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Award Number: DAMD17-98-1-8049

TITLE: Selective Chemosensitization of Rb Mutant Cells

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CONTRACTING ORGANIZATION: Cold Spring Harbor Laboratory
Cold Spring Harbor, New York 11724

REPORT DATE: July 2001

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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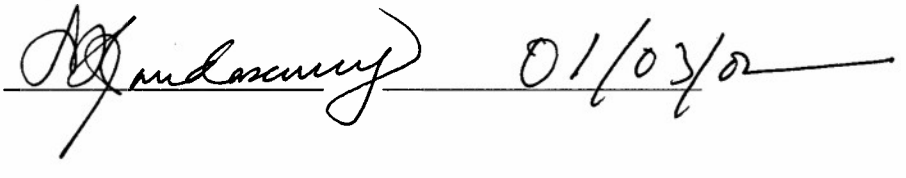

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual Summary (1 Jun 98 - 1 Jun 01)

4. TITLE AND SUBTITLE
Selective Chemosensitization of Rb Mutant Cells

5. FUNDING NUMBERS
DAMD17-98-1-8049

6. AUTHOR(S)
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Cold Spring Harbor Laboratory
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The primary objective of this project ultimately is to develop strategies that specifically kill breast cancer cells. The adenoviral oncoprotein E1A serves as a tool to understand how both normal and tumor cells become chemosensitive. E1A confers chemosensitivity and p53 potentiates E1A function. Signaling to p53 involves the $p19^{ARF}$ tumor suppressor gene. Furthermore, at least two functions within the N-terminal region of E1A are required to confer chemosensitivity and stabilize p53 in normal cells. One of these functions is to inactivate the retinoblastoma gene product and E1A mutants unable to inactivate Rb but retaining the second N-terminal function selectively promote chemosensitivity in cells lacking an intact Rb pathway. The second N-terminal function correlates with E1A's ability to bind the p400/TRRAP complex, occurring independently of binding p300/CBP. This function is a "myc-like" function as c-myc also binds the p400/TRRAP complex, and c-myc can specifically synergize to fully restore chemosensitivity with an E1A mutant unable promote apoptosis or bind p400. Ongoing studies to further elucidate the mechanism of how E1A enhances chemosensitivity may provide a pathway to target that improves the likelihood of successfully treating breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
(1) apoptosis; (2) chemotherapy; (3) Rb mutation; (4) F1A; (5) chemosensitivity	37
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Reportable Outcomes

Manuscripts:

de Stanchina E. et. al; *E1A signaling to p53 involves the p19(ARF) tumor suppressor.* Genes Dev 1998 Aug 1;12(15):2434-42

Samuelson and Lowe; *Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins.* Proc Natl Acad Sci U S A 1997 Oct 28;94(22):12094-9

Presentations, Posters, and Abstracts:

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (poster). Cold Spring Harbor Laboratory Meeting on Cancer Genetics and Tumor Suppressor Genes (1):183, Cold Spring Harbor NY, August 16-20, 2000.

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (oral presentation and poster). Era of Hope Department of Defense Breast Cancer Research Program Meeting (1): 301, Atlanta GA, June 8-11, 2000.

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (poster). Cold Spring Harbor Laboratory Meeting on The Cell Cycle (1):163, Cold Spring Harbor NY, May 17-21, 2000.

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (poster). Keystone Symposium on Cancer, Cell Cycle and Therapeutics (1): 69(#230), Steamboat Springs CO, January 8-13, 2000.

de Stanchina E., McCurrach M.E., Zindy F., Shieh S.Y., Ferbeyre G., Samuelson A.V., Prives C., Roussel M.F., Sherr C.J., and Lowe S.W.; *E1A signaling to p53 involves the p19(ARF) tumor suppressor.* Cold Spring Harbor Laboratory Meeting on Cancer Genetics and Tumor Suppressor Genes (1):111, Cold Spring Harbor NY, August 19-23, 1998.

Samuelson and Lowe; *Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the retinoblastoma-related proteins* (oral presentation and poster). Cold Spring Harbor Laboratory Meeting on Programmed Cell Death (1):259, Cold Spring Harbor NY, September 17-21, 1997.

Conclusions

Our early data and other studies [7] [8] [9] provided genetic evidence that E1A's interaction with the p300/CBP proteins were critical for chemosensitivity. However, subsequent results demonstrate it is highly *unlikely* that E1A's interaction with p300/CBP is the relevant cellular target that promotes chemosensitivity. In fact, E1A's interaction with p400, presumably to disrupt the normal function of the p400/TRRAP complex, is required for stabilizing p53 and enhancing chemosensitivity. Furthermore, this function of E1A is a "myc-like" function as ectopic c-myc can restore chemosensitivity to an E1A mutant unable to bind p400. Future studies will be aimed at further deciphering the nature of this function of E1A.

We show that amino acids eleven to 143 are sufficient to confer chemosensitivity in normal cells. Furthermore, we have shown that two separable functions of E1A are necessary to enhance chemosensitivity. Future experiments will determine whether these two functions are sufficient or whether additional functions within E1A are required to enhance chemosensitivity. Additional experiments will define the minimal domain of E1A required to selectively kill Rb-deficient cells. Based on the outcomes of these experiments we envision developing small peptide sequences that mimic the N-terminal functions of E1A. This may determine whether small molecules can be envisioned to work in conjunction with conventional chemotheraputic treatments to improve the successful treatment of breast cancer.

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July 14, 2001

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Sincerely,

Andrew V. Samuelson

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Introduction

The primary objective of this project is to develop strategies to specifically kill breast cancer cells with mutations either specifically in the retinoblastoma tumor suppressor gene product Rb, or more generally within the Rb-pathway. This strategy is being developed based on our previous results that examined chemosensitivity in a highly defined system using the adenoviral oncoprotein E1A. E1A sensitizes primary cells to the induction of apoptosis by diverse stimuli, including many agents used in cancer therapy.

We examined how E1A promotes chemosensitivity by expressing E1A or a series of E1A mutants in primary human and mouse fibroblasts using high-titer recombinant retroviral vectors. Hence, E1A was studied in genetically-normal cells outside the context of adenovirus infection. Using this approach, we genetically defined two distinct E1A activities that act in concert to promote chemosensitivity, and prove that *one of these functions is to inactivate the retinoblastoma gene product.* Since breast cancer cells frequently have either Rb mutations, or more generally within the Rb-pathway, an E1A mutant unable to inactivate Rb may selectively enhance chemosensitivity in breast cancer cells while remaining defective in normal cells. Last year I reported that the ability of E1A to bind the p300/CBP transcriptional co-activators and block p300 histone acetyl-transferase (HAT) activity is not required for E1A to confer chemosensitivity or stabilize p53. Elucidating the cellular proteins targeted by E1A to promote apoptosis may reveal cellular pathways that are good targets for small molecule drugs to enhance the efficacy of chemotheraputic drugs in treating breast cancer.

Body of Annual Report

The objectives outlined in my original research proposal consisted of four specific aims designed primarily to elucidate the mechanism of E1A-mediated chemosensitivity. Two of these aims were designed to determine whether E1A mutants could selectively mediate chemosensitivity in various knockout primary cells and in breast tumor cell lines. The final aim was to use this knowledge to selectively enhance chemosensitivity in tumor cells. The focus of my research has since evolved to identify the mechanism by which E1A promotes apoptosis by continuing an extensive structure/function mutational analysis. By discovering the relevant cellular protein targets and the pathways these targets modulate it may be conceivable to develop small molecule drugs that selectively enhance chemosensitivity in breast cancer.

The regions within E1A required for chemosensitivity mapped between amino acids 15 to 60 and 122 to 140. Furthermore, at least two separable functions within these N-terminal regions are required for E1A to mediate chemosensitivity. One function is to inactivate of the Rb tumor suppressor gene product ([1], *see Appendices*). Contrary to what was previously thought, the ability of E1A to bind p300/CBP or to block p300 HAT activity is *not* required for E1A to confer chemosensitivity. Thus, the cellular target(s) of the N-terminal region of E1A remained to be determined. Two possible candidates are p400 and the transformation/transcription domain associated protein (TRRAP). p400 was originally characterized by immunoprecipitation as a doublet [2]. The lower molecular weight band has since been shown to be TRRAP (David Livingston, personal communication and unpublished results). TRRAP has been shown to be an essential cofactor for c-myc and E2F-1 transformation. Furthermore, anti-sense TRRAP mRNA can block transformation by E1A and ras [3]. Additionally, p400 and TRRAP form a complex targeted by E1A to promote transformation (Miriam Fuchs, personal communication and unpublished result). I collaborated with Miriam Fuchs in David Livingston's lab to test whether the ability of E1A to target the p400/TRRAP complex correlates with the ability of E1A to confer chemosensitivity.

The regions of E1A that are required to bind p400 were determined in U-2 OS cells and normal diploid fibroblasts (IMR90s). U-2 OS cells were transiently transfected with proretroviral plasmids. IMR90s were retrovirally infected with various E1A constructs and selected to remove uninfected cells. Cells were collected for immunoprecipitation analysis. Lysates were normalized for equal amounts of protein by Bradford analysis. E1A vectors were expressed at similar levels (*Figure 1, bottom*). Immunoprecipitates were examined by immunoblot for p400 (*Figure 1, top*). Of note, the antibody cross-reacts with p300 (*lower bands, Figure 1 top*) and p400 (*upper bands, Figure 1 top*). An antibody that immunoprecipitates p400 serves as a positive control (*Figure 1 top, lanes 2*). In contrast αp300 antibodies immunoprecipitate p300 but not p400 (*lanes 3*). αTAg serves as another control for specificity as U-2 OS and IMR90s do not express TAg (*lanes 4*). αE1A co-immunoprecipitates p400 and p300 in cells expressing E1A (*lanes 5*) while cells expressing empty vector do not (*lanes 1*). E1A mutants fell into four classes: unable to bind p400 and p300 (ΔN^1, *lanes 6*), able to bind

[1] Deletes amino acids 2 to 36, fails to bind p300, binds Rb, and defective in apoptosis, previously described.

p400 but not p300 (*RG2*[2], *lanes 7*), able to bind p300 but not p400 (*Δ26-35*[3], *lanes 8*), and able to bind p400 and p300 (*ΔCR2*[4], *lanes 9*). Significantly the ability of E1A to bind to p400 correlates with the ability of E1A to promote apoptosis as mutants that failed to bind p400 failed to promote apoptosis and mutants that retain binding to p400 promote apoptosis[5]. Furthermore, an extended analysis of E1A mutants for binding to cellular proteins revealed that the N-terminal regions of E1A required to bind p400 were inseparable from those required to promote apoptosis (summarized in *Figures 2 and 3*). Therefore, it is likely that the p400/TRRAP complex is the cellular target of the E1A N-terminal region required to promote chemosensitivity and stabilize p53. How E1A modulates p400/TRRAP activity to promote apoptosis remains to be determined. However, p400 fragments that disrupt the p400/TRRAP complex can rescue the transformation defect of E1A Δ26-35 (Miriam Fuchs, personal communication). Thus, it is likely that E1A disrupts normal function of the p400/TRRAP complex to promote transformation. I am in the process of testing how p400 fragments can effect E1A-mediated chemosensitivity. If the function of E1A that promotes chemosensitivity is to disrupt the normal function of the p400/TRRAP complex, then p400 fragments that disrupt the p400/TRRAP complex should rescue the apoptotic defects of the E1A Δ26-35 mutant. Furthermore p400 fragments that bind E1A but unable to bind TRRAP should block the ability of E1A to both bind this complex and promote chemosensitivity. Experiments to test both of these hypothesis are underway.

