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E2F activity is essential for survival of Myc-overexpressing human cancer cells

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Effective cell cycle completion requires both Myc and E2F activities. However, whether these two activities interact to regulate cell survival remains to be tested. Here we have analysed survival of inducible c-Myc-overexpressing cell lines derived from U2OS human osteosarcoma cells, which carry wild-type pRb and p53 and are deficient for p16 and ARF expression. Induced U2OS-Myc cells neither underwent apoptosis spontaneously nor upon reconstitution of the ARF-p53 axis and/or serum-starvation. However, they died massively when concomitantly exposed to inhibitors of E2F activity, including a constitutively active pRb (RbΔcdk) mutant, p16, a stable p27 (p27T187A) mutant, a dominant-negative (dn) CDK2, or dnDP-1. Similar apoptotic effect was observed upon down-modulation of endogenous E2Fs through overexpression of E2F binding site oligonucleotides in U2OS-Myc cells, upon expression of RbΔcdk or dnDP-1 in the *Myc*-amplified HL-60 (ARF−; p53−) human leukemia cells, and upon co-transfection of Myc and RbΔcdk in SAOS-2 (ARF+; p53−) human osteosarcoma cells but not in human primary fibroblasts. Consistent with these results, a dnp53 mutant did not abrogate the Myc-induced apoptotic phenotype, which instead strictly depended on caspase-3-like proteases and on Myc transcriptional activity. Our data indicate that in contrast to normal cells, Myc-overexpressing human cancer cells need E2F activity for their survival, regardless of their ARF and p53 status, a notion that may have important implications for antineoplastic treatment strategies.
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Introduction

The tumor suppressors p16INK4A (p16) and p14/p19ARF (ARF) are two alternative and structurally unrelated products encoded by the *INK4A/ARF* genetic locus (Duro *et al*., 1995; Quelle *et al*., 1995), a frequent target of inactivation in tumorigenesis (Roussel, 1999). While p16 inhibits the phosphorylation of the retinoblastoma protein (pRb), ARF stabilizes p53 and activates p53-mediated growth-inhibitory responses (reviewed in Sherr and Weber, 2000). Deregulated expression of distinct cell cycle stimulating oncogenes, such as Myc, E2F-1, E1A, and Ras can upregulate ARF gene expression and result in p53-mediated cell cycle arrest or apoptosis (Sherr and Weber, 2000). This appears to be an important fail-safe mechanism to guard cells that do not carry deletions or mutations of *ARF* or *p53* from abnormal proliferative signals. Consistent with that, ARF- or p53-null MEFs are similarly resistant to Myc-induced apoptosis and when wild-type or ARF-hemizygote MEFs are immortalized by Myc, they escape from apoptosis by usually sustaining mutation of either ARF or p53 but not of both (Zindy *et al*., 1998). Moreover, reintroduction of the missing protein in knockout MEFs resensitizes them to apoptosis. It has also been shown that B-cell lymphomas arising in Eu-*Myc*-transgenic mice are characterized by *ARF* deletion or *p53* mutation (Eischen *et al*., 1999). Crossing Eu-*Myc* mice with *ARF*-null or *p53*-deficient mice, results in similarly accelerated malignant phenotype, characterized by aggressive lymphomas with low apoptotic rates and resistance to chemotherapeutic drugs (Schmitt *et al*., 1999). All these results have lead to the notion that ARF, as a sensor of oncogenic insults such as Myc overexpression, regulates a tumor suppressor pathway operating via p53-dependent apoptosis.

However, it remains unclear how closely this model is also applicable to human tumor cells. In keeping with the functional link between ARF and p53, a p53-mediated cell cycle arrest in G1 and G2/M was elicited by ectopic ARF expression in ARF-negative human tumor cells (Stott *et al*., 1998), but whether ARF-mediated activation of p53 might also result in apoptosis was not investigated. Moreover, an inverse correlation between ARF expression and p53 status

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was observed in several human tumor cell lines (Stott *et al*., 1998). More recently, either mutually exclusive (Fulci *et al*., 2000; Pinyol *et al*., 2000) or coexisting (Gazzeri *et al*., 1998; Markl and Jones, 1998; Sanchez-Cespedes *et al*., 1999) alterations of p53 and ARF have been reported in human tumors. While the former studies are consistent with these genes acting along the same pathway, the latter, together with the fact that murine ARF-p53 double-null preB cells are more resistant to Myc-induced apoptosis than cells lacking p53 or ARF alone (Eischen *et al*., 1999), support the idea of additional, p53-independent tumor suppressive functions of ARF (Weber *et al*., 2000). Alternatively, tumor cells may first undergo p53 mutation and then, despite little selection against ARF, may sustain deletions targeting p16 that coincidentally co-delete ARF (Stott *et al*., 1998; Sanchez-Cespedes *et al*., 1999).

