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Overexpression of p21$^{waf1/cip1}$ arrests the growth of chicken embryo fibroblasts that overexpress E2F1

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P21$^{waf1/cip1}$ is a potent inhibitor of cell cycle progression and can inhibit the growth of both normal cells and cells transformed by a number of oncogenes. However, the ability of p21$^{waf1/cip1}$ to inhibit the growth of cells that overexpress the transcriptional transactivator E2F1 is controversial: it has been reported both that E2F1 can and cannot overcome the block in the cell cycle induced by p21$^{waf1/cip1}$. To avoid the complications that arise when such experiments are done with permanent cell lines, we tested the effects of overexpressing p21$^{waf1/cip1}$ and E2F1 in primary chicken embryo fibroblasts. In this system very high levels of E2F1 overexpression cause considerable apoptosis; however, the surviving cells still overexpress E2F1. These cells are transformed and their growth is blocked by overexpression of p21$^{waf1/cip1}$.

Keywords: retrovirus; growth suppressor; cell cycle; E2F; waf1/cip1

Introduction

Progression of eukaryotic cells through the cell cycle is regulated by external stimuli and internal checkpoints. An important step in the cell cycle is the transition from G_1 into S phase. This transition is primarily controlled by the G_1 cyclins and their partners, cyclin-dependent kinases (cdks) (for review see Sherr, 1994). A major phosphorylation target for the cyclin-cdk complex is the tumor suppressor pRb and the related family members, p130 and p107 (reviewed by Weinberg, 1995). When pRb is hyperphosphorylated, the transcription factors that are bound to it are released. E2F is the best characterized of the Rb associated transcription factors. The active form of the E2F transcription factors are heterodimers that contain an E2F subunit and a DP subunit. Both E2F and DP are gene families; five E2F family members and three DP family members have been cloned. When E2F is released from pRb, the transcription of S phase genes is increased. This is a key element of the progression to S phase (reviewed in Farnham *et al*., 1993; Helin and Harlow, 1993; Hollingsworth *et al*., 1993; see also La Thangue, 1994; Nevins, 1994; DeGregori *et al*., 1995a).

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E2F was first identified by its ability to bind to sequences in the adenovirus E2 promoter (Kovesdi *et al*., 1986). Many cellular genes contain E2F binding sites and the transcription of such genes is regulated by E2F (for reviews see Helin and Harlow, 1993; La Thangue, 1994; Nevins, 1994). The first member of the E2F family to be cloned, E2F1, was isolated because it can associated directly with pRb (Chellappan *et al*., 1991; Helin *et al*., 1992; Kaelin *et al*., 1992; Shan *et al*., 1992). pRb suppresses transcriptional activation mediated by E2F1 through a direct interaction with E2F1; the transactivating domain of E2F1 and its pRb binding domain overlap (Flemington *et al*., 1993; Helin *et al*., 1993; Shan *et al*., 1994) and overexpression of pRb abrogates the ability of E2F1 to stimulate transcription (Helin and Harlow, 1993). The inhibitory effect of pRb on E2F1 can be overcome by an inactivating mutation in pRb, by phosphorylation of pRb (Cress *et al*., 1993; Flemington *et al*., 1993; Goodrich and Lee, 1993; Hiebert, 1993; Dynlacht *et al*., 1994) or by pRb interactions with viral oncoproteins (La Thangue, 1994; Nevins, 1994). The expression of E2F1 is cell cycle dependent, the highest levels are seen at the G_1/S boundary (Shan *et al*., 1992). E2F1 can induce entry of quiescent cells into S phase (Johnson *et al*., 1993) and has been shown to be both an oncogene (Johnson *et al*., 1994) and a tumor suppressor (Field *et al*., 1996; Ymasaki *et al*., 1996). Overexpression of E2F1 transforms a rat embryo fibroblast cell line (Singh *et al*., 1994) and causes rat fibroblasts to enter S phase and induces apoptosis (Qin *et al*., 1994; Shan and Lee, 1994; DeGregori *et al*., 1995a; Kowalik *et al*., 1995).

The kinase activity of the cyclin-cdk complexes is regulated by cdk inhibitors (CKIs) that bind both to cyclin and cdk in the complex and inhibit the kinase activity of the holoenzyme (for review see Sherr and Roberts, 1995; Morgan, 1996). Two families of inhibitors play important roles at the G_1/S transition. The INK4 family members (p15, p16, p18 and p19) specifically inhibit cdk4 and cdk6. The other family of CKIs include p21^{waf1}, p27^{kip1} and p57^{kip2}. They bind to cyclins and inhibit a greater variety of cyclin/cdk complexes. While the roles of the CKIs are complex, some aspects of their action(s) are clear. For example, one effect of the overexpression of p21$^{waf1/cip1}$ or p27^{kip1} is to inhibit the phosphorylation of pRb. This, in turn, prevents the release of E2F from a complex with (hypophosphorylated) pRb and interferes with the entry of the cell into S phase (El-Deiry *et al*., 1993; Xiong *et al*., 1993; Peter and Herskowitz, 1994; Toyoshima and Hunter, 1994; Zhang *et al*., 1994; Harper *et al*., 1995).

