p73β-Mediated Apoptosis Requires p57^{kip2} Induction and IEX-1 Inhibition

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Abstract

Similarly to p53, p73α and p73β induce growth arrest and/or apoptosis in response to DNA damage or when exogenously expressed. However, how they trigger apoptosis remains unresolved. After stable transduction of either p73α or p73β, a greater apoptotic response was observed for p73β in both primary and tumor cells. Consistently, blocking ectopic and endogenous p73β expression by specific shRNA significantly decreased apoptotic levels after DNA damage. We found that p73β targets the apoptotic program at multiple levels: (i) facilitating caspase activation through p53-dependent signals and (ii) inducing p57^{KIP2}, while down-regulating c-IPA1 and IEX1 through a p53-independent mechanism. p73β-mediated apoptosis was considerably reduced after inhibition of p57^{KIP2} by small interfering RNA, IEX-1 overexpression, and in mouse embryo fibroblasts derived from p57$^{-/-}$ mice. Data from this study offer evidence for the apoptotic activity exclusive of p73β. In the clinical context, these results might have potential therapeutic implications, because p73β could induce alternative apoptotic responses in tumors harboring p53 mutations. (Cancer Res 2005; 65(6): 2186-92)

Introduction

p73α and *p73β*, besides having close homology to *p53*, also share certain p53 functions, including their role on transcriptional regulation and their ability to induce cell death following transient overexpression (1–4). In addition, several tumor-associated stress signals, such as DNA damage, or known drugs that induce the expression of p53-responsive genes, also activate p73α and p73β (5–12). Studies conducted using knockout mice revealed that the induction of apoptosis by p53 requires the presence of an active p73 (13). It has also been reported that p73 can induce apoptosis in tumor cells lacking functional p53 (14–16). The involvement of p73α and p73β in mediating chemosensitivity has been studied by specific p73 expression knockdown, revealing a decrease in apoptosis after treatment (14). These results imply that p73 might function as a signal transducer of drug-induced DNA damage to the apoptotic machinery of the cells. In consequence, inactivation of p73 function may reduce the apoptotic response, as well as decrease the cytotoxicity induced by anticancer agents.

Distinct p73 isoforms, varying in their COOH termini, have been reported to be functionally different. For example, the ability of p73β to inhibit cell growth in p53-deficient cells was found to be larger than that of p73α (17–20). Although the functional differences among these splice variants are not well understood, these observations suggest that the COOH-terminal region of p73α may play a regulatory role, modulating its ability to induce gene expression.

Therefore, it is necessary to identify novel p73 target genes that might mediate and distinguish p73α- from p73β-induced apoptosis, shared or not by p53. In this report, stable tumor and primary cell lines exogenously expressing either p73α or p73β were generated. A stronger apoptotic induction was observed for p73β when compared with that of p73α. To further dissect the mechanisms and target genes involved in either p73α- or p73β-mediated apoptosis, we studied the specific p73α- and p73β-apoptotic activities. We found that p73β targets p53-dependent and p53-independent pathways affecting apoptosis.

Materials and Methods

Cells and Gene Transfer. H1299, HCT116, MEF3T3-Tet^{off} (Clontech Labs, Inc., Palo Alto, CA), and mouse embryo fibroblasts (MEF) were grown in DMEM medium supplemented with 10% FCS. H1299-Tet^{off} and HCT116-Tet^{off} were developed by stably transfected with the regulator plasmid (pTet-Off), following the manufacturer's instructions (Clontech Labs) and supplemented G418 (50 μg/mL). H1299-Tet^{off}, HCT116-Tet^{off}, and MEF3T3-Tet^{off} cells were infected with recombinant retroviruses expressing HAp73α, HAp73β, HAp73β292, or HAp73α292 under the control of a tetracycline-regulated promoter of low copy number (pMSCV^{puro}-TREp73α, -p73β, -p73β292 and -p73α292) and selected with puromicin (2 μg/mL). In a Tet-off system, the cells are maintained in the presence of tetracycline (t = 0). Following withdrawal of tetracycline, cells were induced to selectively express ectopic p73α, p73β, p73β292, or p73α292 proteins at different times. DBD-shRNA and Ct-shRNA were transfected into H1299 and HCT116 cells plated at 0.6 × 10^5 per 35-mm dish, using recombinant retrovirus (pMSCV^{puro}-DBDshRNA and pMSCV^{puro}-SAMshRNA, encoding IRES-GFP 3′), whereas specific p73β-siRNA was transient transfected, at the amounts indicated in the corresponding figure legends. Cloning strategies and primer sequences are available from the authors upon request or from Dharmacon Research (Lafayette, CO).

Small Interfering RNA. The small interfering RNA (siRNA) duplexes were synthesized by using protocols supplied by Dharmacon Research. The control Luciferase-siRNA (Luc-siRNA) was 5′-gccattctatcctctagaggatg-3′. siRNA duplexes were transient transfected by using Oligofectamine (Invitrogen, San Diego, CA) following the manufacturer's instructions, and analyzed 36 hours post-transfection.

