

Myc Requires Distinct E2F Activities to Induce S Phase and Apoptosis

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Summary

Previous work has shown that the Myc transcription factor induces transcription of the E2F1, E2F2, and E2F3 genes. Using primary mouse embryo fibroblasts deleted for individual E2F genes, we now show that Myc-induced S phase and apoptosis requires distinct E2F activities. The ability of Myc to induce S phase is impaired in the absence of either E2F2 or E2F3 but not E2F1 or E2F4. In contrast, the ability of Myc to induce apoptosis is markedly reduced in cells deleted for E2F1 but not E2F2 or E2F3. From this data, we propose that the induction of specific E2F activities is an essential component in the Myc pathways that control cell proliferation and cell fate decisions.

Introduction

The stimulation of cell proliferation involves the induction of immediate early gene products such as c-Myc, as well as the subsequent induction of the G1 cyclin-dependent kinases. The activation of these G1 cyclin-dependent kinases leads to phosphorylation of the retinoblastoma tumor-suppressor protein, which then allows an accumulation of E2F transcription factors. Numerous experiments have now shown that the activity of this Cdk/Rb/E2F pathway is critical for normal cell cycle progression from G1 into S phase (Nevins, 1998; Dyson, 1998). In addition, the Cdk/Rb/E2F pathway is disrupted in virtually all human cancers. Likewise, dereg-

ulation of Myc, either through gene amplification or chromosomal rearrangements, is a common event in many human cancers, highlighting the critical role of this activity for normal cell growth regulation.

E2F activity controls the expression of a variety of genes that encode proteins essential for DNA replication and cell cycle progression (Dyson, 1998; Nevins, 1998). Numerous experiments have shown that E2F plays a critical role in cell cycle control as well as apoptosis. Inhibition or lack of E2F activity will block G1 to S phase progression in mammalian cells, and ectopic expression of several E2F proteins is sufficient to induce S phase in otherwise quiescent cells. E2F activity is composed of six different heterodimers made up of six distinct E2F proteins dimerized with one of two DP proteins. Various properties of the individual E2F family members suggest distinct functional roles for the proteins. In particular, the E2F1, E2F2, and E2F3 activities appear to play a positive role in cell cycle progression, while E2F4, E2F5, and E2F6 most likely contribute to the repression of cell growth. In addition, the Rb/E2F pathway integrates the proliferative response with cell survival pathways through the ability of one E2F family member, the E2F1 product, to induce p53 accumulation and apoptosis (Wu and Levine, 1994; Kowalik et al., 1995, 1998; Qin et al., 1994; Shan and Lee, 1994).

Like E2F, the c-Myc protein has also been shown to play a critical role in the control of cell proliferation and to serve as a link between proliferation and cell fate by inducing p53-dependent apoptosis (Evan et al., 1992; Hermeking and Eick, 1994; Wagner et al., 1994; Lüscher and Eisenman, 1990). Myc, in conjunction with the Max heterodimer partner protein, is known to function as a transcription factor through the recognition of E box sequence elements (Blackwell et al., 1990; Blackwood and Eisenman, 1991). A number of target genes have been identified that could play a role in the action of Myc in cell proliferation control. These include genes that encode proteins involved in DNA replication, such as ornithine decarboxylase (Wagner et al., 1993; Bello-Fernandez et al., 1993), proteins that function in the G1 transition, such as Cul1, cdk4, and the Cdc25A phosphatase (O'Hagan et al., 2000; Hermeking et al., 2000; Galaktionov et al., 1996), and genes encoding proteins working later in the cell cycle, such as the catalytic subunit of telomerase (Wang et al., 1998). In addition to these targets, recent work has provided evidence for the Id2 gene as a Myc target (Lasorella et al., 2000).

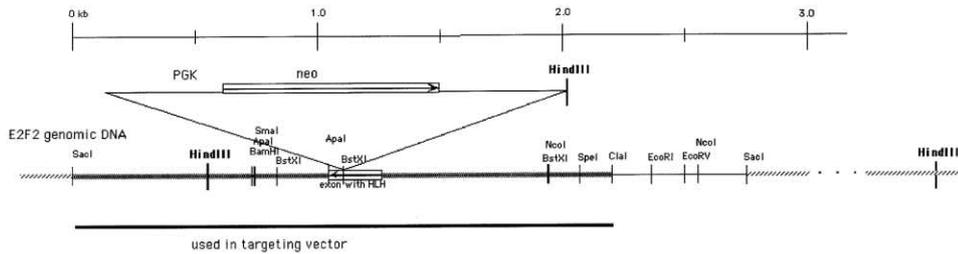
In addition to a role of Myc in cell proliferation control, a number of studies have documented the capacity of Myc, when expressed in the absence of survival signals, to induce apoptosis (Evan et al., 1992; Hermeking and Eick, 1994; Wagner et al., 1994). Although the precise nature of the relevant targets is largely unknown, recent work has shown that Myc-induced apoptosis operates through at least two pathways, one involving the stimulation of cytochrome C release (Juin et al., 1999), and the other involving activation of the ARF/p53 pathway (Zindy et al., 1998).

