaling). Alexa Fluor 488- or 594-conjugated anti-IgG were used as secondary antibodies (Molecular Probes). Paraformaldehyde-fixed cells and sections were blocked in HEPES, 3% bovine serum albumin, 0.3% Triton and incubated with primary (4 °C, overnight) and secondary antibodies (room temperature, 1 h). Cells nuclei were counterstained with DAPI (1 μg/ml). Samples were mounted with 90% glycerol in phosphate-buffered saline and analyzed under Leica TCS SP2 AOBS confocal laser scanning microscopy (Leica Microsystems, Heidelberg, Germany) equipped with an inverted Leica DM IFR microscope with an ×63 oil immersion objective. Mix dyes were acquired by sequential multiple channel fluorescence scanning to avoid bleedthrough. Routinely, 0.15–0.20-μm thick focal planes were scanned.

Assessment of Mitochondrial Depolarization—After treatment at the indicated time points, the mitochondrial transmembrane potential was determined using MitoTracker Red CM-H₂XRos (Molecular Probes), a cationic, lipophilic fluorochrome dye that is retained in the negatively charged mitochondrial matrix but is lost if the mitochondrial inner membrane potential is lost. Staining was done following the manufacturer’s instructions. Briefly, cells were incubated for 30 min under growing conditions with 200 nM MitoTracker Red CM-H₂XRos. Cells were washed in growth medium, and fixed in the same medium containing 3.7% formaldehyde for 15 min. After fixation, cytosin preparations were made and immunofluorescence assay for p73 was performed as described above.

Cytocfluorometric analysis of mitochondrial membrane depolarization was assessed by uptake of TMRE (Molecular Probes), a lipophilic, cationic fluorochrome dye that is only taken up by mitochondria having an intact electrochemical gradient. Following TMRE exposure (added 30 min before harvesting to a final concentration of 25 nM), cells were centrifuged and resuspended in a TMRE-containing buffer (10 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, and 25 mM TMRE). Analysis was carried out on 10,000 gated EGFP-expressing cells using a FACSAbudor flow cytometer equipped with CellQuest software (BD Biosciences).

Flow Cytometric Analysis of Bax Activation—Bax-associated conformational changes were assessed as previously described (17). Briefly, after fixation (0.25% paraformaldehyde, 5 min) and washing, cells were incubated for 30 min in the presence of digitonin (100 μg/ml) with antibodies recognizing NH₂-terminal epitopes of Bax (clone 6A7; BD Pharmingen). After incubation with Alexa Fluor 488-conjugated antimouse antibody for 30 min, cells (10,000 per sample) were analyzed on a FACScan flow cytometer, using CellQuest software.

Caspase Activity Assay—Activities of caspase-3-like enzymes and caspase-9 were determined fluorometrically using DEVD-AMC and LEHD-AMC, respectively, as substrates in a buffer containing 100 mM HEPES, 0.1% CHAPS, 5 mM dithiothreitol, 10% Nonidet P-40, and 1% sucrose (DEVD-AMC) or 10% polyethylene glycol (LEHD-AMC), as previously described (18).
RESULTS

p73α Expression Inhibits Drug-induced Apoptosis in SCLC Cells—At present, at least seven alternatively spliced p73 mRNAs named α, β, γ, δ, ε, ζ, and η have been identified. However, at the protein level α and β variants are the most abundant (for review see Ref. 7). To investigate the ability of these two most expressed p73 isoforms to modulate apoptotic cell death we used a panel of cell lines, namely, SCLC H82 and U1285, non-SCLC H1299, and osteocarcinoma Saos2. H82, U1285, H1299, and Saos2 are p53-null cells, and do not show detectable levels of p73 (Refs. 19 and 20, and data not shown). These cells are therefore a convenient experimental model for evaluation of induction of apoptosis by p73. The different cell lines were transiently transfected with expression vector for p73α, p73β, and ΔNp73α. H82, U1285, and H1299 cells were then treated for 24 h with etoposide (VP16), and Saos2 cells (insensitive to VP16 treatment) for 4 h with STS. Full-length p73 isoforms induced apoptosis, and potentiated drug-induced cell death in H1299 and Saos2 cells. On the contrary, ΔNp73α reduced both spontaneous and drug-induced cell death in these cells (Fig. 1, C and D). Similar effects were observed with embryonic kidney 293 and neuroblastoma SH-SY5Y cells (data not shown). p73α, p73β, and ΔNp73α expression had only minor effects on the basal level of apoptosis in the H82 and U1285 cells. However, whereas p73β potentiated VP16-induced cell death, both the amino terminus-truncated ΔNp73α and the full-length p73α isoforms exhibited inhibitory effects (Fig. 1, A and B). The contrasting effects of p73α, p73β, and ΔNp73α on apoptosis were not because of differences in their level of expression or subcellular localization (Fig. 2, and data not shown). p73α appears to act in a cell type-specific manner, acting either as a pro-apoptotic (in Saos2, H1299, HEK-293, and SH-SY5Y cells), or as an anti-apoptotic molecule (in H82 and U1285 cells). Next we examined whether the inhibitory effect of p73α, observed in SCLC cells, was dependent on the type of treatment used. p73α was able to reduce the ability of cisplatin and STS to induce cell death in H82 cells, suggesting that it had a broad range inhibitory effect (Fig. 1, E and F).

