p73 induces apoptosis via PUMA transactivation and Bax mitochondrial translocation

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Abbreviations: TA, transactivating isoform; ΔN, amino terminus deleted isoform; Δ84, 84 amino terminus residues deleted isoform; GFP, green fluorescent protein; pDsRed1-Mito, plasmid encoding a red protein tagging mitochondria; Dox, doxycycline; DBD, DNA binding domain; TAD, transactivation domain; FBS, fetal bovine serum; PBS, phosphate buffered saline; Luc, luciferase; MEF, mouse embryo fibroblasts; OD, optical density; PI, propidium iodide; SD, standard deviation;
SUMMARY

p73, an important developmental gene, shares a high sequence homology with p53, and induces both G1 cell cycle arrest and apoptosis. However, the molecular mechanisms through which p73 induces apoptosis are unclear. By using an inducible model of p73 (isoforms α, β, γ) expressed in Saos-2 cells, we found that p73-induced apoptosis is mediated by PUMA induction, which in turn causes Bax mitochondrial translocation and cytochrome c release. Overexpression of p73 isoforms promote cell death and Bax promoter transactivation in a time-dependent manner, with the p73γ isoform being the most effective. However, the kinetics of apoptosis do not correlate with the increase of Bax protein levels. Instead, p73-induced mitochondrial translocation of Bax is kinetically compatible with the induction of cell death. p73 is localised in the nucleus, and remains nuclear during the induction of cell death, indicating that the effect of p73 on Bax translocation is indirect. The ability of p73 to directly transactivate PUMA, as compared to other BH3 proteins, and the direct effect of PUMA on Bax conformation and mitochondrial relocalization, suggests a molecular link between p73 and the mitochondrial apoptotic pathway. Our data therefore indicate that PUMA-mediated Bax mitochondrial translocation, rather than its direct transactivation, correlates with cell death. Finally, human ΔNp73 inhibits TAp73- as well as p53-induced apoptosis. The ΔNp73 isoforms, controlled by a second distinct promoter, seem therefore to act as dominant negatives, repressing the PUMA/Bax system, thus finely tuning p73-induced apoptosis. Our findings demonstrate that p73 elicits apoptosis via the mitochondrial pathway using PUMA and Bax as mediators.

Keywords: Apoptosis, p73, ΔNp73, Bax, PUMA.
INTRODUCTION

p73 is a member of the p53 family (1). The two proteins show a high degree of sequence homology, particularly in the central sequence-specific DNA binding domain (DBD), the amino terminal activation domain (TAD), and the carboxyl terminal oligomerization domain (1).

Even though p73 shows an evident developmental role, the strong structural similarity suggests that at least in part the function of p73 may therefore closely resemble that of p53 (2, 3). Indeed, like p53, p73 induces G1 cell growth arrest (1, 4), activates the transcription of some endogenous p53 target genes, such as p21Waf1/Cip1, RGC (ribosomal gene cluster), Mdm2, Bax, cyclin G, GADD45, IGF-BP3 (insulin-like growth factor binding protein 3) and 14-3-3σ (4-8), and induces apoptosis irrespective of p53 status (1, 4). The structural integrity of the p73 DBD is required for these activities, suggesting that p73 recognizes the p53-responsive DNA elements.

The levels of p73 are not changed by exposure to DNA-damaging agents such as actinomycin D or UV irradiation, which increase p53 levels (1) Moreover, steady-state levels of p73 are not reduced by complex formation with Mdm2 (7, 8), which targets p53 for ubiquitin-mediated proteolysis (9-11). Recent studies have shown that p73 can be stabilized and tyrosine phosphorylated by c-Abl following DNA damage, leading to an enhanced p73-mediated apoptotic response (12-14). Therefore there are both similarities and differences in the mechanisms of p53- and p73-mediated apoptosis.

Bax, a pro-apoptotic Bel-2 family member, is a p53 and p73 target gene (15, 16). In unstressed cells, Bax protein exists as an inactive monomer in the cytosol and is induced to homo-oligomerize and translocate to mitochondria upon death stimuli, thus leading to cytochrome c release and caspase activation (17-20). Cytochrome c release results from the induction of the mitochondrial permeability transition, an event associated with disruption of the mitochondrial inner transmembrane potential ΔΨm (21), and which has been implicated in a variety of apoptotic phenomena (22, 23). Unfortunately however, very little is known on the molecular events through which p73 induces apoptosis.

