Rb Inhibits E2F-1-induced Cell Death in a LXCXE-dependent Manner by Active Repression*

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Rb (retinoblastoma protein) inhibits E2F-1-induced cell death. We now show that the ability of Rb to inhibit E2F-1-induced cell death is dependent on a functional LXCXE-binding site in Rb, thereby suggesting that proteins that bind the LXCXE-binding site in Rb may regulate the anti-apoptotic activity of Rb. HDAC1, an LXCXE protein that plays a critical role in Rb-mediated transcription repression, abrogates the effect of Rb on E2F-1-induced cell death. In contrast, RF-Cp145, another LXCXE protein, cooperates with Rb to inhibit E2F-1-induced cell death. Both proteins exert their effect in an LXCXE-dependent manner. Rb regulates E2F-induced cell death by acting upstream of p73. Rb represses the p73 promoter. Our results further suggest a model in which Rb-E2F-1 complexes mediate the anti-apoptotic activity of Rb through active repression of target genes without recruiting HDAC1.

The Rb tumor suppressor gene is frequently mutated in many kinds of tumors. Rb functions in part through interaction with E2F family of transcription factors. Rb represses the transcription of E2F-responsive genes by at least two mechanisms. Rb binds to E2F transcription factor family members such as E2F-1 and directly represses transcriptional activation by E2F. In addition, the Rb-E2F complex that forms at the promoter actively represses transcription by recruiting chromatin modeling enzymes (1–4). The second mechanism is termed active repression and is invoked to explain transcriptional repression of activator proteins bound to the proximal promoter. Rb can repress transcription by recruiting class I histone deacetylases such as HDAC1 (4–7) and other proteins like RBP1 (8, 9). Histone deacetylases facilitate condensation of chromatin, thereby conferring transcriptional repression.

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Rb regulates cell cycle progression from the G1 to S phase and in the S phase (Refs. 10 and 11; and reviewed in Ref. 12). In addition, Rb inhibits cell death induced by DNA-damaging agents (13, 14) or E2F-1 (15). The demonstration that Rb null mice die at 13–15 days of gestation accompanied by increased cell death in multiple organs (18–19) suggests that Rb plays a critical role in regulating apoptosis. Inactivation of the E2F-1 gene in Rb null embryos partially rescues the apoptosis seen in Rb−/− embryos (19) and implicates E2F-1 as a critical mediator of apoptosis observed in Rb−/− embryos.

The AB pocket residues 379–772 of Rb are highly conserved through evolution. The majority of germ line mutations found in hereditary retinoblastoma and most tumor-derived mutations in the Rb gene map to the AB pocket. Viral oncoproteins also bind to the AB pocket and inactivate Rb (20–22). A conserved LXCXE motif in these viral oncoproteins is critical for their ability to bind Rb (23). Many cellular Rb-binding proteins, which regulate Rb activity including HDAC1 (7), BRG1 (24), and RF-Cp145 (14), contain an LXCXE motif. The three-dimensional crystal structure of the AB pocket of Rb interacting with the LXCXE peptide revealed residues in Rb that contact the LXCXE peptide (25). Accordingly, mutation(s) of these contact residues suppressed the ability of Rb to bind LXCXE-containing viral proteins. These Rb mutants retain the ability to bind E2F, repress E2F-dependent transcription, and inhibit growth comparable with wild type Rb (26, 27) but fail to protect cells from apoptosis (14).

The transcription factor E2F-1 induces both cell cycle progression and apoptosis. E2F-1-induced cell death acts via two parallel pathways. In the p53-dependent pathway, E2F activates p19ARF transcription. p19ARF binds MDM2 and relieves p53 from MDM2-mediated degradation (28, 29). These events thus enable p53 to induce cell death. This pathway is therefore impaired by inactivation of either the INK4a or the p53 locus. The second pathway is p53-independent and has been suggested to act via the p53-related gene p73 (30). E2F-1 transactivates the p73 gene (31). Disruption of p73 function impairs E2F-1-induced apoptosis in p53−/− cells (30), thus suggesting that activation of p73 is required for p53-independent E2F-1-induced cell death.