E1A promotes apoptosis and p53 potentiates E1A function. E1A-mediated apoptosis requires both Rb inactivation and binding to p400/TRRAP. c-myc also binds TRRAP and promotes p53-dependent apoptosis [3, 4]. Furthermore, both c-myc and E1A signaling to p53 require ARF [5, 6]. However, the ability of c-myc to confer chemosensitivity in normal cells is not as great as E1A (*Figure 4, left*). One possible explanation is that c-myc does not inactivate Rb. Another possibility is that E1A possesses additional functions that enhance chemosensitivity. Nevertheless, the ability of c-myc to induce p53 and promote chemosensitivity is dependent on c-myc levels as "weak" c-myc retroviral vectors (those without a Kozak signal) express c-myc poorly (*Figure 4, right*) and do not induce p53 or chemosensitivity (*Figure 4, left*). In contrast, "strong" c-myc retroviral vectors express c-myc, induce p53 and have some ability to promote chemosensitivity (*Figure 4*). Thus, E1A and c-myc share the common characteristics of targeting p400/TRRAP, promoting apoptosis, and increasing p53 stability.

Since E1A and c-myc both target the p400/TRRAP complex and promote apoptosis, I tested whether c-myc could specifically synergize with an E1A mutant unable to bind p400. IMR90s and primary mouse embryo fibroblasts (MEFs) were assayed for chemosensitivity and p53 levels after being retrovirally transduced with either: empty vector, E1A, E1A Δ26-35, c-myc, or both E1A Δ26-35 and c-myc. Cells co-expressing E1A Δ26-35 and c-myc synergize to induce apoptosis and p53 at similar

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[2] Arginine to glycine at amino acid 2, fails to bind p300, binds Rb, and promotes apoptosis, previously described.

[3] Deletes amino acids 26 to 35, binds p300, binds Rb, defective in apoptosis, previously described.

[4] Deletes amino acids 122 to 140, binds p300, fails to bind Rb, defective in apoptosis in wild type but not Rb-/- cells, previously described.

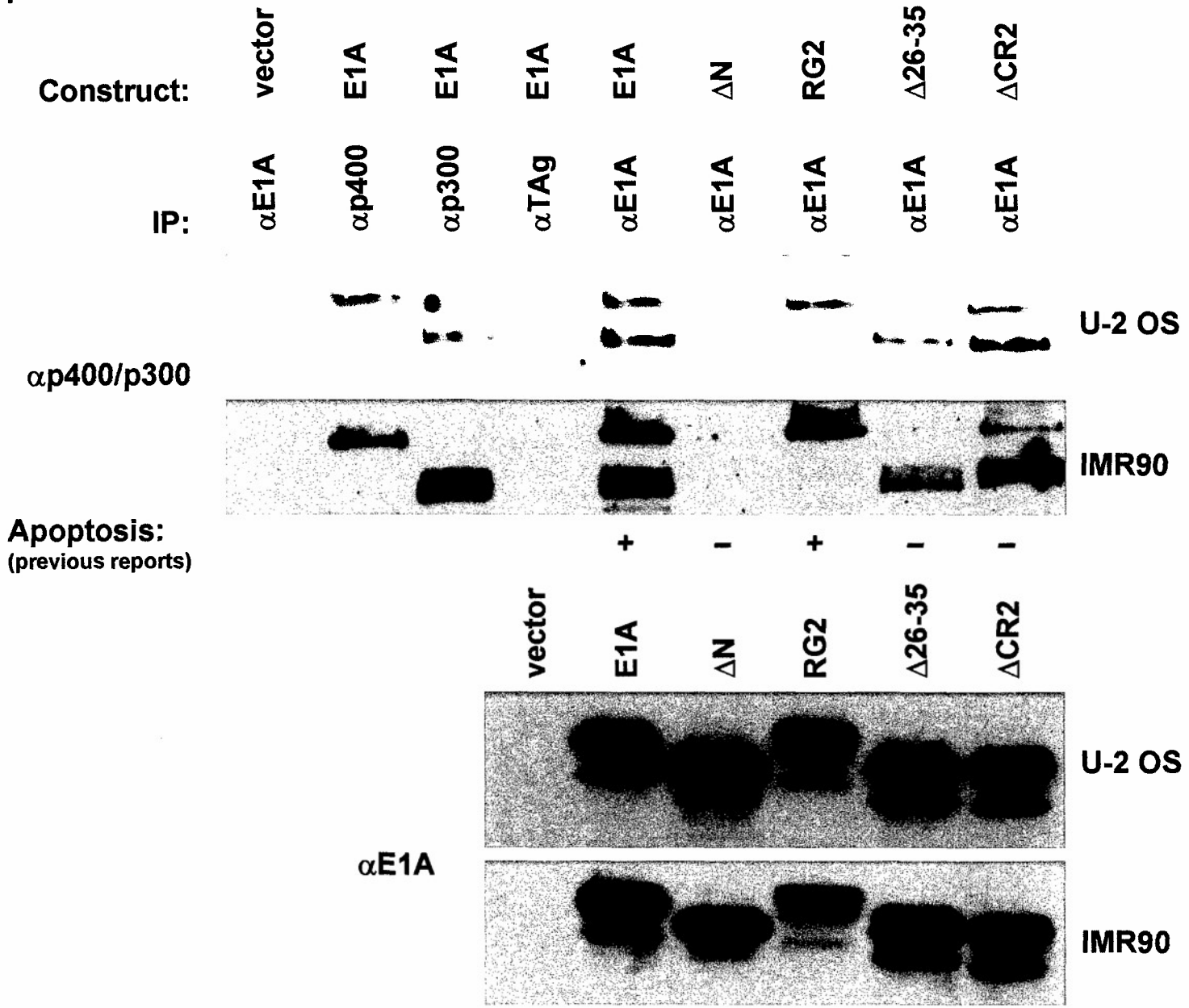
[5] Rb binding is also required for apoptosis

levels to full length E1A (*Figure 5*). In contrast, cells expressing only E1A D26-35 or c-myc remain unable to induce p53 or chemosensitivity. Synergy was specific as cells expressing E1A ΔCR2 (binds p400 but not Rb) and c-myc remain unable to induce p53 and apoptosis (data not shown). This data suggests that E1A and c-myc promote apoptosis through a common mechanism by targeting the p400/TRRAP complex. Thus, the N-terminal function of E1A required for apoptosis can be thought of as a "myc-like" function. Future studies will be aimed at further deciphering the nature of this function of E1A.

Key Accomplishments

- E1A signaling to p53 involves the $p19^{ARF}$ tumor suppressor
- Inactivating Rb is required for E1A-mediated chemosensitivity
- An E1A mutant unable to interact with Rb and full length E1A confers similar levels of chemosensitivity in breast and other cancer cell lines.
- The ability of E1A to confer chemosensitivity does not correlate with the ability of E1A to interact with p300/CBP or to block p300 HAT activity
- The first 143 amino acids of E1A are sufficient to confer chemosensitivity
- A refined mutational analysis of E1A shows that the regions within E1A that are required for chemosensitivity are between amino acids 15 to 60 and 122 to 140.
- E1A-mediated apoptosis and stabilizing p53 correlate with the ability of E1A to bind p400 and map between amino acids 15 to 48 of E1A.
- c-myc can rescue the E1A Δ26-35 defect, thus the N-terminal function of E1A is a "myc-like" function.
- Chemosensitivity correlates with elevated p53 protein levels

Figure 1


Construct:
vector
E1A
E1A
E1A
E1A
ΔN
RG2
Δ26-35
ΔCR2
IP:
αE1A
αp400
αp300
αTAg
αE1A
αE1A
αE1A
αE1A
αE1A
αp400/p300
U-2 OS
IMR90
Apoptosis:
(previous reports)
+
-
+
-
-
vector
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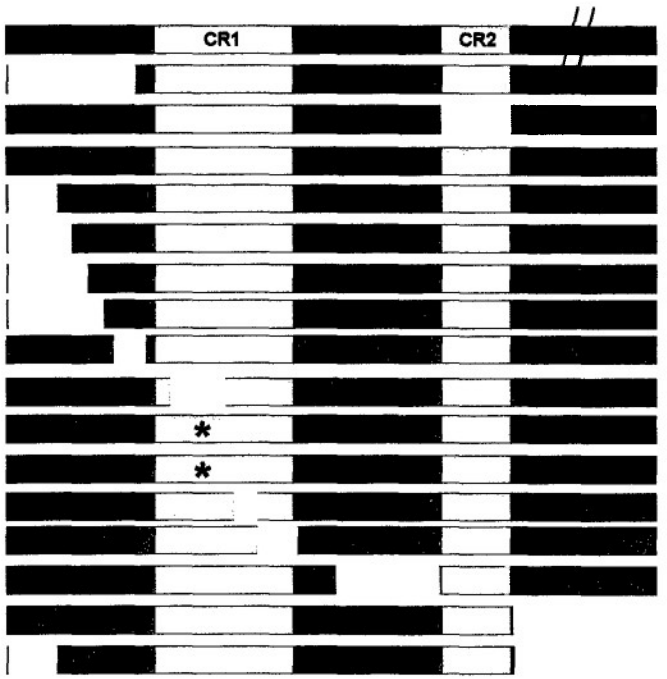
IMR90