Gene Expression and Protein Analysis. For p73 activation, H1299 and HCT116 cells were treated with 15 μmol/L cisplatin (Sigma, St. Louis, MO) for 24 hours. Cellular extracts (40 μg) were resolved on 4% to 20% SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were incubated with the following primary antibodies: for specific p73 detection, anti-p73 antibodies (C17, Santa Cruz Biotechnology, Santa Cruz, CA; ER13, Cell Signaling, Beverly, MA); for HA-p73 proteins 12CA5Mab (BabCo,

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Richmond, CA); for p21 detection, anti-p21 antibodies (Ab-1, Oncogene Research Products, Uniondale, NY). Anti-Ran antibody (Santa Cruz Biotechnology) was used to normalize the amount of loaded proteins. Bax, Noxa, Bak, Bid, Apaf1, Casp9, and PIG3 were detected with anti-Bax (Cell Signaling), anti-Noxa (Ab1; Oncogene Research Products), anti-Bak (Santa Cruz Biotechnology), anti-Bid (Santa Cruz Biotechnology), anti-Apaf1 (Upstate), anti-Casp9 (Cell Signaling), and anti-PIG3 (Ab1; Oncogene Research Products) antibodies, respectively. Mdm2, p57, IEX-1, and cIAP1 were detected by using anti-Mdm2 (clone 2A10; a gift from A. Levine, Cancer Institute of New Jersey, University of Medicine and Dentistry of New Jersey, New Brunswick, NJ), anti-p57^{KIP2} (C20; Santa Cruz Biotechnology), anti-IEX-1 (C20; Santa Cruz Biotechnology), and anti-cIAP1 (H83; Santa Cruz Biotechnology) antibodies, respectively. Horseradish peroxidase–conjugated secondary antibodies were used to detect the bound primary antibodies. Immune complexes were visualized with an Enhanced Chemiluminescence detection system (Amersham, Arlington Heights, IL), and further quantificated by ImageQuant software (Phosphorimager, Molecular Dynamics, Amersham Biosciences Corp., Piscataway, NJ).

Apoptosis Assays. For Annexin V-propidium iodide (PI) analysis, floating and adherent cells were collected, washed with cold PBS, and resuspended in 200 μL of binding buffer containing Annexin V-FITC (0.5 μg/mL; Clontech Labs) and 5 μg/mL PI (Sigma), in accordance with the manufacturer's instructions. The percentage of apoptotic cells was calculated by scoring for cells that were initially PI-positive and further positive for Annexin V. Stained cells were analyzed in a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson, Mountain View, CA).

For terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay, the cells were previously fixed at the indicated times with 70% ethanol overnight, washed with PBS, and terminal transferase reaction was done following the manufacturer's instructions (*In situ* Cell Death Detection Kit, Fluorescein, Roche, Nutley, NJ). Briefly, the assay is based on the labeling of DNA strand breaks by terminal transferase, which catalyzes polymerization of labeled fluorescein-dUTP to free 3′-OH DNA ends (TUNEL reaction). The percentage of apoptotic cells was calculated by scoring for cells that were initially PI-positive and positive for the fluorescein labels, or TUNEL-positive cells by flow cytometry. Data shown are representative of three independent experiments.

Results

Enhanced Apoptotic Response of Ectopic p73β Expression when Compared with that of p73α. The lack of tumor-associated stress signals, such as DNA damage, which could selectively activate and stabilize specific p73 isoforms does not allow to analyze the apoptotic role of individual p73 proteins. To understand how different p73 isoforms induce cell death, a p53 apoptotic-like response was reproduced using retrovirally mediated stable transduction of either *p73α* or *p73β* under the control of a tetracycline-inducible promoter in H1299- (p53-null), HCT116- (wt p53), and MEF3T3-Tet^{off} (wt p53) cells. As a control, p73α292 mutant defective for DNA binding and transcriptional activation (R292H, homologous to p53R273H), was tested for its proapoptotic ability, as well as p73β292 (data not shown; ref. 2). After induction in H1299 cells, the expression of p73 proteins was analyzed after 36, 60, and 96 hours by immunoblotting using anti-HA-specific antibodies. p73 proteins peaked at 36 hours post-induction to levels similar to those detected after treatment with cisplatin for 24 hours, decreasing at 96 hours after induction (Fig. 1*A*). Western blot analyses done with both HCT116 and MEF3T3 cells following p73α or p73β induction revealed similar kinetics (data not shown). To verify whether the induced proteins were transcriptionally active, we checked the level of endogenous of p21, a known target of p53 and p73, and found it similarly induced by both p73α and p73β (Fig. 1*A*).

To characterize the time-dependent apoptotic effects after selective p73α, p73β, or p73α292 induction, H1299 cells were harvested and subsequently analyzed for apoptosis by TUNEL assay. Flow cytometric analysis showed that the percentage of apoptotic cells increased from 1.1% to 10.5% after 96 hours of p73α induction (Fig. 1*B*, *top*). Strikingly, p73β produced a significantly higher apoptotic response, up to 21.1% after 96 hours (Fig. 1*B*, *middle*). Cells transduced with the transcriptionally inactive mutants, either p73α292 or p73β292 (data not shown) showed lack of both cell cycle arrest and apoptotic functions, supporting the importance of transcriptional regulation in these responses (Fig. 1*B*, *bottom*). Additionally, we did simultaneous Annexin V and PI staining on the inducible HCT116 cells. At 96 hours after p73β induction, 32.6% of cells were Annexin V positive. A fraction of those Annexin V–positive cells was also PI positive (11.4%), suggesting that at 96 hours some cells had already entered into a late apoptotic stage (Fig. 1*C*). The effect of a time-dependent expression of p73α and p73β was also tested on primary cells. MEF3T3-Tet^{off} cells transduced with p73α, p73β, or p73α292 were maintained in the absence of tetracycline for varying periods, and the number of cells undergoing cell death was studied by TUNEL assay. p73β-induction of led to a substantially enhanced apoptotic response (1.4-17%; Fig. 1*D*). Taken together, these results indicate that tumor and primary cells exogenously expressing p73β undergo apoptosis to a greater extent than those expressing p73α.