To investigate the relation between c-Myc and the ARF-p53 apoptotic pathway in human tumor cells we analysed the survival properties of inducible, c-Myc-overexpressing cell lines derived from osteosarcoma U2OS cells (Santoni-Rugiu *et al*., 2000), which carry wild-type pRb and p53 (Diller *et al*., 1990) and are deficient for p16 and ARF expression (Stott *et al*., 1998; present study and data not shown). Induction of Myc alone or after reintroduction of ARF did not result in death of U2OS cells, even when these cells were concomitantly serum-starved, suggesting that they may differ from MEFs and primary murine lymphocytes in their response to the activation of the ARF-p53 pathway in presence of deregulated Myc expression. Thus, this type of human tumor cells may not necessarily rely on disruption of this pathway to counteract Myc-derived apoptotic signals. In an attempt to identify other potential regulators of survival in Myc-overexpressing tumor cells, we tested whether E2F activity would represent one of these. We have recently shown that E2F and Myc activities are concomitantly required to ensure timely and proper levels of DNA synthesis and orderly completion of cell cycles, despite these activities being able to promote the G1/S transition independently of each other (Lukas *et al*., 1999a; Santoni-Rugiu *et al*., 2000). Therefore, we investigated whether survival of Myc-overexpressing tumor cells may involve endogenous E2F activity. Our results indicate that this activity is critical for the survival of human tumor cells with deregulated Myc expression, regardless of the intactness of the ARF-p53 pathway, a notion that may have important implications for antineoplastic therapeutic strategies.

Results

Expression of ARF does not sensitize U2OS cells to Myc-induced apoptotic signals

It is known from studies in different cell types that increased Myc expression is able to stimulate either cell cycle progression or apoptosis, if the former is perturbed by serum starvation or by other types of cell-stress (reviewed in Prendergast, 1999). In this respect, flow cytometric analysis of DNA content showed that stable induction of Myc by culturing U2OS-Myc cells in tetracycline (TET)-free medium stimulated progression through all phases of the cell cycle (Table 1 and Santoni-Rugiu *et al*., 2000), without provoking a significant increase in the sub-G1 cell compartment (Table 1). In agreement with this, the viability of derepressed U2OS-Myc cells monitored over 6 days by the trypan blue dye exclusion method was virtually unaffected in presence of serum and very modestly perturbed under serum-starvation (Figure 1a). As this could be explained, at least in part, by the lack of ARF gene expression in these cells (see Introduction and Figure 1b), we tested whether ectopic HA-tagged ARF would sensitize them to potential apoptotic signals prompted by Myc (Juin *et al*., 1999). Transfection efficiency was monitored by anti-HA immunostaining as reported (Santoni-Rugiu *et al*., 2000). However, following Myc-induction, no significant difference in the amount of apoptotic sub-G1 fraction was observed between U2OS-Myc cells proficiently transfected with HA-ARF and those transfected with empty-vector, even in conditions of serum-starvation (Table 1). Nevertheless, exogenous ARF was functional, as its expression resulted in appreciable stabilization of p53 (Figure 1b) and arrest in G1 and G2/M when cells were kept in serum-containing medium (Table 1), consistent with previous observations in U2OS cells (Stott *et al*., 1998). In serum-starved U2OS-Myc cells, ARF expression reinforced the G1 arrest induced by serum deprivation (Table 1). Taken together these data imply that human tumor cells may, at least to some extent, respond

Table 1 Flow cytometric analysis of DNA content in propidium iodide-stained U20S-Myc cells[a]

