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p53 and its homologues, p63 and p73, induce a replicative senescence through inactivation of NF-Y transcription factor

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Recent studies have identified two p53 homologues, p63 and p73. They activate p53-responsive promoters and induce apoptosis when overexpressed in certain human tumors. Here, we report that p63, like p53 and p73, induces replicative senescence when expressed in a tetracycline-regulated manner in EJ cells lacking a functional p53. In addition to transcription activation of p53-responsive genes, we found that p63 and p73 repress transcription of the cdk1 and cyclin B genes, both of which are irreversibly repressed in senescent human fibroblast. In transient transfection assay, p63 and p73 repress the cdk1 promoter regardless of the presence of a dominant negative mutant form of p53. Furthermore, we found that DNA binding activity of NF-Y transcription factor, which is essential for transcription of the cdk1 and cyclin B genes and inactivated in senescent fibroblast, is significantly decreased by expression of either of p53, p63, or p73. Since NF-Y binds to many promoters besides the cdk1 and cyclin B promoters, inactivation of NF-Y by p53 family genes may be a general mechanism for transcription repression in replicative senescence. *Oncogene* (2001) **20,** 5818–5825.

Keywords: p53; p63; p73; cdk1; cyclin B; NF-Y; senescence

Introduction

The proliferative lifespan of normal mammalian cells is limited by replicative senescence (Hayflick and Moorhead, 1961; Campisi, 1997), a process that appears to be primarily mediated by gradual shortening of telomeres (Shay, 1997). Senescent cells withdraw irreversibly from the cell cycle, but remain viable indefinitely, and display characteristic phenotypic markers, such as enlarged and flattened morphology, irreversible growth arrest, and expression of the senescence-associate β-galactosidase activity at pH 6.0 (Dimri *et al*., 1995). It has been shown that expression of *ras* and *raf* oncogenes can induce a rapid onset of replicative senescence in normal fibroblast cells associated with induction of p53, p16, p19 and hypophosphorylation of Rb protein (Serrano *et al*., 1997; Zhu *et al*., 1998). Recently, promyelocytic leukemia (PML) protein appears to be increased in and required for *ras*-induced senescence (Pearson *et al*., 2000; Ferbeyre *et al*., 2000). These studies suggest that replicative senescence, like apoptosis, is a programmed response of the organism to potentially oncogenic impact.

Tumor cells are capable of extended proliferation as if the capability to become senescent has been somehow repressed or lost. It has been shown that certain tumor cells enter into senescence state by expression of tumor suppressor genes such as p53 (Shin *et al*., 1997), Rb (Xu *et al*., 1997), p21 (Fang *et al*., 1999) and p16 (Uhrbom *et al*., 1997). These results suggest that p53 and Rb signaling pathways can trigger replicative senescence program which is inactivated in human tumor cells. However, a detailed mechanism by which tumor suppressor genes trigger replicative senescence is largely unknown.

Mutations in the p53 tumor suppressor gene are the most frequently found genetic alterations in human cancers, implying that a loss of this gene represents a fundamentally important step in genomic instability and susceptibility to malignant transformation (Hollstein *et al*., 1994; Kinzler and Vogelstein, 1996). Wild type p53 limits cellular proliferation by inducing a transient cell cycle arrest, apoptosis, or senescence, depending on the expression level or cellular context (Chen *et al*., 1996; Levine, 1997). The underlying mechanism of tumor suppressor activity of p53 resides in part in its ability to bind DNA in a sequence-specific manner to activate gene transcription (Juven *et al*., 1993). The identification of two p53 homologues, p63 and p73, revealed that p53 is a member of a family of related transcription factors (Kaghad *et al*., 1997; Osada *et al*., 1998; Yang *et al*., 1998; Jost *et al*., 1997). p53 and its two homologues share sequence

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similarity with highest degree of the homology in the DNA binding domain. Like p53, p63 and p73 proteins are capable of binding to p53-responsive elements and can activate p53-responsive genes. When overexpressed p63 and p73 can induce cell cycle arrest and apoptosis, suggesting a potential for a therapeutic approach using expression of p63 or p73. Other evidence, however, suggest that p63 and p73 are important for regulation of normal development (Yang *et al*., 1999, 2000; Mills *et al*., 1999). An extended C-terminal region, not found in p53, is alternatively spliced in p63 and p73 (Kaghad *et al*., 1997; Osada *et al*., 1998; Yang *et al*., 1998). The p63 and p73 genes are rarely mutated in human cancer, although p73 loss is observed in neuroblastoma and a subtype of T-cell lymphoma (Ichimiya *et al*., 1999; Osada *et al*., 1998). Thus, p53, p63 and p73 appear to have overlapping and distinct functions: p53 regulates the stress response to suppress tumors; p63 is essential for ectoderm development; and p73 might regulate both the stress response and development. Because p53 and p73 are linked to different upstream pathways, this family of transcription factors might regulate a common set of genes in response to different extracellular signals and developmental cues.