Our previous work has shown that the three E2F genes

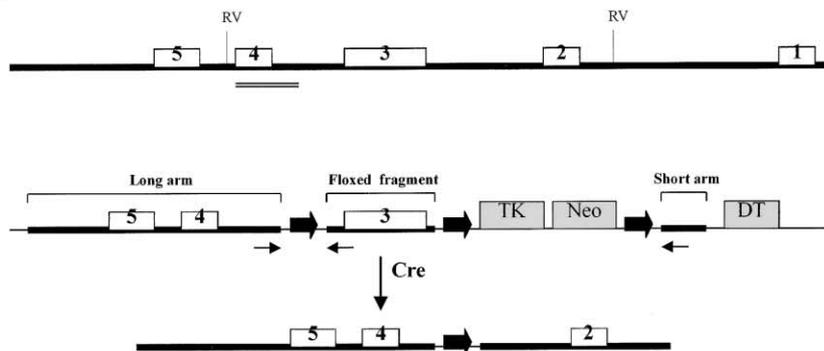
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A.

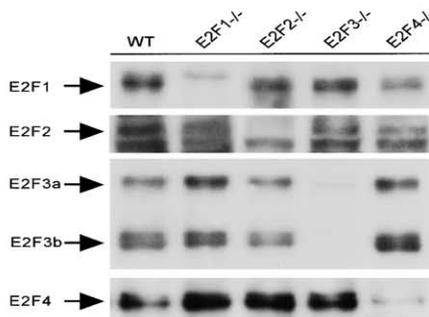
E2F2



E2F3



B.



C.

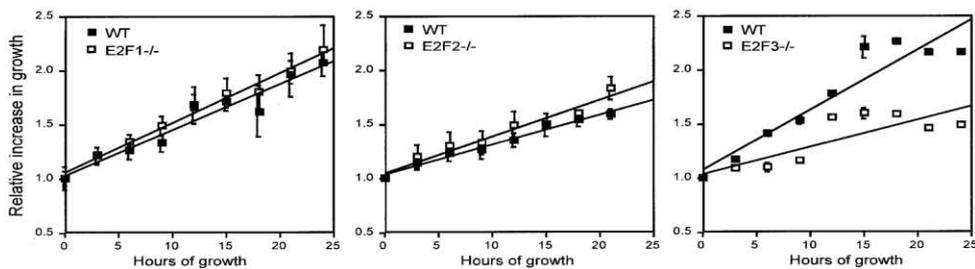


Figure 1. Characterization of E2F1, E2F2, and E2F3 Null Fibroblasts

(A) Disruption of the *E2F2* and *E2F3* genes. A schematic diagram of the *E2F2* genomic sequence is shown together with those sequences included in the targeting vector (depicted by the bold solid line). Flanking genomic sequences are indicated by the hatched lines. Restriction enzyme sites are indicated. In the *E2F2* knockout, the exon encoding the DNA binding domain (HLH) is disrupted by neo insertion. The *E2F3* genomic structure, including exons 2–5, is depicted above the sequence used for homologous recombination. The positions of TK, neo, and DT resistance genes, as well as loxP sites (heavy arrows) are indicated. Primers used for PCR (small arrows under sequence) and probe used for Southern analysis (double line under genomic sequence) are indicated. In the *E2F3* knockout, exon 3, which encodes most of the DNA binding domain, is deleted.

normally activated in G1 of the cell cycle, E2F1, E2F2, and E2F3a, can also be activated by ectopic Myc expression in a quiescent cell without cdk activation or G1 progression to S phase (Sears et al., 1997, 1999; Leone et al., 1997; Adams et al., 2000). In contrast, the expression of the E2F4 or E2F5 genes is not induced by Myc (data not shown). Further, analysis of the E2F2 and the E2F3 promoters has provided evidence that the induction of E2F2 and E2F3 by Myc could be direct, since a series of Myc binding sites are essential for this induction as well as for the normal induction of E2F2 and E2F3 expression following serum stimulation (Sears et al., 1997; Adams et al., 2000). The fact that Myc and E2F genes share functional properties, including their roles in activating cell proliferation as well as apoptosis, together with the observation that Myc can induce E2F gene expression raises the possibility that Myc function might be mediated at least in part through the action of the E2F transcription factors. We now use a genetic approach to provide evidence of a functional link between Myc and various E2F activities.

Results

To directly explore the potential connection between E2F gene activation and Myc function, we have made use of mice that are deficient in either E2F1, E2F2, or E2F3. The *E2F1*^{-/-} mice have been described (Field et al., 1996; Yamasaki et al., 1996). The *E2F2*^{-/-} and *E2F3*^{-/-} mice have recently been generated using the targeting constructs shown in Figure 1A, and detailed phenotypic analysis is in progress (S.J.F., unpublished data; L.W. et al., submitted). For our experiments, we prepared primary mouse embryo fibroblasts (MEFs) from 13.5 day embryos. Embryo genotypes were determined by PCR or Southern analysis. Analysis of E2F proteins in the cells derived from embryos with the indicated genotypes yielded the expected absence of the relevant E2F protein as measured by Western blot analysis (the faint bands appearing in a few of the knockout samples have been determined by further analysis to be nonspecific) (Figure 1B). Moreover, the expression of the various E2F proteins was not substantially altered by the absence of any one family member. We have also measured growth rates as one measure of the properties of these cells. As shown in Figure 1C, the *E2F1*^{-/-} and *E2F2*^{-/-} cells grow at the same rate as wild-type cells in medium containing 15% fetal calf serum. In contrast, *E2F3*^{-/-} cells grow at a reduced rate in these cell culture assays, although *E2F3*^{-/-} mice are born (with reduced frequency) and can survive to adulthood and are fertile (data not shown), consistent with other recent analyses

of *E2F3*^{-/-} mice (Humbert et al., 2000b). We have now used these cells to examine a functional link between Myc and these E2F genes, employing two assays for Myc function: the ability of Myc to induce entry into S phase and the ability of Myc to induce apoptosis. In all experiments, we tested multiple MEF preparations individually to ensure the reproducibility of the results.

