E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction

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E2F1 overexpression has been shown to induce apoptosis in cooperation with p53. Using Saos-2 cells, which are null for p53 and lack functional Rb, we have demonstrated that E2F1 overexpression can also induce apoptosis in the absence of p53 and retinoblastoma protein (Rb). E2F1-induced apoptosis can be specifically inhibited by Rb but not mdm2, which is known for its ability to inhibit p53-induced apoptosis. Through the study of the apoptotic function of a set of E2F1 mutants, it was clear that the transactivation and the apoptotic function of E2F1 are uncoupled. The transactivation-defective E2F1 mutants E2F1(1–374), E2F1(390–1)DF(Δ mdm2), and E2F1(406–415)(Δ Rb) can induce apoptosis as effectively as wild-type E2F1. In contrast to E2F1 transactivation, the DNA-binding activity of E2F1 was proven to be essential for its apoptotic function, as the DNA-binding-defective mutants E2F1(132) and E2F1(132)(1–374) failed to induce apoptosis. Therefore Rb may inhibit E2F1-induced apoptosis by mechanisms other than the suppression of the transactivation of E2F1. This hypothesis was supported by our observation that although Rb overexpression can specifically repress the apoptosis induced by wild-type E2F1 and a Rb-binding-competent E2F1 mutant E2F1(390–1)DF(Δ mdm2), it failed to inhibit the apoptosis induced by mutants E2F1(1–374) and E2F1(Δ 406–415)(Δ Rb), which are defective or reduced in Rb binding and transactivation. All of these points argue for a novel function for E2F1 and Rb in controlling apoptosis. The results also indicate that transcriptional repression rather than the transactivation function of E2F1 may be involved in its apoptotic function. The results presented here may provide us some physiological implication of the repression function of the Rb–E2F1 complex.

[**Key Words:** Apoptosis; E2F1 overexpression; p53; Rb; transactivation]

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The E2F transcription factor is a key regulator of cell cycle progression. Increasing E2F transcriptional activity by introducing exogenous E2F1 protein or an expression plasmid is sufficient to drive quiescent cells into the S-phase of the cell cycle (Johnson et al. 1993). Cooperation with *ras* oncogene to transform rat embryo fibroblast cells (REFs) and generate tumors in nude mice provided experimental evidence that E2F1 can act as a oncogene (Johnson et al. 1994; Singh et al. 1994). However, the high incidence of a variety of tumors that occur in E2F1 knockout mice argues strongly that E2F1 is also a tumor suppressor (Field et al. 1996; Yamasaki et al. 1996). Nothing is known about the mechanisms through which E2F1 suppresses tumour growth.

E2F transcription factor activity consists of a heterodimer between a member of the E2F family and a mem-

ber of the DP family. Five members of the E2F family have been identified (E2F1, E2F2, E2F3, E2F4, and E2F5) (Lam and La Thangue 1994; Hijmans et al. 1995; Sardet et al. 1995), and two members of the DP family have also been isolated (DP1 and DP2) (Lam and La Thangue 1994; Zhang and Chellappan 1995). It is generally believed that E2F family proteins, but not DP, can bind to DNA with sequence specificity as homodimers. However, a significant increase in DNA-binding activity can be achieved when an E2F family member heterodimerizes with a DP family member. This stimulation of DNA-binding activity is reflected in the increase in E2F-dependent transcription when E2F is cotransfected with DP1 (Lam and La Thangue 1994; Wu et al. 1995). In addition to association with DP family proteins, the transcriptional activity of E2F can be regulated in vivo by members of pocket binding proteins, including retinoblastoma (Rb) and Rb-related proteins p107 and p130. Direct protein–protein interactions between members of the E2F family and Rb or Rb-related proteins result in down-regulation

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of E2F-dependent transactivation. Binding to E2F and resultant repression of E2F-dependent transcription is thought to be the pathway through which Rb or the other members of the Rb-related protein negatively regulate cell cycle progression in mammalian cells. It is generally believed that E2F1, E2F2, and E2F3 favor binding with Rb while E2F4 and E2F5 tend to bind to p107 or p130, respectively. In contrast to Rb, the transactivation function of E2F1 can be stimulated through its binding to the oncoprotein mdm2 (Martin et al. 1995).

It has been shown recently that E2F1 overexpression can induce apoptosis in cooperation with p53 (Qin et al. 1994; Wu and Levine 1994; Kowalik et al. 1995). Thus, the ability to induce apoptosis may be responsible for the tumor suppressor function of E2F1. To understand the role E2F1 plays in tumor development, it is essential for us to know how E2F1 induces apoptosis. It has been recently reported that an increase in DNA binding is accompanied by a dramatic increase in apoptosis in cells overexpressing E2F1/DP1 or a cyclin A-binding-defective mutant of E2F1, E2FΔ24 (Hiebert et al. 1995; Krek et al. 1995; Shan et al. 1996b). The increase in apoptosis seen with E2F1/DP1 and the cyclin A-binding-defective mutant E2FΔ24 was interpreted as the result of an increase in the transcriptional activity of E2F1. The transactivation function of E2F1 has therefore been thought to be crucial for its apoptotic function. Nevertheless, the mechanisms through which E2F1 induces apoptosis remain unclear.