Modulation of Apoptotic Response by Specific p73α and p73β Expression Knockdown. To confirm whether the greater p73β apoptotic response was specific at the endogenous level, retroviral vectors containing short hairpin RNAs (shRNAs) targeting specific p73 isoforms were generated and assessed for their ability to impact on p73 and apoptotic levels. These shRNAs were designed either against the DNA binding domain (DBD-shRNA), inhibiting the expression of all *p73* isoforms, or the SAM domain (SAM-shRNA), specifically blocking *p73α* but not *p73β*. In addition, a specific p73β-siRNA was designed to selectively silence *p73β* but not *p73α*. After transient transfection of these shRNAs or siRNAs into either HCT116-p73α or HCT116-p73β inducible cells, p73 protein levels were analyzed by immunoblotting using anti-p73 antibodies (Fig. 2*A*). Treatment with DBD-shRNA resulted in reduced to undetectable levels of p73α and p73β, whereas cells transfected with SAM-shRNAs showed reduced p73α levels but maintained p73β expression (Fig. 2*A*). As expected, p73β-siRNA knocked down only p73β levels. As controls, neither GFP-shRNA nor Luc-siRNA had effect on p73 expression. TUNEL assay revealed that the percentage of cells undergoing apoptosis in the presence of DBD-shRNAs decreased from 11% to 2.3% for HCT116-p73α, and from 16.9% to 3% for HCT116-p73β (Fig. 2*B*). However, whereas the transient expression of SAM-shRNA against *p73α* decreased the apoptotic percentage to control levels, the apoptotic rate remained 15.6% in cells overexpressing p73β (Fig. 2*B*). This result was reversed in the presence of p73β-siRNA, indicating an important and specific apoptotic activity of p73β.

We then analyzed whether these p73-specific shRNAs produced the expected effect on cells whose endogenous p73α expression was induced in response to DNA damage (21). Parental H1299 or HCT116 cells were treated without (−) or with cisplatin (30 μmol/L) for 24 hours and further transfected with DBD-shRNA, SAM-shRNA, or p73β-siRNA, and as control GFP-shRNA, for additional 24 hours and then examined by TUNEL assay. As shown in Fig. 2*C*, both p73α and p73β levels increase after cisplatin treatment. The cell death level induced by this apoptotic stimulus decreased after