E2F2 and E2F3 but Not E2F1 or E2F4 Are Required for Myc to Induce S Phase in Quiescent Fibroblasts

Previous work has shown that ectopic expression of Myc protein in otherwise quiescent cells can lead to an induction of S phase (Eilers et al., 1991; Vlach et al., 1996). We used a recombinant adenovirus to express Myc in quiescent primary MEF cells with either wild-type or various *E2F*^{-/-} genotypes. As shown in Figure 2, infection of quiescent wild-type MEFs (WT) with increasing doses of the Ad-Myc virus led to a substantial induction of BrdU incorporation. The ability of Myc to induce S phase in the *E2F1*^{-/-} fibroblasts was largely unimpaired when compared to the activity of Myc in wild-type cells. In contrast, the capacity of Myc to induce S phase in the *E2F2*^{-/-} cells or the *E2F3*^{-/-} cells was essentially abolished. As one further control, we also assayed the ability of *E2F4*^{-/-} cells to respond to Myc, since E2F4 is not normally induced by Myc expression. As shown in Figure 2, the *E2F4*^{-/-} cells responded equally well to Myc expression as did wild-type cells. Western blot assays demonstrated equivalent production of Myc protein from the recombinant adenovirus in each of the cell types (data not shown).

To demonstrate that the failure of the *E2F2*^{-/-} cells or the *E2F3*^{-/-} cells to respond to Myc was indeed due to the absence of these proteins and not some other defect in these primary MEFs, we assayed the ability of ectopic expression of the appropriate E2Fs to render the respective cells responsive to Myc. Thus, the *E2F2*^{-/-} cells were infected with a low dose of Ad-E2F2 virus insufficient to significantly induce S phase and then assayed for their ability to respond to Myc expression. Similarly, E2F3 was reintroduced in E2F3 null cells and assayed for a Myc-induced response. As shown in Figure 3, expression of E2F2 in the *E2F2*^{-/-} cells or E2F3 in the *E2F3*^{-/-} cells completely restored the responsiveness of these cells to Myc, resulting in an induction of BrdU incorporation similar to that seen in wild-type cells.

Based on these results, we conclude that E2F activity, particularly E2F2 and E2F3, is required for the ability of Myc to stimulate quiescent fibroblasts to progress into S phase. We do not mean to suggest from these results that E2F2 or E2F3 activation is solely responsible for entry to S phase. Indeed, it is clearly evident that the

(B) *E2F1*, *E2F2*, *E2F3*, or *E2F4* heterozygous mice were mated, and mouse embryo fibroblasts (MEFs) were prepared from 13.5 day embryos (Kamijo et al., 1997). Embryo genotypes were determined by PCR analysis. Passage 3 primary MEFs grown in DMEM/15% fetal calf serum with either wild-type, *E2F1*^{-/-}, *E2F2*^{-/-}, *E2F3*^{-/-}, or *E2F4*^{-/-} genotypes were harvested, nuclear extracts were prepared, and the expression of either E2F1, E2F2, E2F3, or E2F4 was determined by Western blot analysis as described in Experimental Procedures.

(C) Proliferation rates of wild-type and E2F-deficient MEFs. MEFs derived from *E2F1*^{-/-}, *E2F2*^{-/-}, or *E2F3*^{-/-} 13.5 day embryos and their respective wild-type littermates were plated in duplicate at a density of 2.5×10^5 cells per 60 mm tissue culture plate containing DMEM/15% FCS. Twelve hours after plating, cells were trypsinized, and live cells, as determined by trypan blue exclusion, were counted using a hemocytometer in 3 hr intervals for 21 or 24 hr. Each time point represents the average of nine independent cell density measurements from each of the duplicate plates. The y axis represents the fold-induction of cell growth relative to the initial time point.

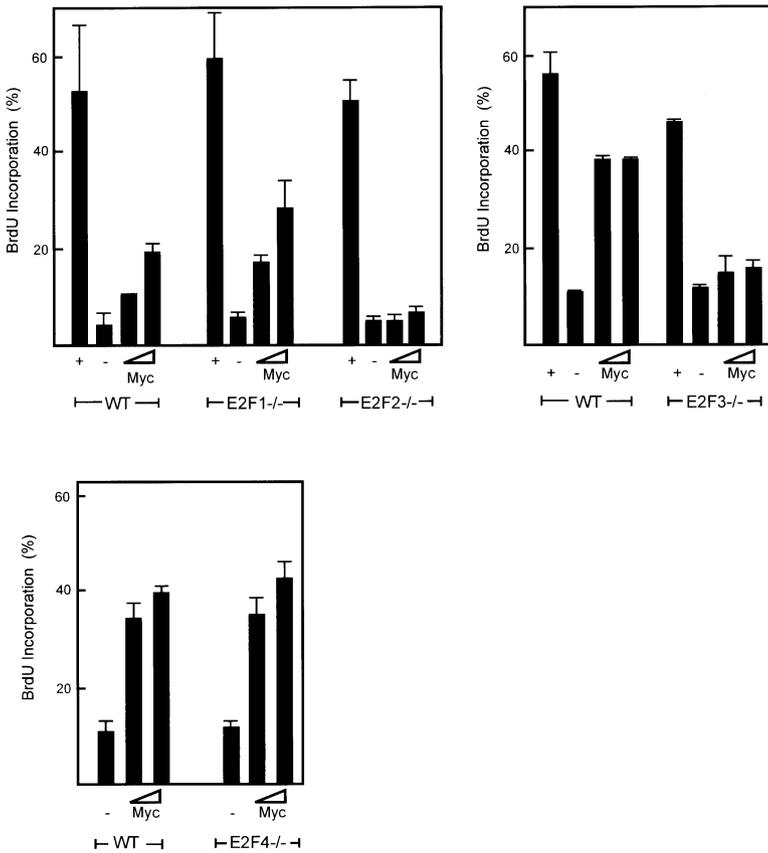


Figure 2. The Ability of Myc to Induce S Phase Is Dependent on E2F2 and E2F3 Activity