We have previously shown that the LXCXE interaction is critical for the ability of Rb to inhibit cell death induced by a variety of DNA-damaging agents (14). Proteins that bind the LXCXE-binding site in Rb would be expected to regulate the anti-apoptotic activity of Rb. Indeed, we have shown that RF-Cp145, an LXCXE-containing protein, cooperates with Rb to mediate cell survival after DNA damage. In this paper we have investigated the mechanism of Rb-mediated inhibition of E2F-1-induced cell death. We find that in contrast to the wild type Rb, which promotes cell survival, the LXCXE nonbinding
N757F Rb mutant fails to inhibit E2F-1-induced cell death. Unlike RF-Cp145, HDAC1, which also interacts with the LXCXE-binding site in Rb, does not cooperate with Rb to inhibit E2F-1-induced cell death. In contrast, HDAC1 inhibits Rb-mediated cell survival, which is independent of its deacetylase activity but dependent on the LIXCXE motif in HDAC1. Interestingly, Rb-E2F-1 complexes mediate the anti-apoptotic activity of Rb through active repression without recruiting HDAC1.

MATERIALS AND METHODS

Plasmids, Cell Culture, and Transfection—The mammalian expression vectors for wild type Rb, the N757F Rb mutant, RF-Cp145, BRG1, and HDAC1 have been described (14). The H141A HDAC1 mutant was generated by site-specific mutagenesis by standard methods, and the mutation was verified by sequencing of the entire coding sequence of the cDNA. The HDAC1 ΔLXCXE mutant has been described (7). The mammalian expression vectors of p73α, p73β, the p73 dominant negative mutant (p73 DN1) and point mutant of the p73 dominant negative mutant (p73 DN1 L-P) were as described (30). The mammalian expression vector of N-terminal DNA-binding domain of E2F-1 (1–368) and the chimeric E2F-1/Rb protein (N-terminal DNA-binding domain of E2F-1 (1–368) and the pocket domain of Rb (379–792)) have been described (2, 32). The N757F mutant of the E2F-1/Rb chimeric protein was generated by PCR, and its sequence was verified by sequencing. C33A cells (Rb null human cervical carcinoma) from ATCC were maintained in Dulbecco’s modified Eagle’s medium complemented with 10% fetal bovine serum. C33A cells were transfected using the calcium phosphate transfection method. The cells co-transfected with puromycin.

ABE vector (where indicated) were selected with 2.5 μg/ml of puromycin for 3 days as described previously (14).

Survival Assay—The cells were transfected with E2F and other vectors. The calcium phosphate precipitates were washed off 24 h later, the cells were washed, and fresh medium was added, and cell survival was determined 17 h later. Cell survival was measured 17 h after UV irradiation. In all survival assays, the cells were co-transfected with β-galactosidase expression vector. The cells were then fixed with 0.1% glutaraldehyde and incubated for 12 h in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 1 mg/ml) to detect β-galactosidase activity as described (14). The relative number of surviving cells in the presence of the appropriate transfected vector was determined by counting the number of blue cells in >200 nonoverlapping fields in the presence or absence of transfected E2F-1. We compared the number of surviving cells in the absence or presence of E2F-1 for each transfection condition to determine the percentage of survival (Supplementary Figure III). The data shown (means ± S.D.) are derived from five different experiments done at different times.

Antibodies, Immunoblotting, and Immunoprecipitation—Anti-pRb antibody (IF-8) (Santa Cruz Biotechnology), anti-HA1 antibody (BabCo), anti-E2F-1 antibody (Santa Cruz), and a polyclonal Rb specific antibody 851 (33) were used. Whole cell extracts were prepared in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 10 mM EDTA, 1% Nonidet P-40, and protease inhibitors. The proteins were separated on 7.5% polyacrylamide gels and detected by immunoblotting using the ECL detection reagent (Amersham Biosciences).

1 The abbreviation used is: HA, hemagglutinin.
RESULTS

The LXCXE-binding Site in Rb Is Essential for Rb-mediated Inhibition of E2F-1-induced Cell Death—To investigate the role of the LXCXE-binding site in Rb in regulating E2F-1-mediated cell death, we utilized the β-galactosidase assay (14).

In this assay, the cells are co-transfected with a vector expressing β-galactosidase, and the percentage of surviving β-galactosidase-positive cells (blue cells) is determined (Fig. 1D). We compared the number of surviving cells in the absence or presence of E2F-1 for each transfection condition to determine the percentage of survival. The data shown (means ± S.D.) are derived from five different experiments done at different times. Transfection of the Rb-deficient C33A cell line with vectors expressing E2F-1 induces cell death. Increasing amounts of wild type Rb inhibit E2F-1-induced cell death in a dose-dependent manner (Fig. 1A). In contrast, the LXCXE nonbinding N757F Rb mutant (hereafter referred to as Rb N757F mutant) fails to inhibit E2F-1-induced cell death (Fig. 1A). Increasing amounts of E2F-1 were also less efficient at inducing cell death in the presence of Rb as compared with the Rb N757F mutant (Fig. 1B). The selective inhibition of E2F-1-induced cell death by wild type Rb but not the Rb N757F mutant is not due to an effect on E2F-1 protein levels (Fig. 1C). Both wild type Rb and the Rb N757F mutant stabilize E2F-1 protein levels with similar efficacy (Fig. 1C) as shown previously (34–36).