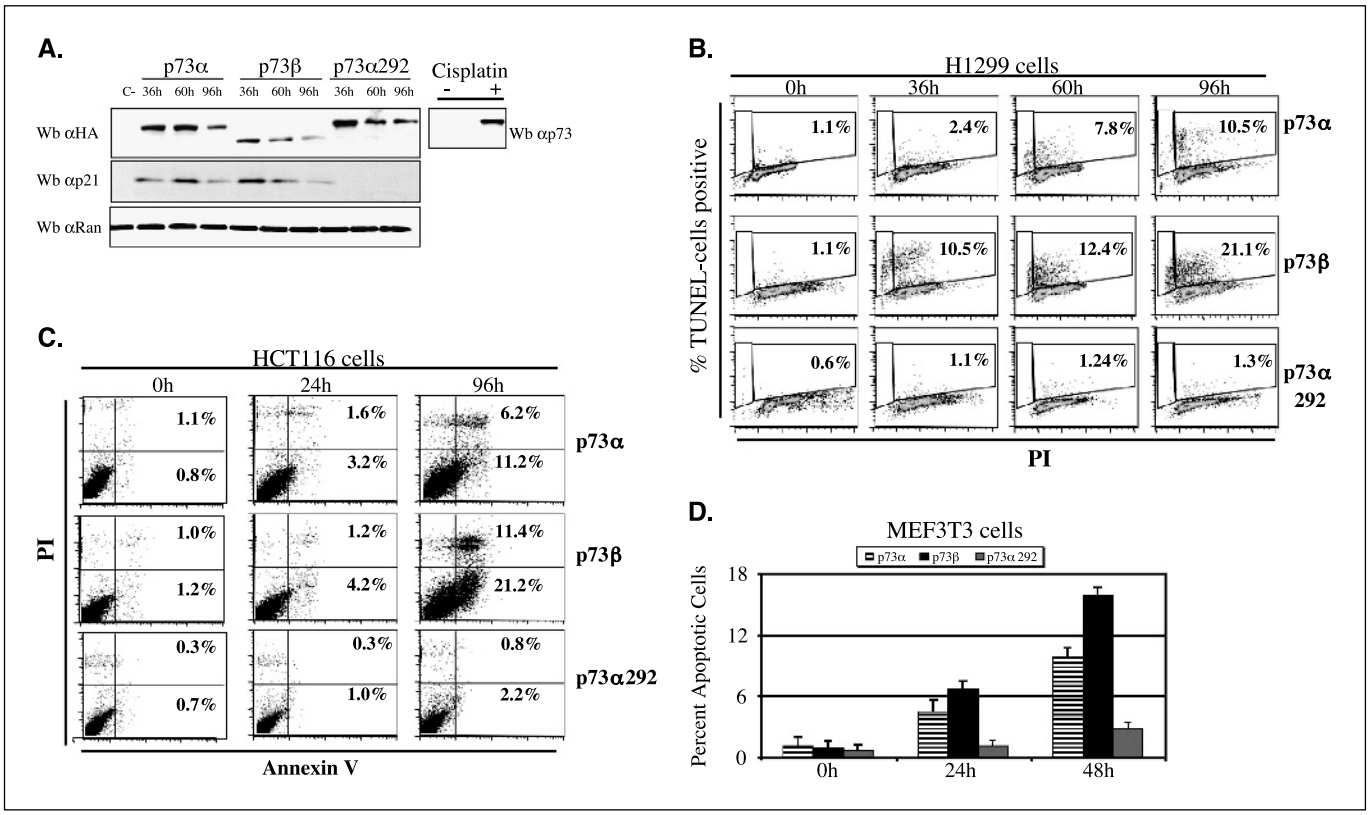

Figure 1. Inducible expression of p73α, p73β, and p73α292 proteins and subsequent cellular apoptotic responses. *A,* levels of p73 protein were assessed on tetracycline-inducible H1299 cells stably transduced with recombinant retroviruses expressing p73α, p73β, and p73α292 proteins. Western blot (*Wb*) analysis showed either induced expression of HA-tagged p73α, p73β, and p73α292 following by withdrawal of tetracycline at the indicated time points, or in the presence of tetracycline (*C-*) as a control. Ran was examined as a loading control, and endogenous p21 expression, as a control of p73 transcriptional activity. H1299 cells were untreated (−) or treated (+) with cisplatin for 24 hours and cellular extracts were prepared. Endogenous p73α proteins were revealed by Western blot analysis using anti-p73 (ER13; *right*). *B,* % apoptotic tetracycline-inducible H1299 cells expressing p73α, p73β, and p73α292 before (t = 0) and after removal of tetracycline at different times. Quantification was done by biparametric analysis of TUNEL-positive cells levels versus DNA content by flow cytometry (see details in Materials and Methods). *C,* apoptotic levels of tetracycline-inducible HCT116 cells after ectopic overexpression of p73α, p73β, and p73α292 as control before (t = 0) and after removal of tetracycline at different times. % Apoptotic cells was determined after Annexin V-PI (see details in Materials and Methods). *D,* % apoptotic cells from tetracycline-inducible MEF3T3 cells upon ectopic overexpression of p73α, p73β, and p73α292 before (t = 0) and after removal of tetracycline at different times. Quantification was done by TUNEL assay and flow cytometric analysis. *Columns*, means of triplicate in one of three independent experiments; *bars*, ±SD.

DBD-shRNAs expression from 30% to 13% and from 38% to 15% on H1299 and HCT116 cells, respectively (Fig. 2*D*). However, SAM-shRNAs treatment did not significantly modify the expected apoptotic levels in DNA-damaged H1299 and HCT116 cells, whereas p73β-specific siRNAs were able to decrease the cell death levels to 17% and 20%, respectively (Fig. 2*D*). These results further support a more prominent proapoptotic role *in vivo* for p73β than for its p73α homologue.

Specific Induction of p73β Expression Results in Activation of Apoptotic Target Genes Activated in p53-Dependent and p53-Independent Pathways. In performing a series of microarray experiments, we dissected target genes involved in either p73α- or p73β-mediated apoptosis.[3] We then decide to investigate and validate whether the observed differences in the expression of regulated targets accounted for the functional differences between p73α- and p73β-apoptotic activities. After ectopic p73α, p73α292, and p73β expression, the expression of selected targets was analyzed by Western blotting and further quantified using Image-Quant software (Fig. 3*A* and S1). Following the induction of the apoptotic cascade associated with mitochondrial release (22), we found that proapoptotic targets of p53 such as Noxa, Bak, Bid, and Bax were up-regulated by p73β. However, when p73α was overexpressed, Noxa levels were reduced after 60 hours post-induction (49.5%) and even lower with p73α292 (22%). Whereas Bid and Bax were weakly up-regulated by p73α after 60 hours, Bak levels were similar after forced expression of either p73α or p73α292 (29% and 25% at 60 hours, respectively). These proteins localize at the mitochondria, promoting loss of mitochondrial membrane potential and cytochrome *c* release, thereby activating the Apaf-1/caspase 9 dead-effector complex. These latter events were also analyzed by immunoblotting and further quantificated, revealing enhanced caspase 9 processing, PIG3 activation, and Apaf-1 induction by p73β when compared with that produced by p73α. Altogether, these findings strongly suggest that Bax, Noxa, Bak, Bid, PIG3, Apaf1, and Casp9 are mediators of p73β-induced apoptosis.

A time course of p73β induction revealed the consistent enhanced expression of Mdm2, decreasing in a dose-dependent manner with p73β levels, whereas it was barely detectable after p73α induction (Fig. 3*B*). Moreover, the cyclin-dependent kinase inhibitor $p57^{KIP2}$ was significantly induced by p73β. Furthermore,

[3] S. Gonzalez and C. Cordon-Cardo, unpublished data.

the inhibitor of apoptosis protein c-IAP1, which suppresses apoptosis by preventing procaspase activation and inhibiting mature caspase activity, and the immediate early gene 1 (*IEX-1*) were considerably lower in cells overexpressing p73β than in those overexpressing p73α (60% versus 30% and 60% versus 10% at 90 hours, respectively). These findings suggest that p73β proapoptotic function is not only shared with the p53-dependent apoptotic pathway, but it is also regulated through p53-independent mechanisms.

Contribution of New Apoptotic Target Genes on p73β-Mediated Apoptotic Response: *p57KIP2* and *IEX-1*. To further study the p57^{KIP2} role on p73β-mediated apoptosis, we sought to selectively silence *p57^{KIP2}* gene transcripts. H1299 cells were stably transduced with retroviral vectors expressing either p73α or p73β for 24 hours and transiently transfected with increasing amounts of p57^{KIP2}-siRNA for additional 24 hours (Fig. 4*A*). The effects of p57^{KIP2} silencing were then evaluated with regard to the proapoptotic activity of either p73α or p73β by TUNEL assay. Because p73β induces p57^{KIP2}-mediated transcription (23), it was of interest to test whether its absence would repress p73β-induced apoptosis. As illustrated in Fig. 4*B*, increasing p57^{KIP2}-siRNA levels led to a substantial reduction of p73β-induced cell death (22-7%). These data suggested that the proapoptotic ability of p73β is likely mediated by p57^{KIP2} activity. Additional support for p57^{KIP2} and p73β connection was shown by cisplatin treatment (Fig. 4*C*). We thus analyzed whether p57^{KIP2} silencing produced the expected results in cells wherein endogenous p73α and p73β expression was induced in response to DNA damage. H1299 and HCT116 cells were treated with cisplatin for 24 hours, further transiently transfected with p57^{KIP2}-siRNA, and then examined by TUNEL assay. The cell death level induced by an apoptotic stimulus decreased after p57^{KIP2}-siRNA expression from 35% to 12% and from 47% to 15% using H1299 and HCT116 cells, respectively (Fig. 4*C*). These data suggest that the apoptotic effect caused by cisplatin action requires p57^{KIP2} induction, likely mediated by p73β.