It has been reported that overexpression of E2F1 can overcome a G_1 block caused by p21$^{waf1/cip1}$ inhibition of cyclin/cdk activity (DeGregori *et al*., 1995b; Dimri *et al*., 1996). This supports the idea that one of the effects of the G_1 cyclins-cdks is to release active E2F which then activates genes involved in S phase transition. However, it has also been reported that overexpression of p21$^{waf1/cip1}$ or p27^{kip1} blocks the cell cycle in cells that overexpress E2F1 (Mann and Jones, 1996).

We developed replication competent retroviral vectors that express high levels of human E2F1 protein in chicken embryo fibroblasts (CEF). Vectors are available with different envelope specificities so that the same cell can be infected with viruses expressing two different proteins. We have previously shown that overexpression of p21$^{waf1/cip1}$ (which will be referred to as p21) can arrest the growth of primary CEF transformed by a variety of oncogenes, both nuclear and cytoplasmic (Givol *et al*., 1995). This system has the advantage that we are using primary cells, and that we can control the level of expression. Since overexpression of p21 leads to growth arrest at G_1 and since E2F1 has been reported both to be able and to be unable to overcome this arrest, we wished to test the effects of p21 in CEF that overexpress E2F1. In this report we show that CEF overexpressing human E2F1 are morphologically transformed and high levels of human p21 can arrest the growth of these cells. We also found that high levels of expression of human E2F1 causes apoptosis in CEF and that this effect can be blocked by high levels of human Bcl-2. This may have implications as to whether E2F1 functions as an oncogene or a tumor suppressor, which may be related to whether its expression does or does not lead to apoptosis.

Results

Description of avian retroviral vectors

The replication competent retroviral vector RCASBP is a derivative of RCAS (Hughes *et al*., 1987). RCASBP contains the *pol* gene from the Bryan high titer strain of Rous Sarcoma Virus (RSV) and grows to a higher titer than the parental RCAS (Petropoulos and Hughes, 1991). In both vectors the expression of the inserted genes is under the control of the viral LTR promoter; inserted genes are expressed at higher levels in CEF infected with RCASBP than with RCAS. We have developed RCAS and RCASBP vectors that express E2F1 (Figure 1). The human E2F1 cDNA (Kaelin *et al*., 1992) was inserted into the RCAS(A) and RCASBP(A) retroviral vectors (as described in Materials and methods) to generate RCAS(A)E2F1 (this vector will be referred to as E2F1) and RCASBP(A)E2F1 (which will be referred to as BP-E2F1). Both of the vectors have the same envelope specificity (A); the difference between them is that they induce the expression of different levels of both viral proteins and E2F1. As expected, CEF infected with the BP-E2F1 virus express higher levels of E2F1 than cells infected with the E2F1 virus (data not shown).

The RCASBP(B)p21 and RCASBP(B)bcl-2 vectors (Figure 1) have been described previously (Givol *et al*., 1994, 1995). Because these viruses have a subgroup B envelope, they can be used to infect CEF that express E2F1 (subgroup A).

Expression of human E2F1 protein in CEF using retroviral vectors

CEF were transfected with plasmids that encode the E2F1 and BP-E2F1 viruses. We used two different vectors to try to express different levels of E2F1 protein to ask whether, if the level of E2F1 was high enough, the p21 block could be overcome. At passage two after transfection, CEF infected with BP-E2F1 were morphologically transformed (Figure 2a). CEF infected with E2F1 were transformed at passage three post-transfection. This correlates with the difference in growth rate of these two viruses, which is related to the difference in the level of expression of viral proteins. Western blot analysis of E2F1 protein showed that the infected cells expressed an immunoreactive protein of the correct size (Figure 3, lanes 2 and 4).

The BP-E2F1 virus induces apoptosis in CEF

The initial effect of the BP-E2F1 virus on the morphology of CEF is more striking than that of the E2F1 virus (data not shown). Furthermore, in contrast to what is seen with the cells infected with E2F1 (data not shown), a large number of the cells infected with the BP-E2F1 virus undergo apoptosis (Figure 4). Apoptosis was measured on both the floating and adherent cells at an early passage by FACS analysis (Figure 4a), by DNA fragmentation (Figure 4b) and by DAPI staining of the floating cells (data not shown). Because so many cells undergo apoptosis and detach from the plate, the cells were passaged at high density (1:2) for a period of approximately 4–6 days. The surviving cells could then be passaged normally (1:3 every 2 days). The cells that survived retained a transformed phenotype but showed little apoptosis. The levels of expression of retroviruses are modulated by the site of integration. We believe that the surviving

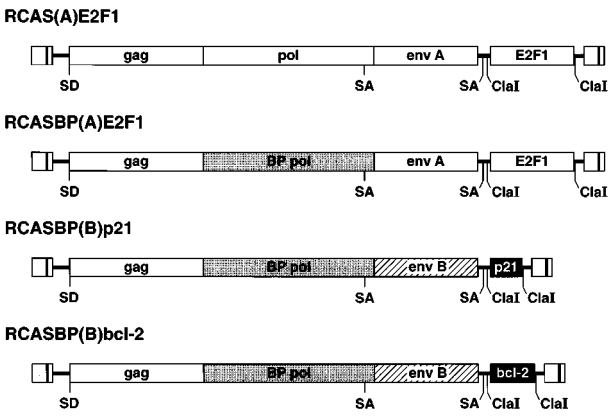

Figure 1 Schematic diagram of the vectors. RCASBP and RCAS express cDNA inserts as spliced subgenomic mRNA. Location of the splice donor site (SD), splice acceptor site (SA) and the recognition site for the restriction endonuclease *Cla*I are shown. The vectors differ in the envelope subgroups A or B (hatched), in the polymerase region BP and the inserts cloned in the *Cla*I site. RCAS(A)E2F1 is referred to as E2F1 and RCASBP(A)E2F1 is referred to as BP-E2F1 in the text. RCASBP(B)p21 is called BP-p21 and RCASBP(B)bcl-2 is called BP-bcl-2 in the text