Figure 2



				IMR90s		MEFs	
	p300	Rb	p400	% apoptosis	p53	% apoptosis	p53
E1A	+	+	+	100	+	100	+
ΔN	–	+	–	7	–	24	–
ΔCR2	+	–	+	24	–	51	–
RG2	–	+	+	102	+	84	+
Δ2-11	–	+	+	95	+	81	+
Δ2-15	–	(+)	+	88	+	65	nd
Δ2-20	–	(+)	–	43	±	30	nd
Δ2-24	–	(+)	–	13	–	20	nd
Δ26-35	+	+	–	29	–	49	–
Δ48-60	±	–	+	19	–	44	nd
E55	nd	nd	nd	57	nd	75	+
M9	±	+	+	68	±	68	±
Δ64-68	±	+	+	90	+	72	+
Δ70-81	–	+	+	86	+	91	+
Δ85-119	+	(+)	+	120	+	74	nd
143	+	(+)	+	90	+	95	+
Δ2-11.143	(–)	(+)	(+)	106	+	93	nd

Figure 3

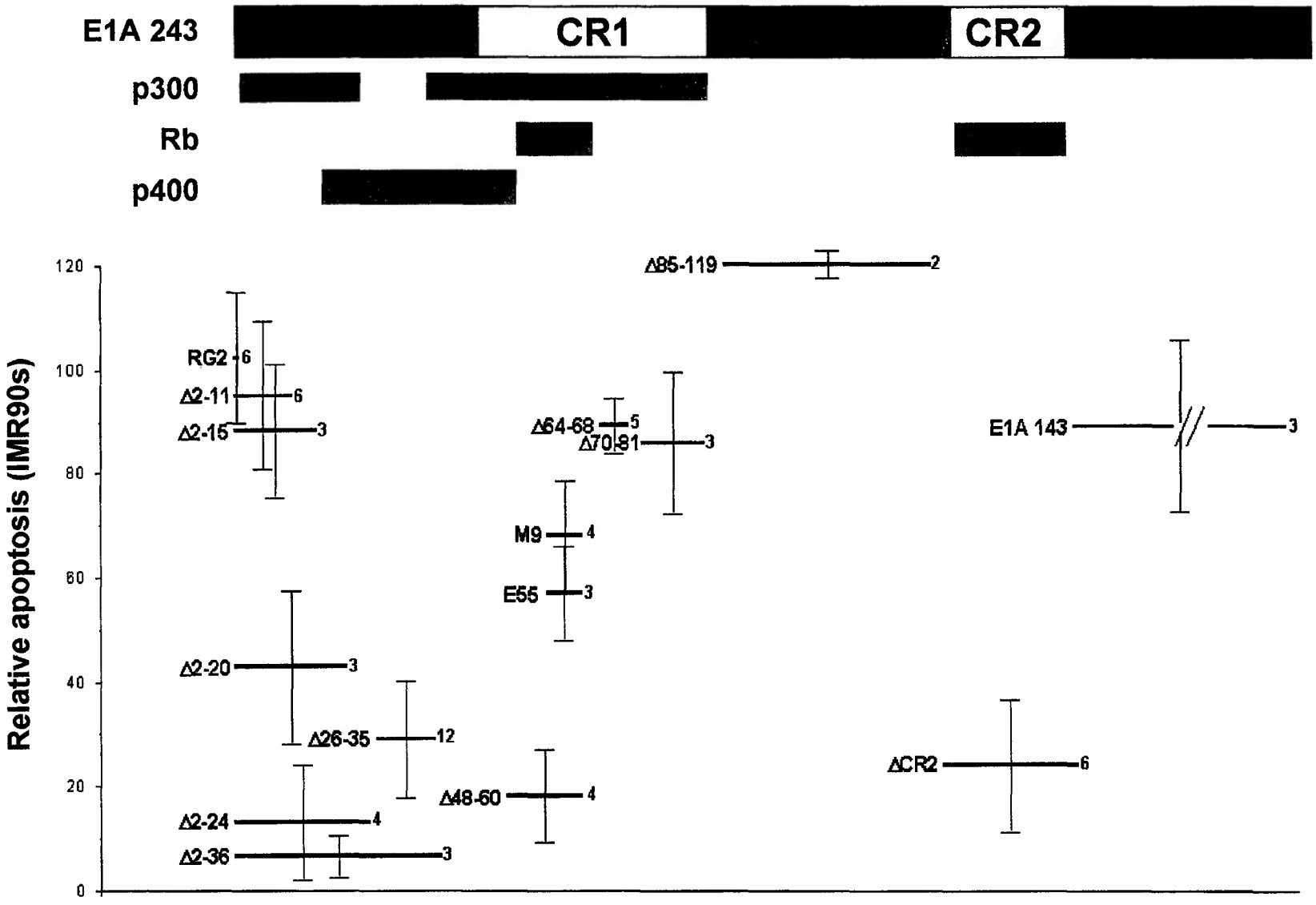
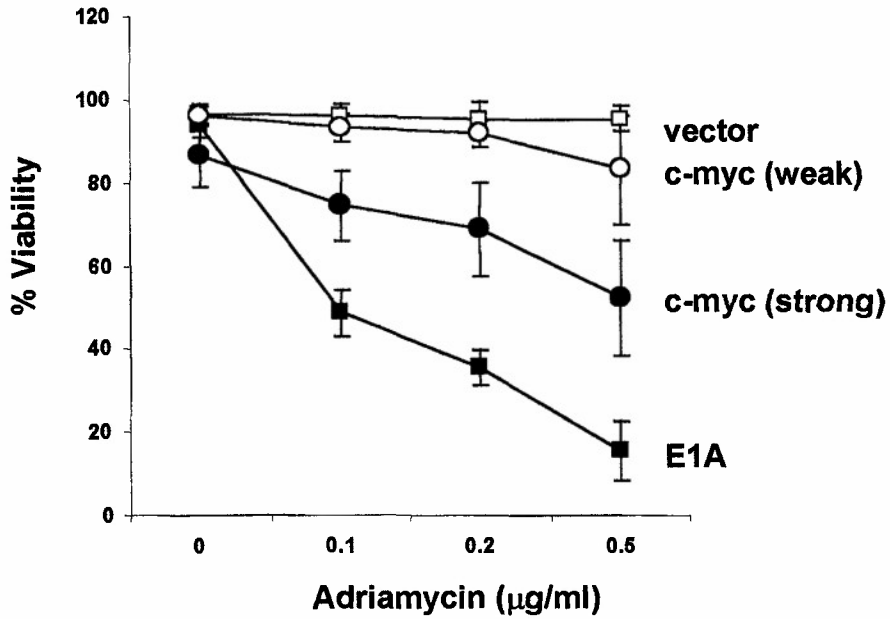

E1A 243
CR1
CR2
p300
Rb
p400
Relative apoptosis (IMR90s)
120
100
80
60
40
20
0
RG2 6
Δ2-11 6
Δ2-15 3
Δ2-20 3
Δ26-35 12
Δ2-24 4
Δ2-36 3
Δ85-119 2
Δ64-68 5
Δ70-81 3
M9 4
E55 3
Δ48-60 4
E1A 143 3
ΔCR2 6

Figure 4


120
100
80
60
40
20
0
% Viability
0
0.1
0.2
0.5
Adriamycin (μg/ml)
vector
c-myc (weak)
c-myc (strong)
E1A