Primary MEF preparations from each genotype, either wild-type, *E2F1^{-/-}*, *E2F2^{-/-}*, *E2F3^{-/-}*, or *E2F4^{-/-}*, were assayed for BrdU incorporation in response to Myc. Cells were plated, brought to quiescence, and then infected with either a control adenovirus lacking an insert (- and + lanes multiplicity of infection [moi] equal 2000) or increasing doses of a recombinant virus containing the *c-myc* gene (moi of 1000 or 2000). Infected cells were maintained in low serum, except for one sample of the control virus infection in which fetal calf serum was added to 15% (+). BrdU incorporation was assayed as described in Experimental Procedures. At least 300 cells were counted per plate. The mean values from either two to three independent experiments (left panel) or triplicate plates from a representative experiment (right and lower panels) are shown with standard deviations.

E2F2^{-/-} and the *E2F3^{-/-}* fibroblasts are capable of proliferating in response to serum (Figure 2, +), although it is true that the *E2F3^{-/-}* cells are somewhat impaired in their growth capacity (Figure 1C) (Humbert et al., 2000b) (L.W. et al., submitted). We interpret our results to suggest that proliferation normally involves synergistic, overlapping regulatory events. By isolating the proliferative response to Myc alone, it seems likely that we have accentuated the role of E2F2 as well as E2F3 in the

process by which Myc contributes to cell proliferation control. As such, we propose that E2F2 and E2F3 are important functional targets for Myc-mediated stimulation of cell proliferation.

A Specific Role for E2F1 in Myc-Induced Apoptosis

In addition to the role of Myc in stimulating cell proliferation, other studies have shown that the Myc protein induces apoptosis when produced in the absence of

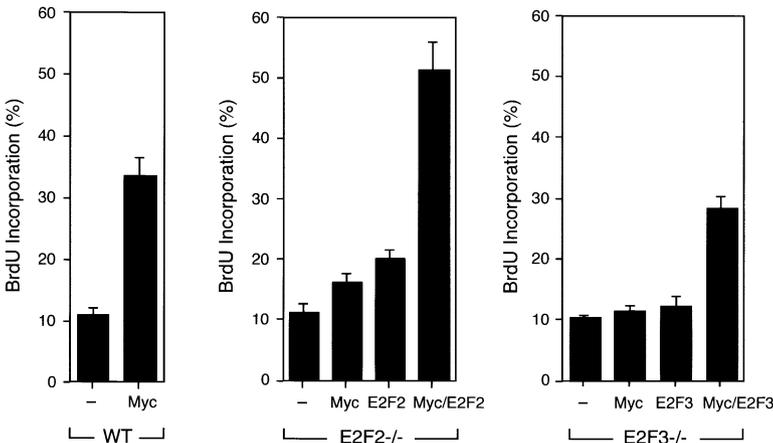


Figure 3. E2F Proteins Restore the Response to Myc

Primary MEFs, either wild-type, *E2F2^{-/-}*, or *E2F3^{-/-}*, were brought to quiescence and then infected with recombinant adenoviruses as follows. Wild-type cells (left panel) were infected with either Ad-Con (control virus) moi 2000 (-) or Ad-Myc moi 1000 (Myc). The *E2F2^{-/-}* cells (middle panel) were infected with either Ad-Con moi 2000, Ad-Myc moi 1000, Ad-E2F2 moi 200, or with a combination of Ad-E2F2 and Ad-Myc at the same moi. The *E2F3^{-/-}* cells (right panel) were treated similarly except for infection with Ad-E2F3 moi 300 (E2F3) rather than Ad-E2F2. Infected cells were maintained in low serum medium, and at 20 hr postinfection BrdU was added to all plates. Cells were harvested at 30 hr postinfection, fixed, and assayed for BrdU incorporation. Approximately 300–500 cells were counted per plate, and the mean value from triplicate plates was calculated along with standard deviations.