cells are those that, by virtue of the integration of the provirus, express lower levels of E2F1. Western blot analysis of E2F1 protein in CEF infected with BP-E2F1 at passage 3 and passage 8 post-transfection showed that the average level of E2F1 protein was higher at passage 3 than at passage 8 (Figure 3, lanes 2 and 3). Furthermore, the level of E2F1 expression in cells infected with BP-E2F1 at passage 8 was comparable to the level of expression in CEF infected with E2F1 at passage 8 (Figure 3, lanes 2 and 4). We believe that the lower levels of E2F1 protein seen in cells infected with BP-E2F1 at passage 8 is due to a selection for cells that express lower levels of E2F1 and are therefore less apoptotic. The ability of E2F1 to drive cells into S phase and induce apoptosis has been shown to depend on the levels of E2F1 in other systems (Shan and Lee, 1994; Asano *et al*., 1996).

Overexpression of p21 causes growth arrest of CEF morphologically transformed by E2F1

To determine whether p21 can inhibit the growth of CEF transformed by E2F1, CEF infected with either the BP-E2F1 or E2F1 virus (both of which were morphologically transformed, Figure 2b, upper panels), were infected with the p21 virus (Givol *et al*., 1995). Both of the viruses expressing E2F1 are subgroup A, so that the p21 virus, which is subgroup B, was able to infect the E2F1 expressing cells. As shown in Figure 2b lower panels, overexpression of p21 causes growth