Here we report a study on p73-induced apoptosis in Saos-2 cells demonstrating that the γ isoform is the most effective both in the induction of apoptosis and in Bax transactivation. However, at least in this model, Bax transactivation does not seems to be crucial for the inducion of death. We show that in cells undergoing p73-dependent apoptosis, p73 displays a nuclear localization pattern, while Bax translocates from the cytosol to mitochondria, thus causing cytochrome c release. The BH3-only protein PUMA is transcriptionally induced during p73-mediated cell death and favours Bax’s conformational change and relocalization to the mitochondrial membrane.
mitochondria. Moreover, ΔNp73, a recently cloned p73 isoform (24), which lacks the TAD domain and acts as a p73 dominant negative, inhibits p73-induced apoptosis. Here we provide evidence for a transcription-independent effect of p73 on Bax, able to activate the mitochondrial pathway during cell death.

**EXPERIMENTAL PROCEDURES**

*Cell culture* - Saos-2 and HeLa cells stably expressing Bax-GFP or cytochrome c-GFP fusion protein were cultured in a 1:1 mixture Ham’s F-12:D-Minimal Essential Medium supplemented with 10% heat-inactivated FBS at 37°C in a humified atmosphere of 5% CO2 in air. Saos-2 cells with doxycycline (dox)-inducible expression of p73 isoforms (25) or PUMA (prepared as in reference 25) were cultured in the same medium supplemented with 10% heat-inactivated tetracycline-free FBS (Tet system approved foetal bovine serum, Clontech). Both p73 and PUMA inducible cell lines were HA-tagged in order to monitor the steady state levels of protein induction by Western and laser densitometry. MEFs for null p53, Bax, Bak, Bax/Bak, and U2OS cells were grown in similar conditions. To induce protein expression, the tetracycline inducible cell lines were treated with the tetracycline analog dox at 2.5μg/ml as indicated.

*Plasmids* - Human p73 isoforms (including Δ84p73β) and p53 cDNAs in pCDNA3 (18, 26), ΔNp73 (24) and PUMA cDNA (27, 28) have been previously described.

*Western Blot Analysis* - Saos-2 subclones with dox inducible expression of p73 isoforms were treated with dox (2.5μg/ml) for 48 h and lysed in buffer A (50mM Tris, pH 8.0, 150mM NaCl, 0.5% NP-40, 0.5mg/ml leupeptin, 1mg/ml aprotinin and 0.5mM PMSF) for 1h on ice. The lysate was cleared by centrifugation, and 20μg aliquots of cell extract, as determined by the Bradford method, were resolved by electrophoresis in a 12% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) to quantitate the steady state levels of protein expression.

In order to analyze the expression of Bax in the mitochondrial fraction, cells were resuspended in 3 ml ice cold buffer B (250 mM sucrose, 20 mM HEPES, 10mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 μg/ml PMSF, 8 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.4. Cells were passed through an ice cold cylinder cell homogenizer (H&Y Enterprise, Redwood City, CA).
Intact cells and nuclei were pelletted for 10 min at 750 x g. The supernatant was spun at 10,000 x g for 20 min. The pellet was lysed in buffer A as reported above and represents the mitochondrial fraction. 20μg of this lysate was resolved by electrophoresis in a 12% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with anti-Bax, anti-procaspase antibody (Pharmingen) or anti-Bax conformational epitope (antibody 6A7, by C Thompson). Immunocomplexes were detected by a chemiluminescence-based system (Amersham Pharmacia Bio-tech, Inc., Piscataway, NJ) according to the manufacturer’s instructions. Densitometric analysis was carried out using Kodak 1D 2.0 software.

**Determination of Apoptosis** - To estimate DNA fragmentation, Saos-2 cells with dox-inducible expression of p73 isoforms were treated with dox (2.5μg/ml) for 48h, collected at 800 x g for 10min and fixed with a 1:1 phosphate-buffered saline and methanol-acetone (4:1 (v/v)) solution at -20 C. Hypodiploid sub-G1 events were evaluated by flow cytometry after propidium iodide (PI) staining, as previously described (13, 24, 26). For each point 20,000 events were collected, excluding doublets and aggregates by electronic gate.

**Bax staining** - Saos–2 cells with inducible expression of p73γ and p53 were stimulated to produce the protein for the indicated times using dox (2.5μg/ml) and then fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15min. Cells were then permeabilised for 2min in 0.1 % Triton X-100 in PBS and incubated for 1h with anti-Bax antibody followed by 30min incubation with anti-rabbit ALEXA 488-antibody (Molecular Probes). Ten thousand events were collected monitoring 530nm fluorescence on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA), as indicated above.

