Differential response of p53 target genes to p73 overexpression in SH-SY5Y neuroblastoma cell line

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Summary

***p73*, the first *p53* gene homologue, encodes an array of p73 proteins including p73α full-length (TAp73α) and amino-truncated isoforms (ΔNp73α), two proteins with opposite biological functions. TAp73α can induce tumor suppressive properties, while ΔNp73α antagonizes p53 as well as TAp73 in a dominant-negative manner. In human malignant neuroblasts, p53 protein is wild-type but known to be excluded from the nucleus, therefore disabling its function as a tumor suppressor. The present study investigates whether there is a functional link between p73 isoforms and p53 in neuroblastoma. Experiments were performed on two neuroblastoma cell lines differing in their p53 status, e.g. wild-type p53 SH-5Y5Y cells and mutated p53 IGR-N-91 cells. Data indicate that (i) both TA- and ΔN-p73α enhance p53 protein level in SH-SY5Y cells, whereas level remains unchanged in IGR-N-91 cells; (ii) only in SH-SY5Y cells does forced TAp73α overexpression markedly induce nuclear accumulation of p53 protein; (iii) p21 protein expression is increased in both cell lines infected with TAp73, suggesting that, in IGR-N-91 cells, p21 is induced by p73 through a p53-independent pathway; (iv) in the SH-SY5Y cell line, Btg2 expression is strongly enhanced in cells overexpressing TA, and to a lesser extent in cells overexpressing ΔN. Taken together our results suggest that TAp73 may restore p53 function in NB with wild-type nonfunctional p53, but not in NB with mutated p53.**

Key words: Neuroblastoma, p73, p53, Apoptosis, Differentiation

Introduction

p53 is the most frequently altered tumor suppressor gene in a wide spectrum of human cancers (Hollstein et al., 1991; Levine et al., 1991) with a mutation frequency of up to 50% (Caron de Fromentel and Soussi, 1992). It is now well established that loss of p53 function by gene mutation, deletion, protein degradation, or viral oncogene binding plays a central role in cancer development. However, the emerging molecular complexity resulting from various signals makes the known transduction pathways intriguing (Giaccia and Kastan, 1998). For instance, in neuroblastoma (NB), p53 is not mutated but high levels of p53 wild-type protein are retained in the cytoplasm of undifferentiated cells (Moll et al., 1995). The unusual accumulation of p53 in such cells is the result of a marked resistance to MDM2-mediated degradation since MDM2 overexpression in neuroblastoma cell lines fails to decrease the high steady-state levels of endogenous p53 (Zaika et al., 1999). This resistance to MDM2 degradation may be due to a covalent modification of p53. Recently, it was reported that wild-type p53 accumulates in the nucleus as an inactive conformation, refractory to integration into the transcriptional complex (Wolff et al., 2001).

p73 was recently identified as structurally and functionally homologous to tumor suppressor protein p53 from its sequence homology and gene organization: a transactivation domain (TAD), a DNA binding domain (DBD) and an oligomerization domain (OD) (Kaghad et al., 1997). As such, p73 can exert p53-like activities in various contexts, including transactivating p53 target genes like *p21*, *MDM2* or *BAX*, leading to apoptosis and cell growth arrest (Jost et al., 1997). In contrast to the *p53* gene, which encodes one major protein, *p73* encodes a variety of protein isoforms through using alternative promoters at the 5′-end or differential splicing at the 3′-end (Yang et al., 2002; Stiewe and Pützer, 2002). At the 5′-end, two major isoforms are generated: a full-length (TAp73α) and an amino-terminal-truncated, TAD-lacking isoform (ΔNp73α). At the 3′-end, p73β isoform, lacking exon 13, is the most studied. TAp73α expression is thought to be required for the neurogenesis of specific neural structures – such as pheromone signaling (Yang et al., 2000) – and appears necessary in neuronal differentiation in mice (De Laurenzi et al., 2000), whereas ΔN-p73α plays an essential anti-apoptotic role in vivo (Pozniak et al., 2000).

In undifferentiated NB, only ΔNp73α was observed by western blotting in tumors (Douc-Rasy et al., 2002) and this isoform appeared to be associated with poor prognosis (Casciano et al., 2002). This suggests that, in NB, ΔNp73α acts as a dominant-negative agent in the transactivation by p73α, while TAp73α would be transiently expressed and rapidly degraded. Apart from the anti-apoptotic effect of ΔNp73α on p53 in neurons, so far the functional link between TAp73 and p53 has not been elucidated. In order to determine whether p73 isoforms are capable of restoring p53 biological activities in NB, we studied the forced expression of TA- and ΔN-p73α in two NB cell lines with wild-type and mutated p53.

Materials and Methods

RT-PCR, obtaining restriction enzyme fragments, and p53 sequencing

cDNA was obtained by reverse transcription (RT) of 1 μg of total RNA using Moloney Murine Leukemia Virus Reverse transcriptase (Superscript II TM RNase H-; Gibco-BRL Kit) and random hexamers to prime the synthesis in conditions specified by the manufacturer. Then cDNA was submitted to PCR within the coding region (from exon 1 to exon 11) of the *p53* mRNA human gene (GenBank accession number KO3199). Primer sequences were as follow: for the fragment covering the complete p53 cDNA: primer sense: 5′-GCTTTCCACGACGGTGACA-3′, primer antisense: 5′-CCC-ACAACAAAACACCAGTGC-3′); for exons 6-10, primer sense at the junction of exon 6 and 7: 5′-CTATGAGCCGCCTGAGGTTG-3′, primer antisense (10AS): 5′-CTCACGCCCACGGATCTG-3′; to detect an accurate nucleotide sequence 7,8,9, primer sense specifying the nucleotides at the end of exon 9 linked with the beginning of exon 7 (underline) was: 5′-AATATTTCACCCTTCAGGTTG-3′ and primer antisense was: 10AS described above. The PCR reaction was conducted in the presence of pfu Hotstart DNA polymerase (Stratagene) for 30 cycles of 1 minute at 90°C, 1 minute at 65°C and 2 minutes 30 seconds at 72°C using a Perkin-Elmer Cetus thermocycler. The RT-PCR products were purified then digested with three restriction endonucleases: *Sty*I, *Rsa*I and *Taq*I, to first localize the insertion fragment. An aliquot (1/10) of the PCR products was analyzed on a 2% agarose gel or a 7.5% polyacrylamide gel and visualized by UV illumination after ethidium bromide staining.