In this study, we set out to gain insight into the process of p53-induced senescence in human tumor cells. We first examined whether p63, like p53 and p73, induce replicative senescence in human tumor cells lacking functional p53. We hypothesized that the onset of senescence involves a transcription repression of proliferation-related genes that give rise to the irreversible growth arrest. We found that p53 family proteins repress transcription of cyclin B and cdk1 genes through inactivation of NF-Y transcription factor. Since NF-Y binds to many promoters besides the cyclin B and cdk1 promoters, we propose that transcription repression in senescent cells is mediated, at least in part, by p53-dependent inactivation of NF-Y.

Results

Inducible expression of p63 isoforms in p53-deficient EJ cells

We first examined the effects of inducible expression of p63 isoforms using a tetracycline-regulated gene expression system (Gossen and Bujard, 1992) in EJ cells that lack a functional p53 protein. EJ cells were transfected either with ptet-p53, ptet-p63α, or ptet-p63γ containing a HA-tag and a neomycin-resistant marker. More than 10 stable clones were obtained, which were subsequently infected with a recombinant adenovirus encoding tTA that binds to and activates the tetracycline-regulated promoter in the absence of tetracycline (Gossen and Bujard, 1992). Expression of p53 and two p63 isoforms was induced in more than two clones for each gene following infection with the tTA-encoding adenovirus in the absence of tetracycline. As shown in Figure 1a, HA-tagged p53, p63α, and p63γ were undetectable in the absence of tTA expression. After infection with the tTA virus, expressions of p53 and the p63 isoforms all reached a maximum at 48 h. As expected from the inability of the viral DNA to replicate in EJ cells, p53 and the p63 isoforms decreased to hardly detectable levels 6 days after the infection (Figure 1a). Expressions of the p53-inducible genes, p21 and mdm2, were induced after infection with the tTA virus (Figure 1a). Consistent with the results of p21 expression, cdk2 kinase activity decreased in cells expressing the p63 isoforms (Figure 1b).

p63 induces irreversible growth arrest with markers of replicative senescence

Figure 2 shows the effects of inducible expression of p63 isoforms on proliferation of EJ cells. While EJ cells infected with the virus encoding tTA grew as fast as with uninfected cells, proliferation of cells expressing p53, p63α or p63γ was inhibited (Figure 2a). Consistent with this, incorporation of BrdU into cells expressing p53, p63α, or p63γ decreased after induction of their expression (Figure 2b). However, there was no evidence of apoptosis in EJ cells expressing one of the p63 isoforms (data not shown). We previously reported that p53 expression does not induce apoptosis in EJ cells, but induces permanent growth arrest and a replicative senescence (Shin *et al*., 1997). p63-expressing cells became flattened and enlarged just like EJ cells made senescent by p53 expression as previously shown (Figure 3b). To examine the reversibility of p63-induced growth arrest in EJ cells, we performed a

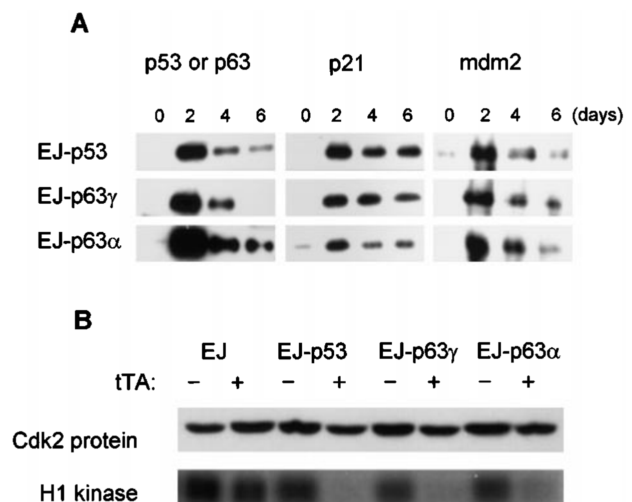

Figure 1 Inducible expression of p53 and p63 isoforms in EJ cells. (**a**) Protein analyses by immunoblotting. Cells containing ptet-p53, ptet-p63γ, or ptet-p63α were infected with a recombinant adenovirus encoding tTA. Protein lysates were prepared at the indicated times. (**b**) Inhibition of Cdk2 associated histone H1 kinase activity following induction of p63 in EJ cells. The upper panel shows the result of immunoblot analysis of the cdk2 protein for each sample. The lower panel is the result of a histone H1 phosphorylation by Cdk2, which was immunoprecipitated from cells infected with the tTA adenovirus for 0 (−) or 2 days (+)

