E1A signaling to p53 involves the p19^{ARF} tumor suppressor

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The adenovirus *E1A* oncogene activates p53 through a signaling pathway involving the retinoblastoma protein and the tumor suppressor p19^{ARF}. The ability of E1A to induce p53 and its transcriptional targets is severely compromised in *ARF*-null cells, which remain resistant to apoptosis following serum depletion or adriamycin treatment. Reintroduction of p19^{ARF} restores p53 accumulation and resensitizes *ARF*-null cells to apoptotic signals. Therefore, p19^{ARF} functions as part of a p53-dependent failsafe mechanism to counter uncontrolled proliferation. Synergistic effects between the p19^{ARF} and DNA damage pathways in inducing p53 may contribute to E1A's ability to enhance radio- and chemosensitivity.

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Tumor-specific mutations identify genes essential for normal growth control and reveal fundamental processes involved in tumorigenesis. Similarly, viral oncoproteins target cellular proteins critical for malignant transformation—often the same activities altered by spontaneous mutation in cancer cells. For example, many DNA tumor viruses encode proteins that bind and inactivate both p53 and the retinoblastoma (Rb) protein, and inactivation of both is essential for viral transformation (Lane and Crawford 1979; Linzer and Levine 1979; DeCaprio et al. 1988; Whyte et al. 1988a; Dyson et al. 1989; Werness et al. 1990). Consistent with the relevance of these interactions, *p53* and *Rb* are frequently mutated in human tumors (for review, see Greenblatt et al. 1994; Weinberg 1995).

Although the high frequency of *p53* mutations in human cancer implies a central role for p53 in tumorigenesis, the signals that trigger p53 in suppressing tumor growth remain poorly defined. p53 is a sequence-specific DNA-binding protein that promotes cell-cycle arrest or apoptosis in response to a variety of cellular stresses (for examples, see Kastan et al. 1991; Graeber et al. 1994; Linke et al. 1996; for review, see Ko and Prives 1996; Levine 1997). For example, p53 levels and activity increase following DNA damage owing, in part, to de novo phosphorylation and the accompanying conformational changes (Shieh et al. 1997; Siliciano et al. 1997). Phosphorylation at serine-15 prevents p53's interaction with Mdm2 (Shieh et al. 1997), a protein that can down-regulate p53 via ubiquitin-mediated proteolysis (Haupt et al. 1997; Kubbutat et al. 1997). In principle, failure of p53 to suppress proliferation following DNA damage might indirectly promote tumor development by allowing the growth and survival of cells with mutations (Livingstone et al. 1992; Yin et al. 1992; Griffiths et al. 1997), but whether this provides the primary driving force for *p53* mutation in tumors is unclear.

Oncogenes can also induce p53, leading to increased apoptosis or premature senescence (Lowe and Ruley 1993; Hermeking and Eick 1994; Wagner et al. 1994; Serrano et al. 1997). For example, the adenovirus *E1A* oncogene induces p53 and promotes apoptosis in primary cells (Debbas and White 1993; Lowe and Ruley 1993; Querido et al. 1997; Samuelson and Lowe 1997), which is reflected by E1A's remarkable ability to enhance radio- and chemosensitivity (Lowe et al. 1993). Although *E1A* is a mitogenic oncogene, p53 acts to limit its oncogenic potential. Thus, *p53*-deficient primary fibroblasts expressing *E1A* are resistant to apoptosis and become oncogenically transformed (Lowe et al. 1994b). Two E1A domains act in concert to promote p53 accumulation and apoptosis in primary cells; the first inactivates Rb, whereas the second binds the p300/CBP transcriptional coactivators (Samuelson and Lowe 1997). Interestingly, the integrity of both domains is required for E1A's oncogenic potential (Whyte et al. 1988b, 1989). The ability of E1A to activate p53 is not unique, as c-Myc activates p53 to promote apoptosis (Hermeking and Eick 1994;

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Wagner et al. 1994) and oncogenic *ras* induces p53 leading to premature senescence (Serrano et al. 1997). How oncogenic signals activate p53 is not known, although it is conceivable that they induce p53 by inadvertently damaging DNA. Nevertheless, the general involvement of p53 in the cellular response to oncogenes raises the possibility that these stimuli are fundamental to p53's tumor suppressor activity.

The *INK4a/ARF* locus is second only to *p53* in the frequency of its disruption in human cancer (for review, see Haber 1997). This locus encodes p16^{INK4a}, a cyclin-dependent kinase inhibitor (CDKI) that acts upstream of Rb to promote cell-cycle arrest (Serrano et al. 1993). Although compelling evidence indicates that p16^{INK4a} is an important tumor suppressor, the *INK4a/ARF* locus encodes a second protein translated in an alternate reading frame, designated p19^{ARF} (Quelle et al. 1995). p19^{ARF} and p16^{INK4a} are often codeleted in tumor cells, but mice lacking p19^{ARF} alone are highly cancer prone (Kamijo et al. 1997; for review, see Haber 1997). p19^{ARF} promotes cell-cycle arrest (Quelle et al. 1995), whereas *ARF*-null primary mouse embryo fibroblasts (MEFs) do not undergo replicative senescence and are transformed by oncogenic *ras* alone (Kamijo et al. 1997). Thus, *ARF* is a bona fide tumor suppressor.