Rb Does Not Inhibit p73-induced Cell Death—p73 is an inducer of cell death in many cell lines (30). It has been suggested that E2F-1-induced cell death in p53−/− cells is mediated in a p73-dependent manner (30). Because C33A cells lack p53, we determined whether Rb inhibits E2F-1-induced cell death by regulating the ability of p73 to induce cell death. We first determined whether E2F-1-induced cell death in C33A cells is p73-dependent. We used a dominant negative mutant of p73 (p73 DNLM) that selectively inhibits p73-mediated transactivation but not p53-dependent transactivation (30). This p73 DNLM inhibited E2F-1-induced cell death in C33A cells (Fig. 2A). A point mutant of the p73 dominant negative mutant (p73 DNLM L-P) had no effect on E2F-1-induced cell death in C33A cells
A. Percentage surviving cells (A) or pH positive cells (B). The effect of increasing concentrations (125, 250, and 500 ng) of either control vector or Rb. The effect of increasing concentrations (125, 250, and 500 ng) of RF-Cp145, HDAC1, or BRG1 to cooperate with Rb to inhibit E2F-1-induced cell death was determined. All of the cells were transfected with a β-galactosidase plasmid (200 ng). The results from three experiments are shown. B, protein expression by various vectors used in A was determined by immunoblotting lysates from transfected cells with Rb, HA, or E2F-1-specific antibodies. HA-specific antibody detects transfected BRG1, HDAC1, and RF-Cp145.

The inhibition of E2F-1-induced cell death by p73 is not due to its effect on E2F-1 protein levels (Fig. 2D). Because p73 has been shown to induce cell death (30), we determined whether Rb inhibits p73α- or p73β-induced cell death. Rb did not inhibit p73α-induced (Fig. 2B) or p73β-induced (Fig. 2C) cell death in C33A cells. Rb had no effect on p73 protein levels (Fig. 2E). Because E2F-1 activates the p73 promoter (Ref. 30 and data not shown), our results suggest that Rb and E2F regulate cell death by acting upstream of p73.

Effect of HDAC1, BRG1, and RF-Cp145 on Rb-mediated Inhibition of E2F-1-induced Cell Death—Our demonstration that a functional LXCXE-binding site is required for Rb to inhibit E2F-1-induced cell death prompted us to investigate whether cellular LXCXE proteins such as HDAC1 (7), BRG1 (37), or RF-Cp145 (14) cooperate with Rb to inhibit E2F-1-induced cell death. HDAC1, a histone deacetylase, contains an LXCXE motif that is essential to bind and cooperate with Rb in mediating optimal transcriptional repression of promoters containing E2F-binding sites (7). BRG1, a member of the SW12/SNF2 family of chromatin remodeling ATPases contains an LXCXE motif, binds Rb (4, 24), and is required for Rb to inhibit cell cycle progression. The large subunit of RF-C (RF-Cp145) enhances cell survival after DNA damage in Rb null cells in a strict Rb- and LXCXE-dependent manner (14).

C33A cells, in addition to lacking Rb, lack BRG1 and functional Rb-HDAC1 complexes and have been used to clarify the role of BRG1 and HDAC1 in Rb function (4). RF-Cp145, the large subunit of RF-C, enhances the ability of Rb to inhibit E2F-1-induced cell death in a dose-dependent manner (Fig. 3A). In the absence of Rb, RF-Cp145 by itself was unable to inhibit E2F-1-induced cell death (Fig. 3A), thus showing that RF-Cp145 enhances cell survival only in the presence of Rb. BRG1 on the other hand had no significant effect in either enhancing or inhibiting the ability of Rb to inhibit E2F-1-induced cell death (Fig. 3A). BRG1 also had no effect on E2F-1-induced cell death in the absence of Rb (Fig. 3A). In contrast, HDAC1 abrogates the ability of Rb to inhibit E2F-1-induced cell death in a dose-dependent fashion but had no effect on E2F-1-induced cell death in the absence of Rb (Fig. 3A). BRG1, HDAC1, and RF-Cp145 had no effect on E2F-1 or Rb protein levels (Fig. 3B).