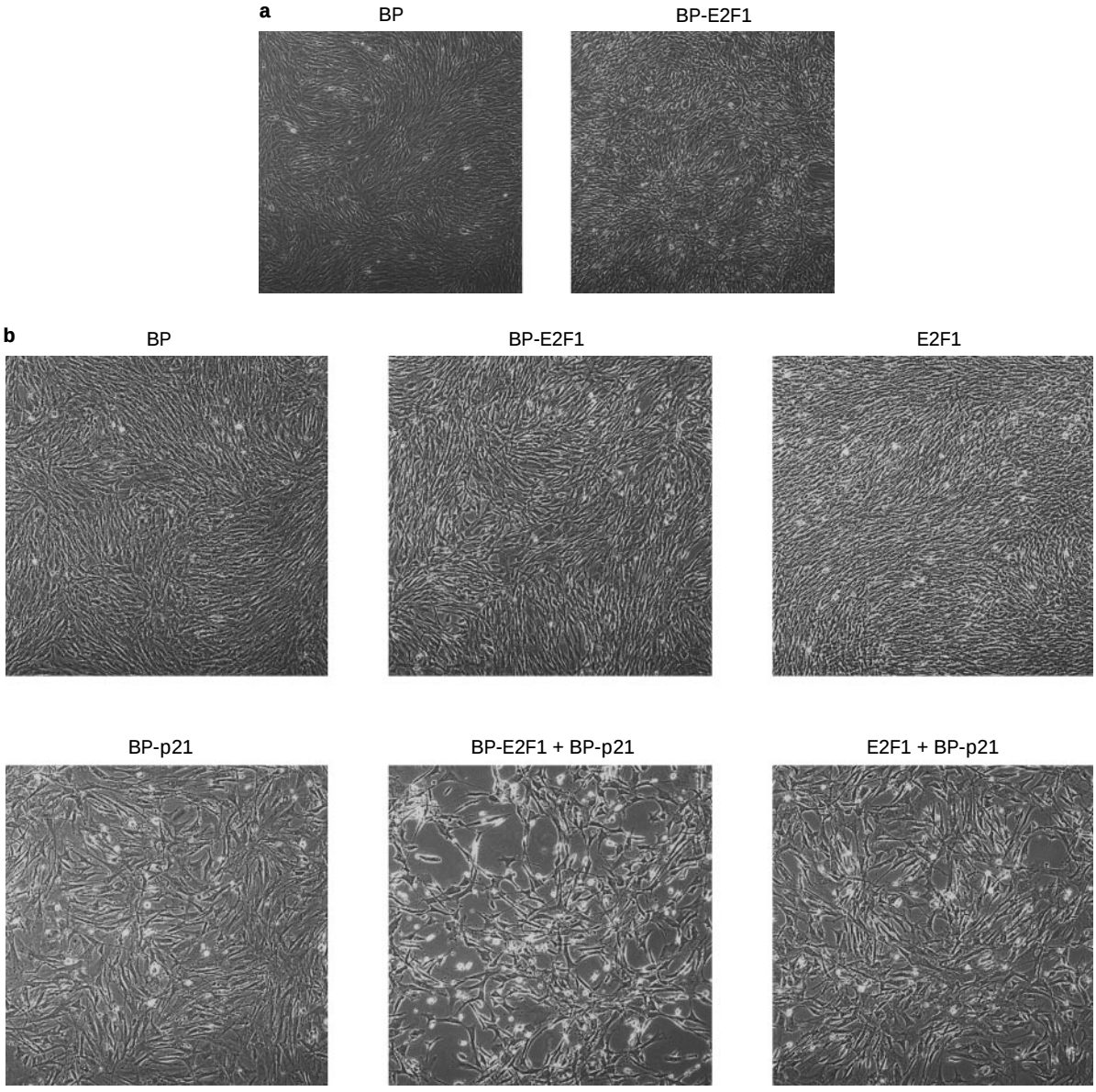

Figure 2 (**a**) The effect of human E2F1 expression on the morphology of CEF. Non-adherent cells were removed and adherent cells were viewed using an inverted confocal microscope (see Materials and methods). CEF infected with the vector (BP) show a normal morphology; CEF infected with BP-E2F1 are morphologically transformed. The images were taken 8 days after transfection. Magnification: ×100. (**b**) Effects of human p21 expression on the morphology of CEF and CEF expressing E2F1. The upper panels show CEF infected with the vector (BP) and CEF that are morphologically transformed by the BP-E2F1 and E2F1 viruses. Images of fixed cells were taken 12 days after transfection. The lower panels show the effects of the p21 virus on these cells. Images were taken 4–5 days after infection with the p21 virus. BP-E2F1 + BP-p21 infected cells were passaged once and refed every 2 days while the other cells were passaged twice before being fixed and photographed. Magnification: ×100

arrest of CEF expressing E2F1; cells that were not infected with the p21 virus (upper panels) continued to divide. These growth arrested cells express p21 as shown by Western blot analysis (Figure 5a, lane 3). Cultures infected with both the E2F1 and p21 viruses showed some growth after 10 days of arrest (indicated by the cultures reaching confluence). However, examination of these growing cells by Southern blot (data not shown) and Western blot analysis showed that they did not express p21 protein (Figure 5a, lane 4) but that they did express E2F1 protein (Figure 5b, lane 4). Similar results were observed for CEF infected with both the BP-E2F1 and p21 viruses (data not shown). This is similar to our previous observations in CEF that were infected with both the Src and p21 viruses or with the Myc and p21 viruses (Givol *et al*., 1995). In those experiments, transformed cultures that were growth arrested by p21 also showed some growth after 10 days of arrest. The growing cells, however, did not overexpress human p21.

Growth arrest, by p21, of CEF transformed by E2F1 is at the G_1 checkpoint

To determine at what stage of the cell cycle p21 arrests the growth of CEF transformed by E2F1, we analysed the DNA content of infected cells. Fluorescence-activated cell sorter (FACS) analysis was used to determine the DNA content and the cell cycle distribution of infected cells. Non-adherent and adherent cells were pooled, fixed, stained with propidium iodide and scanned. The effects of E2F1

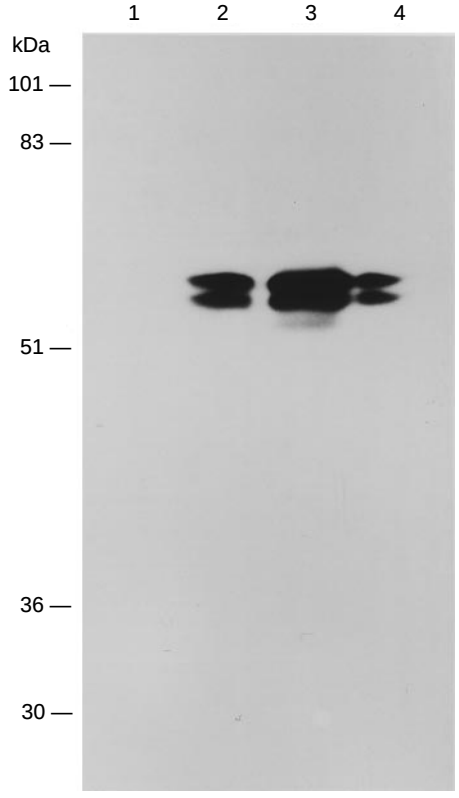

Figure 3 Immunoblot analysis showing levels of expression of human E2F1 in CEF. Cell lysates were analysed by gel electrophoresis and immunoblotting as described in Materials and methods. (1) Vector (BP) infected cells. (2) CEF infected with BP-E2F1, 16 days after transfection (passage 8). (3) CEF infected with BP-E2F1, 6 days after transfection. (passage 3). (4) CEF infected with E2F1, 16 days after transfection. (passage 8)