p19^{ARF} may function in a genetic and biochemical pathway that involves p53. At the organismal level, the consequences of deleting *p53* and *ARF* are remarkably similar (Donehower et al. 1992; Kamijo et al. 1997). In either case, the mutant mouse develops normally but is highly predisposed to malignant tumors of a similar overall pattern and latency. At the cellular level, enforced expression of p19^{ARF} can induce cell-cycle arrest in cells harboring wild-type but not mutant p53 (Kamijo et al. 1997). In turn, p19^{ARF} can physically associate with p53 itself and/or Mdm2 to alter p53 levels and activity (Kamijo et al. 1998; Pomerantz et al. 1998; Zhang et al. 1998). Nevertheless, *ARF* is not required for the p53 response following DNA damage, as radiation induces G_1 arrest in *ARF*-deficient fibroblasts and apoptosis in *ARF*-deficient thymocytes (Kamijo et al. 1997, 1998). Thus, an understanding of the signals that activate p19^{ARF} may help to explain its role as a tumor suppressor as well as that of p53.

In this study we compared the mechanism whereby DNA damaging agents and the *E1A* oncogene activate p53. We demonstrate that E1A activates p53 through a fundamentally different mechanism than DNA damage, which is dependent on the presence of p19^{ARF}. Furthermore, simultaneous activation of p53 through oncogenes and DNA damage synergize to promote apoptosis and thereby enhance radio- and chemosensitivity. These data imply that p19^{ARF} acts to suppress tumor growth in response to hyperproliferative signals. Conversely, as p19^{ARF} mediates activation of p53 by an oncogene and is frequently lost in human tumors, these data strongly support the view that p53's tumor suppressor activity can arise from its ability to eliminate oncogene-expressing cells.

Results

E1A and DNA damage induce p53 through distinct mechanisms

The *E1A* oncogene induces p53 through a mechanism involving inactivation of Rb gene product, and up-regulation of p53 correlates with the ability of E1A to promote apoptosis (Lowe and Ruley 1993; Lowe et al. 1994b; Samuelson and Lowe 1997). DNA damage produced by radiation and certain cytotoxic drugs also activates p53, at least in part, through a kinase that phosphorylates p53 on serine-15 (Shieh et al. 1997; Siliciano et al. 1997). To determine whether DNA damage and E1A induce p53 through similar mechanisms, we examined the phosphorylation status of p53 on serine-15 in cells expressing or lacking *E1A*. *E1A* was introduced into normal diploid human fibroblasts (IMR90 cells) by retroviral-mediated gene transfer. After a 3-day drug selection to eliminate uninfected cells, p53 levels and phosphorylation status were assessed by Western blot analysis using antibodies that recognize total p53 or only that fraction phosphorylated on serine-15 (Shieh et al. 1997; Siliciano et al. 1997). For comparison, IMR90 cells were treated with ionizing radiation or with the calpain/proteosome inhibitor LLnL, both of which are also known to stabilize p53 (Maki et al. 1996). Total p53 was examined by Western blotting; alternatively, p53 was immunoprecipitated and scored for the presence of serine-15 phosphate using antibodies that detect this epitope.

As expected, ionizing radiation produced a large increase in p53 protein (Fig. 1A, lane 2) accompanied by p53 phosphorylation on serine 15 (Fig. 1B, lane 2). LLnL also induced p53 but without serine-15 phosphorylation (Fig. 1, A, lane 3, and B, lane 1). E1A produced even greater increases in p53 levels (Fig. 1A, lane 4) without detectable phosphorylation of p53 on serine 15 (Fig. 1B, lane 3). However, E1A did not inhibit p53 phosphorylation on serine-15, as γ-irradiation of cells expressing *E1A* produced little, if any, additional increase in p53 protein (Fig. 1A, lane 5) but led to a marked increase in antiphosphoserine-15 reactivity (Fig. 1B, lane 5). Induction of p53 in the absence of serine-15 phosphorylation argues that E1A does not produce DNA damage indirectly but, rather, suggests that E1A and ionizing radiation activate p53 through distinct mechanisms.

E1A induces p19^{ARF} through domains required for p53 accumulation and apoptosis

Enforced expression of p19^{ARF} stabilizes p53 and arrests proliferation in a p53-dependent manner, yet *ARF* is not required for radiation-induced cell-cycle arrest or apoptosis (Kamijo et al. 1997; Pomerantz et al. 1998; Zhang et al. 1998). The fact that E1A also stabilizes p53 through a DNA damage-independent mechanism is consistent with the possibility that E1A acts through p19^{ARF} to induce p53. *E1A* or various *E1A* mutants were introduced into primary MEFs, and p19^{ARF} expression was monitored 3 days later. E1A caused a dramatic induction of p19^{ARF}, correlating with p53 accumulation (Fig. 2, A

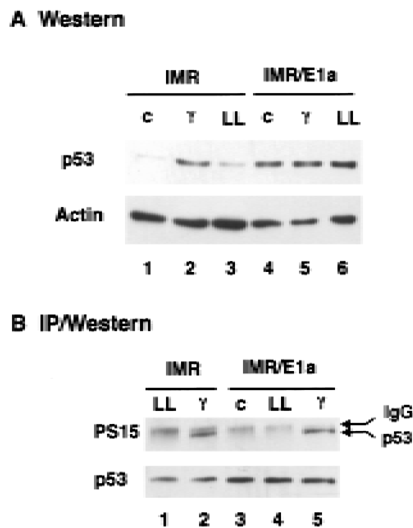

Figure 1. E1A induces p53 in the absence of phosphorylation on serine-15. IMR90 fibroblasts were infected with control (IMR) or *E1A*-expressing (IMR/E1A) retroviruses. Extracts were prepared from untreated cells (c), or from cells treated 3 hr earlier with 7 Gy γ radiation (γ) or 2 hr earlier with 50 μM LLnL (LL). (*A*) p53 levels were determined by Western blot analysis using pAb 1801 and DO1. Equal loading of the gel was confirmed by stripping the blot and reprobing with anti-β-actin antiserum. (*B*) p53 was immunoprecipitated from extracts corresponding to 100 μg (IMR) or 35 μg (IMR/E1A) total protein using pAb 1801, and Western blots were probed with antibodies specific for p53 phosphoserine-15 (αp53-P–Ser-15).