U20S-Myc cells	*Apoptotic* (%)	*G0/G1* (%)	*S* (%)	*G2/M* (%)
+Tet	2	56	34	10
+Tet+Vector[b]	3	58	28	14
+Tet+ARF	2	69	9	22
+Tet−FCS[c]	3	72	18	10
+Tet+ARF−FCS	2	82	8	10
−Tet	3	34	39	27
−Tet+Vector	2	36	38	26
−Tet+ARF	3	45	19	36
−Tet−FCS	4	63	23	14
−Tet+ARF−FCS	2	71	11	18

[a]Each profile was assessed at least three times and in independent clones with similar results. Untransfected cells were cultured in TET-containing (+Tet) or -free (−Tet) medium for 4 days and cell cycle distribution analysed by flow cytometry with CellQuest software and quantified with ModFit software. Sub-G1 cells were gated and counted separately by using CellQuest and expressed as percentage of total number of cells. [b]Cells transfected with 5 μg empty pCMV or ARF vector and 1 μg pCMV-CD20 were cultured in the same conditions and CD20-positive cells analysed as above. Empty vector was added to a total of 25 μg DNA/10-cm-diameter dish. [c]Cultured in absence of fetal calf serum (−FCS)

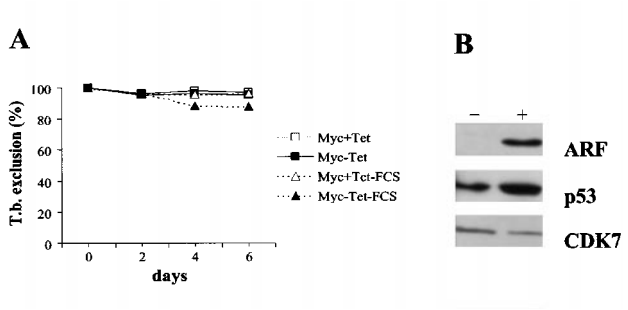

Figure 1 (**a**) Viability of U2OS-Myc cells. Trypan blue exclusion test performed at the indicated time-points after plating and culturing the cells in TET-containing (+Tet) or -free (−Tet) medium. Myc induction caused virtually no cell death in presence of FCS and minimal toxicity in FCS-deprived cells (−FCS). The experiment was repeated at least three times with comparable results and reproduced in independent clones. (**b**) Ectopic ARF stabilizes endogenous p53 expression in U2OS-Myc cells. Cells were transfected with ARF expressing vector (+) or empty vector (−) and Myc transgene was derepressed by TET removal. Cells were harvested 2 days later and 50 μg protein lysate/lane were electrophoretically resolved and immunoblotted with mAb DCS-240.1 to human ARF and DO-1 to p53. CDK7 expression was assessed with MO-1 mAb and used as loading control

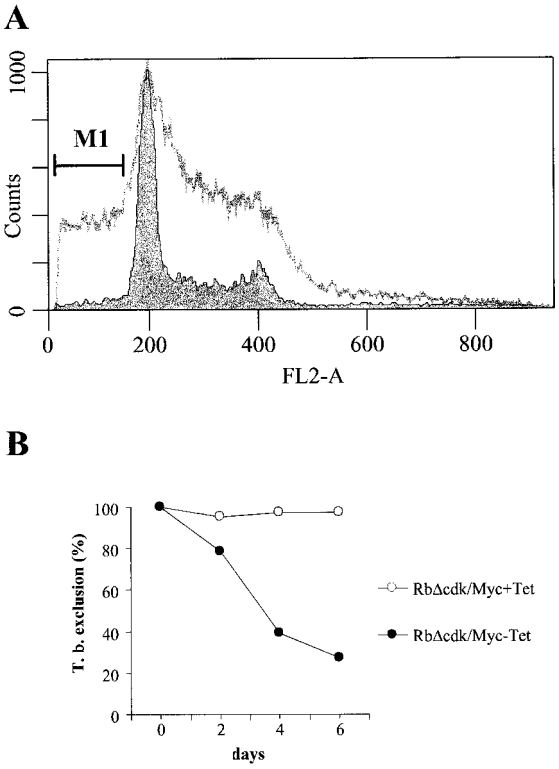

Figure 2 Coexpression of Myc and constitutively active Rb causes severe cell death of U2OS cells. (**a**) Representative flow-cytometric DNA histograms of PI-stained U2OS-RbΔcdk/Myc cells, grown in medium with (filled area) or without (empty area) TET for 4 days. Note the massive entry and accumulation of cells in S phase after transgene induction, as originally described (Santoni-Rugiu *et al*., 2000) as well as the massive presence of sub-G1 (apoptotic) cells (M1 gate). The result was reproduced multiple times in this and other independent U2OS-RbΔcdk/Myc clones. (**b**) Progressive loss of viability of U2OS-RbΔcdk/Myc cells after induction of the transgenes. Cell integrity was evaluated by trypan blue exclusion as described in Figure 1a. The experiment was repeated at least three times, with comparable results