Recent studies showed that p53-induced apoptosis can be specifically inhibited by mdm2 through direct protein-protein interaction (Haupt et al. 1996). It has also been shown that Rb can inhibit apoptosis induced by various agents including γ radiation, transformation growth factor β 1 (TGF- β 1) and γ -interferon (IFN- γ) (Haas-Kogan et al. 1995; Berry et al. 1996; Fan et al. 1996). Rb is known for its ability to interact with E2F1 and negatively regulate its transactivation function. Thus, it was thought that Rb may inhibit E2F1-mediated apoptosis through its inhibition on the transcriptional activity of E2F1 (Qin et al. 1994). Such a hypothesis was used to explain the observation that increased apoptosis was seen in the cells expressing a transactivation-competent and Rb-binding-defective E2F1 mutant (Shan et al. 1996a).

To understand how E2F1 induces apoptosis and through which mechanisms Rb inhibits E2F1-induced apoptosis, we investigated the apoptotic function of a number of E2F1 mutants in Saos-2 cells which are null for p53 and lack functional Rb. The ability of Rb to inhibit the apoptosis induced by wild-type E2F1 and its mutants was also studied.

Results

E2F1 can induce apoptosis independent of p53 and is inhibited by Rb but not mdm2

We used Saos-2 cells to address the issue of whether E2F1 can induce apoptosis independently of p53. Saos-2

cells are null for p53 and also express a carboxy-terminal truncated Rb that is defective for E2F binding (Shaw and Tegtmeyer 1981). Thus Saos-2 cells are also ideal for investigating the effects that Rb may have on the apoptotic function of E2F1 and its mutants. When an E2F1 expression plasmid (pCMV-E2F1) was introduced into Saos-2 cells, DNA fragmentation typical of apoptotic cells was seen in the cells transfected with the E2F1 expression plasmid but not with the control vector (Fig. 1A). The ability to induce apoptosis by E2F1 in transiently transfected Saos-2 cells was also confirmed by immunofluorescence labeling of the cells expressing exogenous E2F1 in conjunction with DAPI staining to identify apoptotic cells with condensed nuclei (data not shown). The apoptotic cells in the transfected Saos-2 cell population were also quantitated using flow cytometry analysis of propidium iodide-labeled cells. There was a three- to fourfold increase in apoptotic cells in E2F1-transfected Saos-2 cells compared to cells transfected with a control vector (Fig. 1B-D). Similar results were obtained using time-lapse microscopy to measure apoptosis (data not shown).

Existing data imply that E2F1 transcriptional activity may be important for its apoptotic function (Shan and Lee 1994; Krek et al. 1995; Shan et al. 1996a,b). If so, Rb would inhibit E2F1-induced apoptosis by suppressing its transactivation function. In contrast, mdm2 should promote E2F1-induced apoptosis, as it has been shown to stimulate the transactivation of E2F1 (Martin et al. 1995). The effects of Rb and mdm2 were investigated by introducing wild-type Rb or mdm2 into Saos-2 cells together with E2F1. Interestingly E2F1-induced apoptosis was specifically suppressed by the coexpression of Rb but not mdm2 (Fig. 2A,B). This is in contrast to observations with p53 under the same conditions. p53-induced apoptosis was significantly inhibited by the coexpression of mdm2 but not Rb (Fig. 2C,D). DNA fragmentation data also confirm the FACS results that E2F1-induced apoptosis can be specifically inhibited by Rb (Fig. 2E). The specificity of Rb to inhibit apoptosis induced by E2F1 but not by p53 in the transfected Saos-2 cells is not attributable to a lack of Rb expression. The difference between the expression level of transfected Rb detected in E2F1 + Rb and p53+ Rb cotransfected cells was very small (Fig. 3A). Failure to show any effect of mdm2 on E2F1-induced apoptosis was also not attributable to the lack of protein expression, as similar levels of exogenous mdm2 expression were detected in E2F1 + mdm2- and p53 + mdm2-transfected cells (Fig. 3B).

The fact that E2F1-induced apoptosis was only inhibited by coexpression of Rb but not mdm2 argues that the inhibition is specific. Furthermore, inhibition of E2F1-induced apoptosis by Rb is not attributable to a simple repression of E2F1 expression, as in Rb and E2F1 cotransfected cells, the expression level of E2F1 is always higher than that seen in cells transfected with E2F1 alone (Fig. 3C). The increase in the level of E2F1 when coexpressed with Rb suggests that Rb might induce E2F1 expression through its ability to protect E2F1 from ubiquitin degradation (Hateboer et al. 1996; Hofmann et al. 1996). It also

Figure 1. Overexpression of E2F1 can induce apoptosis in Saos-2 cells independent of p53. DNA fragmentation (A) and FACS analysis (B–D) of E2F1 and control plasmid-transfected Saos-2 cells (VECTOR). For FACS analysis, transfected cells were gated based on the expression of CD20 (B). Apoptosis was measured by the accumulation of cells with a sub-G₁ DNA content, and they are in the region labeled as M1 (C). The graph (D) represents the percentage of cells with sub-G₁ DNA content (apoptotic cells) in transiently transfected Saos-2 cells expressing wild-type E2F1 or control plasmid. The mean values were derived from four independent experiments.

indicates that the inhibition of E2F1-induced apoptosis by Rb would be underestimated in the experimental system used in this study. In contrast to E2F1, coexpressing Rb did not significantly alter the expression level of p53 (Fig. 3D).