and B, cf. lanes 2 and 1). A similar increase was also observed in *ARF* mRNA expression, indicating that E1A was affecting *ARF* transcription or message stability (Fig. 2B, cf. lanes 2 and 1). As demonstrated previously (Kamijo et al. 1997), *ARF* is constitutively upregulated in *p53*$^{-/-}$ MEFs (Fig. 2B, lane 5), suggesting the presence of a negative feedback loop. However, E1A still induced p19^{ARF} expression in *p53*-deficient cells (two- to threefold), implying that p53 is not required for p19^{ARF} upregulation by E1A (Fig. 2B, lane 6).

E1A associates with a series of cellular proteins, including Rb, the Rb-related proteins p107 and p130, and the transcriptional coactivators p300 and CBP (for review, see Flint and Shenk 1997). E1A mutants unable to bind either p300/CBP (E1A ΔN) or the Rb-family proteins (E1A ΔCR2) were impaired in their ability to induce p19^{ARF} and p53 (Fig. 2A, lanes 3,4), implying that E1A's ability to bind both sets of cellular proteins is required for maximal p19^{ARF} accumulation. In agreement, p19^{ARF} protein induction was restored in cells coinfected with both *E1A* mutants (data not shown). p19^{ARF} levels were slightly elevated in *Rb*-deficient MEFs (Fig. 2A, lane 5) although this difference was more pronounced in later passage MEFs (data not shown; see also Zindy et al. 1998). Importantly, p19^{ARF} levels were further increased by expression of E1A (Fig. 2A, lane 6) or, in contrast to normal cells, the E1A ΔCR2 mutant (Fig. 2A, cf. lanes 4 and 8). However, p19^{ARF} was not elevated in *p107*- and *p130*-deficient MEFs, nor was it induced by E1A ΔCR2 (data not shown). Thus, among the Rb-family proteins that bind E1A, the recognized ability of E1A to inactivate Rb solely contributes to p19^{ARF} accumulation. These data demonstrate that at least two E1A functions contribute to p19^{ARF} induction: inactivation of Rb and, possibly, binding to p300/CBP. Notably, these are the same domains of E1A that are necessary for its ability to induce p53 and promote apoptosis (Samuelson and Lowe 1997).

ARF promotes p53 accumulation in response to E1A

p53 activation is typically accompanied by increased expression of its transcriptional targets, including p21 and Mdm2. p21 is a CDKI involved in p53-dependent cell-cycle arrest (El Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993). Mdm2 acts in a negative feedback loop to down-regulate p53 and is expressed from two promoters, one of which is regulated by p53 (Barak et al. 1993,

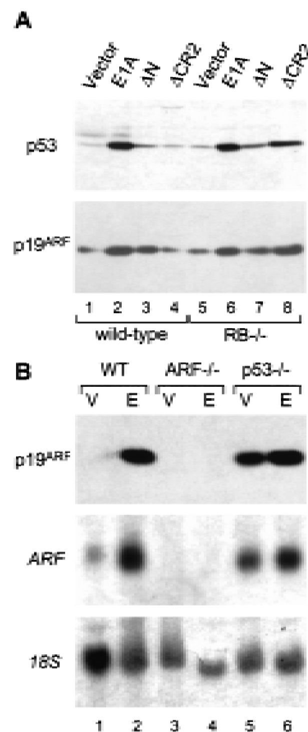

Figure 2. E1A induces p19^{ARF} and p53 through a similar mechanism. (*A*) Early passage (about three to four) wild-type and *Rb*$^{-/-}$ MEFs from littermates embryos were infected with retroviruses expressing full-length *E1A* or *E1A* mutants unable to bind p300/CBP (ΔN) or the Rb-related proteins (ΔCR2). An empty retroviral vector was used as a control (vector). Immunoblotting was performed using polyclonal antibodies against p19^{ARF} or p53. Using this procedure, each E1A mutant is efficiently expressed at comparable levels (Samuelson et al. 1997). (*B*) Wild-type (WT), *ARF*-null (*ARF*$^{-/-}$), and *p53*-null (*p53*$^{-/-}$) MEFs were infected with a control vector (V) or a retrovirus expressing full-length *E1A* (E). Lysates were derived from whole populations passaged minimally in culture (<1 week) and analyzed for ARF protein (*top*) or mRNA (*middle*) expression by Western or Northern blotting, respectively. Northern blots were rehybridized using a probe to the 18S rRNA to confirm equal loading (*bottom*).