Nucleotide sequencing from exon 6 to exon 10 was performed from the 5′- to 3′-end of p53 cDNA and confirmation with antisense sequencing using Model 377 Sequencer (Abiprism). The primers used were located in flanking region exon 6-7 and 9-10 to overlap the mutated region.

Recombinant p73-adenovirus constructs

pcDNA-TAp73α and pcDNA-ΔNp73α plasmid constructs were gifts from Dr Daniel Caput (Sanofi Recherche, Labège). Recombinant adenoviral vector expressing full-length p73α (Ad-p73α) and ΔN-p73α (Ad-ΔNp73α) were produced by pcDNA-p73α or pcDNA-ΔN-p73α in vivo homologous recombination in 293 cells after linearized plasmid co-transfection containing the left adenovirus ITR (inverted terminal repeat) under CMV promoter control, the adenovirus pIX sequence, and d1324 DNA digested with *Cla*I restriction enzyme. The resulting recombinant adenovirus was replication deficient but could be amplified by infecting 293 cells, which transcomplement for the deleted E1 region. Ad-p73 viruses were purified by cesium chloride centrifugation, in 20 μl PBS aliquots (Biorad, France) containing 10% glycerol, and stored at –80°C until use. The presence of the p73 sequence in Ad-p73 was confirmed by restriction enzymes treatment, and virus DNA sequencing determined the nucleotide sequences. The ability of Ad-p73 to induce p73 protein overexpression in infected cells was tested by western blot and nuclear localization was detected by immunocytochemistry.

Cell culture and adeno-recombinant infection

The parental human neuroblastoma SH-SY5Y cell line was purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). The human IGR-N-91 cell line was established in our laboratory from the bone marrow of a patient with metastatic NB after unsuccessful adriamycin–vincristine chemotherapy (Ferrandis et al., 1994). Cells were grown under standard conditions in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine and 10% fetal calf serum. Cells were infected with the recombinant adenovirus vector expressing either full-length p73 (Ad-p73α) or N-terminally truncated p73 (Ad-ΔNp73α) at an MOI of 15. After 48 hours cells were harvested for western blotting, northern blotting, or immunocytochemistry.

Immunocytochemical analyses

Cells grown in Lab-Tek (Polylabo) double chambers per slide were fixed in 4% paraformaldehyde. The fixed cells were rinsed twice in PBS, blocked for 2 hours in PBS containing 0.05% Triton X-100, and finally incubated for 18 hours at 4°C with primary antibody. They were then rinsed twice in PBS and incubated for 2 hours with the second antibody using the streptavidin-biotin peroxidase technique. The rabbit polyclonal p73 antibody (gift from Dr D. Caput) was diluted at 1/280 and the monoclonal p53 antibody, DO-7 (Dako) was diluted at 1/150. A biotinylated anti-rabbit IgG and anti-mouse IgG (Dako) were used as secondary antibodies. Rabbit IgG or normal mouse IgG was used as a negative control in p73 or p53 experiments, respectively.

Western blot analysis

Protein lysates (50 μg) were submitted to 7.5 or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), after which the proteins were electroblotted to nitrocellulose membrane. The blot was blocked with 5% skim milk in PBS (Biorad, France) and submitted to western blotting with polyclonal p73 antibody for p73 expression, using methods previously described (Douc-Rasy et al., 2002), with monoclonal anti-p53 antibody (DO-7 from DAKO) at a dilution of 1/1000, with polyclonal anti-BAX (N20 from Santa Cruz) at a dilution of 1/500, with monoclonal anti-MDM2 (clone IF2, Ab-1 from Oncogene Research) at a dilution of 1/100, and with monoclonal anti-p21 antibody (Oncogene Research) at a dilution of 1/25. Protein detection was carried out using the ECL kit (Amersham Pharmacia Biotech, France). Controls were performed using β-actin antibody mAb 1501 (Chemicon).

Northern blot analysis

Total RNA was isolated from non-infected or infected cells (see cell culture and infection) using techniques from RNeasy Qiagen Kit. Denatured RNA samples (10 μg/well) were subjected to electrophoretic separation and transferred to a Hybond C extrafilter. The quality of the RNA was verified by the integrity of the 28S and 18S rRNA bands stained with ethidium bromide. Prehybridization and hybridization were carried out under standard conditions. For use as probes, p53, a cDNA fragment digested by *Xba*I-*Xba*I; *BAX*, an *Eco*RI-*Eco*RI fragment obtained from plasmid pSFFV-neoBax (a gift from Dr Olivier Brison; pSFFV-neoBax originally provided by Dr Stanley Korsmeyer); *BTG2* (a gift from Prof. Alain Puisieux, Lyon, France); *MDM2* (Oliner et al., 1993); *PUMA*, a cDNA from plasmid pHA-PUMA [a gift from Dr Bert Vogelstein (Yu et al., 2001)]. cDNA fragments were labeled with [α-^{32}P]dCTP (Amersham Biosciences) using NonaPrime Kit (Q.bio gene). Kodak X-OMAT LS films were exposed for various periods of time to the blots.

FACS analysis

SH-SY5Y cells were seeded onto plates at a density of 1×10^4 cells per cm^2. The cell cycle distributions were measured by fluorescence-activated cell sorting (FACS) analysis. Cells were collected and fixed in 4 ml 70% ethanol at –20°C for at least 30 minutes. Cells were washed twice with PBS and incubated for 30 minutes at room temperature in PBS containing 100 μg/ml RNase A and 10 μg/ml propidium iodide. DNA content and cell cycle analysis were assessed by FACScalibur.

Results

TAp73α- and ΔNp73-forced expression enhanced endogenous p53 accumulation in SH-SY5Y cells

The two NB cell lines studied were SH-SY5Y and IGR-N-91.