differently than MEFs and primary mouse lymphocytes (Eischen *et al*., 1999) to the activation of the ARF-p53 pathway in the presence of deregulated Myc expression. The possibility that apoptosis in U2OS-Myc cells could be prevented by ARF-mediated cell cycle arrest or by impairment of the tumor suppressor apoptotic pathway at a level downstream of ARF and p53 (Soengas *et al*., 2001) cannot be ruled out. In any case, the reconstitution of the ARF-p53 axis does not sensitize U2OS cells to Myc-dependent apoptotic signals. We therefore searched for other mechanisms that could do that.

Constitutively active Rb induces death of U2OS-Myc cells

It is still not known whether the effects of Myc on cell cycle and survival are regulated independently (Prendergast, 1999). We have recently shown in a specifically engineered U2OS-derived cell line, U2OS-RbΔcdk/Myc, that Myc can stimulate G1/S transition and persistent DNA replication in cells long-term deprived of E2F activity (Santoni-Rugiu *et al*., 2000) by a constitutively active pRb mutant, pRbΔcdk (Lukas *et al*., 1997, 1999b). However, both E2F and Myc activities are required to ensure timely and proper levels of DNA synthesis and orderly completion of cell cycles (Lukas *et al*., 1999a; Santoni-Rugiu *et al*., 2000). It remains untested, though, whether these activities interact in regulating cell survival as well. Therefore, to assess a possible correlation between E2F function and viability of tumor cells with deregulated Myc activity, we have now investigated the survival properties of U2OS-RbΔcdk/Myc cells. At day 4 after transgene co-induction, in addition to the expected accumulation of cells in S phase (Santoni-Rugiu *et al*., 2000), almost 50% of the total number of U2OS-RbΔcdk/Myc cells displayed sub-G1 DNA content (Figure 2a). Furthermore at the same time-point, more than 60% of the cells were unable to exclude the vital dye trypan blue, indicating that they had lost plasma membrane integrity (Figure 2b). Comparable results were obtained in an independent set of experiments where we transiently transfected RbΔcdk in U2OS-Myc cells (see Figure 4) or co-transfected Myc and RbΔcdk in non-clonal parental U2OS cells (data not shown). Altogether, these data indicate that deregulated expression of Myc in the presence of constitutively active Rb induces apoptosis in U2OS cells. It is noteworthy that decreased survival was not observed in U2OS-RbΔcdk or U2OS-RbΔcdk/CycE cell lines, which express RbΔcdk alone or together with another cell cycle stimulator, cyclin E, respectively (Lukas *et al*., 1999b; Santoni-Rugiu *et al*., 2000 and data not shown). This implies that the induction of apoptosis is a specific response to the coexpression of Myc and RbΔcdk.

The apoptotic phenotype in U2OS-RbΔcdk/Myc cells is dependent on Myc transcriptional activity and is mediated by caspase-3-like proteases

Although the biological functions of Myc cannot be entirely explained by the known Myc target genes, the ability of Myc to induce S phase or cell death has been linked to its capability of regulating key target genes associated with proliferation or apoptosis (Prendergast, 1999). Therefore, we assessed the requirement of Myc transcriptional activity for the apoptotic phenotype observed in U2OS-RbΔcdk/Myc cells. For this purpose we transfected these cells with MadMyc (MM), a previously described chimera that antagonizes Myc by inhibiting its transcriptional regulatory function (Berns *et al*., 1997; Santoni-Rugiu *et al*., 2000). Consistent with our earlier results (Santoni-Rugiu *et al*., 2000), MM abolished the massive entry and accumulation of the U2OS-RbΔcdk/Myc cells in S phase. More importantly, it prevented apoptosis in these cells when the transgenes were co-induced by TET-removal, while U2OS-RbΔcdk/Myc cells transfected with empty vector displayed about the same level of apoptosis as the untransfected population after TET-removal (Table 2 and Figure 2a). Thus, the transcriptional regulatory function of Myc is required for the induction of cell death in U2OS cells with deregulated expression of Myc and constitutively active Rb.