**Subcellular Localization** - Cells were grown overnight on a glass coverslip and, after the indicated treatments, fixed in 4% paraformaldehyde in PBS (pH 7.4) for 15min. Saos-2 p73γ-inducible cells were induced with dox (2.5μg/ml) for 24h and then treated, when indicated, with 25μM cisplatin for 24h. Immunofluorescence was carried out using anti-HA or anti-Bax antibody combined with Tunel reaction according to the manufacturer’s instructions (In situ cell death detection Kit TMR red, Roche). HeLa cells stably expressing Bax-GFP were cotransfected by using Lipofectamine 2000 (Life Technologies) with p73γ and pDS-Red1-Mito (3:1).

Confocal images were acquired by using a PCM-2000 Confocal Microscope (Nikon) and exciting with 488nm argon-ion laser line or 542nm He-Ne laser detecting with the appropriate filter set (515/30 green filter and 595/70 red filter). Images were then processed by using PCM–2000 software (Nikon).
Luciferase assay - Saos-2 cells with dox inducible p73 isoforms or p53 were cotransfected with a reporter plasmid containing luciferase cDNA under the control of the Bax promoter (Bax-Pr/Luc) and Renilla plasmid as internal standard (40:1). p73 or p53 expression was then induced by treatment with dox (2.5μg/ml). Saos-2 cells were cotransfected with expression plasmids encoding p73α or p53, with or without ΔNp73α, ΔNp73β or ΔNp73γ together with Bax-Pr/Luc (bax-Pr/Luc:p73/p53: ΔNp73 : Renilla = 1:1:8:0.25). N1E-115 cells were cotransfected with expression plasmid encoding p73α, p73β, p73γ, p73δ or p53, with or without Δ84p73β together with Bax-Pr/Luc. Luciferase assays were carried out 48 h after transfection with the dual-luciferase assay system (Promega) according to the manufacturer's instructions. Luminescence was detected by using EG&G Berthold Lumat LB 9507 averaging the signal for 10s.

RESULTS AND DISCUSSION

p73 expression induces apoptosis in Saos-2 cells - In order to study the mechanisms through which p73 induces apoptosis, we took advantage of three Saos-2-derived cell lines, stably transfected with the HA-tagged doxycycline(dox)-inducible TAp73 isoforms α, β or γ (25). Saos-2 cells are p53-null and do not express p73 at either mRNA and protein levels, therefore forming a suitable model for evaluation of the induction of apoptosis by p73.

We measured p73 expression in Saos-2 cells incubated with the tetracyclin analog dox (2.5μg/ml). Cells were collected at different time points and assayed for protein expression using anti-HA antibody. p73 protein levels were undetectable in untreated cells, whereas the expression of all the three p73 isoforms was induced in a time-dependent manner following treatment with dox (Fig 1A). Densitometric analysis of p73 induction revealed that β and γ isoforms were the most highly induced upon dox treatment (Fig. 1B). In order to test whether caspases play a role during p73-induced cell death, we measured the proenzyme expression levels of caspase 6, 7 and 8 upon dox stimulation of Saos-2 p73 inducible cell lines (Fig. 1, C-E). We found a significant reduction in procaspase 6 steady state levels (less significant for the other procaspases) 48h after treatment of Saos-2 p73β and p73γ. (Fig 1, D-E).

Consistent with the p73β- and γ- triggered processing of procaspase 6, and to a lesser extent of procaspase 8, both isoforms induced apoptosis (Fig. 1F) after 48h of dox stimulation. Therefore, expression of p73, in particular the isoforms β and γ, induces cell death and caspase activation in Saos-2 cells.
p73-induced Bax expression does not correlate with apoptotic kinetics - To investigate the possible involvement of Bax, in p73-induced apoptosis, we performed a transactivating assay using a reporter plasmid (Bax-Pr/Luc) containing the full-length Bax-promoter placed upstream of a luciferase cDNA. We transfected Saos-2 cells expressing the dox-inducible p73α, β and γ isoforms, or p53 as a positive control, and the luciferase assay was performed after 24h. As shown in Fig. 2A, all the three p73 isoforms transactivated the Bax promoter, although to a lesser degree than p53, with the p73γ isoform being the most potent transcriptional activator. This is in keeping with previous literature.