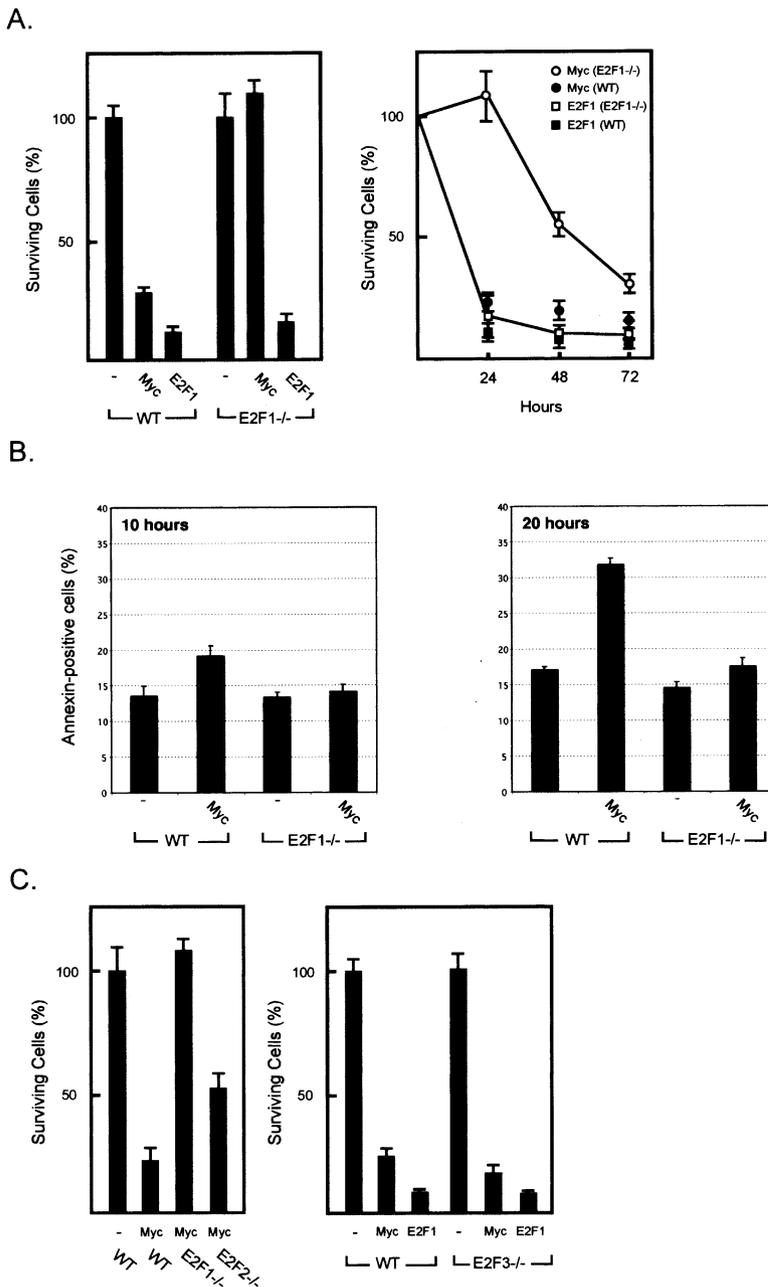


Figure 4. Myc Induction of Apoptosis Requires E2F1 Activity

(A) E2F1 dependence of Myc-induced apoptosis. At left, wild-type and *E2F1*^{-/-} primary mouse embryo fibroblasts were transfected with 1 μ g of CMV-GFP and either 5 μ g of control vector (-), 5 μ g of CMV-Myc (Myc), or 2 μ g of CMV-E2F1 and 2 μ g of CMV-Dp1 (E2F1) as described in Experimental Procedures. Cell survival was measured as described in Experimental Procedures. At right, wild-type MEFs (filled symbols) or *E2F1*^{-/-} MEFs (open symbols) were transfected with either CMV-Myc (circles) or CMV-E2F1 + CMV-DP1 (squares) as described for the left panel. Cells were then washed and incubated in media containing low serum and harvested at the indicated times. The percentage of surviving cells was calculated; the mean values and standard deviations are shown.

(B) Annexin staining assays. Wild-type and *E2F1*^{-/-} MEFs were transfected with 2 μ g of CMV-GFP and either 5 μ g of control vector or 5 μ g of CMV-Myc, washed, and starved for 10 hr or 20 hr. Cells were stained with annexin V and 7-AAD and then analyzed by FACS. The data are the mean and standard deviation of triplicate samples.

(C) Specificity of the E2F requirement for Myc-induced apoptosis. At left, wild-type, *E2F1*^{-/-}, and *E2F2*^{-/-} primary MEFs were transfected with CMV-GFP and either control vector or Myc-expressing plasmid, treated, and processed as described in (A). At right, wild-type and *E2F3*^{-/-} primary MEFs were similarly transfected with control vector, CMV-Myc, or CMV-E2F1 + CMV-DP1 and assayed as described above.

other growth signals (Evan et al., 1992). Like Myc, E2F1 has also been shown to be a potent inducer of apoptosis when expressed in quiescent fibroblasts (Wu and Levine, 1994; Kowalik et al., 1995; Qin et al., 1994; Shan and Lee, 1994), an activity not shared by the other E2F family members (DeGregori et al., 1997). We have thus measured the requirement for various E2F activities in the ability of Myc to induce apoptosis by measuring Myc-induced cell death in primary MEF cells deficient for E2F activities.

Preliminary experiments in primary wild-type MEFs showed that the Myc levels expressed with recombinant adenovirus were insufficient to induce substantial apoptosis. Therefore, for these experiments, primary MEFs with either wild-type, *E2F1*^{-/-}, *E2F2*^{-/-}, or *E2F3*^{-/-} geno-

types were transiently transfected with either a control plasmid or a Myc expression plasmid. Transfected cells were identified by cotransfection with a GFP expression plasmid.

As shown in Figure 4A (left panel), after 24 hr of serum deprivation, expression of Myc in primary wild-type MEFs led to a substantial loss of cell viability (compare control [-] with Myc). In contrast, the induction of cell death by Myc in the primary *E2F1*^{-/-} fibroblasts was severely impaired at this time. Importantly, the *E2F1*^{-/-} cells are not inherently refractory to apoptotic stimuli. They clearly retain the capacity to respond to an apoptotic signal, since cotransfection of an E2F1 expression plasmid together with the E2F heterodimeric partner DP1 (E2F1) did result in *E2F1*^{-/-} cell death equivalent to

that of wild-type cells. As shown in the right-hand panel of Figure 4A, analysis of the effect of Myc over a longer period of time revealed that Myc did eventually induce cell death in the *E2F1*^{-/-} cells (open circles); however, the kinetics of death were drastically different from that which occurs in either wild-type cells (filled circles) or with exogenously added E2F1/DP1 in either wild-type or *E2F1*^{-/-} cells (filled and open squares, respectively). These results show that the acute cell death induced by Myc overexpression, which occurs 24–36 hr after serum deprivation, is largely dependent on the presence of a functional E2F1 protein.