To further characterize the importance of p57^{KIP2} in the apoptotic response elicited by p73β, we did additional experiments using *p57$^{KIP2+/+}$*, *p57$^{KIP2-/-}$*, and *p53$^{-/-}$* MEFs. We transduced these cells with recombinant retroviruses expressing p73β, p57^{KIP2}, or transiently transfected them with IEX-1 or p57^{KIP2}-siRNA. The levels of p73β, IEX-1, and p57^{KIP2} were assayed by Western blot analysis (Fig. 5*A*), and the number of

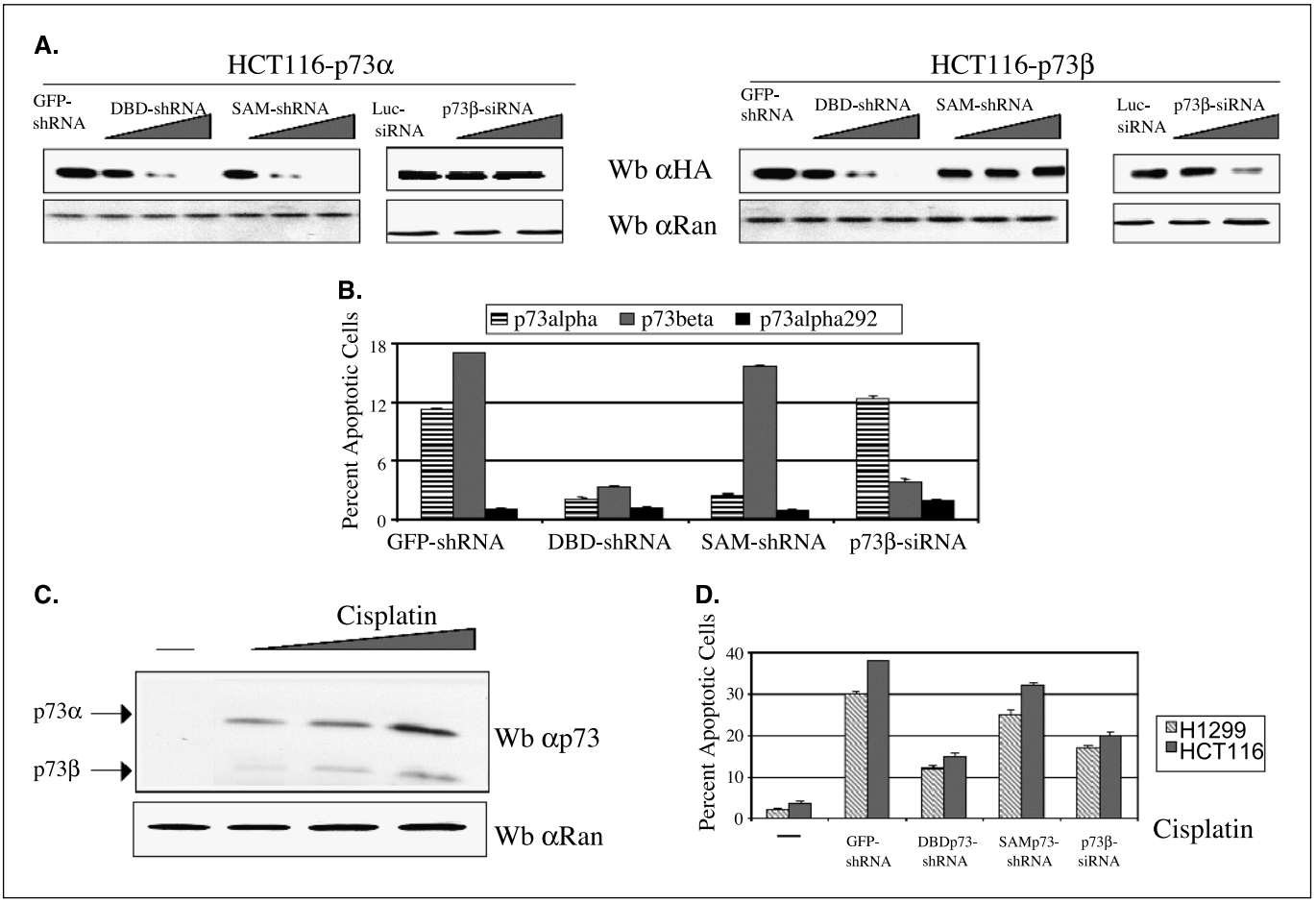

Figure 2. Silencing of ectopic and endogenous p73α and p73β expression by p73-specific shRNAs. *A* and *B,* HCT116 cells plated at 0.6 × 10^5 per 35-mm dish overexpressing p73α (*A*, *left*) or p73β (*B*, *right*) proteins after 24 hours were transfected with increasing amounts of DBD-shRNA, SAM-shRNA, or p73β-siRNA for additional 24 hours (*triangle,* 50, 100, and 500 ng, respectively), and GFP-shRNA (500 ng) or Luc-siRNA (500 ng) as controls. The HAp73 levels were assessed by immunoblotting using anti-HA antibodies, as well as Ran protein as loading control (*A*). % Apoptotic cells treated as described above obtained by TUNEL assay (*B*). *C,* levels of p73 isoforms were analyzed by Western blot (ER13 Ab) detecting endogenous p73α and p73β induction before (−) and after cisplatin treatment (*triangles,* 15, 30, and 60 μmol/L, respectively) in H1299 cells for 36 hours. *D,* parental H1299 or HCT116 cells plated at 0.6 × 10^5 per 35-mm dish were treated without (−) or with cisplatin (30 μmol/L) for 24 hours and further transfected with DBD-shRNA, SAM-shRNA, or p73β-siRNA, and GFP-shRNA as control, for additional 24 hours. The apoptotic levels were obtained by TUNEL assay. *Columns*, means of triplicate in one of three independent experiments; *bars*, ±SD.