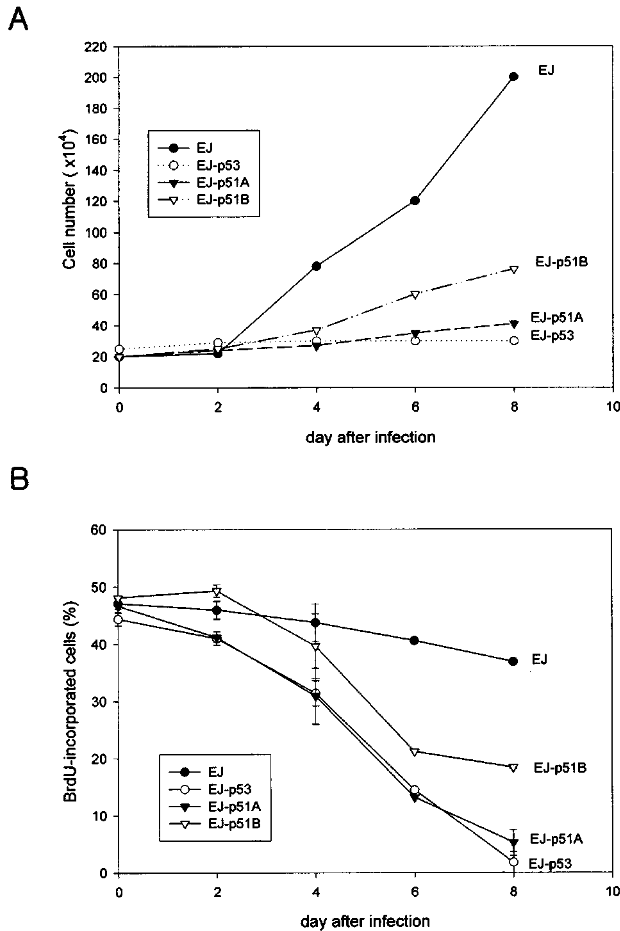

Figure 2 p63-induced growth inhibition. (**a**) Growth curves of EJ cells expressing p53 and one of the p63 isoforms. EJ cells containing ptet-p53, -p63α or -p63γ were infected with the tTA adenovirus. Cell numbers were determined at the times indicated. (**b**) BrdU incorporation assay in EJ cells containing ptet-p53, -p63α or -p63γ. The cells infected with the tTA adenovirus were subjected to BrdU incorporation assay as described in Materials and methods

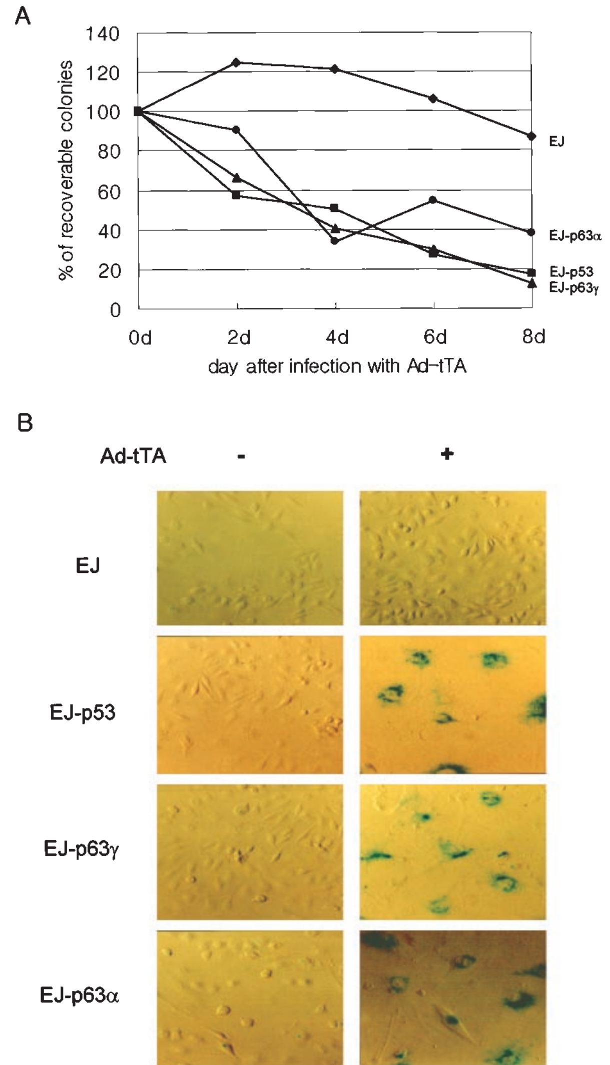

Figure 3 p63-induced senescence. (**a**) Irreversible growth arrest by p63 isoforms. EJ-p63α and -p63γ cells were infected with the tTA virus and seeded at about 100 cells per 60-mm plate and maintained without tetracycline for varying periods followed by an addition of tetracycline. Cultures were maintained in the presence of tetracycline for another 14 days, followed by fixation and Giemsa staining. The numbers of colonies were counted and plotted. (**b**) SA-β-Gal staining following p63 induction. At 8 days after transduction of the adenovirus tTA, cells were stained as described in Materials and methods