These data show that the three LXCXE containing cellular proteins we tested had distinct effects on Rb-mediated inhibition of E2F-1-induced apoptosis. We then determined whether these distinct effects of LXCXE-containing cellular proteins on Rb-mediated inhibition of E2F-1-induced cell death was due to their effect on transcriptional repression. Because E2F-1-induced cell death is p73-dependent (Fig. 2A), we used the p73 promoter (−883 to +77) driving the expression of a luciferase reporter to monitor repression for these studies. This p73 promoter has been used to show that E2F activates the p73 promoter (30). As expected Rb repressed p73 promoter activity (Fig. 2F). We find that RF-Cp145 cooperates with Rb to mediate transcriptional repression, whereas HDAC1 does not (Fig. 2F). We find that RF-Cp145 has no effect on reporter activity in the absence of Rb (Fig. 2F, right panel). These results suggest that the effects of HDAC1 and RF-Cp145 on cell death are probably mediated via effects on Rb-mediated transcriptional repression.

HDAC1 Inhibits the Ability of Rb to Promote Cell Survival in a LXCXE Motif-dependent Manner—To further investigate the effect of HDAC1 on cell death, we compared the ability of wild type HDAC1 and its deacetylase mutant to abrogate Rb mediated inhibition of E2F-1-induced cell death in C33A cells. The removal of acetyl groups from the tails of histone octamers by HDAC1 facilitates condensation of nucleosomes into chromatin, which in turn blocks access of transcription factors, leading to gene repression. The three-dimensional crystal structure of the HDAC1 active site has been resolved (38). Based on these studies the H141A HDAC1 mutant (H141A) was shown to lack deacetylase activity (39). We find that both wild type HDAC1 and the H141A HDAC1 deacetylase mutant are equally effective at abrogating the ability of Rb to inhibit E2F-1-mediated cell death in a dose-dependent manner (Fig. 4A). The ability of HDAC1 to regulate Rb-mediated inhibition of E2F1-induced cell death is therefore not dependent on its catalytic activity.

We next determined whether the LXCXE motif in HDAC1 is required for HDAC1 to abrogate the ability of Rb to inhibit E2F1-induced apoptosis. An HDAC1 mutant in which the LXCXE motif has been deleted (termed HDAC1 ΔLXCXE mutant) has a dramatically reduced ability to bind Rb (7). We therefore compared the ability of wild type HDAC1 with the HDAC1 ΔLXCXE mutant to abrogate Rb-mediated inhibition of E2F-1-induced cell death. We find that although wild type HDAC1 abrogates the ability of Rb to inhibit E2F-1-induced cell death, the HDAC1 ΔLXCXE mutant does not (Fig. 4A).

We show that RF-Cp145 cooperates with Rb to inhibit E2F-1-induced cell death (Fig. 3). We therefore ascertained whether HDAC1 also abrogates the ability of Rb+RF-p145 to inhibit E2F-1-induced cell death. Indeed both wild type HDAC1 and the H141A HDAC1 mutant abrogate the ability of Rb+RF-
results suggest that both the LXCXE-binding site in Rb are required for inhibition of E2F-1-induced cell death.

The RF-C complex has been known to have ATPase activity that is stimulated in the presence of other co-factors like DNA (40–43). The ability to assemble recombinant RF-C subunits in vitro into active RF-C complexes has allowed a characterization of mutants that selectively affect ATPase activity of RF-C. The RF-Cp145 Lys597 residue, which maps to the conserved Walker A motif, is essential for the ATPase activity of the RF-C complex (41). The K657A RF-C p145 mutant has defective ATPase activity but is able to assemble into a five subunit RF-C complex (41). We therefore determined the ability of the K657A RF-Cp145 mutant to enhance Rb-mediated inhibition of E2F-1-induced cell death. We find that whereas wild type RF-Cp145 enhances the ability of Rb to inhibit E2F-1-induced cell death, the K657A RF-Cp145 mutant is unable to enhance the Rb-mediated inhibition of E2F-1-induced cell death (Fig. 5). The K657A RF-Cp145 mutant also does not inhibit E2F-1-induced cell death in the absence of Rb or in the presence of Rb N757F mutant (Fig. 5).