To provide an independent measure of cell death that is characteristic of apoptosis, we have examined wild-type and E2F1 null cells with overexpressed Myc for the presence of phosphatidylserine on the cell surface of intact unfixed cells by staining with the phospholipid binding protein annexin V. As shown in previous work, there is a translocation of phosphatidylserine from the inner surface of the plasma membrane to the exterior during the process of apoptosis, prior to any damage of the outer plasma membrane, which can be detected by staining cells with phycoerythrin-tagged annexin V (Martin et al., 1995). As shown in Figure 4B, Myc expression led to an increase in the percentage of annexin-positive cells that were wild-type for E2F1 (WT). There was a small but reproducible increase in annexin-positive wild-type cells at the earliest time point ($p = 0.0077$) that then increased at the 20 hr time point ($p = 0.0002$). In contrast, expression of Myc in the *E2F1*^{-/-} cells did not show a substantial increase in annexin staining regardless of whether the assay was at 10 hr, 20 hr, or 30 hr (data not shown).

We also examined the ability of Myc to induce cell death in primary MEFs deficient for E2F2 or E2F3. As shown in Figure 4C (left panel), Myc-induced cell death was again impaired in the *E2F1*^{-/-} cells, whereas Myc did induce cell death in the *E2F2*^{-/-} cells, although somewhat less efficiently than in the wild-type cells. Myc was also capable of inducing death in the *E2F3*^{-/-} cells (Figure 4C, right panel) similar to its activity in wild-type cells and similar to the E2F1-induced cell death in both cell types. It thus appears that E2F1 activity is specifically required for Myc to induce apoptosis in fibroblasts and that E2F2 and E2F3 are largely dispensable for this function of Myc.

Discussion

A number of observations have suggested similarities in the biological action of the Myc protein and E2F activities; most striking is the ability of Myc or E2Fs to induce S phase in otherwise quiescent cells and the ability of Myc or E2Fs, particularly E2F1, to induce apoptosis in the absence of normal growth stimuli. Indeed, it has been suggested that the induction of apoptosis by oncoproteins such as Myc or E2F1 results from the generation of an incomplete or conflicting signal for cell growth that is not balanced by the activation of a cell survival signaling pathway (Evan and Littlewood, 1998). Both Myc and E2F1 have been shown to induce the accumulation of p53, and recent evidence shows that these proteins also induce expression of p19^{ARF} (Zindy et al.,

Growth Stimulatory Signals

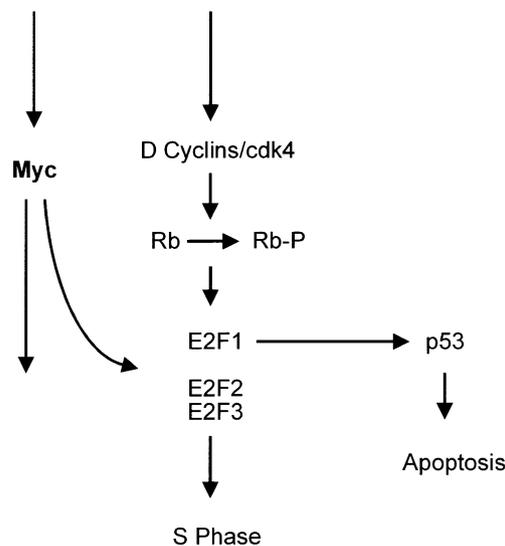


Figure 5. Relationship of E2F Activation and Myc in Cell Growth and Death

The figure depicts pathways controlling the accumulation of E2F1, E2F2, and E2F3 activity as a result of regulation of retinoblastoma protein (Rb) function by the cyclin D/cdk4 kinase as well as the role of Myc in the control of E2F accumulation, as described here and elsewhere. Other experiments have detailed the specific role of E2F1 in the induction of p53 accumulation and the roles of E2F1-3 in the induction of S phase.

1998; DeGregori et al., 1997; Bates et al., 1998; Robertson and Jones, 1998), a protein now known to regulate the ability of Mdm2 to target p53 for degradation (Pomerantz et al., 1998; Zhang et al., 1998; Honda and Yasuda, 1999). The data presented here, showing a genetic requirement for various E2F activities in Myc function, suggest that these previous observations regarding similarities in Myc and E2F activity could be accommodated by a pathway in which Myc functions upstream of E2F, contributing to the accumulation of E2F1, E2F2, and E2F3 activity, and thus inducing both apoptosis and proliferation through distinct E2F family members (Figure 5).

Functional Distinctions in the E2F Family

Previous work has suggested distinct functional roles for the individual E2F activities. E2F1 has been shown to induce apoptosis (Wu and Levine, 1994; Shan and Lee, 1994; Qin et al., 1994; Kowalik et al., 1995, 1998), whereas E2F3 has been shown to be particularly important in cell proliferation (Leone et al., 1998; Humbert et al., 2000b). E2F5 has been implicated in development of the choroid plexus (Lindeman et al., 1998), and recent data implicate E2F4 in differentiation decisions of a broader context (Rempel et al., 2000; Humbert et al., 2000a). The facts that E2F2 and E2F3 are particularly important targets for Myc in a cell proliferation response and that E2F1 is critical for apoptosis provide further evidence that the individual E2F proteins play distinct roles in the control of cell proliferation and cell fate. Moreover, these results also provide evidence for a role

for E2F2 not previously seen in simple proliferation assays, since E2F2^{-/-} MEF cells grow normally in the presence of serum. The underlying basis for these functional differences is not clear but very likely represents the specificity in activation or repression of distinct target genes. Indeed, overexpression of individual E2F proteins has revealed differences in the specificity of E2F target gene activation (DeGregori et al., 1997). We presume that the role of E2F1 in apoptosis versus the role for E2F2 and E2F3 in mediating proliferation likely reflects the activation of distinct groups of genes that perform the necessary functions.