The DNA binding but not the transactivation function of E2F1 is required for E2F1-induced apoptosis

The absence of any effect of mdm2 on E2F1-induced apoptosis suggested that the transcriptional activity of E2F1 might be separable from its apoptotic function. To understand which E2F1 functions are required to induce apoptosis, we tested the apoptotic function of some E2F1 mutants, E2F1(132), E2F1(1–374), E2F1(132)(1–374),

E2F1(390–1)DF(Δmdm2), and E2F1(Δ406–415)(ΔRb) (Fig. 4A). E2F1(132) is a point mutant of E2F1 that is defective for DNA binding. E2F1(1–374) is a carboxy-terminal truncation mutant of E2F1; it lacks the Rb-binding region as well as part of the transactivation domain. E2F1(Δ406–415)(ΔRb) is an internal deletion mutant with reduced transactivation function and Rb binding. E2F1(390–1)DF(Δmdm2) is a double point mutant at the residues 390 and 391, which are critical for mdm2 binding (Martin et al. 1995). It has reduced transactivation function and it is also defective for mdm2 binding; however, this mutant is competent for Rb binding. As shown in Figure 4B, all of the mutants used here are defective or retarded for their transactivation function. Apart from the DNA-binding-defective mutant E2F1(132), the rest of

Figure 2. Effect of Rb or mdm2 on E2F1 or p53-induced apoptosis in Saos-2 cells. A representative FACS analysis (A) and percentage of cells with sub-G₁ DNA content (apoptotic cells) (B) to demonstrate that Rb but not mdm2 can specifically inhibit E2F1-induced apoptosis in transiently transfected Saos-2 cells. C and D demonstrated that under the same conditions, mdm2 but not Rb can specifically inhibit p53-induced apoptosis. (A,C) The apoptotic effect of control, Rb, and mdm2 expression plasmids in Saos-2 cells is shown in the left panel and labeled VECTOR, Rb, and mdm2 respectively. The effect of Rb and mdm2 on E2F1- or p53-induced apoptosis (A and C, respectively) is shown in the right panel and labeled E2F1, E2F1 + Rb, and E2F1 + mdm2 (A) or p53, p53 + Rb, and p53 + mdm2 (C), respectively. Apoptosis is measured by the accumulation of cells with a sub-G₁ DNA content, and they are in the region labeled M1. The graphs (B,D) represent the mean of two to four independent experiments showing the percentage of cells with sub-G₁ DNA content (apoptotic cells) in transiently transfected Saos-2 cells, using the same group of plasmids as those in A or C, respectively. DNA fragmentation (E) also show that Rb can inhibit E2F1 but not p53 induced apoptosis in transfected Saos-2 cells.

Figure 3. Immunoblotting to show the expression level of Rb (A), mdm2 (B), E2F1 (C), or p53 (D) in transfected Saos-2 cells. The plasmids, which were cotransfected with Rb, mdm2, E2F1, or p53, are indicated. Rb protein was detected with an anti-Rb specific rabbit polyclonal antibody C-15 from Santa Cruz (A). mdm2 protein was detected with a monoclonal antibody SMP14 (B). The transfected E2F1 or p53 protein was detected with an anti-E2F1-specific rabbit polyclonal antibody (C-20) from Santa Cruz (C) or anti-p53-specific monoclonal antibody DO.1 (D). The positions of Rb, mdm2, E2F1, and p53 protein are indicated by arrows.

the transactivation-impaired E2F1 mutants, E2F1(1–374), E2F1(390–1)DF (Δ mdm2), and E2F1 Δ 406–415(Δ Rb) have all been shown previously to be active for DNA binding. Using DNA fragmentation and FACS analysis to detect apoptosis, it was clear that the DNA-binding-defective mutant E2F1(132) was incapable of inducing apoptosis in transfected Saos-2 cells (Fig. 5A). Interestingly, the transcriptionally impaired E2F1 mutants, E2F1(390–1)DF(Δ mdm2) and E2F1(Δ 406–415)(Δ Rb) were both competent to induce apoptosis (Fig. 5B,C). Their ability to induce apoptosis is at least as good as that of the wild-type E2F1 and the amount of protein expressed between E2F1 and E2F1(390–1)DF(Δ mdm2) and E2F1(Δ 406–415)(Δ Rb) was very similar (Fig. 5D). It was also of great interest to us that the transactivation-defective mutant E2F1(1–374) also caused apoptosis very efficiently (Fig. 5).