E2F-1/Rb Chimeric Protein Inhibits E2F-1- and UV-induced Cell Death—Rb does not only blocks transcriptional activation by E2F, but the Rb-E2F complex that forms at the promoter actively represses transcription (1, 3). It seems unlikely that Rb inhibits E2F-1-induced cell death by merely blocking transcriptional activation of E2F-1. Both wild type Rb and N757F mutant Rb block transcriptional activation by E2F (data not shown), but only wild type Rb and not the N757F Rb mutant inhibits E2F-1-induced cell death (Fig. 1). It has been previously shown that Rb mutants in which LXCXE binding is selectively inactivated, including the Rb N757F mutant, block E2F-1 transactivation similar to wild type Rb (26, 27, 44). If Rb inhibits E2F-1-induced cell death by blocking E2F-1 dependent transactivation, then the DNA-binding domain of E2F-1 (without a transactivation domain) should inhibit E2F-1 induced cell death. We find that the N-terminal E2F-1 (1–368) DNA-binding domain (E2F-1 DBD) is pro-apoptotic by itself as reported earlier (15, 45) and does not block E2F-1 induced cell death (Fig. 6A). This pro-apoptotic E2F-1 mutant, which lacks the transactivation domain, has been actually shown to inhibit Rb-mediated active repression (46).

We examined whether active repression by Rb-E2F complexes can explain Rb-mediated inhibition of E2F-1-induced cell death. Interestingly, Rb mutants that selectively do not bind LXCXE proteins have been shown to be unable to induce active repression (44). To directly test whether Rb inhibits cell death by active repression, we utilized a chimeric E2F-1/Rb protein that has previously been shown to inhibit E2F-1 transactivation by active repression (2, 32). The E2F/Rb chimera is comprised of an N-terminal E2F-1 DNA-binding domain (1–368) fused to the pocket domain of Rb (379–792). We find that although the DNA-binding domain of E2F-1 is strongly pro-apoptotic (Fig. 6A), the E2F-1/Rb chimera inhibits E2F-1 cell death in a dose-dependent manner (Fig. 6, A and B). In contrast, the E2F-1/Rb (N757F) mutant chimera in which the ability of Rb to bind LXCXE proteins is inactivated is unable to inhibit E2F-1-induced cell death (Fig. 6, A and B). These results suggest that a E2F-1/Rb complex can inhibit E2F-1-induced cell death by a mechanism indistinguishable from active repression.

To determine whether the ability of Rb to inhibit cell death induced by agents other than E2F-1 is also mediated by a similar mechanism, we tested the ability of the E2F-1/Rb chimera to inhibit UV-induced cell death. The E2F-1/Rb chimera inhibits UV-induced cell death in a dose-dependent manner (Fig. 6B), whereas the mutant E2F-1/Rb N757F chimera is
All of the cells were transfected with a
into a five-subunit complex were tested.
Cp145 K657A) but is able to assemble
reconstitute its ATPase activity (RF-
RF-Cp145 mutant that cannot selectively
inhibition of E2F-1-induced cell death.
are unable to enhance the Rb-mediated
X
G
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C33A cells were co-
transfected with E2F-1 (500 ng) and 500
ng of either control vector or wild type Rb
or the N757F Rb mutant. The effect of
increasing concentrations (125, 250, and
500 ng) of wild type RF-Cp145, RF-Cp145
in which the LXCXE motif has been mu-
tated to LXXGK (RF-Cp145 LXGXK), and
RF-Cp145 mutant that cannot selectively
assemble into a five-subunit complex were tested.
All of the cells were transfected with a
β-galactosidase plasmid (200 ng). The res-
results from three experiments are shown.
Although wild type RF-Cp145 enhances
the ability of Rb to inhibit E2F-1-induced
cell death, both the RF-Cp145 K657A mut-
ant and the RF-Cp145 LXGXK mutant are unable to enhance the Rb-mediated
inhibition of E2F-1-induced cell death.

Fig. 5. The ability of RF-Cp145 to
enhance Rb-mediated inhibition of
E2F-1-induced cell death is depend-
ton on an intact LXCXE motif in RF-
Cp145, an intact LXCXE-binding site
in Rb and ATPase activity of the RF-
Cp145 complex. C33A cells were co-
transfected with E2F-1 (500 ng) and 500
ng of either control vector or wild type Rb
or the N757F Rb mutant. The effect of
increasing concentrations (125, 250, and
500 ng) of wild type RF-Cp145, RF-Cp145
in which the LXCXE motif has been mu-
tated to LXXGK (RF-Cp145 LXGXK), and
RF-Cp145 mutant that cannot selectively
reconstitute its ATPase activity (RF-
Cp145 K657A) but is able to assemble
into a five-subunit complex were tested.
All of the cells were transfected with a
β-galactosidase plasmid (200 ng). The res-
results from three experiments are shown.
Although wild type RF-Cp145 enhances
the ability of Rb to inhibit E2F-1-induced
cell death, both the RF-Cp145 K657A mut-
ant and the RF-Cp145 LXGXK mutant are unable to enhance the Rb-mediated
inhibition of E2F-1-induced cell death.