The Dichotomy in Myc Function

Deregulated Myc expression results in both the induction of S phase and the induction of apoptosis if survival factors are limiting. How these processes are linked is not well understood. A variety of possible Myc target genes have been identified, including the recent description of Id2 (Lasorella et al., 2000). Like E2F2 and E2F3, Id2 appears to be essential for Myc-induced cell proliferation but not for Myc-induced apoptosis. In addition, these studies have also identified a role for Id2 in the control of Rb function, since the loss of Id2 function can partially suppress the phenotype resulting from loss of Rb. As such, it has been proposed that one role for Myc in the stimulation of cell growth is the induction of Id2, leading to inactivation of Rb. Nevertheless, since the loss of Id2 does not fully suppress an Rb null phenotype (mice die at birth), and previous work has shown a suppression of Rb phenotype as a result of loss of either E2F1, E2F2, or E2F3 function (Yamasaki et al., 1998; Ziebold et al., 2001) (G.L., unpublished data), it seems likely that the control of E2F proteins and interactions with Id2 are both important for Rb function. Moreover, given the ability of Myc to induce both Id2 and E2Fs, we conclude that the induction of both groups of activities is likely to be an important function of Myc.

In addition to our results showing the requirement for distinct E2Fs to mediate Myc-induced S phase versus apoptosis, other work has also suggested that distinct downstream events mediate these two functions of Myc. In particular, cdk activation was shown to be necessary for the Myc-mediated induction of proliferation but not apoptosis (Rudolph et al., 1996). Although the lack of a cdk requirement for Myc-induced apoptosis might suggest an E2F-independent event, since E2F accumulation is normally regulated by Rb through cdk-mediated phosphorylation of Rb, previous work has demonstrated an ability of Myc to induce E2F1 accumulation in the absence of cdk activity (Leone et al., 1997), presumably by bypassing the normal Rb control. Thus, Myc-induced apoptosis could bypass the need for the cell cycle machinery by directly activating E2F1.

Role of E2F and Myc in the Control of Cell Growth

Several recent studies suggest a role for Myc in the control of cell growth, as opposed to a role in cell cycle progression. In particular, enhanced expression of Myc in B lymphocytes derived from E μ -myc transgenic mice results in an increase in cell size, independent of cell cycle (Eritani and Eisenman, 1999). This coincides with an increase in protein synthesis, an observation consis-

tent with a role for Myc in the control of various genes encoding protein-synthesis components (Guo et al., 2000; Coller et al., 2000). Moreover, studies of *Drosophila* Myc overexpression suggest little effect on cell division but a significant effect on cell growth (Johnston et al., 1999). It thus appears that a major function for Myc in the stimulation of cell growth is the activation of the general apparatus for cellular metabolism, so as to prepare the cell for continued proliferation. In this context, the participation of Myc in the induction of E2F, which regulates the expression of critical proteins to allow induction of the DNA replication machinery, might be an additional part of the process to prepare a cell for proliferation.

Deregulation of Myc can be found in a wide variety of human tumors (Nesbit et al., 1999). In addition, the deregulation of the Rb/E2F pathway, whether involving the loss of a CKI or the amplification of a D cyclin, is a common event in the vast majority of human cancers (Sherr, 1996; Nevins, 2001). Moreover, evidence for deregulation of Myc together with components of the Cdk/Rb pathway has been found in a number of human tumors, including breast carcinoma, melanoma, and small cell lung carcinomas (Nesbit et al., 1999). These observations together with the data presented here suggest a functional synergy between the action of Myc and the regulation of the Rb/E2F pathway. This synergy can be seen as an ability of Myc to activate the genes encoding cyclin D1, cdk4, Cdc25A, and now, as we show here, the E2F1, E2F2, and E2F3 genes.

Experimental Procedures

Generation of E2F2 and E2F3 Knockout Mice

The E2F2-targeting vector contains an approximately 1.7 kb neomycin resistance gene inserted into the exon which encodes the helix-loop-helix and leucine zipper regions of the E2F2 gene. The neomycin gene is transcribed in the opposite orientation with respect to the direction of transcription of the E2F2 exon (Figure 1, arrows indicate direction of transcription). The insertion of the neomycin gene (neo) interrupts the normal transcription of the E2F2 gene in the region encoding the protein domains required for dimerization and DNA binding. The targeting vector also includes a thymidine kinase (TK) gene for negative selection (not shown). For genotyping, genomic DNA was digested with HindIII and probed with a ClaI/SacI external probe. The wild-type allele generates a 3.7 kb band, while the targeted allele generates a 3.2 kb band as a result of the inserted neomycin gene. A 15 kb EcoRI genomic mouse fragment subcloned into pBluescript (Leone et al., 2000) was used to generate the E2F3-targeting vector. The short arm was a 1.6 kb EcoRV/SpeI fragment containing exon 2 that was cloned upstream of a loxP site and a TK/neo cassette and downstream of a diphtheria toxin resistance gene. A 2 kb SpeI fragment containing exon 3 was inserted downstream of the TK/neo cassette, and it was flanked by loxP sites. A 4.5 kb SpeI fragment containing exons 4 and 5 was inserted downstream of the loxP sites and was used as the long arm. A Cre recombinase gene was introduced into ES cell clones that had homologously recombined the targeting vector at the E2F3 locus. Gancyclovir was used for negative selection, allowing only cells that had lost the TK/neo cassette to survive. Clones that exhibited loss of E2F3 exon 3 and the TK/neo cassette were identified by Southern analysis using the probe depicted in Figure 1. PCR using the following primers, which are pictured in Figure 1, also identified clones: long arm, GATTGATTCTGGGTTGTCAGG; floxed, TGAATCATGGACAGAGCCAGG; and short arm, GTGGCTGGAAGGTGCCAAG. 129SV/CJ7 embryonic stem (ES) cell culture, electroporation, and selection of subclones were performed as described (Swiatek and Gridley, 1993). Injection of ES cells into host blasto-