colony-forming assay (Figure 3a). EJ-p63γ and -p63α cells were infected with tTA virus and seeded at about 100 cells per 60-mm plate and maintained without tetracycline for varying periods followed by a tetracycline addition. Cultures were maintained in the absence of tetracycline for another 14 days, followed by fixation and Giemsa staining. The numbers of colonies were determined and plotted in Figure 3a. Colony forming ability was markedly reduced in cells maintained in the absence of tetracycline for 4 or more days, indicating that growth arrest caused by the p63 isoforms became irreversible within 4 days. Although the two isoforms of p63 showed similar effects on growth inhibition, their abilities to inhibit cell growth and induce irreversible growth arrest were different, p63γ being stronger than p63α (Figures 2 and 3a).

It has been shown that senescent, but not quiescent or terminally differentiated cells express SA-β-gal activity, which can be detected by incubating cells at pH 6.0 with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Dimri *et al*., 1995). Since cells expressing p53 or one of the p63 isoforms did not reinitiate their growth after decrease in their expression (Figure 1a), we examined whether cells express this senescence-specific marker. As shown in Figure 3b, >90% of cells started to express SA-β-gal activity 6 days after infection with the tTA-encoding virus, whereas control cells infected with the ΔE1 control virus showed no activity. These results indicated that expression of the p63 isoforms, like p53 and p73, could trigger the senescence pathway in p53-deficient EJ cells.

Transcription repression by p63 and p73 in the presence of a p53 mutant

It has been reported that p63 and p73 activate previously identified p53-target genes (Osada *et al*., 1998; Yang *et al*., 1998; Shimada *et al*., 1999). In addition to transcription activation, p53 has been shown to repress transcription of certain genes (Mack *et al*., 1993; Seto *et al*., 1992). We previously reported that p53 represses transcription of cdk1 and cyclin B1, both of whose transcriptions are regulated in a cell cycle dependent manner and irreversibly repressed in senescent cells (Yun *et al*., 1999; Park *et al*., 2000). Figure 4 shows that mRNA levels of cdk1 and cyclin B1 were significantly reduced in cells expressing the isoforms of p63 or p73. Kinetics of the reduction paralleled that of induction of the p63 or p73 isoforms (Figure 4).

To examine the effect of p63 and p73 on the cdk1 promoter, which contains NF-Y-binding CCAAT sequences and shares structural similarity with cyclin B promoter (Tommasi and Pfeifer, 1995; Piaggio *et al*., 1995), we transfected a p53-null tumor cells (HCT116 $p53^{-/-}$) with the cdk1 promoter-CAT reporter construct (pcdk1-CAT) and either p53, p63, or p73. Figure 5a shows the relative CAT activity of the p53-responsive promoter-CAT (pG5-CAT) and pcdk1-CAT in the presence of either p53, p63γ, or p73β. Like p53, p63γ and p73β activated the G5 promoter but repressed the cdk1 promoter (Figure 5a). These results suggest that p63 and p73, in addition to their ability to activate transcription of the p53-responsible genes, can repress transcription of the p53-repressible genes. To further examine whether the dominant negative mutant form of p53 abolishes the repressive effect of p63γ and p73β on cdk1 transcription, we co-transfected p53, p63γ, or p73β with the p53 mutant, $p53^{273}$ (Figure 5b). The p53 mutant inhibits both induction of pG5-CAT and repression of pcdk1-CAT by wild type p53 (Figure 5b). However, the effects of the p53 mutant on both induction of pG5-CAT and repression of pcdk1-CAT by p63γ and p73β were significantly weaker than those on wild type p53 (Figure 5b). Low concentration of the p53 mutant plasmid in transfection mixture (1 or 2 μg) was enough to inhibit the effects of wild type p53 (Figure 5b). The p53 mutant efficiently inhibits only when 8 μg of the p53 mutant plasmid is transfected with 1 μg of p63 or