Based on the FACS analysis shown in Figure 5, it appeared that the amount of apoptotic cells seen in E2F1(1–374)-transfected Saos-2 cells was higher than that seen in cells transfected with wild-type E2F1, E2F1(390–1)DF(Δ mdm2) or E2F1(Δ 406–415)(Δ Rb). For E2F1(1–374), 63 amino acids from the carboxy-terminal part of E2F1 has been removed. To investigate further whether there

is a difference in the ability to induce apoptosis by E2F1 and its mutant E2F1(1–374) in a longer term, we performed a colony assay. Based on the observation that overexpression of E2F1 in Saos-2 cells did not arrest cells; thus, it was anticipated that the suppression of colony numbers caused by the overexpression of E2F1 could reflect its apoptotic potential. In contrast to E2F1, the overexpression of mutant E2F1(1–374), could cause G₁ arrest (Phillips et al., this issue) as well as apoptosis. Therefore, if the mutant E2F1(1–374) is more active in inducing cell death than wild-type E2F1, one would expect to see fewer colonies derived from Saos-2 cells transfected with E2F1(1–374) than from those transfected with wild-type E2F1. However, the results shown in Figure 6 indicates this is not the case. Although a significant reduction in the number of colonies was seen in both wild-type E2F1- and E2F1(1–374)-transfected Saos-2 cells, but the number of colonies seen in E2F1(1–374)-transfected cells was similar to or slightly higher than that seen in cells transfected with wild-type E2F1 (Fig. 6). A reduced colony number was seen with apoptotic-defective mutant of E2F1, E2F1(132), and this may be attributable to disruption of endogenous E2F1/DP1 complexes and resulting cell cycle arrest (data not shown; Phillips et al., this issue). This phenomenon was seen consistently in two independent experiments. The levels of protein expression for E2F1 and the E2F1 mutants in the transfected cells before selection for stable colonies were similar (data not shown). These results suggest that the mutant E2F1(1–374) is not necessarily more active than that of the wild-type E2F1.

Failure to induce apoptosis by DNA-binding-defective mutant E2F1 has previously been interpreted as a result of a lack of transcriptional activity (Krek et al. 1995). Inability of the DNA-binding-defective mutant E2F1(132) to induce apoptosis is not attributable to a lack of protein expression, as the amount of E2F1 expressed is similar between wild-type E2F1 and E2F1(132) (Fig. 7). The ability of the transactivation-defective or -impaired mutants, E2F1(1–374) E2F1(390–1)DF(Δ mdm2), and E2F1(Δ 406–415)(Δ Rb) to induce apoptosis indicates that the transactivation and the apoptotic function of E2F1 can be uncoupled. Thus, for the first time, it allowed investigation of the role DNA binding rather than transactivation of E2F1 may have in E2F1-induced apoptosis. To test this, we constructed a double mutant of E2F1, E2F1(132)(1–374) that is defective for DNA binding, Rb binding, and transactivation. As shown in Figure 7 (A and B) unlike E2F1(1–374), the double mutant E2F1(132)(1–374) did not induce apoptosis; this was not due to a lack of protein expression of the double mutant (Fig. 7C). These results clearly demonstrated that in contrast to E2F1 transactivation, the E2F1 DNA-binding activity is essential for its apoptotic function.

Rb inhibits E2F1-induced apoptosis through direct binding but not repression of E2F1 transactivation

Because the E2F1 mutant E2F1(1–374) can induce apoptosis without its transactivation function, it is unlikely

Figure 4. A diagram of E2F1 mutants used in this study (A) and a graph to represent their ability to transactivate the E2F1 reporter, 3xwt-luc (B). The results shown in B were derived from two independent experiments, and the transfection efficiency has been normalized using an independent reporter plasmid, CMV-β-gal. Wild-type E2F1 is labeled as E2F1. E2F1(132) is a point mutant of E2F1 defective for DNA binding. E2F1(1-374) is a carboxy-terminal 63 amino acid-truncated E2F1 mutant. It lacks the Rb-binding domain and part of the transactivation domain, and is competent for DNA binding. E2F1(132)(1-374) is a double mutant of E2F1 that is defective for DNA binding, Rb binding, and transcription. E2F1(390-1)DF(Δmdm2) is a double point mutant; residues 390 and 391 were mutated from AA to DF, and it is defective for mdm2 binding. E2F1(Δ406-415)(ΔRb) is an internal deletion mutant; residues 406-415 were deleted. This mutant has much reduced Rb-binding activity. The known functional domains of E2F1 are indicated in Figure A, with the residue numbers in parenthesis (below).

that the inhibition of E2F1-induced apoptosis by Rb occurs through its suppression of E2F1 transactivation. So, through what mechanism might Rb inhibit the apoptotic function of E2F1? In Saos-2 cells, the carboxyl terminus of Rb is deleted and this may be responsible for the sensitivity of Saos-2 cells to radiation (Haas-Kogan et al. 1995) and E2F1-induced apoptosis, as E2F1-induced apoptosis is inhibited by wild-type Rb. Interestingly, also, the carboxy-terminal part of Rb interacts with E2F1 and suppresses its transactivation. This suggests that the carboxy-terminal part of Rb may have an important role in the inhibition of E2F1-induced apoptosis. If the direct binding to E2F1 is crucial for Rb to inhibit E2F1-induced apoptosis, Rb should not be able to inhibit the apoptosis induced by the E2F1 mutants that lack the Rb-binding domain. On the other hand, if the inhibition of E2F1-induced apoptosis is attributable to the cellular changes induced by Rb expression, Rb should inhibit the apoptosis induced by E2F1 and all the mutants regardless of their Rb-binding ability. To test this hypothesis, the effect of Rb on E2F1 and its Rb-binding-defective mutants, E2F1(Δ406-415)(ΔRb) and E2F1(1-374), was investigated using FACS and DNA fragmentation assays. As shown in Figure 8, Rb can only specifically inhibit apoptosis induced by wild-type E2F1 and E2F1(390-1)DF(Δmdm2), which are competent in Rb binding. In contrast, coexpression of Rb failed to inhibit the apoptosis induced by Rb-binding-defective mutants E2F1(Δ406-415)(ΔRb) and E2F1(1-374) (Fig. 8A-C). The inability to inhibit