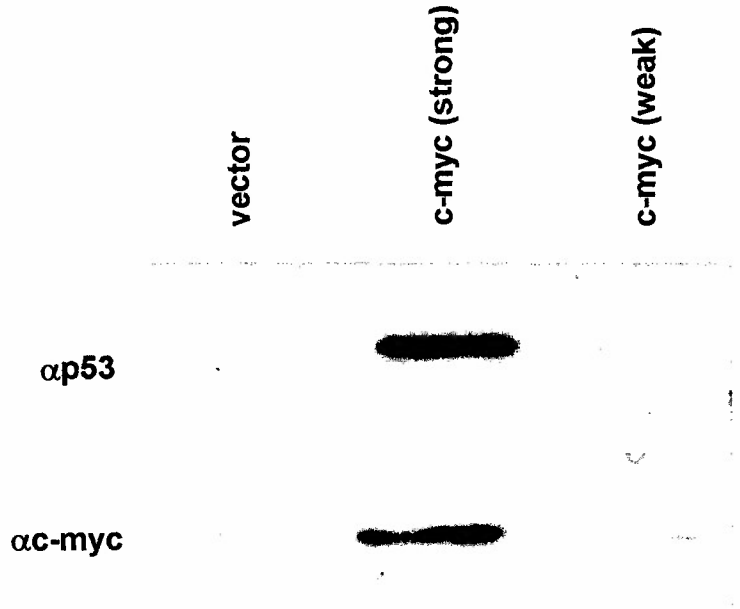

vector
c-myc (strong)
c-myc (weak)
αp53
αc-myc

Figure 5

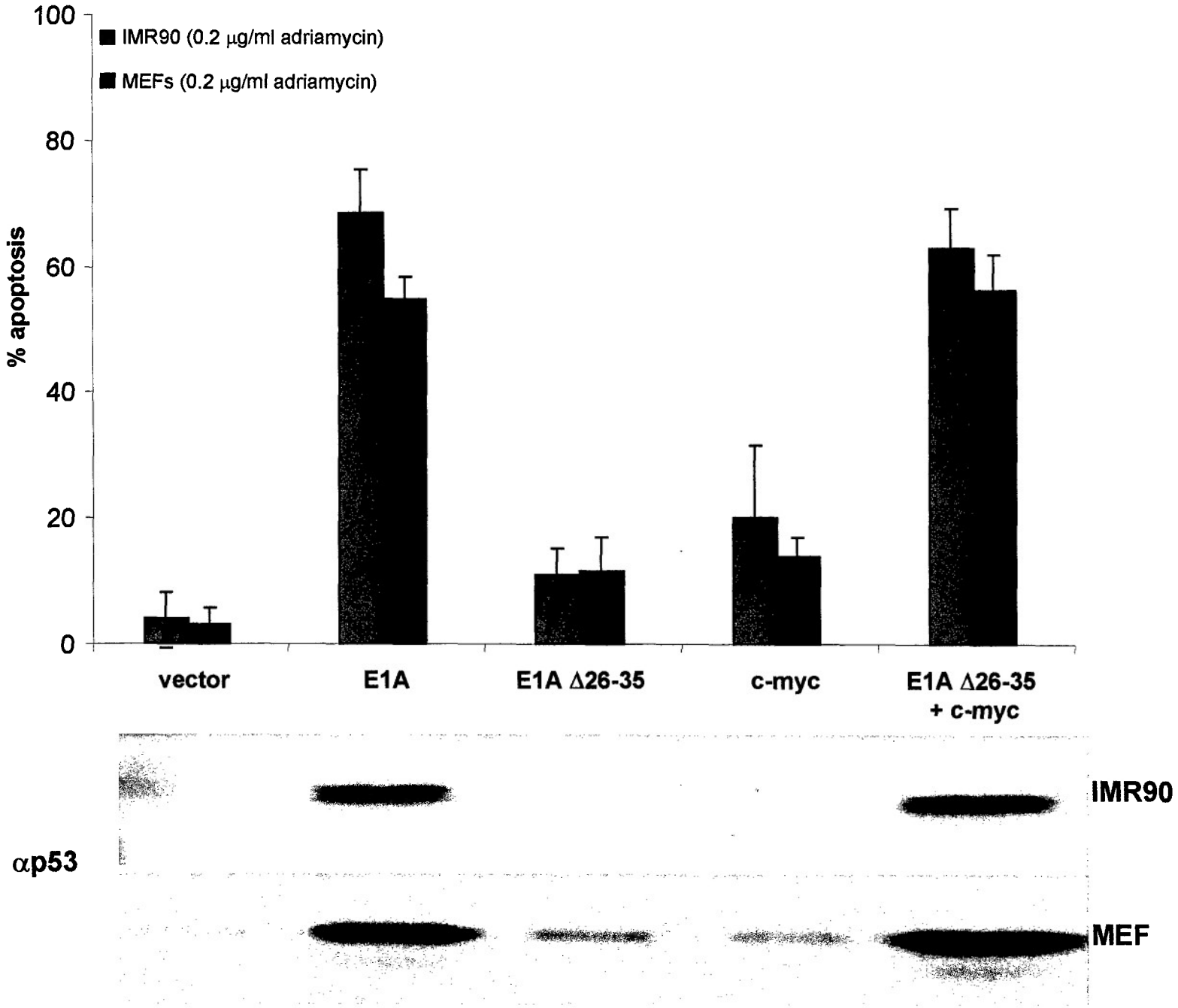

100
80
60
40
20
0
% apoptosis
IMR90 (0.2 μg/ml adriamycin)
MEFs (0.2 μg/ml adriamycin)
vector
E1A
E1A Δ26-35
c-myc
E1A Δ26-35 + c-myc
αp53
IMR90
MEF

Figure 1: Top. Immunoprecipitations and immunoblots to look at E1A interaction with p400 in U-2 OS and IMR90s. Most antibodies were obtained through collaboration with Miriam Fuchs in David Livingston's lab. E1A requires amino acids twenty-six to thirty-five to bind p400. U-2 OS were transfected with 10 μg of construct using Fugene 6 (Roche). 48 hours after transfection, cells were collected for IP-Western. IMR90s were infected three times with retrovirus constructs, selected for 24 hours (puromycin 2.0 μg/ml), media was changed overnight, and cells were collected for immunoprecipitation analysis. The antibodies used in immunoprecipitations are: αp400=PO212 (Livingston), αp300=NM11 (Moran/CSHL), αTAg=PAb416 (CSHL), and αE1A=M73 (Harlow/CSHL). The antibody used for immunoblotting p400/p300 is RW144 (Livingston). Bottom. All constructs were expressed equally by immunoblot. The antibody used for immunoblotting E1A is sc430 (Santa Cruz).

Figure 2: Refined mutational analysis of the N-terminal region of E1A. A schematic diagram of E1A N-terminal mutants is shown on the left. All mutants have previously been described and are either point or deletion mutants within the E1A. All E1A mutants were subcloned into retroviral vectors and sequenced through the ORF. The ability of E1A to bind to cellular proteins was mapped by co-immunoprecipitation analysis for p300[6], RB, and p400. For the ability to bind to a given cellular protein: + equal binding, - is no detectable binding, ± indicates some binding, nd is not determined, and symbols in parenthesis indicate predicted results. The ability of a given mutant to induce apoptosis in either IMR90s or MEFs after adriamycin treatment (24 hours at 0.5 μg/ml) is indicated as percentage relative apoptosis compared to full length E1A. The ability of a given mutant to stabilize p53 is indicated as a + for p53 levels similar to full length E1A, ± for p53 levels greater than cells infected with empty vector but less than full length E1A, - for p53 levels similar to cells expressing empty vector, and nd for not determined.

Figure 3: Schematic diagrams indicating the regions of E1A required to bind various cellular proteins and relative apoptosis of various E1A mutants. Mutations in regions that are required for either p400 or Rb binding result in an inability to promote apoptosis **Top**. Schematic diagram illustrating the regions of E1A required for binding to various cellular proteins. **Bottom**. Graph showing relative apoptosis of IMR90s expressing various E1A mutants relative to full length E1A after adriamycin treatment. Note that the X-axis indicates the primary sequence of full length E1A (243 amino acids) and is aligned relative to the diagram above. Absolute levels of apoptosis of E1A expressing cells after 0.5 μg/ml adriamycin treatment for 24 hours is 84.3% +/- 7.6% (all trials pooled). The relative apoptosis of every E1A mutant was compared to full length E1A by assuming 100% apoptosis for full length E1A. The number of trials for each mutant is to the right of each mutant. A given mutant was normalized to full length E1A in the same trials (e.g. Consider the RG2 mutant. All of the data (all mutants) comes from a total of 16 different trials, but the RG2 mutant was assayed in 6 trials. In normalizing the RG2 mutant to E1A, the only values for E1A that were used in normalizing were the 6 trials where E1A and RG2 are directly compared).

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[6] Also mapped binding for CBP, correlates with p300 binding in all cases.

Figure 4: Left. Cellular viability assay of IMR90s after retroviral infection of either empty vector, c-myc (weak i.e. no Kozak signal), c-myc (strong), or E1A. Cells were treated for 24 hours with the indicated dosage of adriamycin on the x-axis. Cellular viability is represented on the y-axis. Cellular viability was assessed by trypan blue exclusion. Data represents at least three independent experiments. Cells expressing c-myc vectors do not induce apoptosis as efficiently as those expressing full length E1A. c-myc induced apoptosis depends on c-myc levels. **Right**. Immunoblot of IMR90s expressing empty vector of given c-myc construct. c-myc vectors without a Kozak signal fail to stabilze p53 and have little to no increased c-myc expression relative to cells expressing empty vector. Retroviral vectors that express higher levels of c-myc stabilize p53. The antibodies used for p53 and c-myc were CM1 and N-262 (Santa Cruz), respectively.

Figure 5: Top. Cellular viability assay of IMR90s (blue) and MEFs (red) after retroviral infection of either empty vector, E1A, E1A Δ26-35, c-myc, or E1A Δ26-35 and c-myc. Cells were treated for 24 hours with 0.2 μg/ml of adriamycin. Percent apoptosis is represented on the y-axis. Apoptosis was assessed by trypan blue exclusion. Data represents at least three independent experiments. Cells expressing both E1A Δ26-35 and c-myc undergo apoptosis at levels similar to full length E1A. In contrast, cells expressing either E1A Δ26-35 or c-myc remain largely viable. Bottom. Immunoblot for p53 levels in IMR90s or MEFs expressing various constructs. Cells expressing full length E1A or both E1A D26-35 and c-myc showed elevated p53 levels. In contrast, cells expressing either E1A D26-35 or c-myc alone had little induction of p53. The antibodies used for human and murine p53 were CM1 and CM5, respectively.

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.

[*Key Words:* E1A signaling; p53; p19^{ARF} tumor suppressor]

Received June 23, 1998; accepted in revised form June 29, 1998.

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 anti-phosphoserine-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

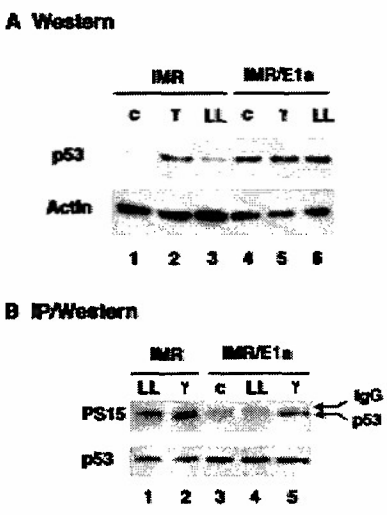

Figure 1. E1A induces p53 in the absence of phosphorylation on serine-15. IMR90 fibroblasts were infected with control (IMR) or *E1A*-expressing (IMR/E1A) retroviruses. Extracts were prepared from untreated cells (c), or from cells treated 3 hr earlier with 7 Gy γ radiation (γ) or 2 hr earlier with 50 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,

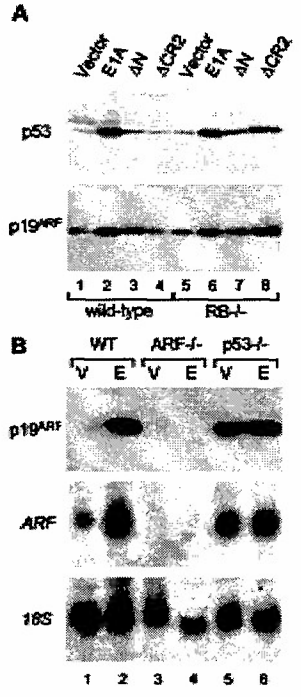

Figure 2. E1A induces $p19^{ARF}$ and p53 through a similar mechanism. (*A*) Early passage (about three to four) wild-type and $Rb^{-/-}$ MEFs from littermates embryos were infected with retroviruses expressing full-length *E1A* or *E1A* mutants unable to bind p300/CBP (ΔN) or the Rb-related proteins (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 (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).

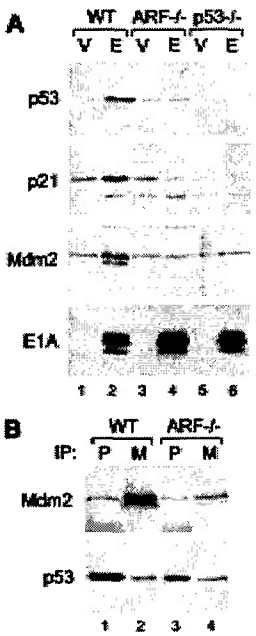

Figure 3. p19^{ARF} mediates p53 induction by E1A and interferes with the p53/Mdm2 interaction. Wild-type (WT), *ARF*-null (*ARF*$^{-/-}$) and *p53*-null (*p53*$^{-/-}$) cell populations harboring a control vector (V) or expressing *E1A* (E) were prepared by retroviral gene transfer. Protein expression 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.