The DNA-

binding domain of E2F-1 as expected is strongly pro-apoptotic
by itself and does not inhibit UV-induced cell death (data not
shown). Thus, E2F-1/Rb complex can inhibit UV- and E2F-1-
induced cell death by active repression.

**DISCUSSION**

In this paper we show that the LXCXE-binding site in Rb is
critical for its ability to inhibit E2F-1-induced cell death. This
result suggests that proteins that bind the LXCXE-binding site
in Rb would regulate the inhibition of E2F-1-induced cell death
by Rb. In this direction we tested the effect of HDAC1 on the
anti-apoptotic activity of Rb. We find that HDAC1 does not
cooperate with Rb to inhibit E2F-1-induced cell death. In fact,
HDAC1 abrogated the inhibition of E2F-1-induced cell death by
Rb. The deacetylase activity of HDAC1 is not required for this
effect. Instead the LXCXE motif in HDAC1 is essential to
abrogate the inhibition of E2F-1-induced cell death. This is in
contrast to HDAC1, another LXCXE protein RF-Cp145, the
large subunit of RF-C, cooperates with Rb to inhibit E2F-1-
induced cell death. The ability of RF-Cp145 to enhance Rb-
mediated inhibition of E2F-1-induced cell death is dependent
on a LXCXE motif in RF-Cp145 and the LXCXE-binding site
in Rb (this paper) as previously demonstrated for UV-induced
cell death (14). Transfection with Rb+RF-Cp145 increased
~7-fold the number of surviving colonies as compared with
control vector transfected cells in long term (7 days) survival
assays. Rb by itself increased surviving colonies by 2-fold.
RF-Cp145 therefore does not just delay but actually inhibits
cell death in a Rb-dependent manner (Supplementary Figure I).

E2F-1-induced cell death has been suggested to act either via
a p53-dependent or p53-independent pathway (30). p73 is tran-
scriptionally activated by E2F-1 and is capable of inducing cell
death independent of p53 status (31). Our data show that Rb
rescues cells from E2F-1-induced cell death but not p73-in-
duced cell death. This result is consistent with a conclusion
that Rb acts upstream of p73 in the p53-independent pathway
of E2F-1-induced cell death.

It seems that the ability of RF-Cp145 and HDAC1 to coop-
erate with Rb to inhibit E2F-1-induced cell death reflects their
effects on Rb-mediated transcriptional repression. This is sup-
ported by our observation that RF-Cp145 enhances Rb medi-
ated repression on the p73 promoter, whereas HDAC1 does not.
This is especially relevant because E2F-1-induced cell death is
p73-dependent.

Rb blocks transcriptional activation not only by E2F but by
the Rb-E2F complex that forms at the promoter actively re-
presses transcription. Rb could inhibit E2F-1-induced apo-
tosis by blocking transcriptional activation by E2F or by the
formation of an active Rb-E2F repressor complex. It appears
unlikely that Rb inhibits E2F-1-induced cell death by blocking
transcriptional activation of E2F-1. Rb mutants defective in
binding LXCXE-containing proteins are unable to inhibit E2F-
1-induced cell death, although such mutants block transcrip-
tional activation by E2F (26, 27, 44) (data not shown). Rb has
been shown to transactivate in some instances. MyoD1, NF-
IL6, CCAAT/enhancer-binding protein, and glucocorticoid re-
ceptor are examples of transcription factors that activate pro-
moters in the presence of Rb (47–51). Because both wild type
Rb and Rb N757F mutant cooperate with MyoD1 to transacti-
vate the MCK promoter (26), it is unlikely that transactivation
by Rb explains its ability to inhibit E2F-1-induced cell death.

We therefore tested whether active repression by Rb-E2F
complexes could explain Rb-mediated inhibition of E2F-1-in-
duced cell death. Indeed, we find that an E2F-1/Rb chimera
that inhibits E2F-1 transactivation by active repression is able
to inhibit E2F-1-induced cell death but only when the LXCXE-
binding site is intact. The mutant E2F-1/Rb (N757F) chimera is
unable to inhibit E2F-1-induced cell death. Our conclusion
that Rb-mediated active repression promotes cell survival explains
why an E2F-1 mutant lacking the transactivation domain
inhibits Rb-mediated active repression (46) and is pro-apoptotic
(15, 45).