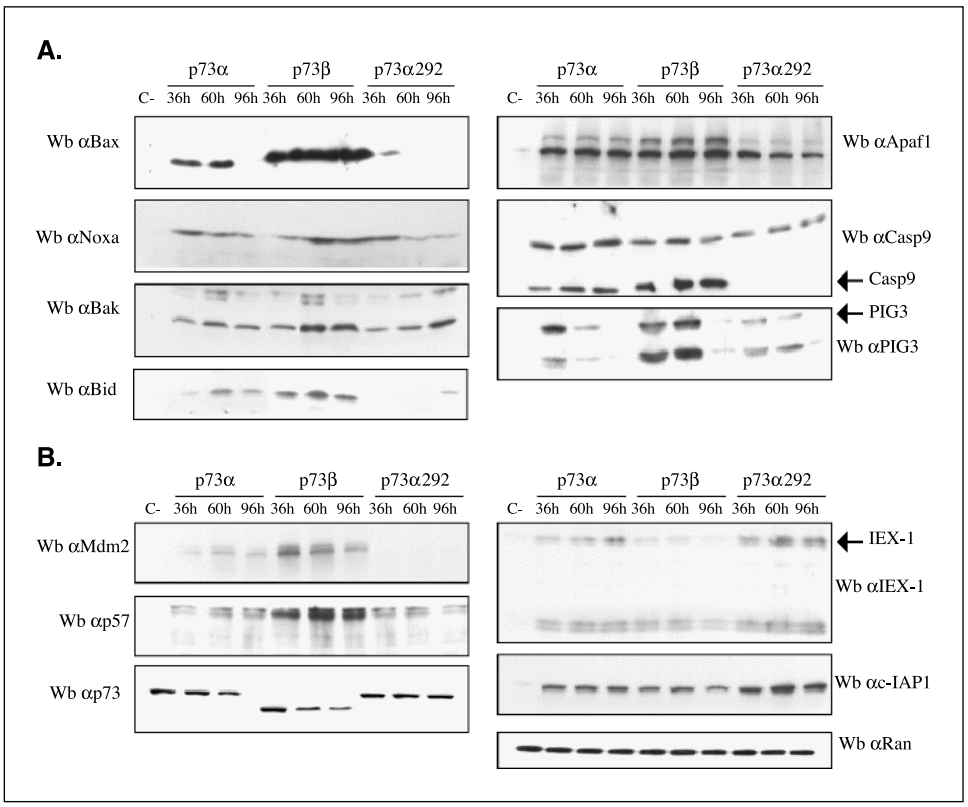

Figure 3. Regulation of apoptotic target genes by p73β and p73α. *A* and *B,* tetracycline-inducible H1299 cells stably tranduced with recombinant retroviruses expressing p73α, p73β, and p73α292 proteins following withdrawal of tetracycline at the indicated time points, or in the presence of tetracycline (*C-*). Total extracts were subjected to immunoblotting for analyzing Bax, Noxa, Bak, Bid, PIG3, Apaf1, Casp9, Mdm2, p57, IEX-1, and c-IAP1 proteins levels. Antibodies were used as described in Materials and Methods. Anti-Ran immunoblotting was performed as loading control.

cells undergoing apoptosis was analyzed by staining with Annexin V-PI (Fig. 5*B-C*). As shown in Fig. 5*B*, ectopic p73β expression induced a significantly higher apoptotic response in $p57^{+/+}$ MEFs (24%) than in $p57^{-/-}$ MEFs (10%). Moreover, similar apoptotic effect was obtained when we forced p73β expression and silenced $p57^{KIP2}$ in $p57^{+/+}$ MEFs (14%; Fig. 5*B*). This result was reversed when coexpressing both p73β and $p57^{KIP2}$ in $p57^{-/-}$ MEFs (26%; Fig. 5*B*). Of note is that forced p73β expression and $p57^{KIP2}$ in the absence of p53 raised the cell death level to 28%. According to our previous results, the proapoptotic ability of p73β is mediated to some extent by $p57^{KIP2}$. Finally, the p73β-specific repression of IEX-1 was also validated by overexpression of IEX-1 in $p57^{+/+}$, $p57^{-/-}$, and $p53^{-/-}$ MEFs, followed by apoptotic analysis. The p73β-specific apoptotic effect observed in $p57^{+/+}$ MEFs was partially reversed by both p73β and IEX-1 coexpression (27% versus 16%, respectively; Fig. 5*C*). Furthermore, forced p73β expression along with IEX-1, in a $p57^{-/-}$ context, raised the apoptotic level to only 7%. Altogether, these results offer evidence for proapoptotic activity of p73β mediated by both $p57^{KIP2}$ induction and IEX-1 inhibition.

Discussion

In contrast to *p53*, *p63*, and *p73* give rise to multiple functionally distinct protein isoforms, some of which differ at their COOH-terminal ends as a result of differential splicing, whereas others lack the NH_2-terminal transactivation domain due to the use of alternative promoters. p73α is the longest form of the p73 family, which contains a sterile α motif (SAM motif), whereas p73β is missing most of the SAM domain. Interestingly, both p73α and p73β have been linked to apoptosis, although the role of p73 in suppressing tumorigenesis is still unclear, because p73-deficient mice are not tumor prone and inactivating mutations in tumors have not been identified (1). Besides, increasing evidence suggests that p73 proteins can regulate genes that do not strictly coincide with p53-responsive genes (14, 16).