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)-

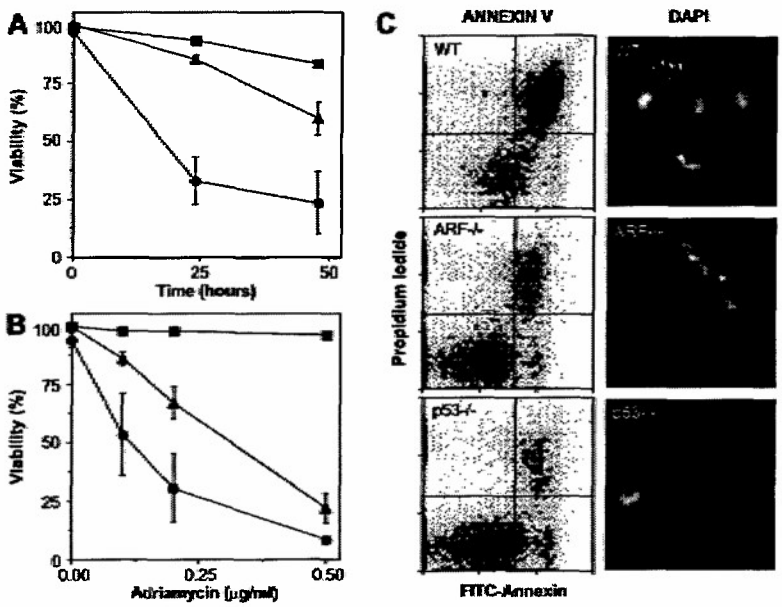

Figure 4. *E1A*-expressing cells lacking *ARF* are defective in apoptosis. Wild-type (●), *ARF*-null (▲), and *p53*-null (■) early passage MEFs were infected with control retroviruses (not shown) or retroviruses expressing *E1A*. Within a week of gene transfer, the resulting cell populations were examined 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.

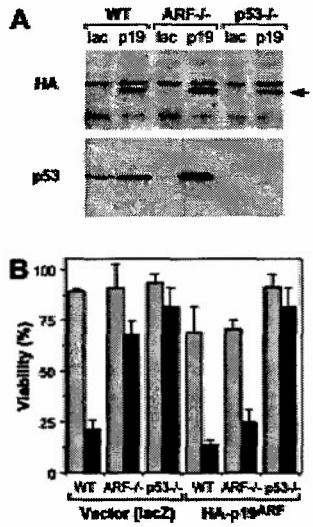

Figure 5. Reintroduction of $p19^{ARF}$ restores apoptosis. Control and *E1A*-expressing populations derived from wild-type (WT), *ARF*-null ($ARF^{-/-}$) and *p53*-null ($p53^{-/-}$) populations were infected with retroviruses expressing *lacZ* or an HA-tagged *ARF* cDNA (Quelle et al. 1995). Thirty-six 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.

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

Because DNA damage and E1A can activate p53 through

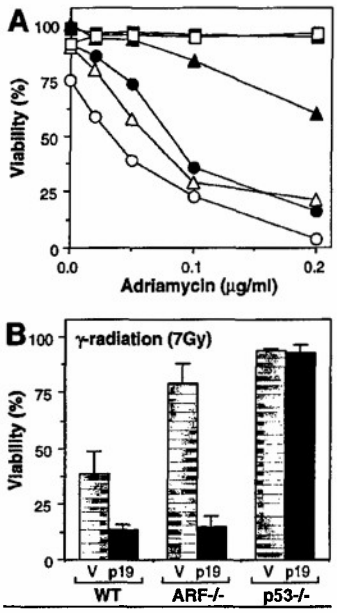

Figure 6. Synergy between p19^{ARF}-dependent and -independent pathways targeting p53. (*A*) *lacZ* (solid symbols)- and *HA-ARF* (open symbols)-expressing cell populations were treated with the indicated doses of adriamycin, and cell viability was determined 24 hr later by trypan blue exclusion. The 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

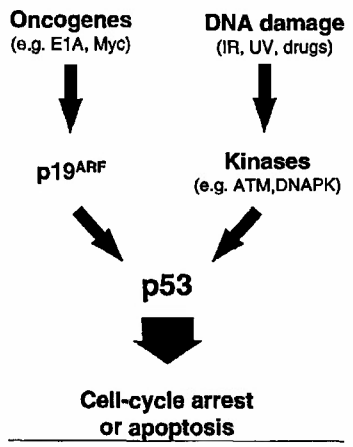

Figure 7. Oncogenes and DNA damage activate p53 through distinct mechanisms. p19^{ARF} acts as an intermediary in p53 activation by mitogenic oncogenes such as *E1A* and *myc*. In contrast, activation of p53 following DNA damage involves de novo phosphorylation of p53 on serine-15 (and other residues) 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.

Acknowledgments

We thank Y. Taya for the generous gift of immunopurified antibodies directed against phosphoserine-15 p53 and N. Dyson for *p107*- and *p130*-deficient MEFs. We also thank Maria Coronesi and Esther Van de Kamp for technical assistance. E.S. is supported by the Italian Ph.D. program and thanks Dr. G. Biamonti and Professor A. Galizzi for support; G.F. is a Tularik postdoctoral fellow; A.V.S. is supported by an Army Breast Cancer Research fellowship; S.W.L. is supported by a Kimmel Scholarship Award. This work was funded in part by American Lebanese Syrian Associated Charities of St. Jude Children's Research Hospital, and by grants CA58316 (C.P.); CA56819, CA71907 (M.F.R.), and CA13106 (S.W.L) from the National Institutes of Health.

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Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 12094–12099, October 1997
Medical Sciences

Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins

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ABSTRACT **The adenovirus E1A oncoprotein renders primary cells sensitive to the induction of apoptosis by diverse stimuli, including many anticancer agents. E1A-expressing cells accumulate p53 protein, and p53 potentiates drug-induced apoptosis. To determine how E1A promotes chemosensitivity, a series of E1A mutants were introduced into primary human and mouse fibroblasts using high-titer recombinant retroviruses, allowing analysis of E1A in genetically normal cells outside the context of adenovirus infection. Mutations that disrupted apoptosis and chemosensitivity separated into two complementation groups, which correlated precisely with the ability of E1A to associate with either the p300/CBP or retinoblastoma protein families. Furthermore, E1A mutants incapable of binding RB, p107, and p130 conferred chemosensitivity to fibroblasts derived from *RB*-deficient mice, but not fibroblasts from mice lacking p107 or p130. Hence, inactivation of RB, but not p107 or p130, is required for chemosensitivity induced by E1A. Finally, the same E1A functions that promote drug-induced apoptosis also induce p53. Together, these data demonstrate that p53 accumulation and chemosensitivity are linked to E1A's oncogenic potential, and identify a strategy to selectively induce apoptosis in RB-deficient tumor cells.**

Despite the widespread use of cytotoxic agents to treat cancer, the molecular mechanisms underlying drug sensitivity and resistance remain poorly understood. Most anticancer agents induce apoptosis, suggesting that tumor-cell chemosensitivity is influenced by the efficiency with which anticancer agents activate apoptotic programs (1, 2). This hypothesis implies that responsive tumors must be more susceptible to apoptosis than normal tissue, and that resistant tumors are unable to efficiently engage apoptotic programs. Tumorigenic mutations can have different effects on apoptosis. For example, activation of the c-*myc* oncogene enhances apoptosis (3) whereas inactivation of the *p53* tumor suppressor gene suppresses cell death (reviewed in ref. 4). These diverse effects suggest that tumor-cell chemosensitivity is determined, in part, by the combined effects of oncogenic mutations on apoptosis (1, 5).

Given the varied impact of oncogenic mutations on apoptosis, it is difficult to study the molecular determinants of chemosensitivity in the unknown genetic background of tumor cells. However, cells expressing the adenovirus early region 1A (*E1A*) oncogene provide a simple model for studying cellular processes that modulate chemosensitivity. E1A promotes apoptosis in nontumorigenic cells (6). As a consequence, E1A-expressing cells become extremely sensitive to toxic agents and readily undergo apoptosis following treatment with anticancer agents (1, 7).

E1A can impinge on a variety of other cellular processes, including transcription, differentiation, and tumor necrosis factor cytolysis (reviewed in ref. 8). During adenovirus infection, E1A makes quiescent cells permissive for virus replication by promoting S phase entry (8). Consequently, E1A has oncogenic potential: E1A facilitates the immortalization of primary rodent cells and cooperates with viral (e.g., *E1B*) or cellular (e.g., oncogenic *ras*) genes to transform primary cells to a tumorigenic state (9). E1B prevents the apoptosis associated with E1A (6), whereas E1A prevents a senescent-like cell cycle arrest provoked by oncogenic Ras (10). Consequently, these transforming interactions illustrate the compensatory mechanisms normal cells possess to suppress transformation (reviewed in ref. 11).

The *E1A* gene expresses several alternatively spliced transcripts, including the 12S and 13S messages encoding 243 (243R) and 289 (289R) amino acid oncoproteins, respectively (reviewed in ref. 12). The 289R protein contains three regions that are conserved between different adenovirus serotypes, designated conserved regions 1, 2, and 3 (CR1, CR2, CR3). CR3 encodes a domain required for transcriptional activation of other viral genes and is absent in the 243R protein, whereas CR1 and CR2 are present in both E1A proteins and are essential for many E1A activities, including oncogenic transformation (13, 14).

E1A 243R associates with a series of cellular proteins, including the retinoblastoma gene product (RB), the RB-related proteins p107 and p130, the p300 and CREB binding protein (CBP) transcriptional coactivators, cyclin A, and certain cyclin-dependent kinases (cdk) (refs. 13 and 14; reviewed in refs. 15 and 16). Because most of these interactions also require residues in CR1 and CR2, the ability of E1A to disrupt the function of these proteins may be crucial for its transforming activities. For example, E1A associates with RB (14, 17) and mutations in either CR1 or CR2 that disrupt this interaction also abolish oncogenicity (13). By binding RB, E1A disrupts RB-E2F heterodimers, thereby relieving repression and promoting transactivation of S phase genes (reviewed in refs. 15 and 16). Mutational inactivation of *RB* achieves a similar effect; consequently, E1A mimics mutational events that occur in familial retinoblastoma and many sporadic tumors (reviewed in ref. 18).

In adenovirus-infected cells, E1A expression appears sufficient for apoptosis (19–21). However, cells tolerate ectopic E1A expression but become extremely prone to apoptosis (1, 7). E1A-expressing cells accumulate p53 protein, and both p53 and Bax—a pro-apoptotic member of the Bcl-2 family—contribute to apoptosis in this setting (1, 7, 22–25). p53 and Bax are inefficient at inducing apoptosis in normal cells lacking E1A (7); indeed, p53 functions to promote cell-cycle arrest (26). Furthermore, E1A-expressing cells possess a discrete factor, absent in normal cells, that is capable of activating the

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Abbreviations: CBP, CREB-binding protein; RB, retinoblastoma; CR1, -2, -3, conserved regions 1, 2, and 3; MEF, mouse embryonic fibroblast; puro, puromycin; hygro, hygromycin.