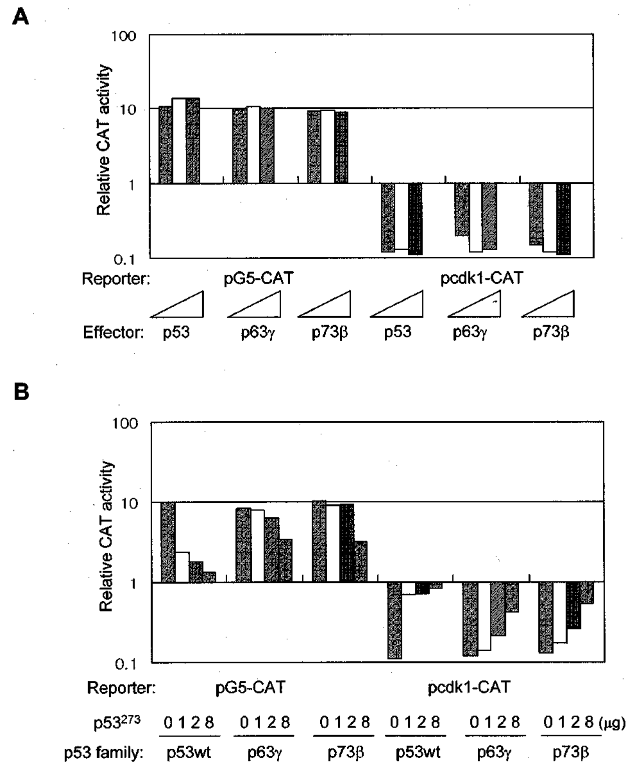

Figure 5 Inhibition of the cdk1 promoter by p63 and p73. (**a**) HCT116 cells with disrupted p53 gene ($HCT116p53^{-/-}$) were co-transfected with 1 μg of either the pG5-CAT or pcdk1-CAT reporter plasmid and varying amounts (0.5, 1, 4 μg) of either p53, p63γ, or p73β. The CAT activities expressed in the co-transfected cells with each reporter plasmid and the pCMV vector were defined as unity. The data represent the means of three independent transfections. (**b**) $HCT116p53^{-/-}$ cells were co-transfected with three different plasmids; 1 μg of either the pG5-CAT or pcdk1-CAT reporter plasmid, 1 μg of either the p53, p63γ, or p73β, and varying amounts (1, 2, 8 μg) of the p53 mutant, $p53^{273}$. The CAT activity expressed in cells co-transfected with each reporter plasmid and pCMV was defined as unity

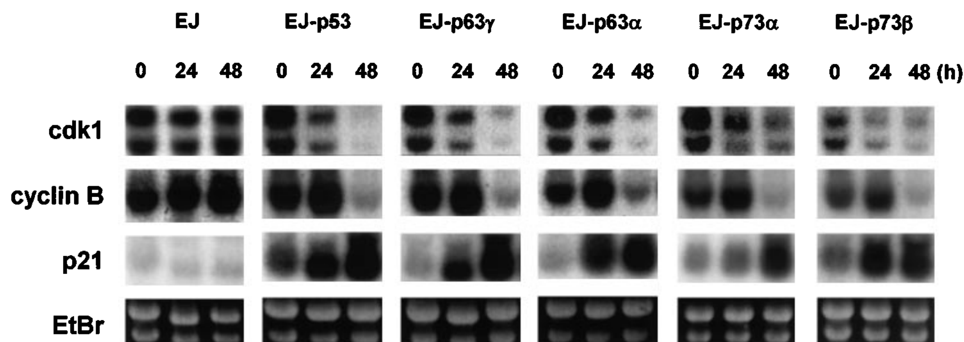

Figure 4 Transcription repression of the cdk1 and cyclin B genes by p63 and p73. RNA samples prepared at the indicated times were analysed as described in Materials and methods. EtBr-stained gels were used as loading controls

p73 plasmid (Figure 5b). These results suggest that p63γ and p73β can activate and repress the p53-regulatable genes in human tumor cells expressing the endogenous p53 mutant gene.

p53 family proteins inactivate DNA binding activity of NF-Y transcription factor

Transcription of the cdk1 and cyclin B genes requires NF-Y transcription factor that binds to CCAAT sequences on the promoters (Yun *et al*., 1999; Bolognese *et al*., 1999). NF-Y consists of three different subunits, YA, YB and YC, all of which are needed to form a NF-Y/DNA complex (Sinha *et al*., 1995). In initial studies, NF-Y has been found in essentially all cell types, suggesting that it represents a prototypic example of the immutable, constitutive transcription factor. However, several recent studies demonstrated that the DNA binding activity of NF-Y is changed during the development (Chang and Liu, 1994; Good and Chen, 1996), senescence (Chen, 1997), or differentiation process (Marziali *et al*., 1997; Farina *et al*., 1999). These results lead us to examine a possibility that p53 and its homologues inactivate NF-Y transcription factor. We examined whether p53 affect to expression of NF-Y subunits, YA, YB, and YC. Immunoblot analysis showed that protein levels of all three subunits of NF-Y were unaffected by expression of p53 (Figure 6a). To examine whether p53 family proteins affect to DNA binding activity of NF-Y, we performed a gel shift assay using CCAAT sequence that is found in the cdk1 promoter. NF-Y/DNA complex was detected as described previously (Yun *et al*., 1999). DNA binding activity of NF-Y was significantly reduced by the expression of either p53, p63γ, or p73β (Figure 5). These results suggest that inactivation of DNA binding activity of NF-Y mediates transcription repression of the cyclin B and cdk1 genes in p53-induced senescence.