Saos-2 cells inducible for p73γ and p53 expression were also analyzed for Bax protein levels following stimulation with dox by flow cytometric analysis. Interestingly, Bax upregulation was induced in a time-dependent manner reaching a maximum at 96h (Fig. 2B, C), thus indicating that the kinetics of Bax expression did not correlate with the induction of apoptosis (Fig. 1F). Although there are structural and functional similarities between p73 and p53, p73 is a less efficient transcriptional activator of the bax promoter (Fig. 2A), and a significantly slower inducer of Bax protein expression (Fig 2B, C) than p53. Hence, at least in our cellular model, the direct transactivation of the bax promoter by p73 does not seems to be the crucial apoptotic effector mechanism driven by p73.

p73 indirectly induces Bax mitochondrial relocalization - Bax translocation from cytosol to mitochondria is a critical step in p53-mediated apoptosis (29). Bax contributes to apoptosis by interacting with Bcl-2/Bcl-xL and thus controlling mitochondrial protein export. Using confocal and subcellular fractionation studies, we investigated whether Bax re-localization could mediate p73-induced apoptosis in cells stably expressing a Bax-GFP fusion protein (Bax-GFP). Bax-GFP HeLa cells were cotransfected with p73γ along with a plasmid encoding a red protein which localizes to the mitochondria (pDsRed1-Mito). Confocal microscopy revealed that Bax-GFP was diffusely distributed throughout the cytosol in the absence of p73 overexpression (green fluorescence, Fig. 3A). However, Bax-GFP moved to a completely punctate distribution colocalizing with mitochondria 48 h after p73 up-regulation (Fig. 3B). To further confirm Bax relocalization into mitochondria in apoptotic cells, we performed a Western blot analysis on the purified mitochondrial fraction from the Saos-2 p73γ subclone, 48h after dox stimulation. As shown in Fig. 3C, induction of p73 revealed a significant increase in Bax protein levels in the mitochondrial fraction as compared to untreated cells. Identical results were obtained using the Bax-GFP HeLa cells (data not shown).

Evidence for transcription-independent p53-mediated apoptosis has been accumulating (30, 31). Since during DNA damage- and hypoxia-stimulated apoptosis a fraction of p53 translocates to
mitochondria and directly induces cytochrome c release, we sought to examine whether p73 undergoes similar distribution changes. At the same time, the experiment would elucidate if Bax translocation is directly induced by the p73 protein per se. Interestingly, p73 (green fluorescence) was clearly restricted to the nuclear compartment and did not re-localize during apoptosis triggered either by p73γ induction (Fig. 3D, E) or by cisplatin treatment of Saos-2 cells overexpressing p73γ (Fig. 3F). Transient transfection of Saos-2 cells with an expression plasmid encoding HA-p73γ gave identical results to those obtained with Saos-2 stably transfected with p73γ (data not shown). Thus, unlike p53, p73 does not translocate to mitochondria in response to death signals. Moreover, Bax translocation to the mitochondria is not caused by a direct p73-Bax interaction.

Finally, we monitored cellular distribution of the endogenous Bax protein in the p73γ-inducible clones 48 h after dox stimulation. Fig. 3G shows that Bax (green fluorescence) was spread throughout the cytosol in control cells. On the contrary, Bax displayed a punctate distribution pattern in p73γ-overexpressing cells, similarly to that observed in Fig. 3B (Fig. 3H). Comparable results were obtained in Saos-2 cells transfected with an expression plasmid encoding HA-p73γ (data not shown). These data indicate that p73 and Bax do not co-localize, even at later stages of p73-induced cell death. In order to more directly investigate the interaction between Bax and p73, we overexpressed both proteins in Saos-2 cells. As shown in Fig. 3I, we did not observe any co-localization of p73 (green fluorescence) in mitochondria (red fluorescence). These results indicate that p73 induces the mitochondrial translocation of Bax through indirect mechanisms.

**PUMA is induced by p73 and causes a conformational change and mitochondrial translocation of Bax** — In order to identify the mediators through which p73 indirectly induces Bax mitochondrial translocation, we examined the modulation of several BH3-only proteins (data not shown), which bind to Bcl2/XL via their BH3 domain, thereby inactivating their protective function (27, 32). The recently identified PUMA gene encodes two BH3 domain–containing proteins (PUMAα and PUMAβ, p53 Upregulated Modulator of Apoptosis) that are induced following p53 activation, and play a role in mediating p53-induced cell death through the cytochrome c/Apaf-1–dependent pathway (27). We therefore investigated the possibility that p73 could directly transactivate PUMA. As shown in Fig. 4A PUMA was transcriptionally induced by p73 within 12h. The specific role of the individual PUMA isoforms is not clear at the moment.