1994; Wu et al. 1993). To determine whether *ARF* is required for p53 induction by E1A, the expression of p53, p21, and Mdm2 were examined in wild-type, *ARF*$^{-/-}$, and *p53*$^{-/-}$ MEFs. In wild-type MEFs, E1A increased p53 protein expression, which was accompanied by accumulation of p21 and several forms of Mdm2 (Fig. 3A, lane 2). Induction of p21 and Mdm2 was p53-dependent, as neither protein was induced by E1A in *p53*-deficient cells (Fig. 3A, lane 6). Remarkably, expression of equivalent levels of E1A did not induce p53 in *ARF*-deficient cells, nor affect its targets p21 and Mdm2 (Fig. 3A, lane 4). Of note, wild-type and *ARF*$^{-/-}$ MEFs infected with a control vector displayed similar p53 levels, indicating that p19^{ARF} loss does not markedly affect basal p53 expression (compare lanes 1 and 3). Therefore, *ARF* facilitates the up-regulation of p53 protein and its associated transcriptional activity following expression of E1A.

When activated by DNA damage, Mdm2 is induced as part of a negative feedback loop that facilitates p53 degradation. However, wild-type MEFs expressing *E1A* accumulate p53 despite a large increase in Mdm2 levels

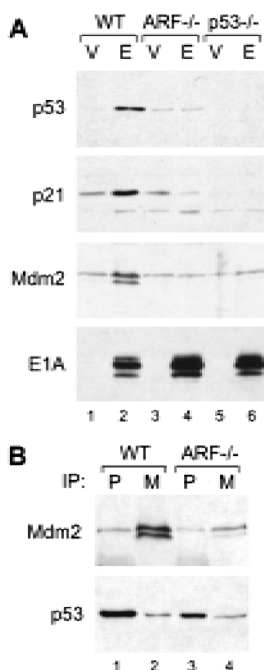

Figure 3. p19^{ARF} mediates p53 induction by E1A and interferes with the p53/Mdm2 interaction. Wild-type (WT), *ARF*-null (*ARF*$^{-/-}$) and *p53*-null (*p53*$^{-/-}$) cell populations harboring a control vector (V) or expressing *E1A* (E) were prepared by retroviral gene transfer. Protein expression was analyzed in whole cell populations passaged minimally in culture (<1 week). (*A*) p53 protein levels along with the levels of its transcriptional targets p21 and Mdm2 were determined by immunoblotting. (*B*) Mdm2/p53 complexes were examined in wild-type and *ARF*-null populations expressing *E1A* by immunoprecipitation with monoclonal antibodies directed against p53 (P) or Mdm2 (M), followed by immunoblotting with a polyclonal rabbit antibody against p53. The blots were then reprobed using the same monoclonal antibody against Mdm2. Note that the p53 blot was overexposed to allow visualization of the amount associated with Mdm2.

(see Fig. 3A, lane 2). We examined the ability of Mdm2 to associate with p53 in MEFs expressing *E1A* by use of sequential immunoprecipitation and Western blotting. Despite the fact that wild-type MEFs expressing *E1A* displayed an ~10-fold increase in p53 and Mdm2 levels as compared to their *ARF*-deficient counterparts, the absolute amount of Mdm2 bound to p53 was comparable in both cell types (Fig. 3B, cf. p53, lanes 2 and 4). Thus, p53 associates poorly with Mdm2 in wild-type cells expressing *E1A*. This implies that p19^{ARF}, either directly or indirectly, contributes to p53 accumulation by preventing Mdm2-mediated degradation of p53 (Pomerantz et al. 1998; Zhang et al. 1998).

Inactivation of ARF attenuates apoptosis

E1A sensitizes primary fibroblasts to apoptosis induced by diverse stimuli, including serum depletion and treatment with chemotherapeutic drugs. The fact that *ARF*-deficient cells are unable to induce p53 in response to E1A suggests that *ARF*$^{-/-}$ MEFs expressing *E1A* might be resistant to apoptosis. Consistent with this possibility, the ability of *Rb* deficiency to trigger apoptosis was attenuated in developing mouse lenses disrupted for both *ARF* and *INK4a* (Pomerantz et al. 1998). Therefore, we compared the sensitivity of various virus-infected populations to cell death following serum withdrawal and treatment with adriamycin, a chemotherapeutic drug that produces double-stranded DNA breaks (Ross and Bradley 1981) and induces p53-dependent apoptosis in this setting. Two criteria were used to monitor apoptosis: annexin V staining followed by flow cytometry to assay membrane changes, and DAPI staining followed by fluorescence microscopy to visualize the characteristic chromatin condensation in apoptotic cells.

Concordant with previous results, wild-type MEFs expressing *E1A* lost viability following serum depletion or adriamycin treatment, whereas *p53*$^{-/-}$ MEFs expressing *E1A* did not (Fig. 4A,B). *ARF*$^{-/-}$ MEFs were significantly more resistant to E1A-induced apoptotic signals as compared to their wild-type counterparts but were somewhat more sensitive than cells lacking p53. In all cases, cell death was due to apoptosis, as measured by annexin V binding as well as chromatin condensation (Fig. 4C). Uninfected MEFs of all genotypes remained viable following serum depletion or adriamycin treatment at these doses, indicating that E1A was required for apoptosis under these conditions (data not shown). Therefore, p19^{ARF} contributes to p53's apoptotic potential in cells expressing *E1A*. However, the fact that *p53* loss is more protective than *ARF* loss implies that some apoptotic signals address p53 through a p19^{ARF}-independent pathway. For example, adriamycin might also exert some of its effects through the DNA damage pathway (see below).