The Amino-terminal Activation Domain Is Not Required for the Anti-apoptotic Activity of p73α in H82 Cells—Full-length p73 isoforms function as transcription factors and regulate the expression of groups of genes by means of direct binding to what was originally identified as the p53-binding site within promoters. To determine whether the transcriptional activity domain of p73α is necessary to inhibit apoptotic cell death, a TAmutp73α construct carrying a point mutation in the TA region, which inactivates this domain, was used (Ref. 3 and data not shown). This mutation did not affect the anti-apoptotic function of p73α. These results pointed out that a functional TA domain is not required for the anti-apoptotic effect of p73α in H82 cells (Fig. 2, A and B). In contrast, TAmutp73β, with a similar mutation was unable to increase VP16-induced cell death, demonstrating that the TA domain of p73β is necessary for its pro-apoptotic function in H82 cells.

The DNA Binding Domain and SAM Motif Containing Carboxyl Terminus Are Required for the Anti-apoptotic Activity of p73α in H82 Cells—Because it appeared that the TA domain of p73α is not required for its anti-apoptotic effect it was important to understand whether binding to DNA is essential for this function. A DBDmutp73α construct with a double point mutation in the DNA binding domain (14) was not able to modulate apoptosis induced by VP16, suggesting that this domain of p73α and possibly its binding to sequences into the promoter region of target genes are necessary for its inhibitory effect.

Because both p73α and ΔNp73α present inhibitory functions, we hypothesized that these functions should be at least in part carried by their common COOH terminus. To prove this idea we took advantage of...
of the deletion construct Δ84p73β, which lacks the TA domain and the carboxyl terminus extension found in p73α. This construct was unable to modulate drug-induced apoptosis, demonstrating that the COOH terminus of p73α and even ΔNp73α are required for their anti-apoptotic function in H82 cells (Fig. 2B). ΔNp73α has been reported to exert a dominant-negative effect on p53 and p73 by blocking their transactivation function hence their ability to induce apoptosis. However, it seems that in H82 cells the anti-apoptotic function of ΔNp73α is largely because of its carboxyl terminus extension, even if transactivation dominant-negative activity may participate. To further investigate the requirement of the p73α carboxyl-terminal region, a series of p73α truncation mutants, including p73αΔC424 (lacking the entire carboxyl-terminal region), p73αΔC505 (lacking the last 131 residues, including part of the SAM domain), and p73αΔC566 (lacking the last 70 residues, deleting an extreme carboxyl-terminal region but with an intact SAM domain), were used (15). Interestingly, whereas p73αΔC566 showed a similar inhibitory effect as p73α, both p73αΔC424 and p73αΔC505, which lack part or the entire SAM motif, were incapable of suppressing VP16-induced apoptosis (Fig. 2, C and D). It is of note that p73αΔC505, which is structurally close to the p73β isoform did not promote apoptosis, in H82 cells. This could be because of different conformations or post-translational modifications inherent to the five unique residues at

FIGURE 2. p73α DNA binding domain and sterile α motif containing carboxyl terminus are required for its anti-apoptotic function in H82 cells. A schematic representation of p73α constructs used and their protein expression levels are depicted. Asterisks (*) localize point mutations (A and C). DNA binding domain is required for p73α anti-apoptotic functions (A and B). H82 cells were transfected with expression vectors encoding p73α, p73β, ΔNp73α, TAmutp73α, TAmutp73β, DBDMutp73α, or Δ84p73β. Twenty-four hours post-transfection cells were treated with 5 μM VP16 for 24 h. Apoptosis was assayed and scored as described in the legend to Fig. 1. The panels represent the mean values of three experiments, each performed in duplicate. SAM containing domain is required for p73α anti-apoptotic functions (C and D). H82 cells were transfected with expression vectors encoding p73α, p73β, p73αΔC566, p73αΔC505, or p73αΔC424. Twenty-four hours post-transfection cells were treated with 5 μM VP16 for 24 h. Apoptosis was assayed and scored as described in the legend to Fig. 1. The panels represent the mean values of three experiments, each performed in duplicate.
the end of the β isoform, or to the 6 last amino acids coming from the carboxyl terminal region of p73α are involved in the inhibition of cell death.