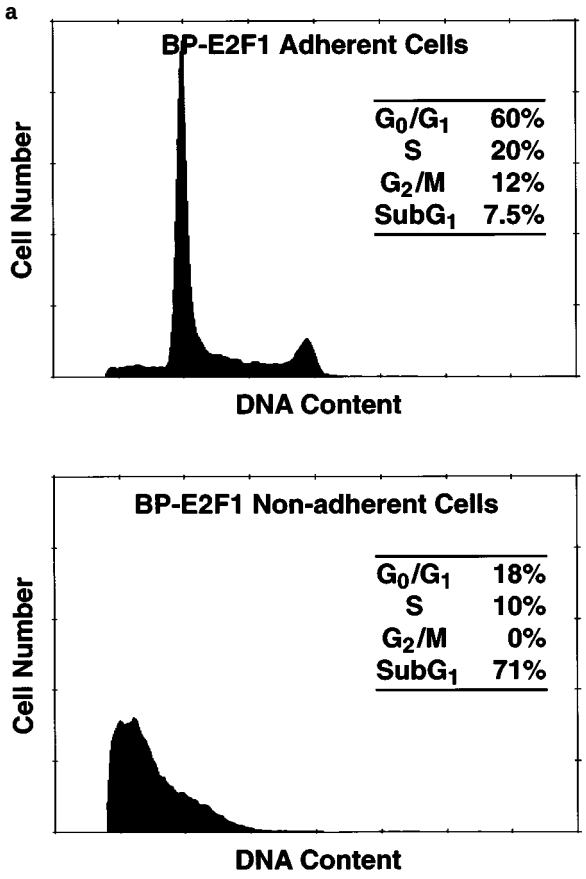

Figure 4 (**a**) Flow cytometric cell cycle analysis of CEF expressing high levels of E2F1. Adherent and non-adherent cells from CEF infected with BP-E2F1 were collected 4 days after transfection. The cells were fixed, stained with propidium iodide, and analysed by flow cytometry. The majority of the non-adherent cells are at a sub G_1 DNA content indicating apoptosis. (**b**) DNA fragmentation typical of apoptotic cells. (1) Marker ΦXHaeIII. (2) DNA of nonadherent cells from CEF infected with the BP-E2F1 virus

overexpression on the distribution of cells in the cell cycle are shown in Figure 6. Twenty-eight percent of cells infected with the BP-E2F1 virus are in S phase as compared with only 20% of cells infected with the BP virus. Some cells had a sub G_1 DNA content, which is indicative of apoptosis. The effects of overexpression of p21 on CEF that express E2F1 are seen in Figure 6, lower panels. There is a dramatic change in the cell cycle distribution. There is an approximately 20% increase in the number of cells in G_0/G_1 and a fourfold decrease in the number of cells in S phase, indicating that, in this system, overexpression of p21 blocks the growth of cells transformed by E2F1.

These results do not rule out the possibility that E2F1 can induce p21 growth arrested cells to enter S phase without completing the cell cycle, as reported previously (DeGregori *et al*., 1995b; Dimri *et al*., 1996).

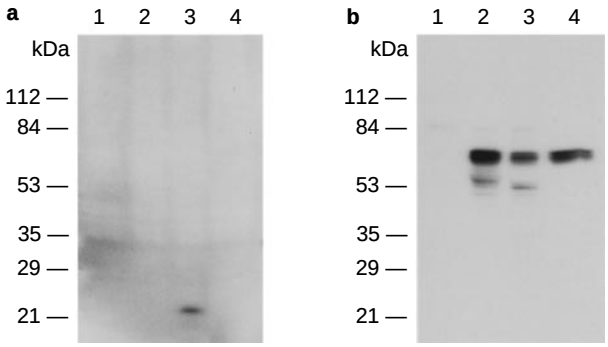

Figure 5 Immunoblot analysis showing expression of p21 (**a**) and E2F1 (**b**) in CEF infected with both the E2F1 and p21 viruses. Duplicate gels were loaded with 15 μl of cell lysate, blotted and reacted with anti-human p21 antibody (**a**) or anti-human E2F1 antibody (**b**). (1) Vector (E2F1-AS) infected CEF. (2) CEF infected with the E2F1 virus. (3) CEF infected with both the E2F1 and p21 viruses. (4) Cells that grew out from the growth arrested culture (lane 3), 16 days after growth arrest was observed

To address this question, vector infected and E2F1 infected CEF were each infected with equal amounts of the p21 virus. After 72 h, the cells were incubated with BrdU for 1 h to label newly synthesized DNA.

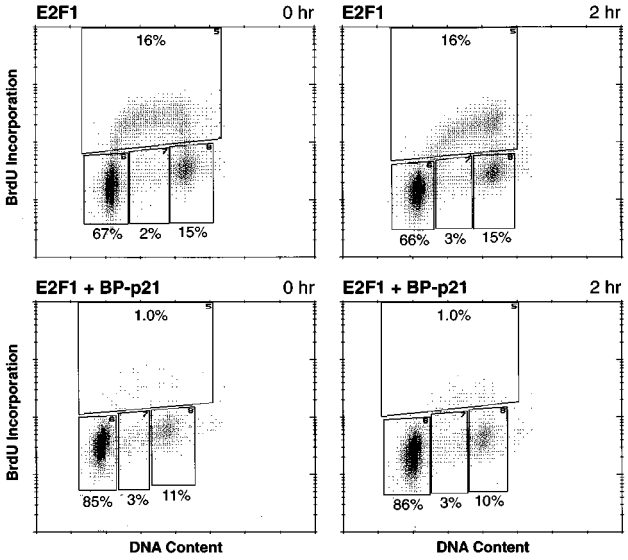

Figure 7 Bivariate flow cytometry of CEF expressing E2F1 and E2F1 + p21. Cells were infected with equal amounts of p21 virus as described in Materials and methods. Seventy-two hours later, cultures were pulse labeled with BrdU for 1 h and harvested at 0, 2, 4, 6 and 24 h. Cells were fixed, denatured and stained with anti-BrdU-FITC monoclonal antibody and propidium iodide. Representative histograms of cells harvested 0 h and 2 h after BrdU labeling are shown. No entry into S phase is seen when cells express p21. Similar results were obtained at the other time points and with CEF infected with p21 virus and a control vector. Numbers represent the percentage of cells in each phase of the cell cycle. Box 5 represents $BrdU^+$ cells. Box 6 represents cells in G_0/G_1. Box 7 represents $BrdU^-$ cells in S phase. Box 8 represents cells in G_2/M