If *ARF* loss protects cells from apoptosis in a p53-dependent manner, a clear prediction is that reintroduction of *ARF* into *E1A*-expressing cells containing wild-type p53 should resensitize them to the effects of serum deprivation and adriamycin. Conversely, cells lacking *p53* should be unaffected by *ARF*. Hemagluttinin (HA)-

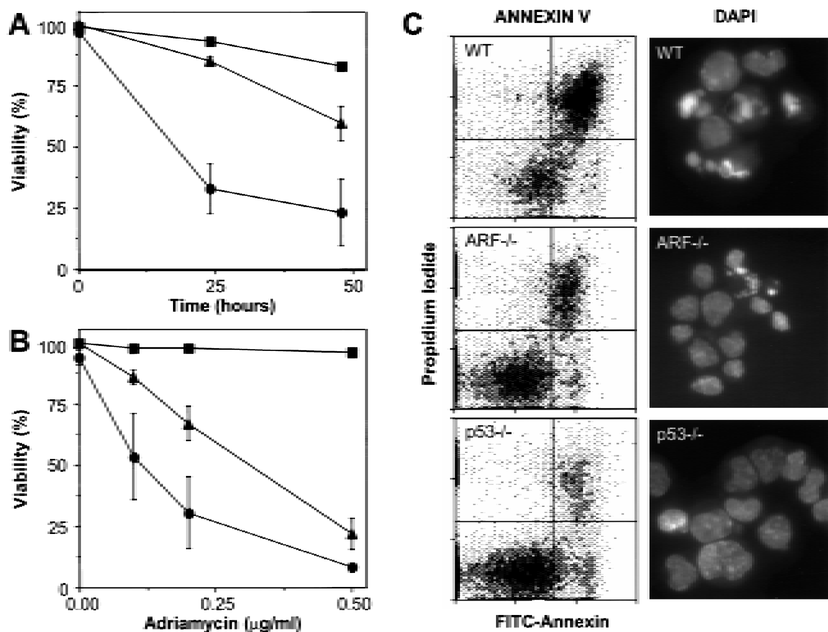

Figure 4. *E1A*-expressing cells lacking *ARF* are defective in apoptosis. Wild-type (●), *ARF*-null (▲), and *p53*-null (■) early passage MEFs were infected with control retroviruses (not shown) or retroviruses expressing *E1A*. Within a week of gene transfer, the resulting cell populations were examined for cell death at various times following serum depletion (*A*) or 24 hr after treatment with the indicated doses of adriamycin (*B*). Cell viability was assessed by trypan blue exclusion. Each point represents the mean±S.D. from at least three separate experiments. Fibroblasts of all genotypes infected with a control vector retained viability (>90%) following serum depletion or adriamycin treatment (data not shown). (*C*) Wild-type (WT), *ARF*-null ($ARF^{-/-}$) and *p53*-null ($p53^{-/-}$) MEFs expressing *E1A* were examined for apoptosis 18 hr after transfer to 0.1% serum conditions. Annexin V binds phosphotidylserine. Apoptotic changes in membrane biochemistry lead to increased concentration of phosphotidylserine on the outer plasma membrane, where it becomes accessible to annexin V (Andree et al. 1990). Propidium iodide fluorescently stains late apoptotic cells that have lost membrane integrity. Shown are representative dot plots from two-color flow cytometry: (*Bottom left* quadrant) Viable; (*bottom right* quadrant) early apoptotic; (*top right* quadrant) late apoptotic. DAPI staining allows visualization of the chromatin condensation characteristic of apoptotic cells. Note that there was little apoptosis in *E1A*-expressing populations in 10% serum nor in vector-only control populations in 0.1% serum (data not shown).

tagged *ARF* was introduced by retroviral gene transfer into wild-type, $ARF^{-/-}$, and $p53^{-/-}$ MEFs expressing *E1A*. Cells were infected at high multiplicity to bypass a need for drug selection. Exogenous $p19^{ARF}$ expression caused a 5- to 10-fold increase in p53 expression in both wild-type and $ARF^{-/-}$ MEFs expressing *E1A* (Fig. 5A), consistent with previous results (Kamijo et al. 1997, 1998). *E1A*-expressing wild-type MEFs infected with a control vector did not undergo apoptosis in high serum conditions but upon transfer to low serum conditions, underwent similar levels of apoptosis as uninfected E1A-expressing MEFs (Fig. 5B). As shown above (see Fig. 4), vector-infected cells lacking *ARF* or *p53* were resistant to apoptosis when transferred to serum-depleted medium (Fig. 5B). Following infection with *ARF* retrovirus, both wild-type and $ARF^{-/-}$ MEFs expressing *E1A* displayed a modest increase in apoptosis when maintained in serum and underwent massive apoptosis upon serum depletion. Importantly, the same levels of exogenous $p19^{ARF}$ had little effect on $p53^{-/-}$ MEFs (Fig. 5B). Hence, depending upon the growth conditions, $p19^{ARF}$ can act upstream of p53 to induce either cell cycle arrest (Kamijo et al. 1997) or apoptosis. The fact that restoration of *ARF* function can resensitize $ARF^{-/-}$ MEFs to the combined effects of E1A and low serum provides compelling evidence that attenuation of apoptosis in $ARF^{-/-}$ cells is a direct consequence of *ARF* loss and not due to additional genetic changes.