**The DNA Binding Domain and Transactivation Domain Are Required for the Pro-apoptotic Activity of p73α in H1299 Cells**—To determine whether the death promoting effects of p73α also depend upon the same domains as the survival ones, we analyzed the effect of p73α constructs with different point mutations and deletions, mentioned above, on VP16-induced apoptosis in H1299 cells. Whereas both DBD and TA domains appeared to be essential for the pro-apoptotic effects of p73α, the unique carboxyl terminus of p73α seems dispensable (Fig. 3).

**p73α Prevents Caspase-3 Activation and PARP Cleavage in H82 Cells**—A set of experiments was performed to identify the level at which p73α inhibited the apoptotic cell death program. Previously we have shown that in SCLC cells the executioner caspase-3 is activated upon VP16 treatment and translocates to the nucleus where it cleaves several substrates, including PARP (21). The resistance of H82 cells to undergo VP16-induced apoptosis in the presence of p73α isoforms could be caused by an inhibition of this execution phase. To confirm this possibility, a staining for p73α/β, active caspase-3, and nuclei followed by confocal immunofluorescence microscopy analysis was performed in H82 cells (Fig. 4, A and B). Whereas, upon VP16 treatment, almost all p73β-transfected cells were characterized by nuclear staining for active caspase-3, only a few ΔNp73α- and p73α-transfected cells showed signs of caspase-3 activation. In addition, a second triple staining with antibodies against cleaved PARP and p73α/β isoforms, and nuclei, was performed to demonstrate the lack of caspase-3 activation. Negative staining for cleaved PARP clearly confirmed the absence of proteolysis of this caspase-3 substrate (Fig. 4, C and D). p73β increased the number of cells with a positive staining for cleaved PARP, whereas both p73α isoforms decreased it. These data demonstrated that the execution phase of the apoptotic program was not initiated in p73α isoforms expressing cells.

**p73α Prevents Mitochondrial Dysfunctions in H82 Cells**—Mitochondria play an important role in the regulation of apoptosis. Specifically, the release of several proteins, normally located in the intermembrane space of mitochondria, has been observed during the early stages of apoptotic cell death (22). Among these proteins is cytochrome c, which being released into the cytosol involves in the activation of caspase cascade and thereby triggers the execution phase of apoptosis. Differences in the regulation of cytochrome c release from mitochondria might explain the different propensities of p73α and p73β overexpressing H82 cells to undergo drug-induced apoptosis. Consequently, the appearance of cytochrome c in the cytosol of these cells was analyzed. Immunofluorescent detection of cytochrome c normally yields a pattern of punctate cytoplasmic staining with some preference for the perinuclear area. This profile of staining is typical for proteins localized in mitochondria. Upon VP16 treatment, cytosolic staining of cytochrome c in H82 cells became diffused, suggesting its release from the intermembrane space of mitochondria to cytosol (Fig. 5A and Ref. 21). Whereas more than 70% of the p73β expressing cells presented a diffuse cytoplasmic staining for cytochrome c upon VP16 treatment, more than 80% of the p73α and ΔNp73α expressing cells kept a punctuate cytoplasmic staining that demonstrated the protection of mitochondria from cytochrome c release.

In various experimental models, the loss of mitochondrial transmembrane potential, ΔΨm, appears to be one of the early apoptotic events (23). AIF is an apoptotic effector protein described recently and defined as a caspase-independent mitochondrial death effector (24, 25). Whereas the translocation of cytochrome c from mitochondria to cytosol does not require a mitochondrial transmembrane depolarization (26), the release of AIF from the mitochondrion has been shown to be dependent on the opening of mitochondrial permeability transition pores (18, 27). Therefore, release of AIF from mitochondria and the loss of ΔΨm in H82 cells upon VP16 treatment were investigated by confocal immunofluorescence microscopy (Fig. 5, B and C). Using antibodies against AIF a punctate cytoplasmic staining pattern with some preference for the perinuclear area was yielded, as shown by simultaneous DAPI staining of the nuclei. Mitochondrial release of AIF in H82 cells was observed upon VP16 treatment, as documented by the loss of punctate cytoplasmic staining and appearance of more diffuse distribution of AIF (Fig. 5C and Ref. 18). p73 isoforms had similar effects on the translocation of this mitochondrial protein as they had on the release of cytochrome c. p73α and ΔNp73α prevented it, whereas p73β potentiated the release of AIF into the cytosol. We have previously shown that VP16 treatment induced ΔΨm disruption in H82 cells (18). Upon VP16 treatment, p73α isoforms expressing cells exhibited mitochondria with an intact membrane potential as seen by the potential sensitive dye CM-H2XRos staining (Fig. 5B).