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apoptotic machinery in a cell-free system (27). Hence, E1A enables cells to more efficiently engage the apoptotic machinery.

In this study, we examined how E1A promotes p53 accumulation and chemosensitivity. To this end, we stably expressed E1A or a series of E1A mutants in primary human and mouse fibroblasts using high-titer recombinant retroviral vectors. Using this approach, we genetically defined multiple E1A activities that act in concert to promote p53 accumulation and chemosensitivity and demonstrate that one of these functions involves inactivation of the *RB* gene product.

MATERIALS AND METHODS

Cells and Cell Culture. Mouse embryonic fibroblasts (MEFs) were isolated as described (10). Cells were maintained in DMEM (GIBCO) supplemented with 10% fetal bovine serum and 1% penicillin-G/streptomycin sulfate (Sigma). $RB^{-/-}$ MEFs were obtained from T. Jacks (28), $p107^{-/-}$ and $p130^{-/-}$ MEFs were from N. Dyson (29, 30). IMR90 cells overexpressed the murine ecotropic receptor, allowing infection with ecotropic retroviruses (10). MEFs were used between passages three and six, IMR90 cells between 20–30 population doublings.

E1A Mutants, Retroviral Vectors, and Infections. The 12S E1A cDNA and 12S E1A deletion or point mutants (31, 32) were subcloned into pLPC (10) or pWZLHygro (unpublished data; J. P. Morgenstern, M. J. Zoller, and J. S. Brugge, Ariad Pharmaceuticals, Cambridge, MA). pLPC-12S coexpresses an E1A 12S cDNA with puromycin phosphotransferase (*puro*) and pWZL-12S coexpresses E1A with hygromycin phosphotransferase (*hygro*). The E1A mutant constructs used in this study were as follows: pLPC 12S.ΔN, pLPC 12S.ΔCR1, pLPC 12S.ΔCR2, pLPC 12S.pm47/124, pWZL 12S.ΔN, pWZL 12S.ΔCR1, and pWZL 12S.ΔCR2.

Ecotropic retroviruses were produced using the Phoenix packaging line (provided by G. Nolan, Stanford University) according to a previously described procedure (10). Cells were placed into medium containing 2.5 μg/ml puromycin (Sigma) or 100 μg/ml hygromycin B (Boehringer Mannheim) to eliminate uninfected cells. When two separate E1A mutants were coexpressed, they were introduced sequentially, the first using LPC and the second using WZLHygro, with drug selection for 2–3 days after each infection.

Cell Viability. Cells (1×10^5) were plated into 12-well plates 24 h before treatment. Twenty-four hours following treatment with adriamycin, or 48 h after serum withdrawal, adherent and nonadherent cells were pooled and analyzed for viability by trypan blue exclusion. At least 200 cells were counted for each point. Null mutant fibroblasts were compared with cells derived from wild-type littermate controls.

Protein Expression. Proteins were extracted in Nonidet P-40 lysis buffer [150 mM NaCl/1% Nonidet P-40/50 mM Tris·HCl, pH 7.5/1 mM phenylmethylsulfonyl fluoride/1 mM EDTA/2 μg/ml CLAP (chymostatin, leupeptin, antipain, and pepstatin)] for 1 h on ice with frequent vortex mixing. Lysates were normalized by Bradford method (Bio-Rad), and 20 μg (for p53) or 10 μg (for E1A) of total protein was loaded in each lane. After electrophoresis, proteins were transferred to Immobilon-P membranes (Millipore) using standard "wet" transfer procedures. E1A was detected using either the M58 or M73 (1:100 dilution) mAbs (33), the latter recognizes an epitope retained in all E1A mutants studied (34). The CM1 and CM5 polyclonal antibodies were used (1:1,000) to detect human and mouse p53, respectively (Novocastra, Newcastle, U.K.). Proteins were visualized by ECL (Amersham), and equal sample loading was confirmed by India Ink.

RESULTS

To determine how E1A promotes chemosensitivity, we began a structure–function analysis to identify the regions of E1A required for this effect. A series of recombinant retrovirus vectors coexpressing various E1A mutants (Fig. 1*A*) with either *puro* or *hygro* were constructed. Earlier studies demonstrated that the 243-amino acid protein encoded by the E1A 12S cDNA was sufficient for apoptosis and chemosensitivity (7, 22); hence, all mutants were derived from an E1A 12S cDNA (31, 32). These mutants were chosen because they are compromised in their ability to physically associate with either the p300/CBP (ΔN and ΔCR1) or RB/p107/p130 (pm47/124 and ΔCR2) family of cellular proteins (Fig. 1*A*) (31).

High-titer ecotropic retroviruses were generated using a transient retrovirus packaging system (35). Virus supernatants were used to infect either normal diploid IMR90 human lung fibroblasts or primary MEFs, and pure populations of E1A-expressing cells were isolated by brief selection in the presence of puromycin or hygromycin B. All E1A mutant proteins were efficiently expressed (Fig. 1*B*). Using this approach, we were able to stably express E1A in primary cell populations in the absence of additional adenoviral proteins—i.e., in a genetically normal background.

Multiple E1A Regions Are Required for Apoptosis and Chemosensitivity. Full-length E1A rendered both human and mouse fibroblasts sensitive to the induction of apoptosis by a variety of agents (Fig. 2; data not shown). As expected, mouse cells expressing E1A lost viability in a dose-dependent manner

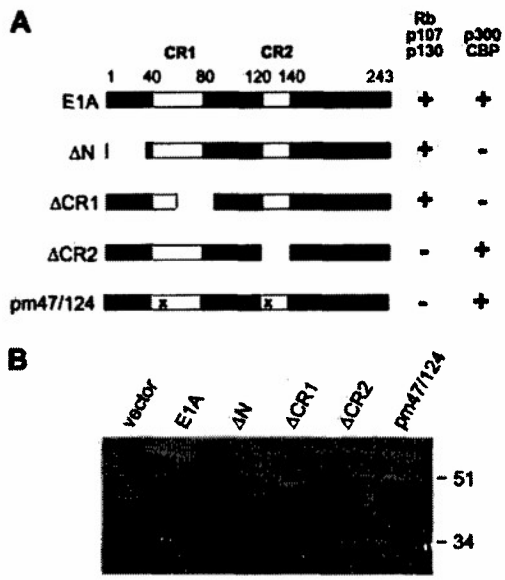

FIG. 1. Structure and expression of E1A mutants. (*A*) The E1A 243R contains two conserved regions (CR1 and CR2, white boxes). In the E1A mutants, deletions are indicated by gaps, and point mutations by an "x." ΔN, ΔCR1, and ΔCR2 are deletions of amino acids 2–36, 68–85, and 120–140, respectively. pm47/124 mutant has a tyrosine to histidine and cysteine to glycine changes at amino acids 47 and 124, respectively. Cellular proteins able to interact with each E1A mutant in coimmunoprecipitations are indicated (31). (*B*) E1A was introduced into IMR90 cells using LPC-based retroviral vectors. After selection in puromycin, E1A levels were determined by Western blot analysis. E1A is highly phosphorylated and undergoes posttranslational modification, which accounts for variable migration in SDS gels (33). Note that the ΔN mutant was able to coimmunoprecipitate similar levels of RB as wild-type E1A (data not shown). Infection efficiencies were >50% before selection and >95% of the cells in the selected cells expressed E1A as determined by immunofluorescence (data not shown). Each E1A mutant localized to the nucleus (data not shown).

following adriamycin treatment or serum withdrawal (Fig. 2 *B* and *C*). Under these conditions cell death is largely p53-dependent, because p53-deficient MEFs expressing E1A remained viable (data not shown; see also refs. 1 and 7). Human cells also lost viability following adriamycin treatment (Fig. 2*A*), but not after serum withdrawal (data not shown). In both cell types, the dying cells displayed features of apoptosis (1, 7). Fibroblasts infected with an empty vector did not undergo apoptosis after either treatment (Fig. 2).

All of the E1A mutants were defective in promoting chemosensitivity in both human and mouse fibroblasts (Fig. 2). IMR90 cells expressing ΔN, pm24/147, or ΔCR2 were completely insensitive to adriamycin treatment (Fig. 2*A*). Although IMR90 cells expressing the ΔCR1 mutant lost viability in a dose-dependent manner, cell death was substantially reduced compared with full-length E1A (35% vs. 11% viable at 0.5 μg/ml, respectively) (Fig. 2*A*). Like IMR90 cells, MEFs expressing ΔN or ΔCR1 remained completely or partially insensitive to adriamycin treatment, respectively (Fig. 2*B*). By contrast, MEFs expressing either the pm47/124 or ΔCR2 mutants displayed modest levels of cell death, but only at the higher doses (Fig. 2*B*). MEFs expressing each E1A mutant were also defective in apoptosis following serum withdrawal, a treatment not known to produce cellular damage (Fig. 2*C*). The behavior of each E1A mutant was independent of the apoptotic stimulus, because similar results were obtained following treatment of human and mouse cells with etoposide, cisplatin, 5-fluorouracil, or γ-radiation (data not shown). Therefore, multiple regions of E1A are required for apoptosis following treatment with diverse agents.

Functionally Distinct Regions of E1A Cooperate to Confer Chemosensitivity. Each E1A mutant defective in apoptosis is also impaired for binding either the p300/CBP or RB-related proteins (see Fig. 1) (31), raising the possibility that these processes are related. However, the observations are correlative, and it is also possible that these mutations affect one or more unknown E1A activities. To establish whether multiple E1A functions contribute to apoptosis, combinations of E1A mutants were expressed in a trans complementation assay. If two E1A mutants were defective because they lacked the same function(s), they would be unable to function in trans to confer chemosensitivity. Conversely, if two mutants were defective owing to loss of *separate* functions, then coexpressing these mutants might restore chemosensitivity. Therefore, E1A mutants were introduced sequentially into IMR90s and MEFs using retroviruses coexpressing different selectable markers (*puro* and *hygro*).

In both human and mouse fibroblasts, E1A mutants that bound different classes of cellular proteins acted in trans to restore chemosensitivity, whereas those that bound the same class did not (Fig. 3). For example, although cells expressing either the ΔN or ΔCR2 mutant alone were insensitive to adriamycin-induced apoptosis, the levels of apoptosis in cells coexpressing these mutants approached those observed in cells expressing full-length E1A (Fig. 3 *A* and *B*). Similar results were observed when cells were treated with other anticancer agents or following serum withdrawal (data not shown). Likewise, cells coexpressing the ΔCR1 and ΔCR2 mutants were as sensitive to adriamycin-induced apoptosis as cells expressing full-length E1A (Fig. 3 *C* and *D*). No increase in chemosensitivity was observed when cells were infected sequentially with the same E1A mutant (e.g., ΔN or ΔCR1) compared with cells infected only once (data not shown). This finding indicates that the cooperativity between ΔN or ΔCR1 with ΔCR2 did not result from increased gene dosage, but rather was due to synergy between separate E1A functions. Thus, multiple E1A activities contribute to chemosensitivity.