Synergy between $p19^{ARF}$-dependent and -independent pathways targeting p53

Because DNA damage and E1A can activate p53 through

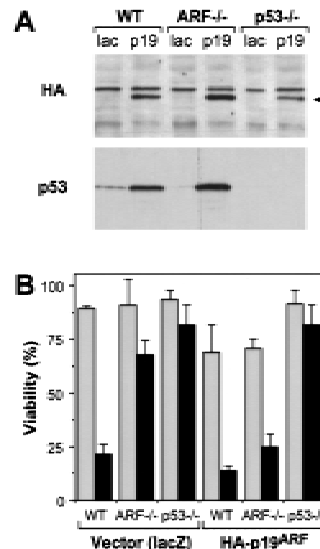

Figure 5. Reintroduction of $p19^{ARF}$ restores apoptosis. Control and *E1A*-expressing populations derived from wild-type (WT), *ARF*-null ($ARF^{-/-}$) and *p53*-null ($p53^{-/-}$) populations were infected with retroviruses expressing *lacZ* or an HA-tagged *ARF* cDNA (Quelle et al. 1995). Thirty-six hours later, the resulting cell populations were analyzed for p53 and exogenous $p19^{ARF}$ protein expression or treated with apoptotic stimuli. (*A*) Immunoblotting of infected populations using a monoclonal antibody recognizing the HA epitope fused to $p19^{ARF}$ or a polyclonal antibody directed against p53. The arrow denotes the migration of HA-tagged $p19^{ARF}$. (*B*) The indicated cell populations were placed in 10% (shaded bars) or 0.1% (solid bars) serum for 24 hr and cell viability was measured by trypan blue exclusion. The values represent the mean and S.D. of at least three separate infections.

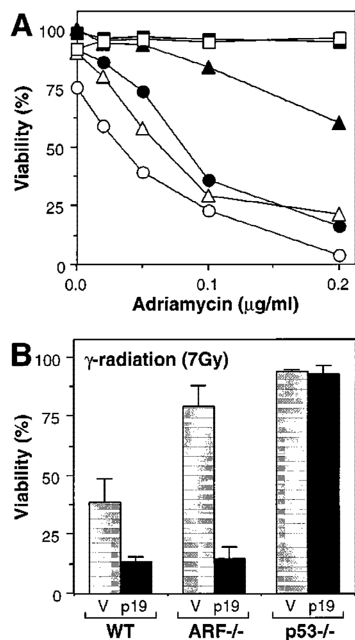

Figure 6. Synergy between $p19^{ARF}$-dependent and -independent pathways targeting p53. (*A*) *lacZ* (solid symbols)- and *HA–ARF* (open symbols)-expressing cell populations were treated with the indicated doses of adriamycin, and cell viability was determined 24 hr later by trypan blue exclusion. The cell populations were as follows: Wild-type MEFs lacking *E1A* (squares); wild-type MEFs expressing E1A (circles); $ARF^{-/-}$ MEFs expressing *E1A* (triangles). Note that $ARF^{-/-}$ and $p53^{-/-}$ MEFs lacking *E1A,* as well as *p53*-deficient MEFs expressing *E1A,* remained viable in adriamycin whether or not they expressed HA–$p19^{ARF}$ (data not shown). (*B*) *lacZ* (V, shaded bars) and HA–$p19^{ARF}$ (p19, solid bars) expressing cell populations were treated with 7 Gy ionizing radiation and cell viability was determined 24 hr later by trypan blue exclusion. The values represent the mean and S.D. of at least three separate populations. MEFs not expressing E1A were resistant to apoptosis under these conditions (data not shown; see also Lowe et al. 1993).

distinct mechanisms, they might act synergistically to enhance cellular chemo- or radiosensitivity. Consistent with this possibility, enforced expression of $p19^{ARF}$ caused a marked increase in apoptosis induced by adriamycin when expressed in either wild-type or $ARF^{-/-}$ MEFs expressing *E1A* (Fig. 6A). Similar results were obtained following treatment of the cells with ionizing radiation (Fig. 6B). Importantly, the enhanced chemosensitivity produced by enforced $p19^{ARF}$ expression required both *E1A* and a cytotoxic insult. Hence, wild-type MEFs lacking E1A did not undergo apoptosis following adriamycin treatment and remained insensitive to low doses of the drug upon enforced expression of $p19^{ARF}$ (Fig. 6A, squares). $ARF^{-/-}$ cells expressing *E1A* were relatively resistant to drug-induced apoptosis (see also Fig. 4) but were resensitized when *ARF* was reintroduced (Fig. 6A, triangles). Importantly, introduction of *ARF* into wild-type cells expressing *E1A* also enhanced apoptosis in response to low doses of adriamycin (Fig. 6A, circles) or ionizing radiation (Fig. 6B), demonstrating that activation of the ARF–p53 pathway promotes both chemo- and radiosensitivity in the face of an oncogenic signal.

Discussion

Oncogenic signaling through the ARF–p53 pathway

A variety of cellular stresses activate p53, including DNA damage, hypoxia, and expression of mitogenic oncogenes (for review, see Ko and Prives 1996; Levine 1997). Following DNA damage, p53 becomes phosphorylated by kinases such as DNA–PK or ATM, leading to changes in p53 conformation and activity. In contrast, the E1A oncogene activates p53 through a fundamentally different mechanism, mediated largely by the tumor suppressor $p19^{ARF}$. Importantly, the DNA damage and E1A signaling pathways act in parallel: E1A does not produce p53 phosphorylation at serine-15 and DNA damage activates p53 independently of $p19^{ARF}$ (Kamijo et al. 1997). Moreover, p53 is phosphorylated on serine-15 following irradiation of ARF-deficient cells (data not shown). Therefore, these data provide a clear example of how p53 integrates upstream signaling pathways emanating from diverse stimuli (Fig. 7).