E2F1(Δ406-415)(ΔRb)- and E2F1(1-374)-induced apoptosis was not because of a lack of Rb expression, as similar amounts of Rb protein were expressed in E2F1 + Rb, E2F1(1-374) + Rb and E2F1(Δ406-415)(ΔRb) + Rb-transfected cells (Fig. 8D; data not shown). As Rb inhibited wild-type E2F1 and E2F1(390-1)DF(Δmdm2) but not E2F1(Δ406-415)(ΔRb)- and E2F1(1-374)-induced apoptosis, this inhibition was probably not caused by cellular changes induced by Rb. These results argue that the direct binding of Rb to E2F1 is required to inhibit the apoptosis induced by E2F1. They also indicate that the suppression of the transcriptional activity of E2F1 is not the mechanism through which Rb inhibits the apoptotic function of E2F1.

Discussion

E2F1 can induce apoptosis independently of p53

Induction of apoptosis may be one of the mechanisms through which E2F1 functions as a tumor suppressor. Previous results suggest that E2F1 and p53 cooperate to mediate apoptosis. However, by introducing E2F1 into Saos-2 cells, we were able to provide direct evidence that E2F1 overexpression can induce apoptosis independently of p53. Such apoptosis can also be inhibited by Rb specifically. What is the physiological relevance of E2F1-induced p53-independent apoptosis? In many tumors, Rb mutations are accompanied by mutations in p53. Thus,

Figure 5. Transactivation-defective E2F1 mutants are competent to induce apoptosis in Saos-2 cells. DNA fragmentation (A), a representative FACS analysis (B), and percentage of cells with sub- G_1 DNA contents (apoptotic cells) (C) to show the apoptotic function of wild-type E2F1 and its mutants in transfected Saos-2 cells. The DNA-binding-defective mutant E2F1(132) is inactive in induction of apoptosis, but the transactivation-defective mutant E2F1(1-374) induces apoptosis. In B and C, the apoptosis was measured by the accumulation of cells with a sub- G_1 DNA content, and they are in the region labeled M1. (The mutants are labeled as in Fig. 4A). The graph represents the mean of two to four independent experiments showing the percentage of cells with sub- G_1 DNA content (apoptotic cells) in transiently transfected Saos-2 cells, using the same group of plasmids as in B. The expression level of E2F1 and its mutants is shown in D.

the results obtained in Saos-2 cells could provide some physiological implications.

It has been reported that Saos-2 cells are sensitive to γ -radiation. Interestingly, the apoptosis induced by γ -radiation in Saos-2 cells can also be inhibited by Rb (Haas-Kogan et al. 1995). Saos-2 cells are null for p53. Carboxy-terminal truncation of the Rb gene results in unrestrained E2F1 activity. Based on the data shown here, it is likely that the deregulated E2F1 activity in Saos-2 cells is responsible for the apoptosis induced by γ -radiation. p53-independent apoptosis induced by E2F1 in cooperation with a DNA-damaging agent, topoisomerase II inhibitor, has also been reported recently to occur in 32D.3 murine myeloid cells (Nip et al. 1997). Interestingly, many lymphoma cells with mutated p53 can still undergo apoptosis in response to DNA damaging agents. As

one of the most common tumors seen in the E2F1 knockout mice are lymphomas, it suggests that there maybe a very important physiological relevance for E2F1 to induce apoptosis in a p53-independent manner.

Apoptotic function of E2F1 requires DNA binding but not transactivation

E2F1 is a transcription factor that can bind to DNA on its own and transactivate gene expression. Thus, the DNA-binding activity can have a significant effect on the transactivation function of E2F1. The DNA-binding activity of E2F1 has been well studied in vitro using gel retardation assays. The DNA-binding activity of E2F1 can be stimulated dramatically through a heterodimerization with its partner protein DP-1. In contrast, bind-

Figure 6. Colony assay to show the fold inhibition of colony number by wild-type E2F1 and its mutants in transfected Saos-2 cells. This graph represents two independent experiments.

ing to cyclin A results in negative regulation of its DNA-binding activity. The increase in apoptosis seen with E2F1 + DP1 or the cyclin A-binding-defective mutant E2FA24 could be interpreted as the result of an increase in DNA-binding or transactivation function of E2F1. The observation that a Rb-binding-defective but transactivation-competent mutant of E2F1 can cause more apoptosis than that of wild-type E2F1 suggested that the transactivation of E2F1 and S-phase entry was crucial for its apoptotic function (Shan et al. 1996a). However, the ability to induce apoptosis by a transactivation-defective mutant, E2F1(1–374), shown here and in Phillips et al. (this issue) argues that the transactivation and apoptotic functions are uncoupled. This was further supported by the fact that two mutants, E2F1(390–1)DF(Δ mdm2) and E2F1(Δ 406–415)(Δ Rb), which are impaired in their transactivation function, can also mediate apoptosis as effectively as wild-type E2F1. This is also in agreement with the observation that although mdm2 is known to stimulate the transactivation of E2F1, it did not affect E2F1-induced apoptosis.