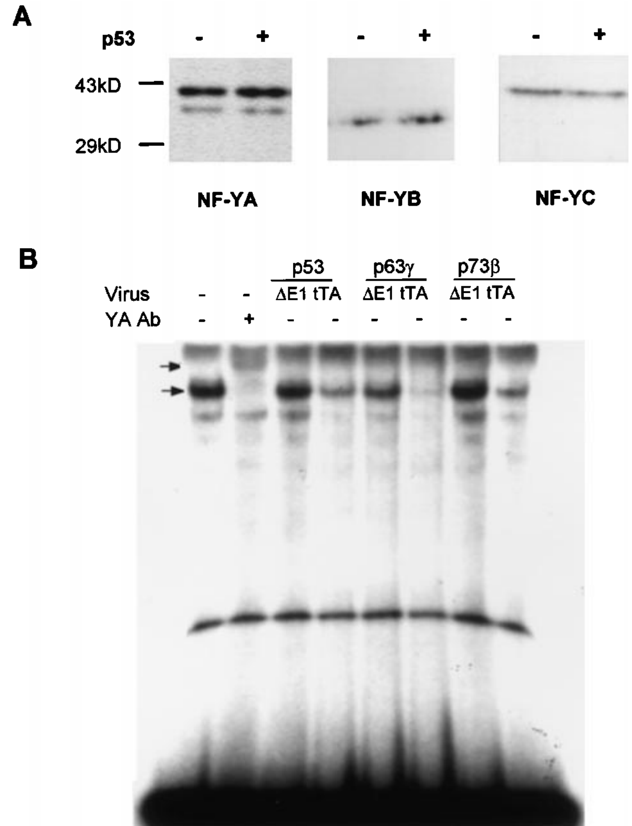

Figure 6 Reduction in DNA binding activity of NF-Y by p53 family genes (**a**) Immunoblot analysis. Lysates of EJ-ptet-p53 infected with the tTA virus for 2 days were subjected to immunoblot with anti-YA, YB, and YC antibodies. (**b**) Electrophoretic mobility shift assays of complexes formed by the DNA probe containing the CCAAT element of the cdk1 promoter (the promoter sequences between −88 and −64). An end-labeled DNA probe was incubated with nuclear lysates of EJ-ptet-p53, p63γ, or -p73β infected with adenovirus encoding tTA (tTA) or the control virus (ΔE1) for 2days. The arrows indicate the NF-Y/DNA complex (lower arrow) and supershifted complex with anti-YA antibody (upper arrow)

Discussion

Many researches reported that p53 homologues, p63 and p73, can activate the p53 pathway, bind and activate p53-responsive promoters, arrest the cell cycle at G1 phase, and induce apoptosis when it is transiently overexpressed (Kaghad *et al*., 1997; Osada *et al*., 1998; Yang *et al*., 1998). Here we presented our findings that p63 isoforms induce a replicative senescence in p53-deficient EJ cells. Following observations supports this conclusion: irreversible growth arrest, flattened and enlarged cell morphology, and SA-β-galactosidase activity at pH 6.0, a specific biochemical marker of senescent cells (Dimri *et al*., 1995). In p63-expressing cells, the commitment to senescence seems to occur within 4 days after infection with the tTA virus (Figures 1 and 2). Recently, it was reported that another p53-homologue, p73, also induces replicative senescence (Fang *et al*., 1999). These results make it apparent that common targets of p53 family proteins trigger a replicative senescence program. Since p21 expression is induced by all p53 family genes and increased in senescent cells, it may play a critical role in p53 family gene-induced senescence. Our results that the p63 induce permanent growth arrest of EJ cells, in combination with previous studies reporting p63-mediated apoptosis in other cell types, imply that p63, like p73, can mimic two major effector functions used in its role of tumor suppression and guardian of the genome.