SH-SY5Y cells harbor wild-type p53 whereas IGR-N-91 cells express a mutated p53 by insertion of exons 7, 8, 9 in tandem. This insertion was detected by western and northern blotting. Western blotting of total protein extracts from IGR-N-91 cells using DO-7 p53 antibody showed a higher relative molecular mass (M_r) in IGR-N-91 cells as compared to wild-type p53 in SH-SY5Y cells (termed IGR and SH respectively, Fig. 1A, left panel). Such a high M_r of p53 might result from either post-translational modifications or long mRNA from primary sequence. The consistency and reproducibility of the band of this high M_r on western blots allow us to exclude the post-translational modifications. Since northern blots probed with p53 cDNA displayed a band of about 300 base pairs, heavier than wild-type p53 (see also Fig. 3B), we suspected a mutation by additional nucleotide insertion, occurring in IGR-N-91 cells. To localize the insertion sequence, RT-PCR, spanning exons from 1 to 11, was performed; PCR products submitted to electrophoresis showed a band of 1750 bp for IGR versus 1432 bp for SH, Fig. 1A, right panel). The digestion pattern with *Sty*I, *Rsa*I and *Taq*I of PCR products had the expected bands for SH but, revealed bands of abnormal size for IGR, consistent with additional nucleotides (Fig. 1B and D), and indicating that the insertion occurred at the end of exon 9. In addition, the fragment amplified by RT-PCR, encompassing exons 6-10, elicited again a band from IGR about 300 bp higher than that from SH (Fig. 1C, left panel). To confirm the insertion by duplication of the incriminated exons 7, 8, 9, we used the nucleotide sequence specifying the junction of exons 9-7 for primer sense, and AS 10 for primer antisense (see Materials and Methods). As expected, the amplified fragment was observed exclusively in IGR but not in SH (Fig. 1C, right panel). Finally, the complete direct (5′- to 3′-end) and reverse sequencing from exons 6 to 10 confirmed the insertion of exons 7, 8, and 9 (109+108+104 nucleotides respectively), resulting in an additional 321 base pairs to the p53 cDNA sequence.

p73 has been shown to harbor two alternative promoters, giving rise to two isoforms with opposite functions in apoptosis and cell cycle arrest: a full length isoform eliciting a transactivating domain (TA) and an amino-terminal truncated isoform, lacking TA (ΔN). We used our own adenoviral recombinant constructs to determine whether a functional link exists between p73 isoforms and p53 expression. Recombinant adenoviral vector expressing TAp73α and ΔNp73α were produced by pcDNA-TAp73α or pcDNA-ΔNp73α in vivo homologous recombination in 293 cells (see Materials and Methods). SH-SY5Y and IGR-N-91 cells were infected with either TAp73α (Ad-TAp73α) or ΔNp73α (Ad-ΔNp73α) recombinant adenovirus at a multiplicity of infection (MOI) of 15 viral particles per cell. Infection efficiency was assessed by immunocytochemistry and western blotting on 48-hour postinfection cells using anti-p73 antibody. As shown in Fig. 2A,B, nearly 100% of recombinant adenovirus-treated cells were stained as compared to almost none of the cells that were either untreated or infected by empty vector. Western blot from 1 μg of total protein extract using p73 antibody confirmed that the Ad-p73 isoform infection was efficient (Fig. 2C, upper-left and middle panel). Under this condition, no endogenous p73 was observed in either untreated cells or cells treated with empty vector. In contrast, using up to 50 μg total protein extract, p73 endogenous protein was observed as follows: SH-SY5Y cells showed two major bands corresponding to TA and ΔNp73, while IGR-N-91 cells exhibited a faint band corresponding to ΔNp73 (Fig. 2C, right panel). Analysis of SH-SY5Y cells revealed a third fast-migrating unidentified band.

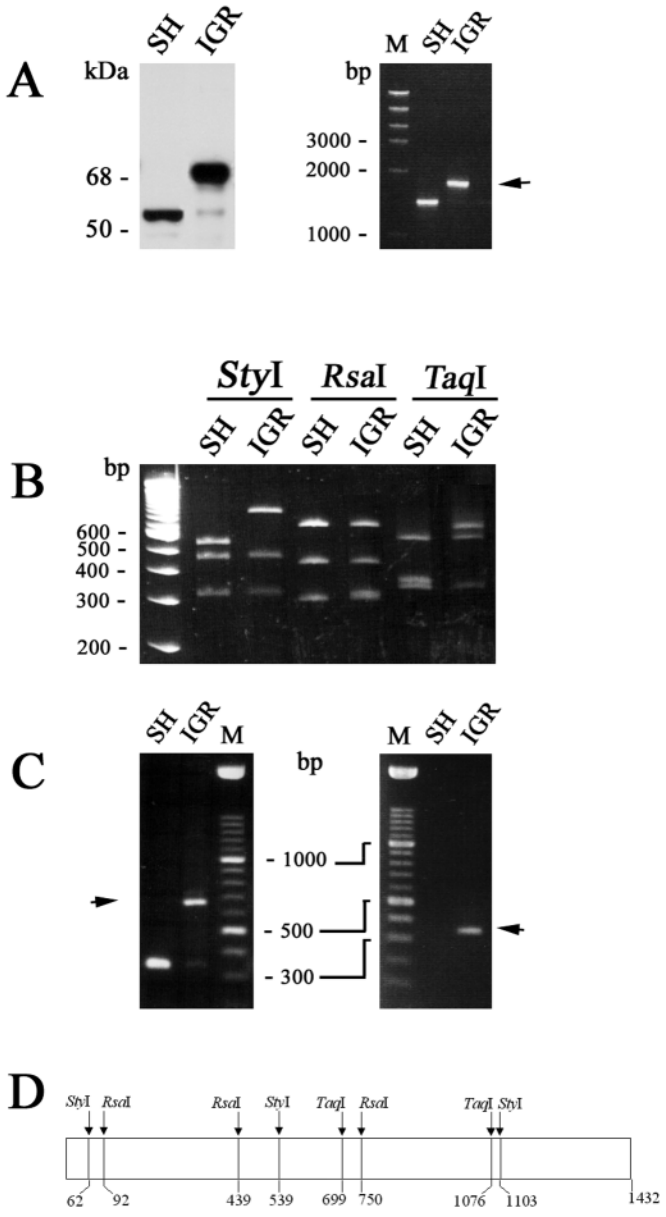


Fig. 1. Identification of the *p53* mutation by insertion in the IGR-N-91 cell line. SH refers to SH-SY5Y cells; IGR refers to IGR-N-91 cells. (A) Western blot (left panel) of protein total lysates showing that the p53 protein band is of a higher molecular mass in IGR cells than in SH cells. Electrophoresis (right panel), in 2% agarose, of RT-PCR products spanning exons 1-11, show an amplified fragment in IGR approximately 300 bp longer than in SH. (B) Restriction endonuclease fragments from *Sty*I, *Rsa*I and *Taq*I. (C) Polyacrylamide electrophoresis of exons 6-10 showing a higher band (~300 bp) in IGR than in SH (left panel); when PCR was performed using the forward primer, spanning the end of exon 9 and the first four nucleotides at the beginning of exon 7, only IGR had an amplified fragment (right panel). (D) Schematic representing the restriction sites of each enzyme in B. Arrowhead, IGR amplified fragment.