We find that Rb-mediated active repression inhibits apo-
tosis induced by other agents. We have previously shown
that cell death induced by DNA-damaging agents like UV, γ-
radiation, and cisplatin is inhibited by Rb but not by Rb mutants
with a selective inability to bind LXCXE proteins (14). We find
that HDAC1 also abrogates the ability of Rb+RF-Cp145 to
promote cell survival after UV (Supplementary Figure II). We
also show that the E2F-1 DNA-binding domain-Rb chimera inhibits UV-induced cell death, whereas the mutant E2F-1/Rb chimera in which the LXCXE-binding site is mutated does not. Active repression of transcription by Rb-E2F complexes plays a critical role in the anti-apoptotic activity of Rb in general.

A number of studies have demonstrated the importance of HDAC1 in active repression by Rb (4, 44). In this paper we show that HDAC1 is not required for Rb-mediated inhibition E2F-1-induced cell death. Our results suggest a model in which Rb-E2F-1 complex-mediated active repression inhibits E2F-induced apoptosis without recruiting HDAC1. We also find that other HDACs like HDAC2, HDAC3, and HDAC4 do not influence Rb-mediated inhibition of E2F-induced cell death (data not shown). Interestingly, active repression by Rb targets two types of promoters (6). Rb-E2F complexes exert active repression in an HDAC-dependent manner on promoters such as the adenovirus major late promoter, whereas Rb-mediated active repression of thymidine kinase promoter and the SV40 enhancer is HDAC-independent (6). This disparity among promoters inhibited by active repression is probably dependent on the transcription factor targeted by the Rb-E2F complexes. The repression of upstream stimulatory factor is dependent on HDAC activity, whereas PU.1 and p65 (NFκB) are repressed by Rb in an HDAC-independent manner (6). Additionally, Rb-mediated active repression in vitro seems to be HDAC1-independent (52). A number of other co-repressors have been implicated in transcriptional repression by Rb (8, 53, 54). Based on our results it is tempting to speculate that Rb-mediated inhibition of cell death by active repression may involve such co-repressors.

We have demonstrated that RF-Cp145 cooperates with Rb-mediated inhibition of E2F-1- (this paper), UV-, ionizing radiation-, and cisplatin-induced (14) cell death. How is the cooperativity between Rb and RF-Cp145 mediated? Our demonstration that Rb/E2F-1-mediated active repression underlies the anti-apoptotic activity of Rb raises the possibility that RF-Cp145 may have some repressor like activity. Consistent with this possibility, RF-Cp145 has been demonstrated to

![Figure 6](http://www.jbc.org/)

**Fig. 6.** E2F-1/Rb chimera inhibits E2F-1- and UV-induced cell death. A, C33A (Rb−/−) cells were co-transfected with E2F-1 (500 ng) and 500 ng of either E2F-1 DNA-binding domain, E2F-1-DBD(1–368), wild type E2F-1(1–368)/Rb(379–792) chimeric protein, or the mutant E2F-1(1–368)/Rb(379–792) N757F chimeric protein. To monitor cell survival all of the cells were transfected with a β-galactosidase plasmid (200 ng). The number of β-galactosidase-positive cells/field are shown as means ± standard deviation from five separate experiments. In all cases >250 fields were counted. B, C33A cells were co-transfected with E2F-1 (500 ng) and increasing amounts (250, 500, and 750 ng) of either wild type E2F-1(1–368)/Rb(379–792) chimeric protein or the mutant E2F-1(1–368)/Rb(379–792) N757F chimeric protein. To monitor cell survival all of the cells were transfected with a β-galactosidase plasmid (200 ng). The results are shown as the means ± standard deviation from five separate experiments. C, protein expression by various vectors used in A and B was determined by immunoblotting lysates from transfected cells with HA-specific antibody to detect E2F-1/Rb chimeric protein expression or with E2F-1-specific antibody. D, C33A cells were co-transfected with increasing amounts (250, 500, and 1000 ng) of either wild type E2F-1(1–368)/Rb(379–792) chimeric protein or the N757F mutant E2F-1(1–368)/Rb(379–792) chimeric protein and 200 ng of a β-galactosidase plasmid. The cells were UV-irradiated (20 J/m²) 36 h after transfection, and cell survival was determined at 17 h after UV irradiation. The results are shown as the means ± standard deviation from five separate experiments.
contain transcriptional repressor domains (55) like those found in Rb. It may be of further value to note that both RF-C (56) and Rb (45) regulate the anti-apoptotic NFκB signaling pathway. Finally, we show that an RF-Cp145 mutant that lacks ATPase activity (41) is unable to enhance the Rb-mediated inhibition of E2F-1-induced cell death. ATPases are essential components of chromatin-modifying protein complexes that regulate Rb activity. The challenge for the future is to determine which biochemical characteristic of RF-C contributes to its ability to cooperate with Rb in inhibiting cell death.