**Bax Overexpression Bypasses p73α Anti-apoptotic Effects**—The anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-XL have been shown to block release of apoptosis-promoting proteins, e.g. AIF, and cytochrome c from the mitochondrial intermembrane space, whereas the pro-apoptotic members Bak and Bax promote it (28). Regulation of Bax activity is complex and at present is still unclear. However, it is known to involve conformational changes, translocation to mitochondria, and oligomerization of Bax. Conformational changes upon pro-apoptotic stimulation have been shown to lead to exposure of NH2-terminal epitopes. Using antibodies specific for these epitopes, the transformation of Bax to its active conformations can be visualized (29). Bax activation and relocation to the mitochondria were observed in VP16-treated H82 cells and overexpression of p73α repressed activation and translocation of Bax to the mitochondria (Fig. 6A). Together these results established that p73α prevented the activation of Bax, mitochondrial apoptotic events, executioner caspase activation, and finally, nuclear fragmentation. These findings also suggested that the inhibition of apoptotic cell death by p73α must occur in an upstream/early event of the apoptotic mechanism. To investigate if this action could be bypassed by Bax overexpression, Bax-encoding plasmid was cotransfected with p73α plasmid into H82 cells and confocal microscopy...
p73α Inhibits Apoptosis in SCLC Cells

FIGURE 4. p73α prevents caspase-3 activation and PARP cleavage in H82 cells. H82 cells were transfected with plasmid encoding p73α, p73β, or ΔNp73α. Twenty-four hours post-transfection untreated or treated with 5 μM VP16 cells were incubated for 24 h and then processed for immunofluorescence assay. p73α prevents caspase-3 activation (A and B). Cells were first stained using a mixture of monoclonal antibodies directed against p73 and Alexa 594-conjugated secondary antibody and then with monoclonal antibody directed against active caspase-3 and Alexa 488-conjugated secondary antibody. Nuclei were counterstained with DAPI. p73 expressing cells presenting a staining for active caspase-3 were scored in both VP16-treated and untreated samples (B). Confocal images of immunostaining with active caspase-3 (green) and p73 (red) antibodies and nuclear DAPI staining (blue) of H82 cells after VP16 treatment (A). p73α prevents PARP cleavage (C and D). Cells were first stained using a mixture of monoclonal antibodies directed against p73 and Alexa 594-conjugated secondary antibody and then with monoclonal antibody directed against cleaved PARP and Alexa 488-conjugated secondary antibody. Nuclei were counterstained with DAPI. p73 expressing cells presenting a staining for cleaved PARP were scored in both VP16-treated and untreated samples (C). Confocal images of immunostaining with cleaved PARP (green) and p73 (red) antibodies and nuclear DAPI staining (blue) of H82 cells after VP16 treatment (D). The yellow color is the result of an overlay between the green fluorescence (cleaved PARP) and the red one (p73) determining the colocalization of these proteins. A representative image of the staining pattern is shown. Three distinct experiments were performed with similar results.

analysis was performed (Fig. 6B). In addition, using the EGFP as transfection marker, apoptotic nuclei were counted 24 h post-transfection (Fig. 7A). p73α was unable to inhibit Bax-induced cell death in H82 cells.

p73α Inhibits Pro-apoptotic Effects of PUMA in H82 Cells—The BH3-only protein PUMA (p53 up-regulated modulator of apoptosis) is transcriptionally induced during p73-mediated cell death and favors a conformational change and relocalization of Bax to the mitochondria (30). In addition, PUMA has been shown to play a key role in stress-induced apoptosis, as puma−/− thymocytes are markedly resistant to genotoxic damage (by VP16 and γ-radiation) (31).

We therefore investigated the possibility that p73α counteracts the pro-apoptotic effect of PUMA in SCLC cells. Using four different commercially available antibodies, unfortunately, we were not able to obtain conclusive results concerning the modulation of endogenous PUMA protein levels by p73 isoforms in H82 cells (data not shown). Therefore, we decided to investigate the potential transcriptional regulation of PUMA by p73 isoforms. TAp73 isoforms function as transcription factors and regulate the expression of groups of genes by means of direct binding to what was originally identified as the p53-binding site within promoters. The promoter of the gene encoding the PUMA protein contains two p53 recognition sequences at the 5′ end between nucleotides −229/−209 and −145/−126. Consequently, we investigated the possibility of differential binding of p73α and p73β to the p53-binding sites in the PUMA promoter, resulting in different PUMA gene expression. To examine this idea, ChIP and gene reporter assays were performed (Fig. 6, C and D). As shown in Fig. 6C, ChIP assays demonstrated that both p73α and p73β proteins in H82 cells extracts bound to the p53-binding sites in the PUMA promoter. However, the β isoform was more efficient than the α isoform in binding to the PUMA promoter region. Overexpression of both TA domain-containing p73 isoforms, p73α and p73β, activated the PUMA promoter as determined by the gene reporter assays. Nevertheless, p73β appears to have a higher transcriptional activity than p73α on the PUMA promoter in H82 cells (Fig. 6D). Together these data suggested that p73α does not inhibit drug-induced apoptosis in SCLC cells by repressing PUMA gene expression. Hence, we decided to investigate if p73α can inhibit the function of PUMA protein.