The ability to separate the transactivation and apoptotic functions of E2F1 enabled us for the first time to address the issue of whether DNA binding is essential for the apoptotic function of E2F1. The lack of induction of apoptosis by the DNA-binding-defective mutant E2F1(132) but not E2F1(1–374) allowed us to show definitively that the DNA binding, and not the transactivation of E2F1, is indispensable for its apoptotic function, as the double mutant E2F1(132)(1–374) is defective in inducing apoptosis. The absolute requirement of DNA-binding activity for E2F1 to induce apoptosis provided us with a new interpretation of all the existing data. The conclusion is consistent with the observation that an increase in DNA binding can result in an increase in apoptosis caused by E2F1.

Rb inhibits E2F1-induced apoptosis through direct binding but not suppression of E2F1 transactivation

Rb has been shown recently to inhibit apoptosis induced

by various agents in different cell systems including IFN- γ , TGF- β 1-, and γ -radiation-induced apoptosis (Haas-Kogan et al. 1995; Berry et al. 1996; Fan et al. 1996). Rb has also been shown to be able to inhibit p53-induced apoptosis in HeLa cells (Haupt et al. 1995). However, in this study, Rb only inhibited E2F1- but not p53-induced apoptosis under the same conditions. The inability of Rb to inhibit p53-induced apoptosis in transfected Saos-2 cells used here may be attributable to a difference in cell systems. Cell type-specific inhibition of p53-induced apoptosis has been reported previously. An inhibition of p53-induced apoptosis by mdm2 was only seen in H1299 cells but not in HeLa cells (Haupt et al. 1996). As in H1299 cells, mdm2 was able to inhibit p53-induced apoptosis specifically. Failure to inhibit E2F1-induced apoptosis by mdm2 in Saos-2 cells under the same conditions argues that the inhibition of E2F1-induced apoptosis by Rb is very specific.

Because the transactivation and the apoptotic functions of E2F1 are separable, the inhibition of E2F1-induced apoptosis by Rb cannot be mediated by the suppression of the E2F1 transactivation function. It has been shown recently that when Rb and E2F1 complex together, the Rb–E2F1 complex can act as an active repressor to inhibit transcription of reporters containing E2F-binding sites (Weintraub et al. 1992, 1995; Zacksenhaus et al. 1996). Therefore, the repression function of the Rb–E2F1 complex may be the mechanism through which Rb inhibits E2F1-induced apoptosis. This hypothesis suggests an active role for Rb in E2F1-induced apoptosis (Fig. 9), as well as the necessity for physical interaction between Rb and E2F1.

This hypothesis was supported by the observation that Rb can specifically inhibit E2F1-induced apoptosis in transfected Saos-2 cells, as well as the observation that direct interaction between Rb and E2F1 is essential for the inhibition of E2F1-induced apoptosis by Rb. These results attribute a novel function for the Rb–E2F1 complex. DNA damage-induced p53-independent apoptosis has been seen in HL60 and U937 cells. Interestingly, cleavage of endogenous Rb by interleukin-1 β -converting enzyme (ICE)-like protease was seen in the apoptotic cell populations of these two tumor cell lines (An and Dou 1996). The cleavage of Rb has also been reported in colorectal tumor cell lines when they die of apoptosis independent of p53 (Browne et al. 1994). Therefore, it is possible that the generation of E2F1-binding-defective Rb through the ICE-like protease cleavage may promote apoptosis mediated by E2F1. These hypotheses argue that under physiological conditions, Rb may protect cells from apoptotic death induced by E2F1. Deregulated E2F1 activity caused by inactivation in Rb binding may lead to increased cell proliferation as well as apoptosis. As Rb can only inhibit E2F1-induced apoptosis through direct binding, the stoichiometry of E2F1 and Rb is the key issue. Overexpressing E2F1 in cells containing Rb may still induce apoptosis if there is enough free E2F1 existing.

Saos-2 cells express carboxy-terminal truncated Rb. If the Rb–E2F1 complex is responsible for the inhibition of

Figure 7. DNA-binding but not transactivation function of E2F1 is essential for E2F1-induced apoptosis. A representative FACS analysis (A) and percentage of cells with sub-G₁ DNA contents (apoptotic cells) (B) show the apoptotic function of wild-type E2F1 and its mutants. Apoptosis is measured by the accumulation of cells with a sub-G₁ DNA content and are in the region labeled M1. (The mutants are labeled as in Fig. 4A.) The graph represents the mean of two to four independent experiments to show the percentage of cells with sub-G₁ DNA content (apoptotic cells) in transiently transfected Saos-2 cells, using the same group of plasmids as in A. The expression level of wild-type and mutant E2F1 in transfected Saos-2 cells is shown in C. Wild-type E2F1 and E2F1 mutant proteins were detected with a mixture of two anti-E2F1-specific monoclonal antibodies, XS73-1 and SX54-3, which have different epitopes (S. Fredersdorf, unpubl.).