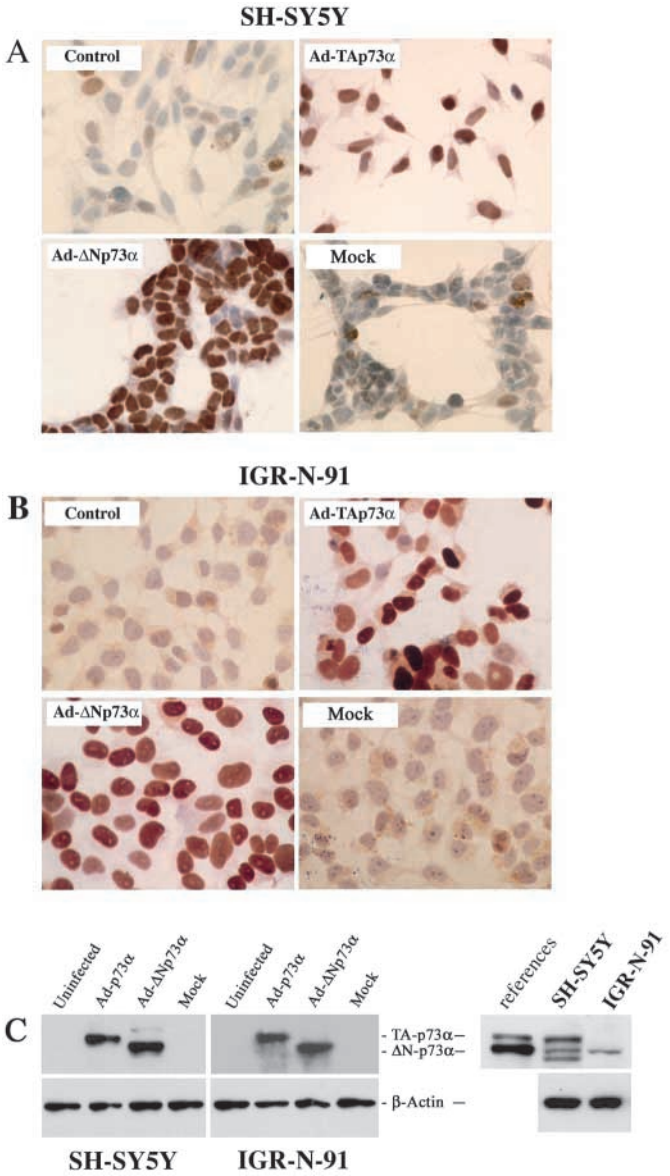


Fig. 2. Immunocytochemistry (ICC) showing high efficacy of recombinant adenovirus infection in SH-SY5Y and IGR-N-91 cells. Untreated or uninfected cells (control of non-infected cells); cells infected by adenovirus expressing full-length p73α (Ad-TAp73α) or ΔNp73α (Ad-ΔNp73α) at MOI of 15 for 48 hours; cells infected by empty vector (Mock). Nearly 100% of treated cells showed p73 expression in the nucleus. (A) ICC of SH-SY5Y cells; (B) ICC of IGR-N-91 cells; (C) western blot of 1 μg of total protein extracts from SH-SY5Y cells (left panel), IGR-N-91 cells (middle panel), and of 50 μg endogenous total protein extracts (right panel); β-actin was used as a protein loading control.

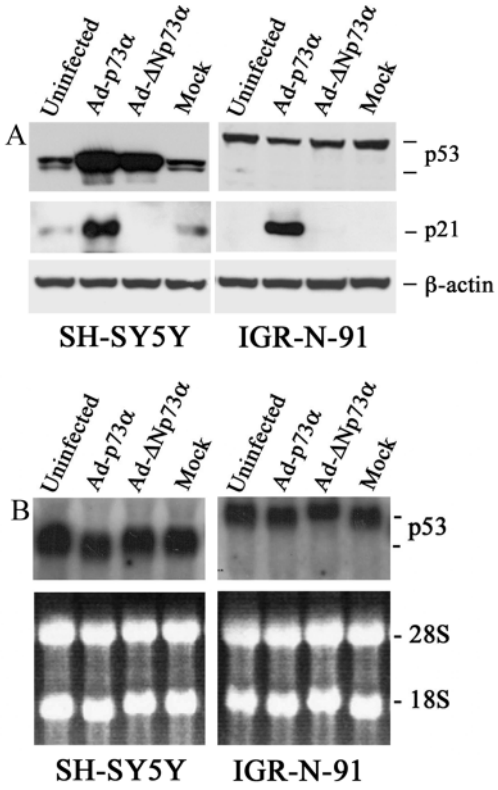


Fig. 3. Induction of endogenous p53 and p21 proteins by p73 ectopic overexpression in SH-SY5Y and IGR-N-91 cells. (A) p53 and p21 proteins were detected by western blot analysis of total protein extracts; the blots were incubated with monoclonal anti-p53 antibody (upper panels) for p53 expression and with monoclonal anti-p21 antibody (middle panels) for p21 expression. Upregulation of p21 protein was observed only with Ad-TAp73α but not with Ad-ΔNp73α. (B) Northern blot showed no induction of p53 mRNA expression. Total RNA (10 μg) was prepared 48 hours after infection and submitted to northern blotting with p53 cDNA probe. Ethidium bromide staining of 28S and 18S rRNAs is used to allow comparison of RNA loaded.