In fact, PUMA overexpression has been shown to induce apoptosis in many cell types (16, 31). To further investigate the possibility that the p73α survival effect is a consequence of an inhibition of pro-apoptotic effect of PUMA, a set of experiments using PUMA overexpression was performed. PUMA-encoding plasmid was transfected with or without p73α into H82 cells. In Fig. 7A, EGFP plasmid was also added to trace the transfected cells. The number of apoptotic nuclei was counted 24 h post-transfection. p73α was able, in a dose-dependent manner, to inhibit PUMA-induced cell death in H82 cells (Figs. 6E and 7A). In addition, we observed that the loss of ΔΨm and the activation of caspase-3-like and caspase-9 enzymes caused by PUMA overexpression in H82 cells were inhibited by p73α expression (Fig. 7, B–D).

We speculated that in H82 cells the functional inhibition of PUMA by p73α might reflect direct protein-protein interaction. To test this hypothesis, coimmunoprecipitation experiments were performed. Total protein extracts from H82 and HEK-293 cell overexpressing HA-immunotagged p73α, FLAG-immunotagged PUMA, or their combination were subjected to immunoprecipitation using p73 antibodies. We were not able to detect PUMA protein in the immune complexes, sug-
suggesting that there is no physical interaction between p73 and PUMA at least in H82 or HEK-293 cells (data not shown).

Recent data implicate Bax as an important mediator of PUMA-activated apoptotic signaling (30, 32). The BH3 domain of PUMA specifically interacts with the first α helix of Bax, which leads to Bax activation (33). In this case PUMA requires functional Bax to initiate apoptosis (30). Because PUMA induces rapid apoptosis through conformational Bax changes and the mitochondria-dependent pathway, we decided to examine whether PUMA overexpression affects Bax activation in H82 cells. To investigate how PUMA regulates Bax activation, we took advantage of the 6A7 anti-Bax antibody, which recognizes the membrane-bound active form of Bax (29). As shown in Fig. 7E, overexpression of PUMA in H82 cells leads to Bax conformational modification/activation. Coexpression of p73α resulted in a decrease of PUMA-induced Bax activation (Fig. 7E). Therefore in SCLC cells, p73α seems to counter the direct effect of PUMA on Bax, and consequently inhibits the mitochondrial cell death pathway activated upon Bax conformational changes. Together these data demonstrated that the indirect inhibition of pro-apoptotic function of PUMA by p73α is important for the reduction of VP16-induced cell death in SCLC cells.

Existence of a Balance between p73α Anti-apoptotic and p73β Pro-apoptotic Functions—ΔNp73 acts as a potent transdominant inhibitor of wild-type p53 and transactivation-competent p73. This suppression is thought to be achieved by the competition for the DNA binding site in the case of p53 and by direct association in the case of p73. ΔNp73 has been found to be frequently overexpressed in a variety of human cancers, but not in normal tissues. This suggests that the balance between ΔNp73 and p73 isoforms may be part of a complex tumor control mechanism. Based on the above finding, we hypothesized that an additional balance between the different full-length p73 isoforms may exist in some cells. To test this idea, H82 cells were co-transfected with p73β and p73α (Fig. 8, A and B). In agreement with the above mentioned hypothesis, p73α in a dose-dependent manner inhibited the pro-apoptotic effect of p73β. As expected, p73β inhibited the anti-apoptotic effect of p73α.

The ability of each p73 isoform to regulate target genes can be achieved thought the intermolecular association of p73 variants. p73β and p73α have been shown to physically interact via their putative OD (15). To determine whether the equilibrium between the pro-apoptotic effects of p73β and the anti-apoptotic effects of p73α is because of direct protein-protein interaction, a deletion mutant of p73 lacking the OD was used. The ΔODp73α variant, which is unable to interact with p73β(15), was inefficient in inhibiting its pro-apoptotic effect (Fig. 8, A and B). These results suggest the existence for a balance between these two p73 isoforms, which influence the outcome of drug-induced apoptosis in H82 cells.

DISCUSSION

p73 has been first described as a structural and functional homolog of the tumor suppressor protein p53. However, the p73 gene is able to encode transcriptionally active p73 isoforms, as well as dominant-negative acting ΔNp73 transcript isoforms. By antagonizing the function of the full-length p73 isoforms, the amino-truncated ΔNp73 isoforms serve as anti-apoptotic proteins. Inherent to this unusual gene structure is the idea of a balance existing between the p73 products containing the important sequences for fulfilling p53-like function, and the ΔNp73 products acting entirely in an opposite way. Therefore, with regard to development of human cancer, it is important to know whether p73 acts like p53 as a tumor suppressor or rather as an oncogene. Overproduction of p73 has been reported to promote apoptosis, similar to the case of p53, suggesting a comparable function as a tumor suppressor gene in human cancers (1, 34). However, these data cannot easily be reconciled with data obtained from knock-out mice. Indeed, in contrast to p53-deficient mice, which develop spontaneous tumors at high frequencies
p73α Inhibits Apoptosis in SCLC Cells