Activation of p53, in turn, can produce several cellular responses, including transient cell-cycle arrest, senescence or apoptosis. Each signaling pathway to p53 may produce subtle differences in p53 activity or function, and perhaps the diversity achieved by a combination of

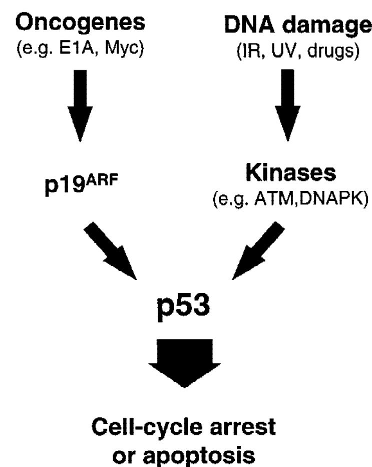

Figure 7. Oncogenes and DNA damage activate p53 through distinct mechanisms. $p19^{ARF}$ acts as an intermediary in p53 activation by mitogenic oncogenes such as *E1A* and *myc.* In contrast, activation of p53 following DNA damage involves de novo phosphorylation of p53 on serine-15 (and other residues) by kinases such as the DNA-dependent protein kinase (DNA–PK) or the product of the ataxia-telangiectasia gene (ATM) (Shieh et al. 1997; Siliciano et al. 1997). Activation of p53 by oncogenes does not involve phosphorylation on serine-15, and both serine-15 phosphorylation (not shown) and p53 activation (Kamijo et al. 1997) following DNA damage are unimpaired in the absence of *ARF.* Therefore, the two upstream signaling pathways to p53 are fundamentally distinct.

these signals accounts for the complex biology of p53. For example, simultaneous activation of p53 by p19^{ARF} and DNA damage synergize to promote apoptosis in the presence of the E1A oncogene (Fig. 6; see also Lowe et al. 1993; Samuelson and Lowe 1997). If similar processes occur in human cancer, therapeutic strategies to exploit p19^{ARF} activation may enhance the radiosensitivity or chemosensitivity of p53-expressing tumors.

Like p53, the outcome of p19^{ARF} activation is dependent on cellular context. For example, enforced *ARF* expression in MEFs induces cell cycle arrest, but cells overexpressing p19^{ARF}, together with E1A or Myc (Zindy et al. 1998), undergo apoptosis, which is potentiated by withdrawal of serum survival factors (Evan et al. 1992; Lowe and Ruley 1993; Lowe et al. 1994b). *ARF*-null MEFs are resistant to both E1A- and Myc-induced apoptosis, bypassing the p53-dependent fail-safe mechanism that normally protects them from these oncogenic signals, and thereby enabling E1A and Myc to function as pure growth promoters. Myc's action as an ''immortalizing gene'' depends in part on its ability to dismantle the ARF–p53 pathway by selecting for surviving cells that have lost either gene (Zindy et al. 1998). In turn, *ARF*-null MEFs do not undergo replicative senescence and can be transformed by oncogenic *ras* alone (Kamijo et al. 1997). We suspect that E1A's immortalizing activity involves similar mechanisms.

Also like *p53, ARF* has no overt role in normal cell cycle control or development; hence, the physiologic circumstances in which it would become activated to inhibit proliferation or suppress tumor growth were not obvious. Studies here with E1A mutants suggest that p19^{ARF} can be activated to suppress proliferation by the *E1A* oncogene through mechanisms that correlate with its binding to both p300/CBP and Rb. These same functions are required for E1A to induce p53 and to promote apoptosis in primary fibroblasts (Samuelson and Lowe 1997) and, remarkably, are also required for E1A's transforming potential (Whyte et al. 1988b, 1989). Loss of *Rb* contributes to ARF induction consistent with the possibility that *ARF* is an E2F-responsive gene (DeGregori et al. 1997). Enforced expression of *E2F-1* induces p19^{ARF}, and conversely, *ARF*-null cells are resistant to E2F-1-induced apoptosis (Zindy et al. 1998). Consequently, p19^{ARF} function, like p53, depends upon the mutational status of *Rb,* and upon both c-*myc* and *ras* proto-oncogene activities. Irrespective of the precise outcome, *ARF* mutations compromise p53 activation and reduce its ability to counter uncontrolled proliferation.

The data presented here provide additional insights into p53's role in tumor suppression. The predominant view of p53 action centers around its ability to function in the cellular response to DNA damage. Although this stimulus is undoubtedly important for p53's tumor suppressor activity and may contribute to the outcome of cancer therapy (Lowe et al. 1993, 1994a), p53 activation in response to oncogenes provides an alternative pressure to mutate *p53* during tumorigenesis (Lowe and Ruley 1993; Lowe et al. 1994b; Symonds et al. 1994). In this view, p53 normally acts to limit the consequences of uncontrolled mitogenesis by promoting cell-cycle arrest or apoptosis, while its loss allows proliferation to continue unabated. The fact that disruption of the ARF-p53 pathway occurs in the majority of human cancers underscores its global importance in suppressing proliferation of oncogene-expressing cells.