A western blot with anti-p53 antibody (DO-7) was performed to detect the effect of p73 isoforms upon p53 expression. Overproduction of Ad-TAp73α markedly enhanced the expression of p53 in SH-SY5Y cells carrying wild-type but non-functional endogenous p53. It is noteworthy that, *p21*, a wildly accepted p53-target gene, was highly upregulated by TAp73α (Fig. 3A). Forced Ad-ΔNp73α overexpression also stimulated p53 upregulation but to a lesser extent (Fig. 3A). Similar experiments have been carried out also in SH-SY5Y cells infected by other constructs: those encoding GFP (green fluorescent protein) and β-galactosidase (βGal). We found that, like empty vector (Mock), neither Ad-GFP nor Ad-βGal induced p53 protein (data not shown). However, in spite of a significant increase in p53 protein level in these cells, the p21 basal expression observed in non-infected cells completely disappeared in Ad-ΔNp73α cells, further supporting an antagonistic role of ΔNp73α towards p53-responsive genes. With regards to IGR-N-91 cells, p73 isoforms had no effect at all on the level of mutated p53 protein (Fig. 3A, right panel). Importantly, p21 up-regulation was noted in Ad-TAp73α-treated cells, therefore supporting the concept that p73α, on its own, can stimulate p21 expression in cells lacking active p53.

p53 upregulation is not occurring at transcriptional level

Since the TA version of p73 is a transcription factor, we further examined whether p73 activates *p53* at the transcriptional level

or not. In the two above-mentioned cell lines, northern blot analysis with a p53 cDNA probe showed no difference in p53 mRNA level of expression between neuroblasts infected with either the recombinant adenovirus encoding for the two p73 isoforms, or with empty vector, as compared to non-infected cells (Fig. 3B). These results suggest that p53 protein is not upregulated at the transcriptional level, as revealed by northern blot.

p53 accumulates in SH-SY5Y nuclei

Shaulsky et al. (Shaulsky et al., 1991) reported that p53 nuclear localization is necessary for its activity. In response to DNA damage-inducing agents, hypoxia, and other cellular stresses, p53 accumulates in the nucleus and is activated as a transcription factor (Prives and Hall, 1999). Immunocytochemistry (ICC) using anti-p53 DO-7 antibody was performed to demonstrate p53 sub-cellular localization. Fig. 4A shows that upon TAp73 adenoviral infection, a strong nuclear p53 staining was observed in almost all SH-SY5Y cells, unlike the non-infected cells (upper panels), and in sharp contrast to IGR-N-91 cells, which all exhibited a diffuse and faint nuclear staining (Fig. 4B). ΔNp73 infection in both cell lines, produced no significant variation of p53 staining compared with uninfected cells (Fig. 4A,B, lane 'uninfected').

TAp73α induces important subG1 population in SH SY5Y cells but G1 arrest in IGR-N-91 cells

It is well-established that G1 cell cycle arrest (G1 checkpoint) is mediated by p53 following DNA damage or irradiation, and that cells with mutant p53 fail to arrest at G1 (Kastan et al., 1991; Kuerbitz et al.,1992). Since p21, the known regulator of cell cycle, was upregulated in both cell lines infected with Ad-TA-p73α expressing either wild-type (SH-SY5Y) or mutant (IGR-N-91) p53, we compared cellular responses with regards to cell cycle distribution. FACS analysis revealed that Ad-TAp73 induced an significant subG1 population (60%) in Ad-TAp73 infected cells unlike Ad-ΔNp73 or non-infected cells (9% and 5%, respectively), therefore indicating that the cells had undergone apoptosis (Fig. 5A,B, left panels).

Interestingly, Ad-TAp73-treated IGR-N-91 cells exhibited a different pattern, with an increase in the number of cells in G1 phase in the population (62% in treated versus 48% in control) as shown in Fig. 5A,B (right panels), which could reflect p21 overexpression as shown by western blotting (Fig. 3A).

ΔNp73 differentially regulates p53-downstream genes

Both p53 and p73 activate the transcription of a number of p53 target genes such as *PUMA*, *BAX*, *MDM2*, and *BTG2* (Zhu et al., 1998). *PUMA* is known to be activated in apoptosis, as its exogenous expression results in an extremely rapid and profound apoptosis (Yu et al., 2001). *BAX*, a downstream effector of p53-induced apoptosis, is p53-regulated at the transcriptional level (Gross et al., 1999). Ad-TAp73 induced significant levels of *PUMA* transcript in SH-SY5Y cells, as analyzed by northern blotting, but nearly undetectable levels in IGR-N-91 cells (Fig. 6). In contrast, variations of *BAX* mRNA expression were more subtle, with high levels observed in control cells of both non-infected or empty vector (Mock), and in Ad-TAp73-treated cells, while a downregulation occurred in both Ad-ΔNp73-infected cell lines. Of note, the *BAX* mRNA level decrease was dramatic in SH-SY5Y cells (Fig. 6), as expected, since ΔNp73 is an antagonist of p53-mediated proapoptosis (Pozniak et al., 2000) whereas it was rather slight in the p53 mutated IGR-N-91 cells, reflecting the lack of ΔNp73.

Next, we examined the mRNA level of *BTG2*, a p53-target gene implicated in neuronal differentiation (Puisieux and Magaud, 1999). In uninfected cells, or in cells infected with