In contrast, the ΔN and ΔCR1 mutants failed to restore chemosensitivity when expressed in trans: cells coexpressing ΔN and ΔCR1 behaved identically to cells expressing the partially defective ΔCR1 mutant alone (Fig. 3 *E* and *F*). As discussed above, both ΔN and ΔCR1 restored chemosensitivity when coexpressed with ΔCR2, implying that the ΔN and ΔCR1 mutations did not produce global aberrations in E1A structure, but rather, disrupted the same function(s). The fact that two E1A mutants that fail to bind p300/CBP (see Fig. 1) (31) are defective for apoptosis because they affect overlapping functions suggests that binding of one or more of these proteins is required for chemosensitivity.

Role of CR2 in Chemosensitivity. CR2 is required for the physical association between E1A and the RB-related proteins (14). In principle, CR2 could contribute to chemosensitivity by inactivating one or more of these proteins or by affecting some other cellular activity. If CR2 promotes chemosensitivity by inactivating a single RB-related protein, then the ΔCR2 mutant should behave like full-length E1A in cells lacking this crucial target. Because all of the *RB*-related genes have been

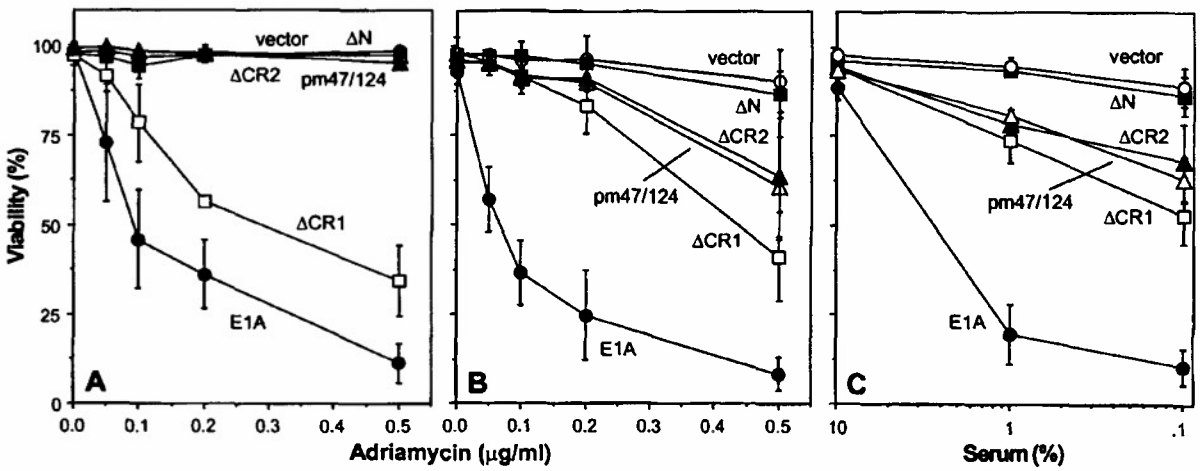

FIG. 2. Multiple regions of E1A are required for chemosensitivity. Primary mouse (MEF) (*A* and *C*) or human (IMR90) (*B*) fibroblasts were infected with an empty vector (vector, ○), vectors expressing full-length E1A (●), or the following mutants: ΔN (■), ΔCR1 (□), ΔCR2 (▲), and pm47/pm124 (△). Infected populations were plated in multiwell dishes and treated with the indicated concentrations of adriamycin (*A* and *B*) or serum (*C*). Cell viability was determined 24 h following adriamycin treatment or 48 h after serum withdrawal. Previous studies demonstrated that cell death under these conditions results from apoptosis (1, 7), and this was confirmed by visualizing chromatin condensation using 4′,6-diamidino-2-phenylindole (data not shown). Each value represents the mean ± SD from at least three separate experiments.

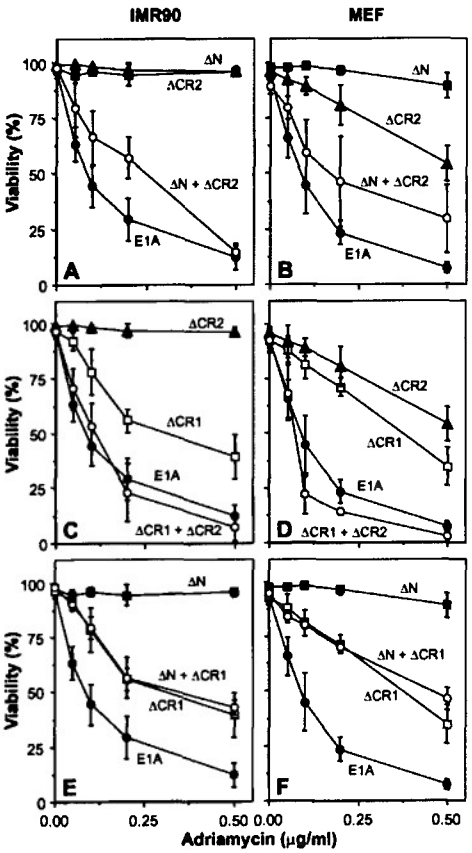

FIG. 3. Separate E1A functions cooperate to confer chemosensitivity. IMR90 or MEF cell populations expressing E1A (●), ΔN (■), ΔCR1 (□), ΔCR2 (▲), ΔN and ΔCR2 (○ in *A* and *B*), ΔCR1 and ΔCR2 (○ in *C* and *D*), or ΔN and ΔCR1 (○ in *E* and *F*) were generated by retroviral infection. Multiple E1A mutants were introduced sequentially as described in *Materials and Methods*. Cell populations were treated with adriamycin and viability was determined 24 h later by trypan blue exclusion. Each value represents the mean ± SD of the data from at least three separate experiments.

disrupted in mice and all are expressed in MEFs (28–30, 36), this hypothesis could be tested definitively.

E1A and the ΔCR2 mutant were introduced into wild-type, $RB^{-/-}$, $p107^{-/-}$, or $p130^{-/-}$ MEFs, and the resulting populations were treated with apoptosis-inducing stimuli (Fig. 4). Adriamycin treatment induced similar levels of apoptosis in cells expressing full-length E1A, irrespective of their genotype. Thus, as expected, loss of the RB-related proteins does not impair apoptosis. Furthermore, MEFs infected with the empty vector were insensitive to adriamycin treatment, demonstrating that loss of either RB, p107, or p130 was not sufficient to produce chemosensitivity (data not shown).

Concordant with previous results, wild-type MEFs expressing ΔCR2 are relatively insensitive to adriamycin treatment (Fig. 4 *Upper Left*). Likewise, $p107^{-/-}$ and $p130^{-/-}$ cells expressing ΔCR2 remained insensitive to adriamycin treatment. By contrast, $RB^{-/-}$ cells expressing ΔCR2 (Fig. 4) or pm47/124 (data not shown) were as sensitive to adriamycin-induced apoptosis as cells expressing full-length E1A. This synergy was specific for ΔCR2 and pm47/124, because the ΔN mutant remained defective in all cell types (Fig. 4 *Lower*). Thus, inactivation of RB—but not p107 or p130—is the critical function of CR2 important for apoptosis. Furthermore, E1A mutants unable to bind RB are defective in normal cells but promote apoptosis in cells with mutant *RB* genes.

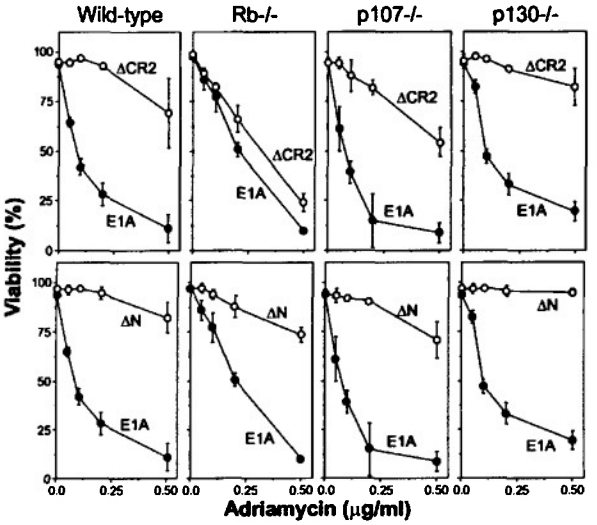

FIG. 4. Inactivation of RB by CR2 is required for chemosensitivity. Wild-type, $RB^{-/-}$, $p107^{-/-}$, and $p130^{-/-}$ MEFs expressing either E1A (●), ΔCR2 (○, *Upper*), or ΔN (○, *Lower*) were generated by retroviral infection. E1A and E1A mutants were expressed at similar levels (data not shown). Cell viability was determined 24 h after adriamycin treatment. Each point represents the mean ± SD from at least three separate experiments.

p53 Accumulation and Chemosensitivity Involve the Same E1A Functions. Cells expressing E1A accumulate p53 protein due, in part, to increased p53 stability (22). To determine whether p53 accumulation and chemosensitivity involve the same E1A functions, we examined the ability of each E1A mutant to induce p53. Cells expressing full-length E1A displayed a 20- to 30-fold increase in steady-state p53 protein levels (Fig. 5*A*). The ΔN and ΔCR2 mutants produced only a slight increase in p53 levels in IMR90 cells, and no increase in MEFs. However, coexpression of both mutants induced p53 to levels observed in cells expressing full-length E1A (Fig. 5*A*). Remarkably, ΔCR2 induced p53 when expressed in $RB^{-/-}$ MEFs (30-fold increase), but not in $p107^{-/-}$ or $p130^{-/-}$ MEFs (Fig. 5*B*). $RB^{-/-}$ cells infected with the empty vector displayed no increase in p53 levels (data not shown). Thus, the same E1A functions that promote apoptosis and chemosensitivity also induce p53.

DISCUSSION

Despite the widespread use of cytotoxic agents to treat cancer, molecular factors that influence tumor-cell chemosensitivity remain largely unknown. The E1A oncoprotein displays a remarkable ability to enhance chemosensitivity, and acts to promote drug-induced apoptosis (1, 7, 37). In this study, we demonstrate that at least two independent E1A functions act in concert to promote apoptosis and chemosensitivity, and that one function involves inactivation of the retinoblastoma gene product. Of note, the regions of E1A that promote apoptosis are similar, if not identical, to those previously shown to facilitate oncogenic transformation (13, 14). Consequently, our results underscore the association between factors that influence tumorigenesis and tumor-cell chemosensitivity.