Materials and methods

Cells and cell culture

IMR90 fibroblasts (early–mid passages) expressed the ecotropic retrovirus receptor to allow infection with murine retroviruses (Serrano et al. 1997). Primary MEFs derived from wild-type, *p53*$^{-/-}$ (Jacks et al. 1994), and *ARF*$^{-/-}$ (Kamijo et al. 1997) day 13.5 embryos were prepared as described previously (Serrano et al. 1997). All cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; Sigma) and 1% penicillin G/streptomycin sulfate (Sigma). To induce DNA damage, cells were either irradiated with 7 Gy ionizing radiation using a J.L. Shepherd Mark I irradiator with a ^{137}Cs source or treated with 0.1–0.5 µg/ml adriamycin. To induce p53 independently of DNA damage, cells were treated for 2 hr with 50 µM LLnL (Sigma).

Retroviral vectors and infection

For most experiments, high-titer ecotropic retroviruses were generated by transient transfection using the Phoenix retrovirus packaging system (G. Nolan, Stanford University, CA) as described previously (Serrano et al. 1997). Virus supernatants were used to infect either IMR90 fibroblasts or early-passage MEFs (≤passage 5), and pure populations of *E1A*-expressing cells were isolated by selection for 2 days in the presence of 2 µg/ml puromycin. Infection was typically between 70% and 90% of cells as judged using a control virus expressing β-galactosidase (not shown). For ectopic expression of p19^{ARF}, a protocol designed to achieve nearly complete infection of cells (Zindy et al. 1998) was used. Retroviral vectors were as follows: LPC, control vector expressing puromycin phosphotransferase (*puro*); LPC-12S, a *12S E1A* cDNA in LPC (McCurrach et al. 1997); LPC-12S.ΔN and LPC-12S.ΔCR2, E1A mutants that fail to associate with p300/CBP or the Rb-related proteins, respectively (Samuelson and Lowe 1997). The retroviral vector encoding HA–p19^{ARF} co-expressed a CD8 cell surface marker (Quelle et al. 1995). pBabePuro–lacZ (a gift of J. Morgenstern, Millenium Pharmaceutical, Cambridge, MA) was used to monitor infection efficiencies and, in some experiments, as a control vector.

Gene expression

Analysis of p53 phosphorylation on serine-15 was performed exactly as described (Shieh et al. 1997). p53 levels were determined by Western blots using PAb1801 and DO1. p53 immunoprecipitations were performed using pAb 1801 followed by immunoblotting with αp53-P–Ser-15 to identify p53 proteins phosphorylated on serine-15. Western blots to detect p19^{ARF} were performed using antibodies to the carboxyl terminus as described (Kamijo et al. 1998); HA-tagged p19^{ARF} was detected using mAb 12CA5 (1:5000 dilution). All other Western blots were carried out as described previously with minor modifications (Serrano et al. 1997). Whole-cell lysates were derived by lysing cell pellets in SDS sample buffer (60 mM Tris-HCl at pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol). Samples corresponding to 30 µg of protein (Bio-Rad protein assay) were

separated on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). p53 was detected using polyclonal antibody CM5 (1:8000 dilution) (a gift of Peter Hall, Dundee University, UK); Mdm2 using mAb 2A10 (provided by G. Zambetti, St. Jude Children's Research Hospital); p21 using polyclonal antibody C-19 (1: 500 dilution) (Santa Cruz), and E1A using mAb M58 (Harlow et al. 1985). Proteins were visualized by ECL (Amersham) and equal sample loading was confirmed by India Ink or Ponseau S staining of the membrane.

For p53/Mdm2 immunoprecipitations, cell pellets were disrupted in ice-cold NP-40 lysis buffer (50 mM Tris-HCl at pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 0.4 U/ml aprotinin, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na_3VO_4) on ice for 1 hr. Cleared lysates were incubated for 2 hr at 4°C with two monoclonal antibodies directed against p53 (pAb 421 and pAb 248) or Mdm2 (2A10), plus 10 mg/ml BSA. Complexes precipitated with protein A–Sepharose (Amersham) were washed three times with ice-cold NP-40 lysis buffer. Immunoprecipitates were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose. Mdm2 was detected by immunoblotting using the same antibody, whereas p53 was detected with CM5 polyclonal antibody as described above.

For Northern blots, total RNA was extracted from cells using RNAzolB (Cinna/Biotecx) ~1 week postinfection and 30 µg was loaded per lane. Following agarose gel electrophoresis and transfer to Hybond membranes (Amersham), blots were hybridized with a ^{32}P-labeled probe specific for *INK4a* exon 1β [the portion of the *INK4a/ARF* locus unique to *ARF* (Quelle et al. 1995)]. A probe specific for 18S rRNA was used to confirm equal loading.

Cell viability and apoptosis

Cells were distributed into 12-well plates (10^5 cells/22-mm well) 12–24 hr prior to serum withdrawal, radiation, or drug treatment. Adherent and nonadherent cells were pooled 24 hr after treatment with γ-radiation, adriamycin, or 0.1% FBS and analyzed for viability by trypan blue exclusion; ⩾200 cells were scored for each point. Apoptotic cell death was confirmed by staining with DAPI or FITC–annexin V. Cells (~1×10^5) were fixed in 5% paraformaldehyde (Mallinckrodt) and DNA was stained with DAPI (1 µg/ml). Images were digitized using a fluorescence microscope coupled to a Photometrics PXL CCD camera (Photometrics Ltd.). For annexin staining, cells were incubated in DMEM with 0.1% FBS for 18 hr, after which adherent and nonadherent cells were pooled. Staining with FITC–annexin V and PI were performed according to the manufacturer's instructions (BioWhitaker) and the cells were analyzed by two-color flow cytometry.

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