Next, we further analysed the mechanism of cell death in U2OS-RbΔcdk/Myc cells. Following derepression of the Myc and RbΔcdk transgenes we detected sustained cleavage of poly(ADP-ribose) polymerase (PARP) into a specific fragment of 85 kD (Figure 3a), a hallmark of caspase-dependent apoptosis (Lazebnik *et al*., 1994). Consistent with this observation, cell death was prevented, in a dose-dependent manner, when cells were treated with the pan-caspase inhibitor ZVAD-fmk or the caspase-3-specific inhibitor DEVD-CHO (Figure 3b and data not shown).

Table 2 Effects of G1/S inhibitors on apoptosis in U2OS-RbΔcdk/Myc cells[a]

Plasmid	*Apoptotic* (%)	*G1* (%)	*S+G2/M* (%)[b]
CMV	45	35	65
p16	49	65	35
p27T187A	54	79	21
dnCDK2	49	89	11

	Apoptotic (%)	*G1* (%)	*S* (%)	*G2/M* (%)
MM	8	51	39	10

[a]CD20-positive cells transfected with the indicated plasmids (CMV, p16, p27T187A at 5 μg; dnK2 and MM at 15 μg) were assayed for cell cycle distribution and apoptosis after a 4-day culture in TET-free medium. Empty vector was added to a total of 25 μg DNA/10-cm-diameter dish. A representative example of three comparable experiments is shown. [b]S and G2/M are represented together because the algorithm was unable to clearly distinguish these two phases in cell transfected with the indicated plasmids except for MM

Furthermore, caspase-3-like activity at day 4 of induction was almost eightfold higher than the activity recorded at day 0 (Figure 3c). In comparison, in U2OS-Myc cells we observed less than twofold increase in caspase-3-like activity at day 4 of induction, suggesting that Myc alone can induce some activity but this is insufficient to produce death of U2OS cells (see Figure 1). Thus, cell death caused by overexpression of Myc and RbΔcdk involves a mechanism dependent in large part on caspase-3-like proteases, in line with other models of Myc-dependent apoptosis (Kagaya *et al*., 1997; Kangas *et al*., 1998; Hotti *et al*., 2000). Consistent with these results, the anti-apoptotic protein Bcl2, known to inhibit the mitochondrial activation of caspase-3-like enzymes and Myc-mediated apoptosis in other systems (Prendergast, 1999), significantly diminished the amount of cell death in U2OS-RbΔcdk/Myc cells (Figure 4).

G1/S inhibitors do not prevent apoptosis in U2OS-RbΔcdk/Myc cells

Inappropriate, sustained expression of the constitutively active RbΔcdk mutant alone in U2OS cells results in temporary G1 arrest followed by gradual entry into S phase due to residual cyclin E-associated kinase activity stimulated by endogenous Myc activity (Lukas *et al*., 1999b; Santoni-Rugiu *et al*., 2000). Instead, cells with forced co-expression of Myc and RbΔcdk, rapidly enter S phase and persistently replicate DNA, owing to the high cyclin E-associated kinase activity induced by ectopic Myc, without, however, being able to productively divide (Santoni-Rugiu *et al*., 2000). Therefore, cell death in U2OS-RbΔcdk/Myc cells could be due to their prominent entry and accumulation in S phase with active cyclin/CDK complexes, as cyclin-CDKs have been implicated in apoptosis of certain cell types (O'Connor *et al*., 2000). In order to test this hypothesis, U2OS-RbΔcdk/Myc cells were transfected with different inhibitors of the G1/S transition, such as the CDK4/6 inhibitor p16, the stable p27T187A mutant of the CDK2 inhibitor p27 (Nguyen *et al*., 1999), and the dominant-negative CDK2 mutant, dnK2 (van den Heuvel and Harlow, 1993) and were further cultured with or without TET. Although capable of significantly reducing the amount of cells in S phase, the expression of each of these G1/S-inhibitors did not decrease the sub-G1 population of induced U2OS-RbΔcdk/Myc cells, but rather increased it (Table 2). This was in contrast to the effect of MM, which as described above, blocked both S phase entry and apoptosis in U2OS-RbΔcdk/Myc cells. Taken together, these data suggest that apoptosis caused by coexpression of Myc and RbΔcdk can not be overcome by the prevention of S phase as such but it is blocked by the inhibition of Myc transcriptional activity. On the other hand, the additional reduction in viability observed in U2OS-RbΔcdk/Myc cells after expression of G1/S inhibitors, supports the idea that E2F activity may be an important regulatory factor for the survival of cells with deregulated Myc expression.