FIGURE 6. p73α prevents PUMA induction and subsequent Bax activation in H82 cells. p73α prevents Bax activation (A). H82 cells were transfected with plasmid encoding p73α, or p73β. Twenty-four hours post-transfection, untreated or treated with 5 μM VP16, cells were incubated for 24 h and then processed for immunofluorescence assay. Cells were first stained using a mixture of monoclonal antibodies directed against p73 and Alexa 488-conjugated secondary antibody. Activated membrane-bound form of Bax was detected by the polyclonal anti-Bax antibody clone 6A7 (Alexa 594). Nuclei were counterstained with DAPI. Confocal images of immunostaining with active Bax (red) and p73 (green) antibodies and nuclear DAPI staining (blue) of H82 cells after VP16 treatment is depicted. Bax overexpression counteracts the p73α anti-apoptotic effect (B). H82 cells were transfected with plasmid encoding p73α, Bax, or both. Overexpressed p73α was detected by immunofluorescence using the monoclonal anti-p73 antibody (Alexa 488). The activated membrane-bound form of Bax was detected with the same polyclonal anti-Bax antibody as in the first panel (Alexa 594). Nuclei were counterstained with DAPI. A representative image of the staining pattern is presented. p73α and p73β bind to the PUMA promoter region (C). Chromatin immunoprecipitation assay using H82 cells transfected with plasmid encoding p73α, or p73β, was performed. A mixture of monoclonal antibodies that recognize p73 was used for the immunoprecipitation of the chromatin. PCR were performed with primers corresponding to the −422 and −211 regions of the PUMA promoter. p73α and p73β transactivate the PUMA promoter (D). H82 cells were transiently transfected with the PUMA-Luc reporter plasmid, containing two p53 binding sites, along with the expression vector for p73α or p73β. Cells were harvested after 24 h incubation and lysed, and cell extracts were assayed for luciferase and β-galactosidase activities. Relative light units were computed after normalization to β-galactosidase. Overexpression of p73α reduces the pro-apoptotic effect of PUMA (E). H82 cells were transfected with plasmid encoding p73α, PUMA, or both. Overexpressed p73α was detected by immunofluorescence using the monoclonal anti-p73 antibody (Alexa 594). PUMA antibody detects endogenous levels of PUMAα and PUMAβ (Alexa 488). Nuclei were counterstained with DAPI. A representative image of the staining pattern is presented. For each panel, three independent experiments were performed showing similar results.

FIGURE 7. p73α inhibits pro-apoptotic function of PUMA in H82 cells. p73α reduces PUMA-induced cell death (A). H82 cells were cotransfected with EGF expression vector together with plasmid encoding Bax or PUMA, both of them in combination with increasing amounts of p73α. Nuclei were counterstained with DAPI and the number of apoptotic EGFP-expressing cells was counted 24 h after transfection. p73α reduces PUMA-induced mitochondrial transmembrane potential disruption (B). Cells were cotransfected with EGF and p73α, PUMA, or both. Cells were harvested after 24 h, and analyzed by fluorescence-activated cell sorter. Transfected cells were sorted by EGFP expression, and their mitochondrial potential was assessed using TMRE staining. Included are also values of the percentage of TMRE-negative cells. p73α decreases PUMA-induced caspases activities (C and D). H82 cells were transfected as in panel A. 24 h post-transfection, cells were harvested and lysed as described under “Materials and Methods.” Caspase-3-like activity (C) and caspase-9 activity (D) were determined by the cleavage of the specific peptide substrates LEHD-AMC and DEVD-AMC, respectively. The maximum linear rate of AMC release (pmol/min) was estimated by linear regression. p73α decreases PUMA-Induced Bax activation (E). H82 cells were transfected with p73α, PUMA, or both. Cells were harvested after 24 h, and analyzed by fluorescence-activated cell sorter, using the polyclonal anti-Bax antibody clone 6A7, which can recognize the activated membrane-bound form of Bax. Percentage of active Bax-positive cells is designated in each quadrant.
thyroid cancer (41). Importantly, p73α overexpression appears to significantly correlate with poor prognosis in ovarian carcinomas (42), and a higher risk in B-cell chronic lymphocytic leukemia (43). These correlations between the high expression level of p73α and prognostic parameters suggest that p73α might be implicated in tumorigenesis and possibly function as a dominant oncogene to enhance tumor progression and therapeutic resistance. The first experimental evidence in this direction comes from the observation that p73α overexpression in a human ovarian cancer cell line is associated with resistance to treatment with DNA-damaging agents (48).