E2F1-induced apoptosis, one would predict that wild-type and Rb-binding-defective mutants of E2F1 should have similar potential to induce apoptosis provided that the protein expression level is the same. However, a higher percentage of apoptotic cells were seen consistently in Saos-2 cells transfected with E2F1(1-374) compared to those transfected by wild-type E2F1 or E2F1(Δ 406-415)(Δ Rb). Interestingly, many proteins such as mdm2, CREB-binding protein (CBP), and Rb interaction sites have been mapped within the last 90 amino acids of E2F1. It is therefore possible that apart from Rb, other protein-protein interactions may also be involved in the inhibition of E2F1-induced apoptosis. It is also

possible that E2F1-induced apoptosis could be inhibited by the expression of endogenous Rb-related proteins such as p107 or p130. Nevertheless, we failed to detect any effect of mdm2 on E2F1-induced apoptosis. In addition, both mdm2 and CBP-binding defective mutants can mediate apoptosis to the same extent as wild-type E2F1 (data not shown). Therefore, it is unlikely that mdm2 or CBP has a role in inhibiting E2F1-induced apoptosis. Whether Rb-related proteins have any effect on E2F1-induced apoptosis in Saos-2 cells remains to be investigated.

Studies of E2F1 expression have shown recently that wild-type E2F1 can undergo ubiquitination and that the

Figure 8. Inhibition of E2F1-induced apoptosis by Rb requires direct binding. DNA fragmentation (A), a representative FACS analysis (B), and percentage of cells with sub-G₁ DNA contents (apoptotic cells) (C) demonstrate that Rb can specifically inhibit apoptosis induced by wild-type E2F1 but not Rb-binding-defective mutants of E2F1. (B) The apoptotic function of wild-type E2F1 and its mutants in Saos-2 cells is shown in the *left* panel and are labeled as indicated. The effect of Rb on the apoptosis induced by wild-type E2F1 and its mutants is shown in the *right* panel and they are labeled accordingly. Apoptosis was measured by the accumulation of cells with a sub-G₁ DNA content and are in the region labeled M1. (C) The graph represents the mean of two to four independent experiments to show the percentage of cells with sub-G₁ DNA content (apoptotic cells) in transiently transfected Saos-2 cells, using the same group of plasmids as in B. The expression level of Rb in Saos-2 cells transfected with wild-type E2F1 or mutant E2F1(1-374) is shown in D. The plasmids that were cotransfected with Rb are indicated. Rb protein was detected with an anti-Rb-specific rabbit polyclonal antibody (C-15).

carboxy-terminal region of E2F1 is responsible for such degradation (Hateboer et al. 1996; Hofmann et al. 1996). An increased stability was seen with E2F1(1-374), which may be responsible for the increased cell death detected in the transiently transfected Saos-2 cells. The ability of E2F1(1-374) to generate a similar number of colonies as wild-type E2F1 in a colony assay provided supporting evidence that E2F1(1-374) is not more active than wild-type E2F1 in inducing apoptosis.

Physiological implication of repression function of the Rb-E2F1 complex: repression model for E2F1-induced apoptosis

Being able to demonstrate that E2F1-induced apoptosis requires DNA binding but not transactivation and can be inhibited by Rb through direct binding allowed us to propose a repression model for E2F1-induced apoptosis (Fig. 9). Recent studies have shown that although it can

Figure 9. A diagram showing the repression model for E2F1-induced apoptosis. (See Discussion for details).

activate gene expression, when E2F1/DP1 complexes with Rb, such a complex can positively suppress transcription and act as a repressor unit (Zacksenhaus et al. 1996). The Rb-E2F1 repressor unit can also inactivate surrounding transcription factors by blocking their interaction with the basal transcription complex (Weintraub et al. 1992, 1995). Therefore, it is possible that E2F1 normally transactivates a set of genes that are required for S-phase entry. In addition, the Rb-E2F1 complex also actively suppresses expression of a set of genes, including the ones involved in apoptosis. The loss of Rb binding will not only result in an increase in transactivation of unrestrained E2F1, it will also result in the loss of repression of E2F1-targeted apoptotic genes and result in an increase in apoptosis induced by unrestrained E2F1. This loss of Rb binding may also allow the interaction of E2F1 with other factors to transactivate apoptotic genes, although there is nothing known about this type of regulation of E2F1 activity. Based on this model, it is the DNA binding but not the transactivation activity of E2F1 that is essential for the induction of apoptosis. The fact that all of the DNA-binding-competent mutants of E2F1, independent of their transactivation function, are equally competent to induce apoptosis as that of wild-type E2F1 in Saos-2 cells supported such a model. The model was also supported by the fact that Rb can specifically inhibit apoptosis induced by wild-type or Rb-binding-competent mutant E2F1(390–1)DF(Δ mdm2), but not apoptosis induced by Rb-binding defective mutant E2F1(1–374) or E2F1(Δ 406–415)(Δ Rb). Furthermore, the model also helps us to explain why a higher level of apoptotic background is often detected in Saos-2 cells, which lack a functional Rb-E2F1 complex because of the carboxy-terminal deletion in Rb. The complex between reintroduced Rb and endogenous E2F1 may be responsible for the profound inhibitory effects of Rb on γ -radiation-induced apoptosis in transfected Saos-2 cells (Haas-Kogan et al. 1995). Apoptosis caused by E2F1 in response to a DNA-damaging agent has also been seen in the other cell system that lacks p53 (Nip et al. 1997); thus, the Rb-E2F1 complex may play a very important role in cellular response to DNA damage. Taken together with the

results shown here, these findings indicate that repression rather than transactivation is important for E2F1-induced apoptosis. It also argues that there are some novel and physiological implications of the repression function of Rb-E2F1 complex.