The present study reveals enhanced apoptotic ability of p73β when compared with that of p73α in both primary and tumor cell lines. Furthermore, shRNA-mediated specific p73α silencing in cells treated with cisplatin does not significantly decrease the apoptotic levels, whereas simultaneous p73α and p73β expression knockdown notably reduced cell death. Moreover, silencing of only p73β, using p73β-specific siRNAs, reduced the apoptosis from 30% to 17% and 38% to 20%, when using H1299 and HCT116 cells, respectively. These results support not only an important and specific proapoptotic role for p73β when compared with its p73α homologue but also a potential critical contribution of p73β to the apoptotic cellular response. The causes for these differences were studied by expression-profiling experiments based on oligonucleotide microarrays, and relevant candidate genes were further analyzed at the protein level.[4] Thus, we have found a strong functional similarity among p73β-induced targets and well-established p53 downstream genes involved in cell death, including the multidomain Bcl-2 family member *Bax*, and "BH3-only" members such as *Puma*, *Noxa*, *Bid*, and *Bak*. p73β, as p53, can also transactivate several components of the apoptotic effector machinery, such as *Apaf-1*, which acts as a coactivator of caspase 9, assisting in the initiation of the caspase cascade (24). In all cases, the promoters of these genes harbor consensus p53 response elements and we postulate that also respond to p73β induction.

[4] S. Gonzalez and C. Cordon-Cardo, unpublished data.

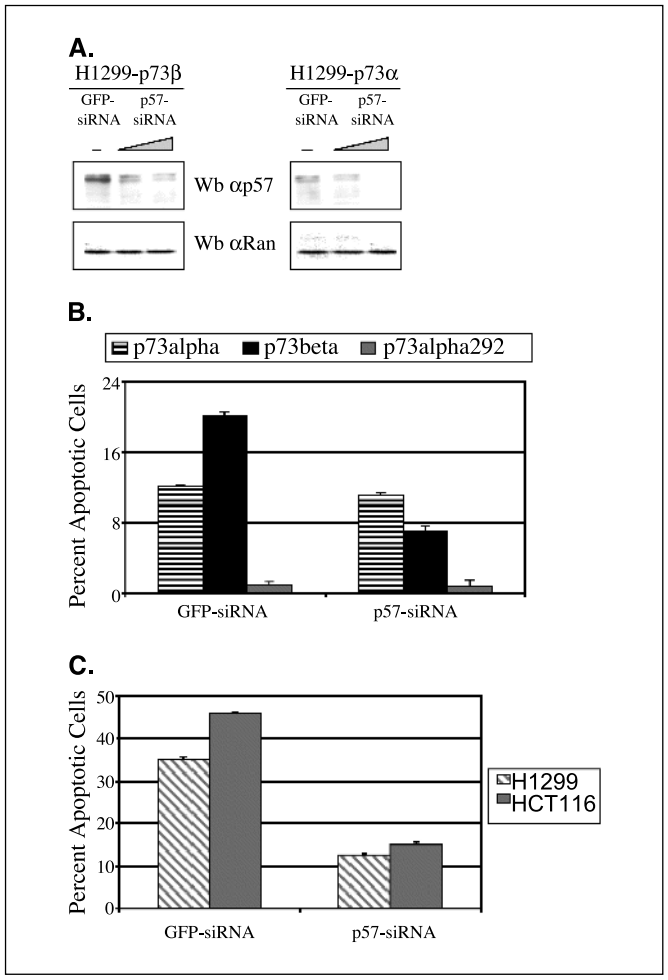

Figure 4. Contribution of p57KIP2 on p73β-mediated apoptosis. *A,* H1299 cells plated at 0.6×10^5 per 35-mm dish were stably transduced with retroviral vectors expressing either p73α or p73β for 24 hours and transiently transfected with increasing amounts of $p57^{KIP2}$-siRNA (*triangle,* 50 and 500 ng, respectively) or GFP-siRNA as control (500 ng) for additional 24 hours. Cellular extracts were prepared, and endogenous $p57^{KIP2}$ proteins were revealed by Western blot analysis using anti-$p57^{KIP2}$ antibodies. *B,* apoptotic levels of H1299 and HCT116 cells treated as described above (*A*) were measured by TUNEL assay. *C,* H1299 and HCT116 cells were treated with cisplatin (30 μmol/L) for 24 hours, further transiently transfected with $p57^{KIP2}$-siRNA (500 ng) or GFP-siRNA as control (500 ng) for additional 24 hours, and then examined by TUNEL assay. *Columns*, means of triplicate in one of three independent experiments; *bars*, ±SD (*B* and *C*).

Surprisingly, p73α-mediated apoptotic response was significantly reduced compared with p73β, probably due to the negative regulatory effect of the COOH-terminal region on transcriptional activities (10). It is conceivable that such domain may influence transcription in a p53-independent manner. Thus, interactions with other proteins through the SAM domain could result in the variability of the regulation observed for Bax, Noxa, Bak, Bid, Apaf1, procaspase 9, and PIG3. In addition, differences in the ability of p73α to activate p53 target genes may also rely on post-translational modifications of the protein (21). It has been reported that p73α can be stabilized by proteosome inhibition, whereas p73β is not, suggesting that sequence elements present in the p73α COOH terminus may target it for degradation (24). Mdm2 binds p73α and reduces its activity as a transcription factor, but unlike p53, the steady-state amount of p73α protein is not reduced in the presence of Mdm2 (25, 26). In the present study, we observed that p73β induction was associated with Mdm2 expression, which decreased in a dose-dependent manner with p73β but not with p73α levels. Prevention of Mdm2-mediated degradation could explain the p73β stability observed. In the absence of p53, we favored the idea that p73β regulation by Mdm2 could lead to p73β protein accumulation and subsequent activation of a p53-like cellular response. Detailed studies are