So far, however, there was no answer on how the p73α isoform might hold oncogenic activity in tumor tissues. In the process of delineating the function of different p73 and ΔNp73 isoforms in a panel of human cancer cell lines, we found that p73α, as ΔNp73α, is able to inhibit apoptosis induced by etoposide, cisplatin, and staurosporine in SCLC cells. Alternatively, p73β exerted pro-apoptotic function in the same cells. The p73α anti-apoptotic function appears to be cell type-specific, as both p73α and p73β exhibited pro-apoptotic function in other cell lines tested (H1299 NSCLC, HEK-293, and Saos-2). The functional diversity between these two carboxyl-terminal variants of p73 also has been recently reported for the regulation of cell differentiation. In fact, whereas p73β lacked any detectable effect on differentiation of C2C12 myoblasts, p73α caused a substantial delay in the expression of muscle-specific genes (2).

Investigation of different biochemical hallmarks of apoptosis revealed that the nuclear events (cleavage of PARP as well as DNA fragmentation) do not occur in the p73α expressing H82 cells. Furthermore, caspase activation was prevented by the overexpression of p73α. In

**FIGURE 8.** Balance between p73α anti-apoptotic and p73β pro-apoptotic effects. A schematic representation of p73 constructs used and their protein expression level is depicted (A). p73α inhibits p73β pro-apoptotic effects (B). H82 cells were co-transfected with 1 µg of plasmid encoding p73α and increasing amounts of p73β (0.5, 1, and 2 µg) or with 1 µg of plasmid encoding p73α and increasing amounts of ΔNp73α (0.5, 1, and 2 µg) or ΔNp73α (0.5, 1, and 2 µg). Twenty-four hours post-transfection, cells were treated with 5 µM VP16 for an additional 24 h. Cells expressing p73 were detected by immunofluorescence and apoptotic cells were scored as described in the legend to Fig. 1.
addition, mitochondria remain intact; neither protein release nor drop in mitochondrial membrane potential were observed when p73α was present in the cell. The translocation of Bax from the cytosol into the mitochondrial outer membrane is a central event during apoptosis. Bax translocation to mitochondria, which is associated with specific changes in the conformation of the protein, was inhibited by p73α. Finally, p73α was also able to repress the pro-apoptotic functions of the BH3-only protein PUMA. The precise mechanisms behind this inhibition are still unclear and await additional investigations. However, the inhibition of PUMA pro-apoptotic function by p73α does not seem to involve other transcriptional repression of the PUMA promoter or direct physical interaction between p73α and PUMA proteins. In addition, we observed that in SCLC cells p73α prevents the direct activation of Bax by PUMA. Altogether these data suggest that p73α has a broad range inhibitory effect preventing the mitochondria- and caspase-dependent cell death pathways. The observed overexpression of p73α in certain tumor types and the anti-apoptotic activity of p73α in certain cells could explain an oncogenic activity for this p73 isoform in tumor tissues.

We also characterized the structure requirement for the anti-apoptotic function of p73α in SCLC cells and found that the DNA binding domain as well as the unique carboxyl terminus residues of p73α are required to suppress cell death. The SAM domain within the carboxyl terminus seems to be essential, because p73α deletion constructs lacking part of or the entire SAM domain were inefficient in inhibiting cell death. Furthermore, we found that the transcriptional domain within the amino residues of p73α did not appear to be essential for its anti-apoptotic function. Indeed, p73α carrying a point mutation in this domain were still able to impede cell death in H82 cells. The unique carboxyl-terminal region of p73α has the function to modulate DNA-binding and transcription activities. In addition this region contains both a proline-rich area and the SAM domain known as interacting motifs. MM1 promotes the pro-apoptotic function by p73α, because MM1 is able to repress the pro-apoptotic activity of the BH3-only protein PUMA. The precise mechanisms behind this inhibition are still unclear and await additional investigations. However, the inhibition of PUMA pro-apoptotic function by p73α does not seem to involve other transcriptional repression of the PUMA promoter or direct physical interaction between p73α and PUMA proteins. In addition, we observed that in SCLC cells p73α prevents the direct activation of Bax by PUMA. Altogether these data suggest that p73α has a broad range inhibitory effect preventing the mitochondria- and caspase-dependent cell death pathways. The observed overexpression of p73α in certain tumor types and the anti-apoptotic activity of p73α in certain cells could explain an oncogenic activity for this p73 isoform in tumor tissues.