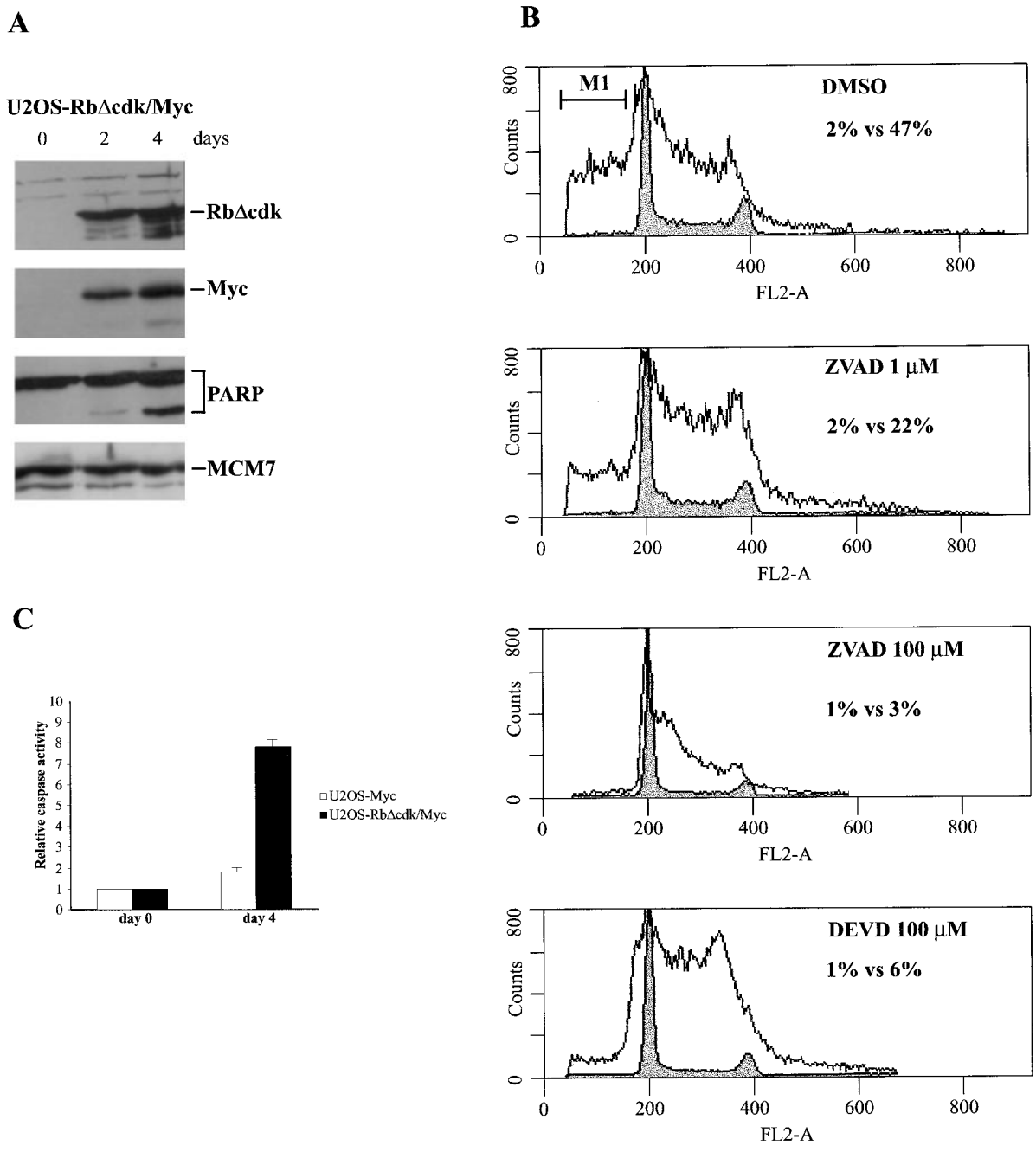

Figure 3 Death of U2OS cells conditionally coexpressing Myc and pRbΔcdk occurs via a caspase-3-like dependent mechanism. (**a**) PARP cleavage after transgenes' induction in U2OS-RbΔcdk/Myc cells. 50 μg protein lysate/lane were electrophoresed and immunoblotted with mAbs to c-Myc (9E10), HA-tagged RbΔcdk (HA12CA5) and PARP (C2-10). MCM7 expression, assessed with DCS-141 mAb, was used as loading control. When expression of the ectopic transgenes is repressed by TET (day 0), PARP is uncleaved. In contrast, sustained expression of transgenes after TET removal (day 2 and 4) causes increasing PARP cleavage. (**b**) Caspase-inhibitors block apoptosis of activated U2OS-RbΔcdk/Myc cells in a dose-dependent manner. Cells (5×10^5) were plated onto 10 cm dishes in TET-containing (filled area) or -free (empty area) medium and after 12 h the medium was supplemented with the pan-caspase inhibitor ZVAD-fmk or the caspase-3-specific inhibitor DEVD-CHO at concentrations from 1 to 100 μM, some of which are omitted in the figure for simplicity. Apoptosis was measured 4 days later by FACS analysis of PI-stained cells. Maximal final concentration of DMSO in the medium was 0.01%. Medium with only 0.01% DMSO was used as a control. The percentages of apoptotic cells in filled areas (transgenes repressed) versus empty areas (transgenes induced) are indicated. (**c**) Caspase-3-like activity in U2OS-Myc and U2OS-RbΔcdk/Myc cells. DEVDase activity was colorimetrically measured in triplicate on cell lysates prepared at day 0 and 4 of transgene induction. After quantification, the activity was expressed as relative to that of TET-repressed cells (day 0), which was set at 1 and previously found in either cell population to be identical to that of parental U2OS cells