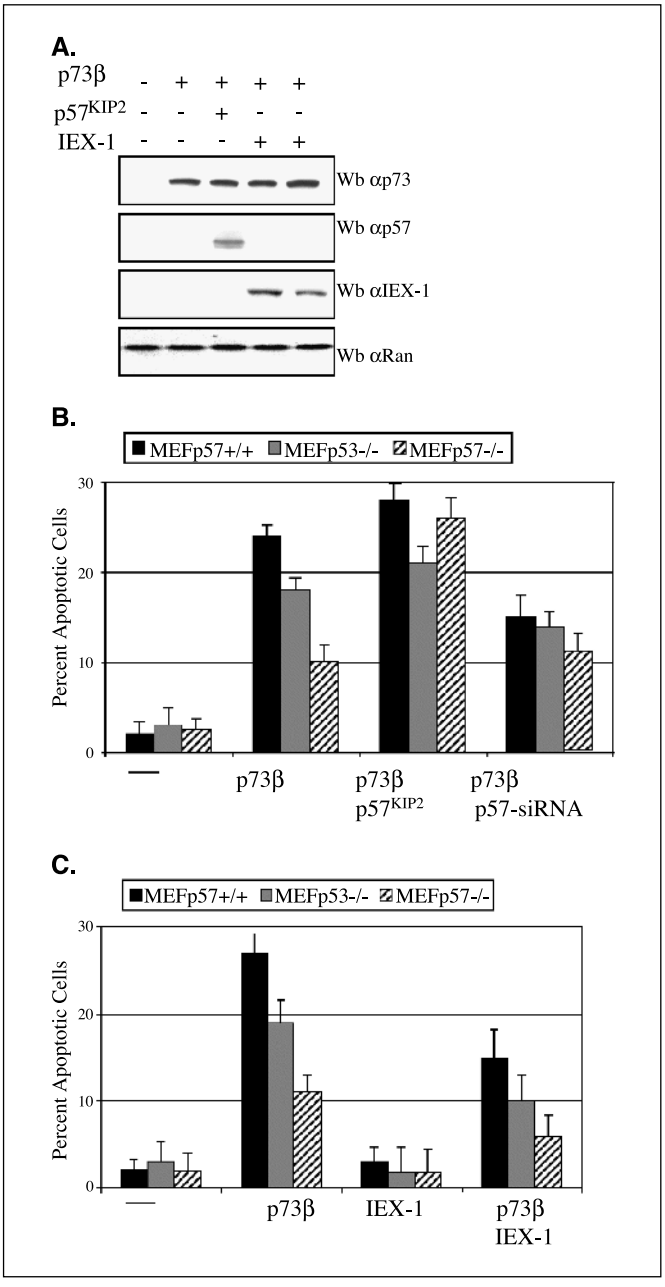

Figure 5. Opposite effect of $p57^{KIP2}$ and IEX-1 on p73β-mediated apoptotic cell death. *A,* MEF cells derived from $p57^{KIP2+/+}$, $p57^{KIP2-/-}$, and $p53^{-/-}$ mice (0.6×10^5 per 35-mm dish) were transduced with p73β, $p57^{KIP2}$, or transiently transfected with IEX-1 or $p57^{KIP2}$-siRNA (500 ng), at the indicated combinations. Levels of p73β, IEX-1, and $p57^{KIP2}$, or Ran as a loading control were assayed by Western blot analysis. Antibodies were used as described in Materials and Methods. *B* and *C,* apoptotic levels of MEFs cells treated as described above (*A*) was quantified by AnnexinV-PI staining and fluorescence-activated cell sorting analysis. *Columns*, means of triplicate in one of three independent experiments; *bars*, ±SD.

needed to elucidate whether p53 family members are regulated through common or distinct pathways. Nevertheless, the distinct phenotypes observed support different biological activities for p73α and p73β.

Approximately 50% of human cancers lack functional p53; however, p73 is not commonly mutated in primary tumors and rather reported as overexpressed in certain neoplasms (27, 28). Therefore, cancer cells harboring an inactive p53 but intact p73 function could still possess tumor sensitivity to DNA-damaging agents, such as those conveyed by radiotherapy and certain chemotherapy regimes (e.g., those containing cisplatin). This is of clinical relevance, because p73α and p73β could induce apoptosis through several mechanisms. In certain cases, p73β and to a less extend p73α may induce a program mechanistically similar to p53-mediated apoptosis. Alternatively, p73β may act through p53-independent pathways to promote cell death, as revealed by IEX-1 down-regulation and increase of $p57^{KIP2}$ expression observed in this study. Consistently with our finding, a recent report established that $p57^{KIP2}$ mRNA can be induced by p73β overexpression (23). At a basic mechanistic level, we propose that $p57^{KIP2}$ could act as a coactivator of p73β-induced apoptosis, allowing a cell cycle arrest before triggering additional apoptotic events, such as IEX-1 down-regulation, leading to cell death. Hence, p73β could serve as a regulator of the apoptotic process, controlling critical genes involved in p53-independent pathways to promote cell death.

Another line of evidence regarding the critical and potentially distinct role of p73 isoforms in human cancer is provided by recent data showing that p53 mutations and loss of p73 expression coexist in certain tumors, such as bladder cancer (29). A significant association between simultaneous p53 and p73 alterations with bladder cancer progression was also observed. Based on these data, it was postulated that p73 could play a tumor suppressor role in bladder cancer, and that its lost had a negative cooperative effect with that of p53 inactivation. This is most probably due to deregulation of apoptotic signals, since proliferative activity was not associated with the different phenotypes observed.

As stated recently, p73 is not a classic tumor suppressor. The existence of inhibitory versions of p73 and the intimate functional cross-talk among all p53 family members provide them with tissue-specific tumor suppressor, differentiation, or pro-oncogenic roles. Tumor cells may then either down- or up-regulate the expression levels of specific isoforms to promote growth, prevent differentiation, or evade apoptosis (30). In this context, data presented in this report offer substantial evidence for additional apoptotic activities of p73β, which in turn might have potential therapeutic implications, since p73β could induce alternative apoptotic responses in tumors harboring p53 mutations.

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