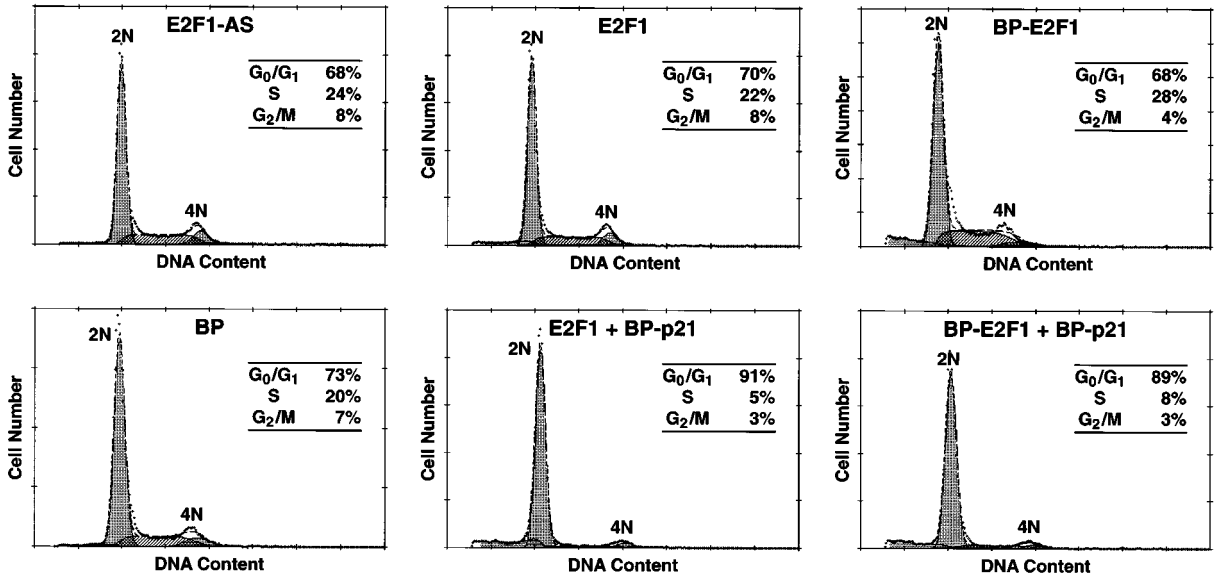

Figure 6 Flow cytometric cell cycle analysis of CEF expressing E2F1 (upper panels) and E2F1 + p21 (lower panels). Adherent and non-adherent cells were pooled and fixed, stained with propidium iodide and analysed by flow cytometry. Cells in G_0/G_1 are represented by the first peak (2N); cells in G_2/M are represented by the second peak (4N) and cells in S phase are represented by the area between the peaks. Quantitation of cell cycle distribution is presented inside each histogram. CEF expressing E2F1 were analysed 6 days after transfection; analysis of the effects of p21 on these transformed cells was performed 3 days after infection with the p21 virus. BP is the vector RCASBP. E2F1-AS is the RCAS vector with E2F1 in the antisense orientation

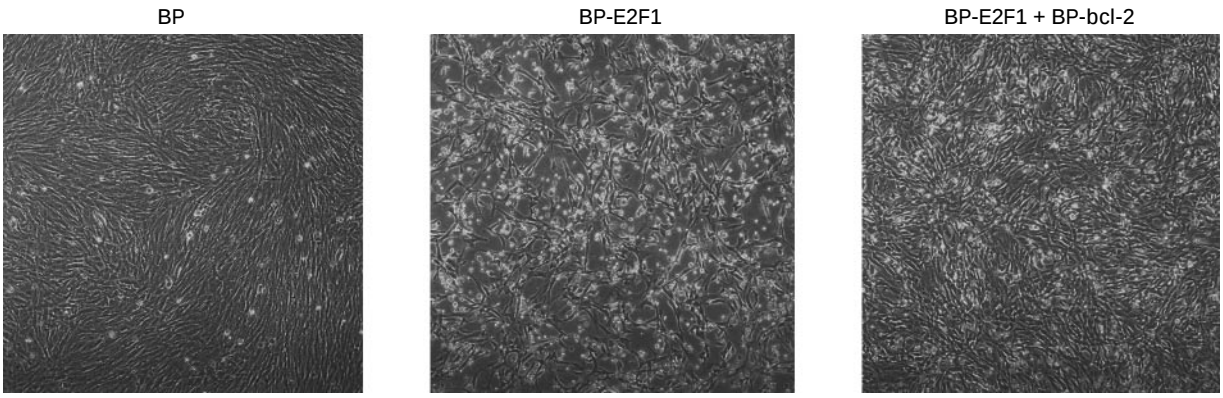

Figure 8 Effects of Bcl-2 on CEF expressing E2F1. CEF infected with RCASBP(A)E2F1 were morphologically transformed and undergoing apoptosis. At passage 4 (8 days posttransfection) cells were split into two and one of the cultures was infected with RCASBP(B)bcl-2 viral stock. The Bcl-2 virus was allowed to spread and after one cell passage (3 days postinfection) the cells were photographed (see Materials and methods). Magnification: ×100.