p53 molecules can oligomerize to form a homotetramer (Friedman *et al*., 1993), and this association is likely to be required for efficient transcription activity. However, the ability to oligomerize can also lead to inactivation of the wild type protein complexed with a mutant protein (Deb *et al*., 1999). This may become important during tumorigenesis, during which process point mutations frequently result in expression of a mutant p53 protein with a single amino acid substitu-

tion in the DNA binding domain (Hachiya *et al*., 1994; Kern *et al*., 1992). Davison *et al*. (1999) reported that the oligomerization domain of p53 does not associate with that of either of p63 and p73 *in vitro*. Moreover, transcription activation function of p73 is not disturbed by the p53 mutants or p53-targeting viral oncogenes such as SVT, E1B-55K and HPV-E6 (Wienzek *et al*., 2000; Marin *et al*., 1998). However, recent studies suggest functional and physical interactions between p53 family proteins. The isoforms of p63 that lack the N-terminal transactivation domain can function as a dominant negative inhibitor of both p53 and p63 (Yang *et al*., 1998). Some tumor-derived p53 mutants can also inhibit the effects of p63 and p73 through a direct interaction (Di Como *et al*., 1999; Gaidon *et al*., 2001). In this study, we found that the p53 mutant can inhibit transcriptional repression function as well as transcriptional activation function of p63 and p73 only when excess molar ratio of plasmid encoding the p53 mutant is transfected (Figure 5b). However, the p53 mutant could not inhibit the functions of p63 and p73 when it was transfected at similar molar ratio with the p63 and p73. These results support previous reports that p63 and p73 can induce cell cycle arrest and apoptosis through activation and repression of the p53-regulatable genes in tumor cells expressing the endogenous p53 mutant gene.

Recent studies implicated the potency of p53 as a transcription repressor in the tumor suppressor function as well as in apoptosis (Shen and Shenk, 1994; Sabbatini *et al*., 1995). Transcription repression function of p53 has been suggested to operate by direct interaction with the TATA-binding protein (TBP) or TBP-associated Factors (TAFs), resulting in inhibition of of the basic transcription machinery (Horikoshi *et al*., 1995; Seto *et al*., 1992). However, other groups beside ours have been reported that p53-dependent repression of certain genes is dependent on the existence of *cis*-acting element on the promoters, at which specific transcription factors bind (Agoff *et al*., 1993; Yun *et al*., 1999), detailed mechanism of which have yet to be made clear. Other groups and we reported that NF-Y mediates transcription repression of the cdk1 and cyclin B genes in response to p53 induction and DNA damage (Yun *et al*., 1999; Bolognese *et al*., 1999). Since NF-Y became to be inactivated in senescent fibroblast cells, it seems to mediate transcription repression of cell proliferation-related genes in senescent cells. These results lead us to examine a possibility that p53 family proteins induce replicative senescence through, at least in part, inactivating NF-Y transcription factor. Here, we propose that p53 family proteins somehow inactivate transactivation function of NF-Y through inhibiting its DNA binding activity. Since many promoters contain NF-Y binding CCAAT sequences (Mantovani, 1999), inactivation of NF-Y by p53 family proteins is one of possible mechanism to explain how cell proliferation-related genes are repressed in senescent cells. Future study should be directed to understand a detailed molecular mechanism by which p53 regulates NF-Y.

Materials and methods

Cell culture, plasmid construction and DNA transfection

The EJ human bladder carcinoma cell line, EJ-p53, -p63α, -p63γ, -p73α, and -p73β cells were maintained in DMEM supplemented with 10% FBS (GIBCO–BRL) and penicillin-streptomycin (50 U/ml). To induce expression of p53 family, we infected cells at 10 m.o.i. of a recombinant adenovirus encoding tTA (obtained from Dr Morgan) for 3–4 h. The NH_2-terminal hemagglutinin (HA)-tagged coding sequences of p63α,γ (obtained from Dr S Ikawa, Sendai, Japan) and p73α, and β (obtained from Dr Uhm, Seoul, Korea) were cloned into pTet-CAT (Shin *et al*., 1997) digested with *Bam*HI and *Sal*I, resulting in plasmid pTet-p63α, -p63γ, -p73α or -p73β, respectively. pTet-p53, p63α, -p63γ, -p73α or -p73β was transfected to EJ cells using the $CaPO_4$ coprecipitation procedure (Graham and van der Eb, 1973). Transfectants were selected in the presence of geneticin (500 μg/ml). Individual clones of stable transfectants, designated EJ-p53, -p63γ, -p63α, -p73α and -p73β, were selected for further analysis.

Northern blot analysis

Total cellular RNA was isolated with the use of Trizol solution (Life Technologies, Inc.), denatured, electrophoresed in a 1% agarose-formaldehyde gel, transferred to a nylon membrane and hybridized as described in the manufacturer's instructions (ExpressHyb, Clontech Inc.). Probes were ^{32}P-labeled using a random primer DNA labeling kit (Bio-Rad). Probes used are: (a) the 1.2-kilobase *Eco*RI/*Bam*HI cdk1 insert fragment containing the complete open reading frame of cdk1; (b) the 1.1-kilobase *Kpn*I/*Xba*I cyclin B1 insert in the pRcCMV plasmid amplified by PCR from human genomic DNA.