Other inhibitors of E2F activity can induce cell death in U2OS-Myc cells

The role of pRb in regulation of apoptosis appears complex. In recent years several studies have focused on apoptosis triggered by loss of Rb function and/or excessive E2F activity (reviewed in Harbour and Dean, 2000; Nevins, 2001). However, pRb has also been reported to activate cell death through diverse and yet unclear mechanisms (Bowen *et al*., 1998; Hsieh *et al*., 1999; Knudsen *et al*., 1999). Thus, we cannot completely rule out that functions of active Rb different from the inhibition of the endogenous E2F activity, could play a causative role in the apoptosis of U2OS cells co-expressing Myc and RbΔcdk. To elucidate this point U2OS-Myc cells were transfected with plasmids encoding a dominant-negative DP-1 mutant (dnDP-1), T187Ap27, dnK2, or p16, all proteins shown to efficiently inhibit E2F activity in U2OS cells (Lukas *et al*., 1997, 1999a,b; Santoni-Rugiu *et al*., 2000). As shown in Figure 5, after removal of TET, a significant percentage of U2OS-Myc cells transfected with these E2F inhibitors, but not with control vector, became apoptotic. Thus, deregulated expression of Myc in U2OS cells deprived of E2F activity by different inhibitors leads to cell death. This supports the notion that apoptosis of U2OS-RbΔcdk/Myc cells is linked to the inhibition of E2F activity by RbΔcdk, rather than to any other potential function of this Rb mutant. Instead, transfection of wild-type Rb did not result in apoptosis of U2OS-Myc cells (Figure 5) most likely because these cells can efficiently inactivate Rb by phosphorylation, consistent with the very modest apoptosis seen in U2OS-Myc cells under conditions of serum-starvation (Figure 1a and Table 1).