Progression of these labeled cells was monitored for 24 h (see Materials and methods). The level of BrdU incorporation as well as the DNA content was analysed by bivariate flow cytometry. As seen in Figure 7, no DNA synthesis is detected in cells expressing both E2F1 and p21 at 0 h and 2 h after BrdU labeling. We see no measurable DNA synthesis at other time points up to 24 h after BrdU labeling (data not shown). We have previously shown, by immunohistochemistry, that in CEF and CEF transformed by a variety of oncogenes, no DNA synthesis is detected in a cell that expresses p21 (Givol *et al*., 1995).

Bcl-2 inhibits apoptosis in CEF that overexpress E2F1

We wished to test whether overexpression of Bcl-2 can inhibit the apoptosis induced by high levels of E2F1. We previously showed that overexpression of Bcl-2 can inhibit apoptosis in CEF that express c-Myc (Givol *et al*., 1994). Although overexpression of Bcl-2 slows the growth of CEF, it does not induce cell cycle arrest in these cells. Following transfection with BP-E2F1 DNA, when all of the cells in the culture were transformed, we infected the culture with the Bcl-2 virus. The Bcl-2 virus completely inhibited the apoptosis caused by overexpression of E2F1 as seen 72 h postinfection (Figure 8).

Discussion

It is clear that the expression of high levels of p21 is sufficient to block the growth of both normal CEF and CEF that express relatively high levels of several oncogenes (Givol *et al*., 1995). These results suggest that oncogenic signals as well as mitogenic pathways converge at the cyclin/cdk complexes and affect the regulation of the cell cycle machinery. The connection between oncogenic signals and cell cycle checkpoints is important for our understanding of cell growth regulation and transformation. For example, the Ras/Raf pathway can activate two opposing signals in primary rat Schwann cells; either cell cycle progression through cooperating oncogenes (Raf + SV40 LTAg or a dominant negative p53 mutant) or cell cycle arrest through elevation of $p21^{waf1/cip1}$ (Raf alone). The presence of a cooperating oncogene is required for shifting the signal from growth arrest towards proliferation (Lloyd *et al*., 1997). The key regulatory step in the G_1 to S transition is thought to be the phosphorylation of pRb (by cyclin/cdks) and the release of E2F. In the original simple version of this model, p21, an inhibitor of cyclin/cdk kinase activity, is placed upstream of the pRb/E2F pathway and it was shown that p21 can inhibit E2F dependent transcriptional activity (Dimri *et al*., 1996; Michieli *et al*., 1996) and that E2F1 overexpression can overcome a p21 mediated growth arrest (DeGregori *et al*., 1995b; Dimri *et al*., 1996). This model has been challenged by experiments which showed, in the pRb positive human osteosarcoma cell line (U2-OS), that overexpression of p21 can block the division of cells that overexpress E2F1 (Mann and Jones, 1996).

Here we show that high levels of p21 can block the growth of CEF that express high levels of E2F1. Our results do not support the simple model which places E2F1 downstream of p21 since, in such a model, overexpression of E2F1 should be able to overcome a p21 block imposed on the pRb/E2F pathway. A particular advantage of the CEF system is that it is possible to test the interactions of proteins (like p21 and E2F1) in cells that are otherwise entirely normal. This eliminates complications that arise when the effects are measured in cell lines, which have additional, usually undefined, genetic changes that make them immortal in culture.

There are two obvious explanations for these results; either there is an additional role (or roles) for p21, which would place it either downstream of E2F1 or in parallel pathways that are also required for cell cycle progression, or alternatively we have not been able to express sufficiently high levels of E2F1 to overcome the p21 block. There does appear to be a limit on the level of E2F1 that CEF will tolerate. Overexpression of E2F1, using either an RCAS or RCASBP vector, induces morphological transformation of CEF. A closer examination of CEF infected with the BP-E2F1 virus showed that there was considerable apoptosis at the earliest passages following infection and that CEF expressing lower levels of E2F1 were selected. This is consistent with results from Shan and Lee (1994).

However the remaining CEF still express substantial amounts of E2F1, and the cells are transformed even at late passages. Therefore, we do not believe that the failure of E2F1 to overcome p21 is a result of inadequate expression of E2F1. Instead, we favor the idea that it is an additional activity (or activities) of p21 that allows it to block the division of cells that overexpress E2F1. Recently, two distinct pathways for the transition from G_1 to S have been reported (Mann and Jones, 1996; Lukas *et al*., 1997). Cyclin E/cdks, but not cyclin D/cdks, can drive cells into S phase by mechanisms that are distinct from E2F1 mediated transactivation (Lukas *et al*., 1997). Cyclin D/cdks have been shown to be upstream of the pRb/E2F pathway (Mann and Jones, 1996) but cyclin E/cdks can drive cells to S phase independently of activating the pRb/E2F pathway (Lukas *et al*., 1997). This implies targets for cyclin E/cdk which are downstream or on a parallel pathway to pRb/E2F and supports a role for p21 activity which is independent of E2F1.