cysts and their implantation into foster mothers was performed as described (Robertson, 1987). The resulting chimeric animals were outbred to C57/BL6 mice, and subsequent generations were propagated by brother-sister matings.

Western Blot Analysis

Aliquots of whole-cell lysates (50 μ g of protein) or nuclear extracts (25 μ g) of protein were separated in a 10% SDS acrylamide gel, blotted to an Immobilon-P membrane, and then incubated with antibodies specific for E2F1 (SC-193 from Santa Cruz at a 1:1000 dilution), E2F2 (SC-633X from Santa Cruz at a 1:1000 dilution), E2F3 (SC-878, Santa Cruz, 1:1000 dilution), E2F4 (SC-866, Santa Cruz, 1:1000 dilution), p53 (a mixture of Oncogene Research Ab-1, 1:300 dilution; and PharMingen 14461C, 1:1000 dilution), or c-Myc (C-33, Santa Cruz, 1:1000 dilution) to detect the respective proteins. Secondary antibodies were HRP conjugated, and proteins were detected with ECL (Amersham) following the manufacturer's instructions.

Assay of BrdU Incorporation

Primary MEFs at passage 3 derived from either wild-type (WT), *E2F1*^{-/-}, *E2F2*^{-/-}, *E2F3*^{-/-}, or *E2F4*^{-/-} embryos were plated onto 35 mm dishes at a density of approximately 5.2×10^4 cells. After 12–18 hr of growth, cells were brought to quiescence by incubation in DMEM containing 0.20%–0.25% fetal calf serum for approximately 48 hr. Quiescent MEFs from each genotype were then infected with either a control recombinant adenovirus containing the CMV enhancer with no insert, a recombinant adenovirus containing the c-myc gene, or recombinant adenoviruses containing the E2F2 or E2F3 genes, as specified in the figure legends. Multiplicities of infection are given in the figure legends. Infected cells were maintained in starvation medium, BrdU was added at 15–20 hr postinfection, and cells were fixed and assayed for BrdU incorporation 30 hr postinfection, as described previously (DeGregori et al., 1995).

Assays of Apoptosis

Wild-type, *E2F1*^{-/-}, *E2F2*^{-/-}, and *E2F3*^{-/-} primary MEFs between passage 3 and 5 were plated in triplicate on 60 mm dishes and transfected by the calcium phosphate precipitation method with CMV-GFP and either control vector pRcCMV (Invitrogen), CMV-Myc, or CMV-E2F1 together with CMV-DP1. Each of the 1 ml transfection mixtures was equilibrated with pBS plasmid so that each contained a total of 25 μ g of DNA; 300 μ l of the transfection mixture was added to each plate. After 12 hr of transfection, cells were washed once, and media containing 15% FCS was added back for an additional 4–6 hr. Cells were then washed again and incubated with media containing 0.25% serum. Adherent cells were trypsinized 24 hr later and fixed in 10% formalin for 15 min, washed twice with PBS containing 1% BSA, and analyzed by flow cytometry. The percentage of GFP-positive cells in each of the Myc- and E2F1-transfected samples is presented as a percentage of GFP-positive cells in the control vector-transfected cells (% surviving cells). Each sample was done in triplicate, and approximately 20,000 cells were analyzed for each condition. For annexin assays, primary MEFs were plated in triplicate to a density of 2.5×10^5 cells per 100 mm plate and then transfected by calcium phosphate precipitation with CMV-GFP plasmid, CMV-Myc, or control (pRc-CMV). Carrier plasmid (pBlue-script) was added up to a total of 20 μ g. Transfection took place for 10 hr, and the plates were subsequently washed with PBS/EGTA (3 mM) to remove precipitate and fed with DMEM containing 15% serum for 4–6 hr. The cells were subsequently washed with DMEM containing 0.2% serum, incubated in DMEM with 0.2% serum for either 10 hr or 20 hr, and then harvested for analysis. Both adherent and floating cells were harvested, washed (in $2 \times$ PBS, $1 \times$ annexin binding buffer), incubated with 10 μ l annexin-PE (PharMingen) and 1 μ g of the vital dye 7-AAD (Molecular Probes), and immediately analyzed by flow cytometry. GFP-positive cells were gated based on a mock-transfected control where >99.5% were gated as GFP negative. GFP-positive cells were analyzed for PE and 7-AAD fluorescence intensity using a Becton Dickinson FACSCalibur equipped with a 488 nm argon laser. Annexin⁺, 7-AAD⁻ and annexin⁺, 7-AAD⁺ cells were scored as apoptotic. Cell Quest (Becton Dickinson) software was used for data acquisition and analysis. Gates for annexin⁺

and 7-AAD⁺ events were based on mock-stained controls where <0.5% of cells were annexin⁺ or 7-AAD⁺.

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