We then examined whether PUMA affects Bax translocation to mitochondria during p73-induced apoptosis. As shown in Fig. 4B-D, overexpression of both PUMA isoforms in HeLa cells stably transfected with Bax-GFP led to Bax movement from the cytosol to mitochondria 24h after transfection. In addition, we found that PUMA caused the release of cytochrome c from mitochondria, as visualised in Hela cells stably transfected with cytochrome c-GFP (Fig. 4E-G).
Therefore, PUMA seems to mediate p73’s effect on Bax, causing mitochondrial release of cytochrome c to activate the final steps of death in the apoptosome.

To investigate how PUMA physiologically regulates Bax cellular distribution during apoptosis, we took advantage of the 6A7 anti-Bax antibody, which recognizes the activated membrane-bound form of Bax (33). As shown in Fig. 5 (panel A), overexpression of PUMA in U2OS cells led to translocation of the endogenous Bax protein to mitochondria. Interestingly, the active membrane-bound conformation of Bax co-localized with PUMA (Fig. 5A). Identical results were obtained in a Tet-on PUMA-inducible H1299-derived cell line. Induction of PUMA by dox stimulation resulted in Bax conformational modification and co-localization with PUMA on the mitochondria (Fig. 5B). The data suggest that PUMA causes a conformational modification of Bax, which then results in its accumulation on the mitochondria.

**PUMA-induced apoptosis requires functional Bax/Bak** – To formally prove that PUMA-induced cytochrome c release and cell death indeed relies on Bax during p73-mediated apoptosis, we first cotransfected Bax−/− mouse embryo fibroblasts (MEF) with p73γ and cytochrome c–GFP. We found that p73-induced cytochrome c release was delayed, but not abrogated, in the absence of Bax (Fig. 6A, and data not shown). When cells were exposed to DNA damage (25 μM cisplatin), apoptosis was indeed slower in Bax −/−, even though the same plateau was reached (Fig. 6B). In this experiment, both in Bax +/+ and Bax −/− cells, p73 steady state protein levels were induced within 12 hours, Fig. 6C. This suggests that indeed p73 is physiologically induced upon DNA damage, and that Bax is not an absolute requirement to induce apoptosis.

We then measured the ability of PUMA to induce cell death in MEFs of different genotypes. We indeed took advantage of either Bax and Bak single knock out cells or Bax/Bak double deficient cells, which are defective in apoptosis initiated by several other BH3-only proteins (34, 35). The overexpression of PUMA in wild type fibroblasts caused significant induction of cell death, that was similar in both Bax or Bak single null MEFs (Fig. 6B). Interestingly, Bax/Bak double null MEFs were completely protected against PUMA-induced apoptosis (Fig. 6D), thus demonstrating that either Bax or Bak are essential for PUMA to induce cell death.

ΔNp73 inhibits p73-induced expression of PUMA and Bax and reduces apoptosis – We next sought to test the effect of the Δ84p73β dominant negative mutant, which lacks transcriptional function (24, 36), on both p73α and p53 transcriptional activity. To this end, we cotransfected murine N1E115 neuroblastoma cells with Bax-Pr/Luc along with expression plasmids encoding p73α, β, γ, δ or p53 with or without Δ84p73β. The results show that Δ84p73β strongly inhibited both p73- and p53-dependent transactivation of the Bax-Pr/Luc (Fig. 7A).
ΔNp73 is a naturally occurring isoform of p73 lacking most of the N-terminal transactivation domain (24). The expression of this isoform is regulated by a distinct promoter located in the third intron (24). This amino-deleted isoform of p73 is highly expressed in tumors, where it seems to play an important role in tumorigenesis (36-37). To investigate the role of the ΔNp73 isoforms, we cotransfected Saos-2 cells with Bax-Pr/Luc, expression plasmids encoding p73α or p53, with or without ΔNp73 isoforms (α, β and γ). As shown in Fig. 7B, ΔNp73 did not have transcriptional activity but efficiently inhibited p73- and p53-dependent transactivation of Bax-Pr/Luc.

In agreement with this results, we found that TAp73-induced PUMA expression in the p73γ-inducible Saos-2 cell line was completely abrogated in cells stably transfected with ΔNp73α and ΔNp73γ (Fig. 7C, lanes 4-6).

We also investigated the effect of ΔNp73α on cell death induced by p73 and p53 overexpression (Fig. 7D). To this aim, we cotransfected Saos-2 cells with expression plasmids encoding p73α, p73γ or p53, with or without ΔNp73α. Cells were harvested after 72h. As shown in Fig. 7D, ΔNp73α efficiently abrogated p73- and p53-induced apoptosis.