Materials and methods

Cell culture, antibodies, and plasmids

Saos-2 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. Anti-E2F1 antibodies SX73-1 and SX54-3 are monoclonal antibodies with two distinct epitopes. The production and characterization of these two antibodies are described elsewhere (S. Fredersdorf, in prep.). Both C-20 and C-15 are rabbit polyclonal antibodies specific to E2F1 and Rb, respectively (purchased from Santa Cruz Biotechnology, Inc.). CD20Leu is an FITC-conjugated monoclonal antibody specific for the cell surface marker CD20 (Becton-Dickinson). DO.1 is a p53-specific monoclonal antibody. All of the expression plasmids used in this study were driven by the cytomegalovirus (CMV) promoter. The E2F1 reporter plasmid was 3xwt-Luc. The double mutant E2F1(132)(1–374) was constructed by PCR using E2F1(132) as template. The mutants E2F1(390–1)DF(Δ mdm2) and E2F1(Δ 406–415)(Δ Rb), were constructed as described (Hagemeier et al. 1993; Martin et al. 1995).

DNA transfection, luciferase assay, DNA fragmentation assay, and colony assay

DNA was transfected into Saos-2 cells using calcium phosphate precipitation as described previously (O'Connor et al. 1995). For the measurement of transcriptional activity, 10^5 cells in a 6-cm dish were transfected with 200 ng of plasmid DNA expressing E2F1 or E2F1 mutants with 2 μ g of E2F1 reporter plasmid 3xwt-Luc. Two micrograms of CMV- β -gal reporter was cotransfected to measure the transfection efficiency. To identify the optimal concentration for the inhibition of Rb on the transactivation function of E2F1, a titrated amount of Rb was also cotransfected. A fivefold excess of Rb plasmid was required to give a significant (90%) repression of the transactivation function of E2F1 (data not shown), and this ratio was used later in the apoptosis inhibition experiment. The transfected cells were then lysed in 100–150 μ l of lysis buffer and assayed as described before (O'Connor et al. 1995). After the normalization of transfection efficiency based on β -gal activity, the mean values that were obtained derived from at least two independent experiments, and two measurements were made for each experiment.

For colony assay, 5 μ g of the *neo* resistant gene containing plasmid DNA expressing E2F1 or its mutants was transfected into 10^6 Saos-2 cells. Twenty hours after transfection, cells were split and grown in the medium containing 1 mg/ml of G418 to select for resistant colonies. The number of resistant colonies were counted 4 weeks after the selection. For FACS and the DNA fragmentation assay, 5 μ g of plasmid DNA expressing p53, 10 μ g for E2F1 or its mutants, 20 μ g for mdm2, or 50 μ g for Rb were used individually or together as indicated to transfect 10^6 cells in a 10-cm dish. The floating cells from each transfected dish were harvested every 24 hr after transfection. Forty-eight hours after the transfection, both floating and attached cells were used for FACS assay (see below). For the DNA fragmentation assay, 2×10^7 cells were transfected with the indicated plasmid DNA using the amount proportional to that used for the FACS assay. Seventy-two after transfection, the collected

cell pellets were used in a DNA fragmentation assay as described previously (Evan et al. 1992)

Flow cytometry

Cells were transfected as described above. Floating cells were harvested every 24 hr after the transfection. Forty-eight hours after the transfection, both attached and floating cells were harvested and stained with FITC-conjugated anti-CD20 antibody. For each experiment, one dish of cells was transfected with the control vector, only without CD20. These cells were later stained with CD20 antibody under the same conditions as those cotransfected with CD20 plasmid to serve as a negative control.

The cells that do not express the CD20 plasmid were used to set the baseline to allow the gating of the CD20-positive cells. One example of such gating is shown in Fig. 1B. After staining with CD20 antibody, the cells were fixed and stained with propidium iodide. The DNA content of all the cells expressing CD20 were analyzed through the flow cytometer (Becton Dickinson) as described (Rowan et al. 1996).

Immunoblotting

The transfected cells were lysed with sample buffer and loaded on SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to nitrocellulose paper and nonspecific binding sites were blocked with a 10% solution of reconstituted dried milk powder for 1 hr at room temperature. Primary antibody was added to the blot and incubated for 2–3 hr at room temperature or 4°C overnight. Finally, a peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulin was incubated with the blot, and bound immunocomplexes detected by the Enhanced Chemiluminescence (ECL) method, as described by the manufacturer (Amersham).

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E2F1-induced apoptosis requires DNA binding but not transactivation and is inhibited by the retinoblastoma protein through direct interaction.

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