Immunoblot analysis

Cells cultured were lysed in EBC lysis buffer (50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 100 mM sodium fluoride, 2 mM sodium vanadate, 2 mM PMSF and 10 μg/ml aprotinin). Protein quantitation was performed using the protein assay kit (Bio-rad). Approximately 40 μg of total cell protein per sample was subjected to SDS–PAGE and transferred to an Immobilon (Millipore) polyvinylidene difluoride filter. The filter was then blocked in 5% nonfat dry milk/0.1% Tween/TBS followed by incubation with each antibody and immunodetection using the ECL system (Amersham). p63γ and p63α were detected using an anti-HA monoclonal antibody (BM, 12CA5), while p53, p21 and hMDM2 were detected by DO-1 (Santa Cruz, p53 specific), WAF-1 (Oncogene Science, p21 specific) and Ab-1 mAb (Oncogene Science, hMDM2 specific), respectively. NF-YA, -YB and -YC were detected by anti-mouse YA and anti-rabbit YB and YC antibodies (obtained from R Mantovani), respectively.

Histone H1 kinase assay

To measure histone H1 kinase activity, we incubated cell lysate (150 μg) for 12 h at 4°C with an anti-cdk2 antibody (M12; Santa Cruz) and protein A-agarose (Boehringer Mannheim Inc.). The immunoprecipitates were washed three times with the lysis buffer and twice with kinase buffer (50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT). The immunoprecipitates in the kinase buffer were mixed with

25 μl of the kinase buffer containing 20 μM ATP, 5 μCi [γ-^{32}P]ATP (3000 mCi/mmol: Amersham), and 5 μg of histone H1 (Boehringer Mannheim Inc.). After 20 min at 30°C, the reaction was terminated with SDS sample buffer, and the products were analysed by SDS–PAGE and autoradiography.

Growth curves and BrdU incorporation assay

EJ-p63 (or EJ-p53) cells infected with adenovirus tTA were splited and seeded at a density of 10^5 cells per 100 mm plate (time = 0 day). Cells were counted on 0, 2, 4, 6 and 8 days by hemocytometer. After transduction with adenovirus tTA, cells were trypsinized and seeded on cover glasses (time = 0 day). At 0, 2, 4, 6 and 8 days, cells on each cover glass were labeled for 1 h with 10 μM BrdU (cell proliferation kit; Boehringer Mannheim) and fixed with acetic acid/ethanol. BrdU-incorporated cells were detected as described in the manufacturer's instructions and counted.

Senescence-associated β-galactosidase (SA-β-Gal) staining

Cells cultured were washed in PBS, and fixed with 0.25% glutaraldehyde in PBS/2 mM $MgCl_2$ for 20 min at room temperature. SA-β-Gal activity at pH 6.0 was detected as reported (Dimri *et al*., 1995).

Transient transfection assay

DNA transfection was performed using the $CaPO_4$ coprecipitation procedure (Graham and van der Eb, 1973). After 48 h of transfection, cells were harvested, and proteins were extracted by three cycles of freeze-thawing. The protein concentration of each cell lysate was determined with the Bio-Rad protein assay kit. In all transfection experiments, β-galactosidase activity derived from pCMV-β-gal was used to monitor and to normalize the transfection efficiency. Chloramphenicol acetyltransferase (CAT) and β-galactosidase assays were carried out according to the reported protocols (Gorman *et al*., 1982). The promoter activities of reporter constructs were assayed by measuring the radioactivity of acetylated forms using a phosphoimage analyzer (Fuji).

Electrophoretic mobility shift assay

Nuclear lysates were prepared from EJ-p53, -p63γ, and -p73β cells which were infected with 10 m.o.i. of a mock adenovirus (ΔE1) or a recombinant adenovirus encoding tTA according to the method described by Dignam *et al*. (1983). A double-stranded, *in vitro* synthesized DNA fragment containing the distal CCAAT motif (−88 to −64) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Nuclear lysates (5 μg) were pre-incubated for 30 min at 0°C with 1 μg of poly [dI-dC] (Pharmacia Biochemical Inc.) in 25 mM HEPES (pH 7.9), 20 mM KCl, 30 mM NaCl, 0.5 mM EDTA, 0.25 mM DTT, and 10% glycerol. The end-labeled probe (about 15 000 c.p.m.) was added and incubated for an additional 20 min at room temperature. The DNA-protein complexes were separated on a 6% polyacrylamide gel in 0.25 × TBE at 15 mA for 2 h. For antibody supershift, 1 μg of antibody for NF-YA (Pharmingen) were included in the pre-incubation mixture.

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