In the context of adenovirus infection, the E1A regions involved in binding the p300/CBP and RB-related proteins have been associated with apoptosis. However, depending on the setting, only the p300/CBP binding region, either the p300/CBP or RB-related protein binding region, or both regions were required (38–40); hence, the results are contradictory. Moreover, in one study, p53 accumulation did not correlate with apoptosis (39). Another study examined the

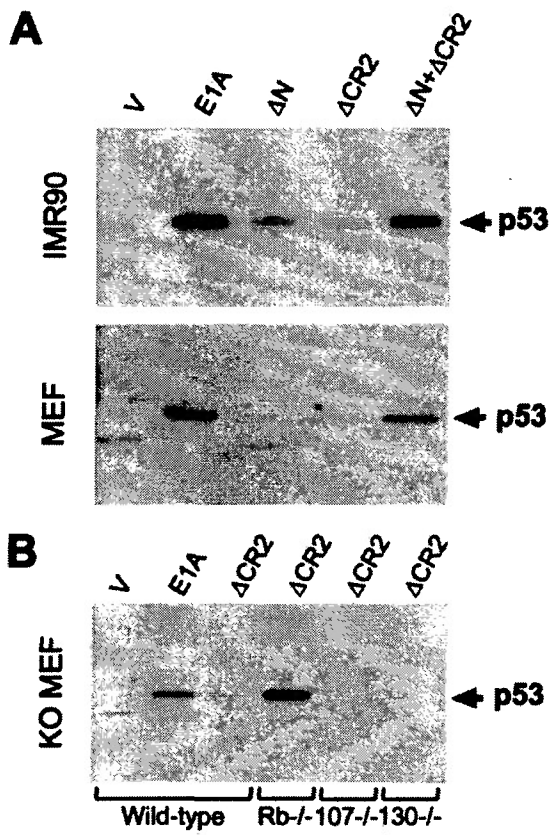

FIG. 5. E1A and p53 accumulation. (*A*) p53 expression in populations of IMR90s or MEFs expressing the empty vector (V), full-length E1A, ΔN, ΔCR2, or coexpressing ΔN and ΔCR2 was examined by immunoblotting using polyclonal antibodies specific for human or mouse p53, respectively. (*B*) p53 expression in wild-type, $RB^{-/-}$, $p107^{-/-}$, or $p130^{-/-}$ MEF populations expressing the indicated E1A proteins. No increase in p53 was observed in $RB^{-/-}$, $p107^{-/-}$, or $p130^{-/-}$ MEFs without E1A, and E1A induced p53 in MEFs of all three genotypes (data not shown.)

relationship between E1A and chemosensitivity in a tumorigenic cell line. Here, the regions of E1A capable of conferring chemosensitivity varied with the agent tested (41). However, even full-length E1A did not substantially enhance apoptosis in these cells, and no correlation between p53 accumulation and chemosensitivity was observed. Furthermore, this study did not support an absolute requirement for RB inactivation for chemosensitivity. All previous studies have been correlative: none provides direct evidence that the cellular proteins targeted by E1A participate in apoptosis.

The studies described above examined E1A in immortal or tumor-derived lines, often in the context of adenovirus infection. However, E1A is a transforming oncogene that itself facilitates immortalization (9); hence, immortal or tumor-derived cells may already have alterations in processes affected by E1A. This may explain why the RB-binding domain of E1A was dispensable for apoptosis in HeLa cells (38, 39)—these tumor cells express papillomavirus E7, an oncoprotein that inactivates RB. Likewise, adenovirus contains several genes that affect apoptosis in addition to EIA (6, 42) and can induce apoptosis in the absence of E1A (39). In this study, retrovirus-mediated gene transfer was used to introduce E1A into whole populations of primary cells, allowing E1A to be studied in the absence of other adenoviral genes and unknown host-cell mutations.

All E1A mutants tested showed marked reduction in apoptotic potential in both primary human and mouse fibroblasts, and the requirement for each E1A region was independent of the apoptotic stimulus. These regions correlated precisely with the ability of E1A to associate with the p300/CBP or RB-related proteins. Coexpression of E1A mutants binding separate classes of cellular proteins functioned in trans to confer chemosensitivity, whereas coexpression of mutants binding the same cellular proteins did not. Thus, this study genetically defines at least two E1A functions that act in concert to promote apoptosis and chemosensitivity.

As has been observed during adenovirus-induced apoptosis (38–40), our results provide genetic evidence that E1A's interaction with the p300/CBP proteins is critical for chemosensitivity. Here, we used a genetic complementation test to demonstrate that two spatially separate E1A mutations, both known to disrupt p300/CBP binding (ΔN and ΔCR1), affect the same E1A function(s) involved in chemosensitivity. Whereas ΔCR1 is unable to associate with p300/CBP in immunoprecipitations, it retains some capacity to affect p300/CBP functions in cells (32, 43). By contrast, the ΔN mutant is completely defective in p300/CBP interaction using both immunoprecipitations and functional assays. Perhaps this explains why the ΔN and ΔCR1 mutants displayed a complete and partial defect in apoptosis, respectively (see Fig. 3). p300 and CBP are both transcriptional coactivators and histone acetyltransferases (ref. 44; reviewed in ref. 45), and E1A binding to p300 produces global changes in transcription (reviewed in ref. 16). Recent studies suggest that p300 and CBP physically associate with p53 and contribute to p53's transcriptional activity, raising the possibility that E1A binding to p300 modulates p53 function to promote apoptosis (46–48). Alternatively, the critical target may not be p300/CBP itself, but another molecule displaced or altered by the E1A–p300/CBP interaction.

In addition to the apparent p300/CBP binding activity, a second E1A function is required for apoptosis and chemosensitivity. Using primary fibroblasts derived from $RB^{-/-}$, $p107^{-/-}$, or $p130^{-/-}$ mice, we conclusively demonstrate that this function involves inactivation of RB, but not p107 or p130. Interestingly, inactivating mutations in the *RB* gene occur in many human cancers; by contrast, mutations in p107 or p130 have not been observed (18). The fact that E1A promotes chemosensitivity by inactivating a tumor suppressor underscores the utility of viral oncogenes to identify processes relevant to human cancer. Furthermore, the critical role of RB inactivation for apoptosis reiterates the fundamental relationship between tumorigenesis and chemosensitivity.

How RB inactivation contributes to apoptosis and chemosensitivity remains to be determined. *RB*-deficient mice display elevated apoptosis in the embryonic lens, fetal liver, and the developing nervous system, implying RB inactivation alone can promote apoptosis in some settings (49, 50). Furthermore, overexpression of RB in HeLa cells can suppress cell death (51). The interaction between E1A and RB releases E2F transcription factors; similarly, overexpression of E2F-1 overcomes RB binding and induces apoptosis in a p53-dependent manner (52). This finding suggests that one or more E2Fs might mediate this aspect of E1A function. However, $E2F\text{-}1^{-/-}$ MEFs expressing E1A display no defects in apoptosis (L. Yamasaki, A.V.S., and S.W.L., unpublished data), indicating that E2F-1 is dispensable for this effect.

We have previously shown that p53 protein accumulates in cells expressing E1A, which correlates with the involvement of p53 in apoptosis (22, 23). Here we demonstrate that the same E1A functions that promote apoptosis and chemosensitivity also induce p53 (see also ref. 38). These regions are also required for E1A's transforming activities (13, 14), implying that p53 accumulation, chemosensitivity, and oncogenic potential arise from the same E1A functions. This suggests that p53 accumulation is a cellular response to oncogenic "stress" rather than a direct effect of E1A on p53. Interestingly, extracts

from E1A-expressing cells possess a discrete factor that reproduces some of the pro-apoptotic activities of E1A in cell-free systems (27). The nature of this factor may shed light on the links between p53, chemosensitivity, and cell-cycle control.

The *RB* gene is mutated in many human cancers, and the RB pathway is disrupted in the vast majority of cancer cells (reviewed in ref. 18). Our results suggest a strategy to specifically kill cancer cells with defective RB function. In normal cells, at least two processes affected by E1A are necessary to promote chemosensitivity—RB inactivation and apparently disruption of some p300/CBP function. The RB-inactivating function of E1A is dispensable for chemosensitivity in RB-deficient cells, consequently such E1A mutants, or small molecules that mimic their action, might synergize with standard chemotherapeutic agents to specifically induce apoptosis in *RB* mutant tumor cells. Although p53 potentiates apoptosis under the conditions used in this study, E1A can promote chemosensitivity in p53-deficient cells (refs. 1 and 7, and unpublished results). Consequently, this therapeutic approach may not strictly depend on the presence of wild-type p53. Experiments to test this strategy are underway.

We thank T. Jacks and N. Dyson for providing mutant fibroblasts, M. Mathews for E1A mutants, and J. Morgenstern for retroviral vectors. M. McCurrach and A. Lin provided helpful assistance and advice. We also thank W. Tansey, M. Soengas, and M. McCurrach for critical reading of the manuscript, and W. Herr and B. Stillman for support. S.W.L. is a Kimmel Scholar. This work was supported by Grant CA 13106 from the National Cancer Institute.

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Publications:

Samuelson A.V. and Lowe S.W. (1997). Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the RB-related proteins. Proc Natl Acad Sci. 94:12094-9.

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Presentations, Posters, and Abstracts:

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (poster). Cold Spring Harbor Laboratory Meeting on Cancer Genetics and Tumor Suppressor Genes (1):183, Cold Spring Harbor NY, August 16-20, 2000.

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (oral presentation and poster). Era of Hope Department of Defense Breast Cancer Research Program Meeting (1): 301, Atlanta GA, June 8-11, 2000.

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (poster). Cold Spring Harbor Laboratory Meeting on The Cell Cycle (1):163, Cold Spring Harbor NY, May 17-21, 2000.

Samuelson and Lowe; *Chemosensitization of Normal and Tumor Cells by Adenoviral E1A* (poster). Keystone Symposium on Cancer, Cell Cycle and Therapeutics (1): 69(#230), Steamboat Springs CO, January 8-13, 2000.

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Samuelson and Lowe; *Selective induction of p53 and chemosensitivity in RB-deficient cells by E1A mutants unable to bind the retinoblastoma-related proteins* (oral presentation and poster). Cold Spring Harbor Laboratory Meeting on Programmed Cell Death (1):259, Cold Spring Harbor NY, September 17-21, 1997.

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REPLY TO ATTENTION OF

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