In this system, E2F1 behaves similarly to Myc. Overexpression causes morphological transformation, and in addition, induces apoptosis. Perhaps this is not surprising since both of these proteins are transcriptional activators. E2F sites have been found in the promoter of c-*myc* (Hiebert *et al*., 1989) and a recent report (Leone *et al*., 1997) showed that E2F1 is a downstream target of c-Myc. In support of the view that there are similarities in the way in which these two transcription factors act, the apoptosis induced by overexpression of E2F1, like the apoptosis induced by overexpression of Myc, can be reversed by Bcl-2 overexpression. However, there are subtle differences; v-Myc is a more potent inducer of apoptosis than c-Myc and drives cells into S phase in the presence of serum (Petropoulos *et al*., 1996). Significant apoptosis is seen seen in CEF expressing c-Myc only in the absence of serum. In this regard E2F1 behaves more like v-Myc that c-Myc. This distinction may be more artificial than real, however, since the induction of apoptosis by E2F1 in the presence of serum clearly depends on the level of E2F1 expression: considerably more apoptosis is seen when E2F1 is inserted in RCASBP than in RCAS. Both the level of E2F1 and the effect of Bcl-2 on the fate of cells that overexpress E2F1 (whether or not the cells will undergo apoptosis) may have implications for the ability of E2F1 to play a direct role in cellular transformation. In cells with relatively low levels of Bcl-2, modest overexpression of E2F1 will lead to apoptosis; cells with high level of Bcl-2 will be transformed by levels of E2F1 overexpression that would otherwise induce apoptosis.

Materials and methods

Construction and growth of RCASBP(A)E2F1 virus

The RCAS and RCASBP vectors have been described elsewhere (Hughes *et al*., 1987; Petropoulos and Hughes, 1991). A 1.3 kb restriction fragment that contains the entire coding sequence for human E2F1 (Kaelin *et al*., 1992) was subcloned into the adaptor plasmid Puc-CLA112NCO. To facilitate cloning the human E2F1 sequences into the adaptor plasmid, PCR was used to create an *Nco*I site at the initiator ATG and to introduce a *Bam*HI site after the TGA termination codon of the E2F1 cDNA. The PCR product was digested with *Nco*I and *Bam*HI and the resulting fragment was cloned into the adaptor. The ends were confirmed by sequencing and a major portion of the coding region was replaced with sequences from the original E2F1 clone (Kaelin *et al*., 1992) by exchanging a 1.238 kb *Sty*I/*Xho*I fragment. This plasmid was digested with *Cla*I and the E2F1 containing fragment was cloned into RCAS(A) or RCASBP(A) to generate RCAS(A)E2F1 or RCASBP(A)E2F1. Ten μg of RCAS(A)E2F1 or RCASBP(A)E2F1 DNAs were transfected by the calcium phosphate method (Wigler *et al*., 1979; Hughes and Kosik, 1984) into CEF prepared from 13-day-old embryos of the EV-0 line. CEF cultures were generated and maintained as described elsewhere (Petropoulos and Hughes, 1991).

Co-infection experiments

The Bcl-2 and p21 viruses have been described previously (Givol *et al*., 1994, 1995). Ten μg of RCASBP(B)bcl-2 or RCASBP(B)p21 DNAs were transfected into CEF and viral stocks were collected after the cultures were completely infected (two or three cell passages). Five ml of fresh or frozen viral stocks were used to superinfect CEF transformed by E2F1. The cells were imaged using a Zeiss (Oberkochen, Germany) confocal laser scanning microscope (CLSM 410). Adobe Photoshop and Macromedia software were used to make a composite of these images. These were printed on a tektronics dye sublimation printer.

Western blotting (immunoblotting)

Infected cells were washed with PBS, scraped off the plate, collected by centrifugation and lysed in protein sample buffer. Cell lysates were boiled and analysed by electrophoresis on a 12% polyacrylamide SDS gel, transferred to a nitrocellulose membrane (BA85; Schleicher & Schuell, Keene, NH) and reacted with a mouse anti-human E2F1 monoclonal antibody, KH95 (PharMingen, San Diego) and anti-human p21 monoclonal antibody Ab-1 (Oncogene Science, Cambridge MA). Protein bands were visualized by using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL).

Fluorescence-activated cell sorter (FACS) analysis

Non-adherent cells were collected by centrifugation of culture supernatants. Adherent cells were trypsinized and collected by centrifugation. Adherent and non-adherent cell fractions were pooled, fixed, stained with propidium iodide and scanned as described previously (Givol *et al*., 1995). Histograms were analysed using the Multiplus AV software (Phoenix Flow Systems, San Diego, CA).

BrdU pulse analysis and FACS analysis

Cells at 50% confluence were labeled for 1 h with 10 mM bromodeoxyuridine (BrdU). The cells were washed twice with PBS and returned to label-free media. At 0, 2, 4, 6, and 24 h after being labeled, the cells were harvested, fixed, and stained with fluorescein isothiocyanate conjugated anti-BrdU antibody (FLUOS, Bhoeringer Mannheim) as specified by the manufacturer. The cells were then treated with RNase and propidium iodide as described previously (Givol *et al*., 1995) and analysed by bivariate flow cytometry on a Coulter EPICS Profile (Miami, Florida) measuring the BrdU-linked green fluorescence (FITC) through a 525 nm bandpass filter and the DNA-linked red fluorescence (PI) through a 610 nm wavelength filter. As a negative control, cells which were not labeled with

BrdU were subjected to this procedure and used as a standard by which to gate the cytometer. Histograms were analysed using Multiplus AV software.

DNA fragmentation

Non-adherent cells from a culture of CEF infected with the BP-E2F1 virus were collected and DNA was extracted as described previously (Laird *et al*., 1991). The DNA was analysed on a 2% agarose gel.

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