CONCLUSIONS

While overexpression of p73 leads to activation of reporter genes containing a p53 responsive sequence, growth arrest, and apoptosis, its physiological significance is still unclear. Recent studies indicate that the differential expression of p73 isoforms, together with the interaction between p73 isoforms themselves and with p53, might be crucial in controlling p53 function and programmed cell death (2-3, 36).

In the present study, we examined the induction of apoptosis by different p73 isoforms (α, β and γ), which occurred 48h after p73 up-regulation. In our system, the γ isoform was the most effective in inducing cell death. We also found that, similarly to p53, all the p73 isoforms were able to transactivate the Bax promoter and induce the expression of the protein in a time-dependent manner. However, transactivation of the Bax promoter by p73γ or p53 was observed only after 72-96h, much later than p73 and p53-mediated induction of apoptosis. Therefore, p73-induced cell death cannot only rely on the ability of p73 to up-regulate Bax expression.

To explain this disparity, we examined the sub-cellular distribution of p73 and Bax during cell death induction. We found that p73 remained in the nucleus during the execution of apoptosis, while Bax assumed a punctate distribution pattern co-localizing with mitochondria. Thus, unlike p53 (30, 31), p73 does not display a mitochondrial, transcription-independent, apoptotic function in response to DNA damage. In addition, since p73 and Bax are distributed in different sub-cellular
compartments upon cell death induction, any interaction between the two proteins must be indirect. Since this function is normally performed by BH3-only proteins, we searched for their modulation in our model, and found that PUMA was the only BH3-only protein significantly modulated by p73 (data not shown). The p53 responsive gene PUMA interacts with Bcl-2, as demonstrated by yeast 2-hybrid screening (27, 28). Moreover, overexpression of PUMA results in mitochondrial translocation of activated endogenous Bax, which partially co-localizes with PUMA, and cytochrome c release, with similar or even more rapid kinetics than those of p73 on apoptosis. Similarly to other BH3-only proteins, such as Bid, Bim and Bad, that bind pro-survival Bcl-2 family members (34, 35), PUMA requires functional Bax and/or Bak to initiate apoptosis. Therefore, PUMA is an intermediate effector of p73-induced Bax translocation to mitochondria, cytochrome c release and apoptosis.

p73 is expressed in at least six alternatively spliced forms, α to ζ (1, 26). Importantly, one spliced variant of p73 (p73Δexon2), which lacks most of the N-terminal transactivation domain, has been identified in cancer (1) but not in normal cells. This isoform inhibits p73-induced apoptosis and competes with p53 for DNA binding. This differential expression of p73 isoforms suggests that interaction between p73 isoforms and with p53 may not only modulate p53 function but also have profound effects in the control of programmed cell death (for review see ref. 36).

Recently, new p73 isoforms lacking the whole transactivation domain, called ΔNp73, were cloned in our laboratory (24) as well as by other groups (38-42), and were shown to inhibit the apoptotic function of p53 and TAp73 (24, reviewed in ref. 36). Although ΔNp73 isoforms have no transcriptional activity, they inhibit TAp73- or p53-dependent Bax transactivation. On the basis of these results, we also examined the effect of ΔNp73 isoforms on cell death induced by the TAp73 isoforms or by p53. Intriguingly, ΔNp73 isoforms did not induce apoptosis but completely abrogated TAp73- and efficiently inhibited p53-induced cell death. Although data reported here would exclude a major role for Bax transactivation in p73-induced apoptosis, the negative effects of ΔNp73 isoforms on p53-mediated Bax-transcription may be regarded as a model for ΔNp73-mediated suppression of p53/TAp73 transactivation in general. Importantly, we found that ΔNp73 also inhibited TAp73-induced PUMA upregulation, in turn blocking Bax translocation to mitochondria and subsequent cytochrome c release.

Together, these data indicate that the regulation of cell death by p73 occurs via the mitochondrial pathway, and is both indirect and complex. The ability of p73 to induce apoptosis is determined by the relative levels of expression of its TAp73 and dominant negative ΔNp73 isoforms. Where TAp73 isoform expression is dominant, the induction of apoptosis appears to be mediated through the transcriptional activation of PUMA, which facilitates mitochondrial translocation of Bax, rather than by direct transcription of Bax itself. The interactions between the
p53 family members now seem much more complex than previously expected (43, 44), resulting in a very tight regulation of the death pathways. The emergence of increasing complexity in cell death pathways enhances options for the manipulation of altered death/survival pathways in pathological conditions.