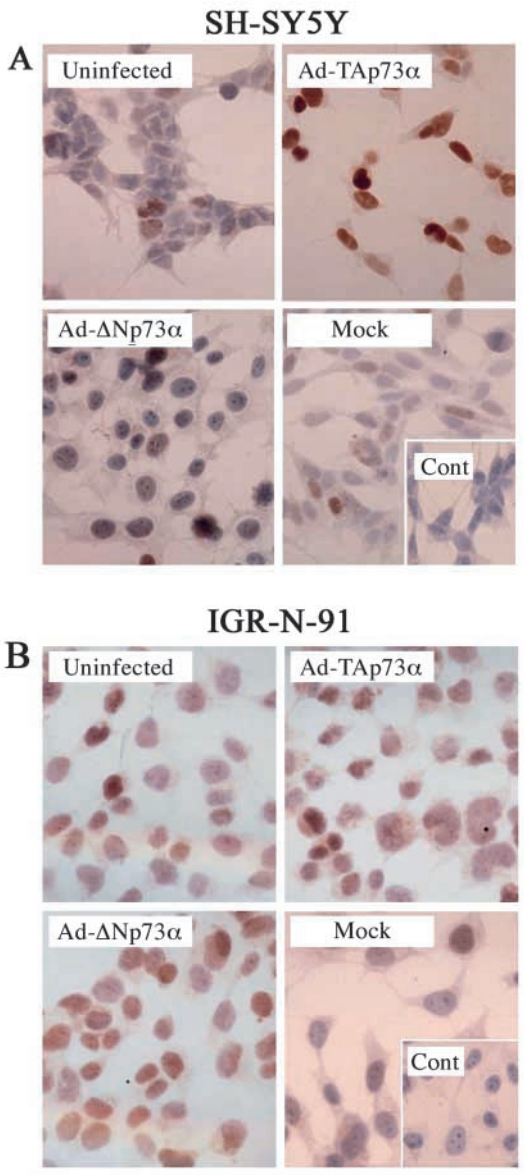


Fig. 4. Immunocytochemistry (ICC) showing strong nuclear staining in Ad-TAp73 SH-SY5Y infected cells. (A) SH-SY5Y cells; (B) IGR-N-91 cells. Parental cells (Uninfected), or cells infected with full-length p73α (Ad-p73α), with ΔNp73a (Ad-ΔNp73α) or with empty vector (Mock). In contrast to SH-SY5Y cells (A), ICC in IGR-N-91 cells led to a diffuse and faint staining in whole cells (B), regardless of the expressed p73 isoform. 'Cont' represents control of second antibody.

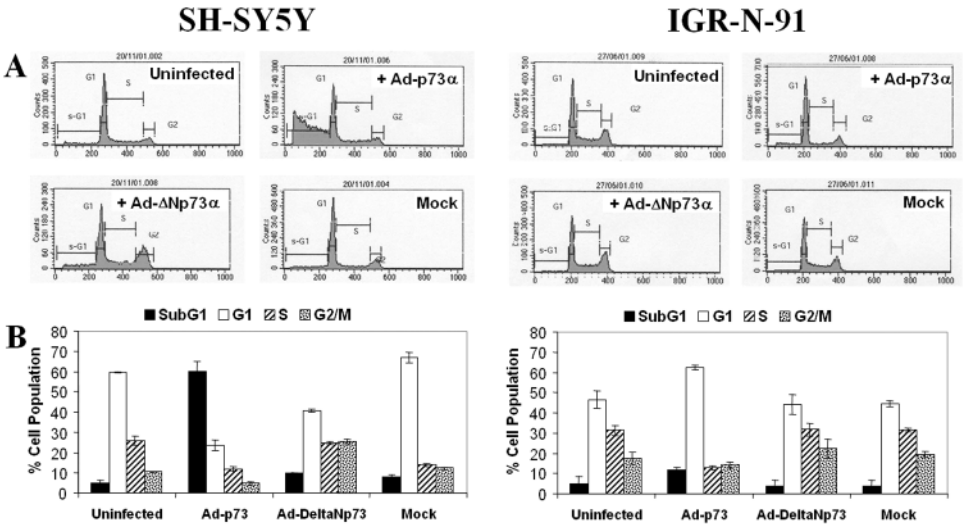
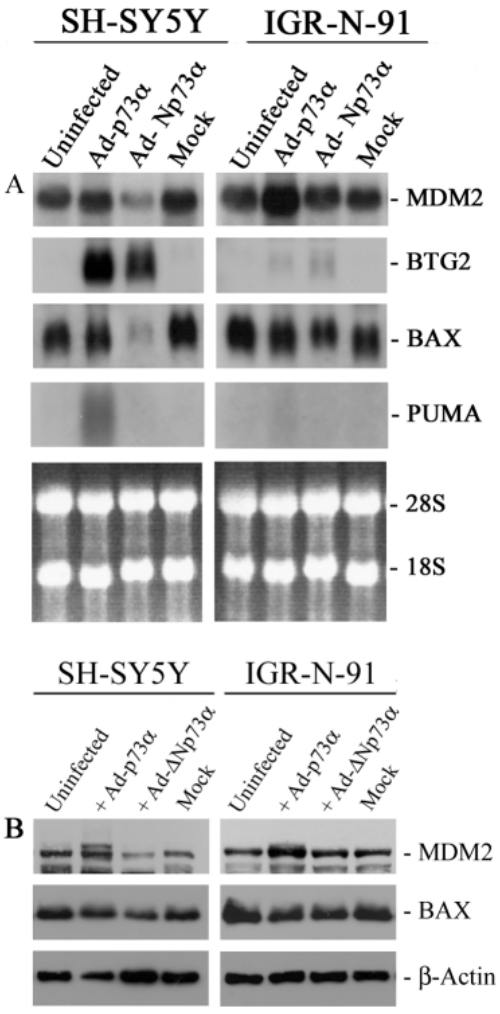


Fig. 5. Cell cycle distribution showing differential patterns induced by TAp73α overexpression. (A) FACS. (B) Histogram representation of cell cycle population: Sub-G1 (black columns); G1 (white columns); S (hatched); G2/M (dots); data are means of three independent experiments ± s.d. (bars). The proportion of Ad-TAp73 SH-SY5Y neuroblasts (Ad-p73α) that underwent Sub-G1 phase increased considerably (60%) compared to Ad-ΔNp73α infected cells (10%) and to the non-infected cells and cells infected by empty vector (5% and 9%, respectively), indicating cells entering apoptosis. Ad-TAp73 IGR-N-91 cells exhibited a cell cycle distribution different from that of SH-SY5Y cells: more of the population underwent G1 phase (62% in treated versus 48% in control) leading to cell cycle arrest mediated by p21 activation as confirmed by western blot analysis shown in Fig. 3A.



empty vector, *BTG2* mRNA was totally absent in both cell lines. In *p73* recombinant adenovirus-infected SH-SY5Y cells, *BTG2* was upregulated, strongly in Ad-TAp73α-infected cells but to a lesser extent in Ad-ΔNp73-infected cells. In contrast, in IGR-N-91 cells, *BTG2* mRNA was very weakly induced in response to either Ad-TAp73 or Ad-ΔNp73 infection, suggesting that either p73 has little effect on *BTG2* at the transcriptional expression level, or that the mutated p53 protein may elicit residual transcriptional activity. Together these results strongly suggest that *BTG2* upregulation detected in SH-SY5Y cells is mediated by wt p53 accumulation resulting from TAp73 and –ΔNp73 overexpression.