The binding of MM1 enhances the transcriptional activity of p73α, c-myc and RACK1 antagonize it. In contrast, SUMO-1 modification does not explain an oncogenic activity for this p73 isoform in tumor tissues. Altogether these data suggest that p73α has a broad range inhibitory effect preventing the mitochondria- and caspase-dependent cell death pathways. The observed overexpression of p73α in certain tumor types and the anti-apoptotic activity of p73α in certain cells could explain an oncogenic activity for this p73 isoform in tumor tissues. Altogether these data suggest that p73α has a broad range inhibitory effect preventing the mitochondria- and caspase-dependent cell death pathways. The observed overexpression of p73α in certain tumor types and the anti-apoptotic activity of p73α in certain cells could explain an oncogenic activity for this p73 isoform in tumor tissues.
29. Hsu, Y. T., and Youle, R. J. (1998) J. Biol. Chem. 273, 10777–10783
30. Melino, G., Bernassola, F., Ranalli, M., Yee, K., Zong, W. X., Corazzari, M., Knight, R. A., Green, D. R., Thompson, C., and Vousden, K. H. (2004) J. Biol. Chem. 279, 8076–8083
31. Villunger, A., Michalak, E. M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M. J., Adams, J. M., and Strasser, A. (2003) Science 302, 1036–1038
32. Liu, F. T., Newland, A. C., and Jia, L. (2003) Biochem. Biophys. Res. Commun. 310, 956–962
33. Cartron, P. F., Gallenne, T., Bougras, G., Gautier, F., Manero, F., Vusio, P., Meftah, K., Vallette, F. M., and Jin, P. (2004) Mol. Cell 16, 807–818
34. Di Como, C. J., Gaiddon, C., and Prives, C. (1999) Mol. Cell Biol. 19, 1438–1449
35. Moll, U. M., Erster, S., and Zaika, A. (2001) Biochim. Biophys. Acta 1552, 47–59
36. Stiewe, T., and Putzer, B. M. (2002) Cell Death Differ. 9, 237–245
37. Zaika, A., Slade, N., Erster, S. H., Sansone, C., Joseph, T. W., Pearl, M., Chalas, E., and Moll, U. M. (2002) J. Exp. Med. 196, 765–780
38. Petrenko, O., Zaika, A., and Moll, U. M. (2003) Mol. Cell Biol. 23, 5540–5555
39. Casciano, I., Mazzocco, K., Boni, L., Pagnan, G., Banelli, B., Allemanni, G., Ponzi, M., Tomini, G. P., and Romani, M. (2002) Cell Death Differ. 9, 246–251
40. Ugor, H., Sayan, A. E., Ozdamar, S. O., Kanpolat, Y., and Ozturk, M. (2004) Oncol. Rep. 11, 1337–1341
41. Frasca, F., Vella, V., Aloisi, A., Mandarino, A., Mazzon, E., Vigneri, R., and Vigneri, P. (2003) Cancer Res. 63, 5829–5837
42. Niyazi, M., Ghazizadeh, M., Konishi, H., Kawanami, O., Sugisaki, Y., and Araki, T. (2003) J. Nippon Med. Sch. 70, 234–242
43. Novak, U., Grob, T. J., Baskaynak, G., Peters, U. R., Aebe, S., Zvählen, D., Tschan, M. P., Kreuzer, K. A., Leibundgut, E. O., Cajot, J. F., Tobler, A., and Fey, M. F. (2001) Ann. Oncol. 12, 981–986
44. Nozaki, M., Tada, M., Kashiwazaki, H., Hamou, M. F., Diserens, A. C., Shinohe, Y., Sawamura, Y., Iwasaki, Y., de Tribolet, N., and Hegi, M. E. (2001) Brain Pathol. 11, 296–305
45. Zaika, A. I., Kovalev, S., Marchenko, N. D., and Moll, U. M. (1999) Cancer Res. 59, 3257–3263
46. Yokomizo, A., Mai, M., Tindall, D. J., Cheng, L., Bostwick, D. G., Naito, S., Smith, D. I., and Liu, W. (1999) Oncogene 18, 1629–1633
47. Kang, M. J., Park, B. J., Byun, D. S., Park, J. I., Kim, H. J., Park, J. H., and Chi, S. G. (2000) Clin. Cancer Res. 6, 1767–1771
48. Vikhanskaia, F., Marchini, S., Marabese, M., Galliera, E., and Broginni, M. (2001) Cancer Res. 61, 935–938
49. Watanabe, K., Ozaki, T., Nakagawa, T., Miyazaki, K., Takahashi, M., Hosoda, M., Hayashi, S., Todo, S., and Nakagawara, A. (2002) J. Biol. Chem. 277, 15113–15123
50. Minty, A., Dumont, X., Kaghad, M., and Caput, D. (2000) J. Biol. Chem. 275, 36316–36323
51. Ozaki, T., Watanabe, K., Nakagawa, T., Miyazaki, K., Takahashi, M., and Nakagawara, A. (2003) Oncogene 22, 3231–3242
52. Hackzell, A., Uramoto, H., Izumi, H., Kohno, K., Funa, K., Ueda, Y., Hijikata, M., Takagi, S., Chiba, T., and Shimotohno, K. (2002) J. Biol. Chem. 277, 39769–39776

p73α Inhibits Apoptosis in SCLC Cells