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FIGURE LEGENDS

FIG. 1. Induction of p73 isoforms in Saos-2 cells induces apoptosis. A, expression of HA-p73α, HA-p73β and HA-p73γ in dox-inducible Saos-2 cells following treatment with 2.5μg/ml dox for the indicated times. Protein expression was determined by Western Blot analysis with anti-HA antibody. One representative experiment out of five performed is shown. B, densitometric analysis of p73 protein levels, normalized to β-tubulin expression and expressed as arbitrary OD units. Values are averages ± SE of seven separate experiments. C-E, Steady state protein levels of pro-caspases 6 (panel C), 7 (panel D) and 8 (panel E) measured in response to dox stimulation of Saos-2 p73α, (C) p73β (D) and p73γ (E) inducible cell lines. The time of induction is indicated. One representative experiment out of three is shown. F, PI staining of Saos-2 cells upon induction of p73 expression for the indicated times. Apoptosis is expressed as percentage of sub-G1 hypodiploid events. Results are means ± SD of duplicate determinations carried out in two different experiments.

FIG. 2. p73 induces Bax up-regulation in Saos-2 cells. A, Bax transcriptional activation by different p73 isoforms. Saos-2 inducible cells were transiently transfected with Bax-Pr/Luc and Renilla plasmid as internal standard, and then either left untreated (-) or stimulated with 2.5μg/ml dox (+). Cells were harvested for luciferase assay 30h after transfection. The histograms show means ± SD of three different experiments each performed in duplicate. B, immunostaining of the Bax protein in p73γ- and p53-inducible Saos-2 cells. Cells were either left untreated (green line) or stimulated with 2.5 μg/ml dox for 72 (red line) and 96h (blue line). Immunostaining was performed with anti-Bax antibody, and Bax expression was analysed by flow cytometry and represented as mean relative fluorescence. Results are means ± SD of triplicate determinations. C, Bax immunostaining after dox-induced p73γ and p53 up-regulation (12-96 h). Data obtained in three separate experiments, each performed in triplicate, were averaged.

FIG. 3. p73 induces Bax translocation from cytosol to mitochondria. HeLa cells stably expressing a Bax-GFP fusion protein (green fluorescence) were either transiently transfected with the pDs-Red1-Mito plasmid alone (A) or cotransfected with the pDs-Red1-Mito plasmid and p73γ for 48h (B). C, subcellular fractionation of Bax protein in the Saos-2 p73γ cells following treatment with 2.5μg/ml dox for 48h. The Bax protein was detected in the mitochondrial fraction by Western Blot. D-F, Subcellular localization of p73 protein in p73γ-inducible Saos-2 cells. Cells were stimulated with 2.5μg/ml dox alone for 6h (D) and 48h (E) or co-incubated with dox and 25μM
cisplatin for 48h (F). The p73 protein (green fluorescence) was stained with anti-HA antibody. G, H, distribution of endogenous Bax protein in the p73γ-inducible clones. Cells were either left untreated (G) or incubated with 2.5μg/ml dox for 48h (H). Bax protein and apoptosis were detected by immunofluorescence using anti-Bax antibody (green fluorescence) and TUNEL assay (red fluorescence), respectively. I, Saos-2 cells were transiently cotransfected with HA-p73γ and pDs-Red1-Mito (red fluorescence) plasmids for 48h and stained with anti-HA antibody to detect p73 (green fluorescence). A representation of the staining pattern is shown. Three distinct experiments were performed. Bars indicate 10μm.

**FIG. 4.** p73 induces PUMA, which in turn causes mitochondrial relocalization of Bax and release of cytochrome c. A, induction of PUMA by p73. Endogenous levels of PUMA were detected by real time RT-PCR in either unstimulated (lane 1) or dox-induced (lanes 2-4) p73 expressing cells as described in Fig. 1. A time course of PUMAα and PUMAβ induction is shown. A representative experiment of two performed is shown. HeLa cells stably expressing either Bax-GFP (B-D) or cytochrome c-GFP (E-G) were transfected with PUMAα (C, F) or β (D, G). GFP was visualised 24h after transfection. Mitochondria were counter-stained with pDs-Red1-Mito plasmid. A representative staining pattern is shown. Three distinct experiments were performed. Bars indicate 10μm.

**FIG. 5.** PUMA expression leads to conformational shift and mitochondrial localization of endogenous Bax. (A) U2OS cells were transfected with PUMAα for 24h and endogenous Bax protein levels were detected by anti-Bax antibody, clone 6A7, which recognises the activated membrane-bound form of Bax. (B) H1299 PUMAα-inducible cells were transiently transfected with Bax-GFP, stimulated with 2.5μg/ml dox for 8h before harvesting, and then subjected to immunofluorescence analysis.