MDM2, a well-known p53 regulator, was nearly unchanged at the transcriptional expression level in Ad-TAp73-infected SH-SY5Y cells compare to empty vector. Like *BAX*, the *MDM2* mRNA level was dramatically downregulated in Ad-

Fig. 6. Northern blots showing mRNA expression level of target genes in response to Ad-p73 SH-SY5Y and IGR-N-91 infected cells. (A) *MDM2*, *BTG2*, *BAX* and *PUMA* mRNA expression. It is noteworthy that in SH-SY5Y cells, *BAX* and *PUMA* mRNA expression is upregulated only in Ad-TAp73α, indicating again the rapid apoptosis observed in such cells (see also FACS in Fig. 5). *BTG2*, the p53 target gene, known to be involved in the regulation of G1/S transition of the cell cycle (Duriez et al., 2002) is strongly stimulated in Ad-TAp73α but also stimulated in Ad-ΔNp73α as compared to uninfected cells or cells infected by empty vector (Mock), or to Ad-TAp73 IGR-N-91 cells, Ad-ΔNp73, whereas *MDM2* mRNA level expression is highly stimulated in Ad-TAp73 IGR-N-91-infected cells compared to Ad-ΔNp73- or control and empty vector-infected cells. Total RNA (10 μg) was prepared 48 hours after infection and submitted to northern blotting with appropriate cDNA probe; ethidium bromide staining of 28S and 18S rRNAs was used as an RNA loading control (A, lower panel). (B) Western blots showing protein expression of MDM2 and BAX. β-Actin was used as protein loading control.

ΔNp73-expressing cells as compared to control or empty vector (Fig. 6A, upper-left panel). In sharp contrast to SH-SY5Y, *MDM2* mRNA levels in IGR-N-91 cells was strongly increased by TAp73 overexpression and downregulated by ΔNp73 overexpression (Fig. 6A, upper-right panel).

We then looked at whether or not BAX and MDM2 protein levels follow those of mRNA levels. Indeed, in each lane (Fig. 6B) the mRNA and the protein expression were totally in parallel.

Taken together, these findings suggest that ΔNp73, but not TAp73 selectively regulates the expression of p53 downstream target genes.

Discussion

In spite of intensive research on p53 function in human cancer, p53 status in human NB tumors remains an open biological enigma. Indeed, p53 protein, not mutated but rather inactive, either because of cytoplasmic 'sequestration' and/or inappropriate conformation, does not function as a transcription factor (Moll et al., 1995; Moll et al., 1996). p53 protein – capable of shuttling between the nucleus and the cytoplasm within minutes in 3T3 cells (Middeler et al., 1997) – must be located in the nucleus in order to activate the targeted genes (Hood and Silver, 2000). We found a strong accumulation of p53 protein only in the nucleus of TAp73-overexpressing cells, which harbor an endogenous wild-type p53 (p53wt). Whether or not this p53 protein was driven with TAp73 from cytoplasm to nucleus in SH-SY5Y cells is still an open question. In contrast in undifferentiated human neuroblastoma (NB) tumors, we have repeatedly found a lack of p53 immunostaining in the cytoplasm of NB cell lines whatever the p53 antibodies used. The reason that p53 is not revealed by immunostaining in the cytoplasm of SH-SY5Y cells may be that endogenous p53wt in NB cells is engaged to a complex protein structure, Parc, a p53-associated, parkin-like cytoplasmic protein (Nikolaev et al., 2003): in such a complex, p53 is anchored in the cytoplasm and its epitope is masked. With this hypothesis, one may speculate that forced expression of TAp73, which is a p53 structural homologue, displaces p53 from the megacomplex and stabilizes it accordingly. Another possible explanation is that endogenous p53 is always in the nucleus but is in a latent form and overexpression of TAp73 can stabilize p53. In p53mut IGR-N-91 NB cells, immunocytochemistry using DO-7, a p53 antibody, showed a discrete staining in the nucleus, but none in the cytoplasm of either cells whether or not they were infected by TAp73 (Fig. 4B). This suggests that endogenous p53mut is always in the nucleus but unable to be in an active conformation as previously reported by Wolff et al., (Wolff et al., 2001) for the AW-1 sub-clone derived from SK-N-SH. The present work indicates that, in NB cells with wild-type but inactive p53, (i) p73 can rescue p53 from cellular 'localization defect' to allow it to function as a transcription factor, and (ii) p53-targeted genes behave differently in response to ΔNp73 isoform overexpression.

Wild-type – but inactive – p53 requires TAp73α to be functional

As evidenced by western blotting, TAp73α and ΔNp73α – the two main isoforms of p73 – were present in SH-SY5Y parental cells when large total protein extracts of up to 50 μg were used (Fig. 2C), whereas ICC showed only faint staining in approximately 10% of nuclei (Fig. 2A, control). TAp73α was not found at all in IGR-N-91 parental cells, but there was a detectable basal level of ΔNp73α, consistent with the ICC negative staining (Fig. 2B, control).

The striking p53 activation in SH-SY5Y cells following exogenous TAp73α overexpression is supported by three lines of evidence: firstly, a high and stable level of p53 protein; secondly, an obvious p53 nuclear localization, and thirdly, an important sub-G1 population associated with high level of *PUMA* transcripts, two indexes of the apoptotic program triggering. From this body of evidence, one can speculate that TAp73, when overexpressed in SH-SY5Y cells, permits p53 protein to form either an active homotetramer or an active hetero-oligomer TAp73/p53 the mechanism of which has to be elucidated. In line with this assumption, ΔNp73 overexpression, although stabilizing p53 (western blot in Fig. 3A), should repress p53 in its tumor suppressor activity.

An additional important player in the p53-directed signaling cascade is p21/WAF1, the cell growth arrest protein whose expression can be regulated directly by p53 and p73. TAp73, but not ΔNp73α, induced a high increase of p21 protein as shown in Fig. 2A. p21 accumulation may be related to p53 induction of TAp73, given that IGR-N-91 cells with constitutively inactive p53 upregulate p21. Nonetheless, it has been recently reported that accumulation of p21 protein does not always result from p53 function, as shown in MCF-7 following TNFα treatment (Drané et al., 2002). Further examination at the transcriptional level will clarify whether p53 or TAp73 induction leads to p21 activation in NB cell lines.