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REFERENCES
1. Weintraub, S. J., Prater, C. A., and Dean, D. C. (1992) Nature 358, 259–261
2. Sellers, W. R., Rodgers, J. W., and Kaelin, W. G., Jr. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11544–11548
3. Weintraub, S. J., Chow, K. S., Luo, R. X., Zhang, S. H., Se, S., and Dean, D. C. (1995) Nature 375, 812–815
4. Zhang, H. S., Gavin, M., Dahiya, A., Postigo, A. A., Ma, D., Luo, R. X., Harbour, J. W., and Dean, D. C. (2000) Cell 101, 79–89
5. Brenham, A., Miska, E. A., McManus, D. J., Reid, J. L., Bannister, A. J., and Kouzarides, T. (1998) Nature 391, 597–603
6. Luo, R. X., Postigo, A. A., and Dean, D. C. (1998) Cell 92, 463–473
7. Magnaghi-Jaulin, L., Grosman, R., Naguibneva, I., Robin, P., Lörain, S., Le Villain, J. P., Troalen, F., Trouche, D., and Harel-Bellan, A. (1998) Nature 391, 601–605
8. Lai, A., Lee, J. M., Yang, W. M., DeCaprio, J. A., Kaelin, W. G., Jr., Seto, E., and Branton, P. E. (1999) Mol. Cell. Biol. 19, 6635–6641
9. Lai, A., Kennedy, B. K., Barbé, D. A., Bertos, N. R., Yang, X. J., and Sancar, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 852–857
10. Knudsen, E. S., Buckmaster, C., Chen, T. T., Feramisco, J. R., and Wang, J. Y. (1995) EMBO J. 14, 461–472
11. Poulsen, C., Schiødt, C., Martin, K., and Lu, X. (1999) Genes Dev. 13, 2227–2232
12. Niculescu, A. B., Ilii, C., Chen, X., Smetas, M., Hengst, L., Prives, C., and Reed, S. I. (1998) Mol. Cell. Biol. 18, 629–643
13. Weinberg, R. A. (1995) Cell 81, 323–330
14. Haas-Kogan, D. A., Kogan, S. C., Levi, D., Dazin, P., Tsai, K. Y., Jacks, T., Vousden, K. H., and Kaelin, W. G., Jr. (2000) Nature 407, 645–648
15. Lai, A., Kennedy, B. K., Barbé, D. A., Bertos, N. R., Yang, X. J., and Sancar, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3519–3524
16. Cai, J., Gibbs, E., Uhmann, F., Phillips, B., Yao, N., O’Dell, M., and Hurwitz, J. (1998) J. Biol. Chem. 273, 18974–18981
17. Chen, P. L., Riley, D. J., Chen, Y., and Lee, W. H. (1996) Genes Dev. 10, 2794–2804
18. Cai, J., Yao, N., Gibbs, E., Finkelstein, J., Phillips, B., O’Dell, M., and Hurwitz, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11607–11612
19. Podust, V. N., Twari, N., Ott, R., and Fanning, E. (1998) J. Biol. Chem. 273, 12835–12842
20. Ellisson, V., and Stillman, B. (1998) J. Biol. Chem. 273, 5979–5987
21. Dahiya, A., Gavin, M., Luo, R. X., and Dean, D. C. (2000) Mol. Cell. Biol. 20, 789–800
22. Phillips, A. C., Ernst, M. K., Bates, S., Rice, N. R., and Vousden, K. H. (1999) Mol. Cell. Biol. 19, 771–781
23. Gu, W., Schneider, J. D., Condorelli, G., Kauhal, S., Mahdavi, V., and Nadal-Ginard, B. (1999) Cell 92, 309–324
24. Singh, P., Cao, J., and Hong, W. (1995) Nature 374, 562–565
25. Cai, J., Yao, N., Gibbs, E., Finkelstein, J., Phillips, B., O’Dell, M., and Hurwitz, J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11607–11612
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