**FIG. 6.** PUMA does not induce apoptosis in the absence of Bax/Bak. (A) Bax−/− mouse embryo fibroblasts (MEF), or the corresponding wild type cells, were cotransfected with HA-p73γ and cytochrome c–GFP expression vectors, and then subjected to immunofluorescence analysis at the times indicated. The nuclear red fluorescence (anti HA) shows cells expressing p73γ. While at 48h Bax−/− cells show a delayed release of cytochrome c (green color), at later time points (60h) there was no difference; at 72h cells were apoptotic. Bar= 10 μm. A representative experiment of three is shown. (B) Bax−/− MEFs show a delayed apoptotic response to DNA damage elicited by 25 μM cisplatin. Apoptosis, evaluated by flow cytometry, is expressed as percentage of sub-G1 hypodiploid events (n= 20,000). Two experiments were performed in triplicate, and represented in
(C) Evaluation of p73 protein levels induced by cisplatin in the same experiment described in panel B. A representative experiment of three is shown. (D) Wild type, Bax 

-/-, Bak 

-/-, Bax 

-/-;Bak 

-/- and p53 

-/- MEFs were transfected with PUMAα for 24h and cell death was measured by flow cytometry after PI staining to quantitate apoptosis. Apoptosis is expressed as percentage of sub-G1 hypodiploid events (n= 20,000). The panel represents the mean of three experiments, each performed in triplicate.

**Fig. 7. p73\textsuperscript{ΔN} isoforms interfere with TAp73 transcriptional and apoptotic activities.** A, mouse neuroblastoma N1E-115 cells were transiently cotransfected with Bax-Pr/Luc and p73α, p73β, p73γ, p73δ or p53 expression vectors in the absence or in the presence of a plasmid encoding Δ84p73β. B, Saos-2 cells were transiently cotransfected with Bax-Pr/Luc, p73α or p53 expression vectors with or without expression plasmids encoding ΔNp73α, ΔNp73β or ΔNp73γ. Cell extracts were prepared 48h later and luciferase activity determined. Results shown in A and B are means ± SD of three different experiments each performed in duplicate. C, ΔNp73 inhibits TAp73-dependent transactivation of PUMA. p73γ-inducible Saos-2 cells were transfected with pcDNA (lane1, 4), ΔNp73α (lanes 2, 5), or ΔNp73γ (lanes 3, 6) together with pBABEpuro. Cells were selected in 1μg/ml puromycin for approximately two weeks to obtain stable transfectants. Stable cell lines were then treated with dox (2μg/ml) for 24h to induce the expression of p73γ (lanes 4-6). Total RNA was isolated and subsequently subjected to RT-PCR using PUMA specific primers. The presence of the ΔN isoforms of both p73α and γ abrogated the induction of all PUMA transcripts upon dox treatment (lanes 5 and 6). The panel show a representative result of three different experiments performed with identical results. D, Saos-2 cells were transiently cotransfected with p73α, p73γ or p53 expression vectors with or without expression plasmids encoding ΔNp73α. PI staining was performed on cells fixed 72h later and apoptosis (expressed as percentage sub-G1 hypodiploid events) was assessed. Histograms show the mean ± SD of three different experiments, each performed in triplicate.

**FIG. 8. p73 induces apoptosis via PUMA-mediated Bax mitochondrial translocation.** Schematic representation of the p73 downstream mediators of cell death. p73 transcriptionally regulates both Bax and PUMA. While Bax induction is not sufficient to trigger apoptosis, PUMA causes mitochondrial relocation of Bax, thus triggering mitochondrial cytochrome c release, in turn leading to apoptotic cell death. The ΔNp73 protein (and similarly, other cancer-specific isoforms with deletion of the TA domain), regulated by a distinct promoter, inhibits TAp73 and p53 transcriptional properties, hence having anti-apoptotic effects.
Melino et al., Fig. 1
Melino et al., Fig. 2
Melino et al., Fig. 3
Melino et al., Fig. 4
Melino et al., Fig. 5

A  PUMA       Bax       merge (+ DAPI)

B  Bax       PUMA       merge (+ DAPI)
Melino et al., Fig. 6
Melino et al.,
Fig. 7
Melino et al., Fig. 8
p73 induces apoptosis via PUMA transactivation and Bax mitochondrial translocation

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