The biological consequence of a DBD repeat, i.e., duplication of exons 7, 8, 9 including a zinc finger, as elicited by IGR-N-91 cells, is so far unknown. In this respect it has been previously reported that alteration of the zinc finger motif located by point mutation at Cys176>Ser, affects the active conformation of p53 because of a conformational shift (Meplan et al., 1999; Wolff et al., 2001). However IGR-N-91 cells elicit a 'gain', not a loss in zinc finger motif. Since, in these cells, TAp73α had no effect on p53 protein level, consistent with a faint and diffuse p53 staining of some nuclei, one can speculate that DBD gain also inactivates p53.

ΔNp73α overexpression transiently induces p53 protein increase and differentially regulates p53-downstream genes

Considering the effect of ΔNp73α in SH-SY5Y cells, in spite of p53 protein upregulation (as revealed by western blotting of cells collected 48 hours post-infection), it is remarkable that neither nuclear, nor cytoplasmic p53 protein accumulation was observed. This lack of nuclear and cytoplasmic p53 accumulation in ΔNp73 infected cells could result either from its association with yet unidentified proteins, preventing antibody epitope recognition, or from the p53 rapid shuttling in fast growing cells. As a matter of fact, experiments with repeated infection revealed that ΔNp73α-overexpressed cells were readily prolific, in full agreement with recent report demonstrating that ΔNp73α is potentially oncogenic (Ishimoto et al., 2002; Stiewe et al., 2002), but also in sharp contrast with

SH-SY5Y cells overexpressing TAp73 and suffering from marked apoptosis, as determined by morphological analysis and a huge sub-G1 cell population. p53-protein-enhanced expression at 48 hours from the transactivating-domain-lacking ΔNp73α was unexpected. We speculate therefore that ΔNp73α might release p53 from its repressor, hence permitting ΔNp73α isoform to exert its dominant negative effect as shown by the downregulation of *BAX* and *MDM2* gene transcripts (Fig. 6). These results are in agreement with a recent report demonstrating that ΔNp73α negatively regulates both p53 and TAp73 and establishes an autoregulatory feedback loop (Nakagawa et al., 2002; Kartasheva et al., 2002).

One of the biochemical functions attributed to p53 is its ability to activate transcription of adjacent genes. As TAp73, but not ΔNp73, appears to enhance p53 expression and to induce its nuclear stabilization in SH-SY5Y cells, a critical issue concerns the selectivity of expression of the target genes, such as *p21, MDM2* and *BAX*, which have, to date, been reported as transactivated by both p53 and TAp73. We reasoned that transcriptional induction of p53 target genes was p73 dependent, since IGR-N-91 cells are consistently p53 inactive (Fig. 6). Comparison between the two TAp73-infected cell lines revealed marked differences between *MDM2* and *BTG2* expression. Undoubtedly, *MDM2* transcription is highly induced by TAp73 in p53-inactive IGR-N-91, and remains unchanged in p53- proficient SH-SY5Y. In normal cells, p53 forms complexes with MDM2 protein for degradation purpose. In contrast, in stressed cells, p53 is activated and subsequently induces the activation of downstream genes including *p21*, *MDM2* and *BAX*, leading to either cell cycle arrest at two main checkpoints (G1/S or G2/M), or a variety of other responses, i.e., DNA repair and apoptotic processes (Giaccia and Kastan, 1998; Apella and Anderson, 2000). In contrast with p53-Mdm2 proteasome complex, Mdm2 protein can also form a complex with p73 protein without degrading it (Dobbelstein et al., 1999; Zeng et al., 1999). Furthermore, the inhibition of Mdm2 activity in p53-proficient SH-SY5Y is consistent with that observed in normal cells, leading to elevated p53 levels and activation of a p53 response (Blaydes and Wynford-Thomas, 1998).

BTG2, one of the p53 target genes, is transactivated after exposure of wild-type p53 to γ radiation, an effect highly correlated with the accumulation of p53 protein and $p21^{WAF1}$ transcripts (Rouault et al., 1996). Accordingly, our results show that *BTG2* transcripts were strongly upregulated in Ad-TAp73-infected SH-SY5Y cells concomitantly with high accumulation of p53 protein. ΔNp73-forced overexpression also significantly upregulated *BTG2* transcript levels, albeit to a lesser extent. This induction was unexpected when one considers the negative dominant effect of ΔNp73 over p53 target genes such as *BAX* or *MDM2*. Interestingly, in TA- or ΔN-p73-infected IGR-N-91 cells, *BTG2* was weakly and equally induced. Such finding deserves a thorough investigation taking into account the kinetics of induction, and using other NB lines with various p53 statuses. Nonetheless, our study shows ΔNp73 selectivity in p53 downstream gene regulation but further studies of p53-adjacent gene promoters as well as physical interactions might elucidate this selectivity.

Taken together, our data underscore p73 involvement in wild-type p53 ability to accumulate in the nucleus of NB cells, a pre-requisite for biological function as a tumor suppressive protein. The p73 effect on p53 activity should be assessed, taking in account the combined activities within the p53 family (Vossio et al., 2002; Yang et al., 2002), and in particular for apoptosis completion, as recently reported (Flores et al., 2002). Our work provides evidence of p53-dependent localization change in wild-type p53-SH-SY5Y cells in response to TAp73 and not in mutated p53-IGR-N-91 cells. Since isogenic cells were not used, one possible experiment to further demonstrate the p53 dependence and the TAp73/p53 cooperativity is an RNAi-p53 approach to SH-SY5Y cells. This technique is under investigation and would clarify this issue. The mechanism by which p73 activates p53 protein from its 'cryptic' conformation remains to be elucidated. It is noteworthy that in normal unstressed cells, wild-type p53 protein is maintained in the cytoplasm as a latent form in a huge complex containing Parc protein (p53-associated, Parkin-like cytoplasmic protein) as shown very recently (Nikolaev et al., 2003). Parc appears to directly interact with p53 in the cytoplasm and prevents its transport to the nucleus. Interference with Parc expression through RNAi results in p53 nuclear translocation and apoptosis. It is not unreasonable to suggest that Parc can also bind to TAp73, a functional homologue of p53. The mechanism by which TAp73 and p53 are liberated from the cytoplasmic complex may be used to develop therapies. Appropriate TAp73 gene therapy might be considered in association with conventional NB treatment to enhance tumor cells apoptosis in patients whose tumor shows wild-type but non-functional p53.

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