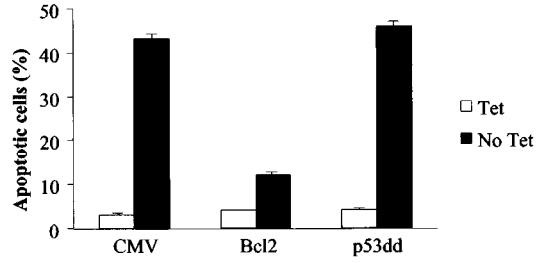

Figure 4 Bcl2 but not a dominant negative p53 molecule (p53dd) inhibits apoptosis in U2OS-RbΔcdk/Myc cells. CD-20-positive cells exposed to 5 μg of the indicated plasmids were cultured for 4 days in TET-containing (white bars) or -free (black bars) medium and assayed for sub-G1 DNA content. Values, expressed as percentage of apoptotic cells, are means ± s.d. of three independent experiments

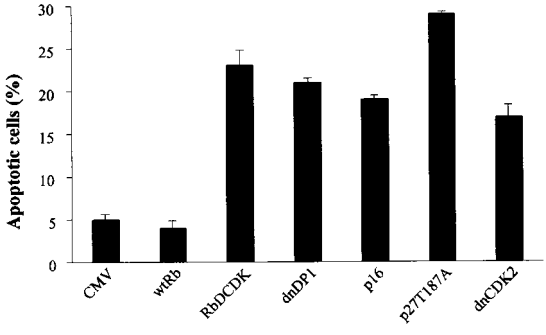

Figure 5 Different inhibitors of E2F activity induce apoptosis of U2OS-Myc cells. Cells were cotransfected with the indicated plasmids (5 μg for each of these plasmids except for dnK2 used at 15 μg), 1 μg CD20 expression vector, and empty vector up to a total of 25 μg DNA/10-cm-diameter dish. Apoptosis was assayed by flow cytometry as sub-G1 DNA content of PI-stained, CD-20-positive cells after a 4-days culture in TET-free medium. Data, expressed as percentage of total number of cells, are means ± s.d. of three independent experiments

Depletion of E2F activity via overexpression of E2F binding site oligonucleotides results in apoptosis of U2OS-Myc cells

To strengthen the concept that deprivation of E2F activity may cause apoptosis of tumor cells with deregulated expression of Myc, we used another approach aimed at sequestering and removing endogenous E2F from its downstream target promoters. U2OS-Myc cells were electroporated with oligonucleotides, containing either a wild-type binding site for E2F transcription factors, E2Fbswt, or a point mutant E2F binding site, E2FbsMut, unable to bind them (Helin *et al*., 1992; Lees *et al*., 1993). Pilot gene-transfer experiments coupled with reporter assays showed that 10 μM was the effective concentration of E2Fbswt resulting in titration of E2Fs and inhibition of E2F activity in U2OS cells without unspecific toxicity while E2FbsMut had no effect on E2F activity at any concentration tested (not shown). When Myc was induced by TET removal, the sub-G1 population remained virtually unchanged in cells electroporated with E2FbsMut or with an oligonucleotide unrelated to E2F (SP1RBF). In contrast, a marked increment of the percentage of U2OS-Myc cells undergoing apoptosis was observed after electroporation of E2Fbswt (Table 3), in keeping with the idea of E2F activity being a critical survival factor for these cells. Importantly, the apoptotic effect of E2Fbswt in induced U2OS-Myc cells was rescued by coelectroporation of the MM chimera (Table 3), a result that resembles the rescue by MM in induced U2OS-RbΔcdk/Myc cells (Table 2)

Table 3 Titration of endogenous E2Fs by forced expression of E2F binding sites causes apoptosis in derepressed U2OS-Myc cells[a]

Oligo	$+Tet$[b] (%)	$-Tet$ (%)
E2Fbswt	5	23
E2FbsMut	4	6
E2Fbswt + MM[c]	3	7
SPIRBF	4	5

[a]Values are percentage of apoptotic cells measured by flow cytometric analysis 4 days after electroporation with the indicated oligonucleotides, as described in Materials and methods. Each measurement was evaluated three times with comparable results. [b]+*Tet* and −*Tet*: cells cultured in TET-containing and -free medium, respectively. [c]Cells co-electroporated with MM

and confirms the requirement for Myc transcriptional activity in this type of cell death. Thus, deregulated Myc transcriptional activity combined with inhibition of E2F activity can lead to cell death of human cancer cells.

p53 is not involved in cell death induced by co-expression of Myc and RbΔcdk

Previous studies have shown that Myc-mediated apoptosis can be p53-dependent or -independent (reviewed in Hoffman and Liebermann, 1998; Prendergast, 1999). In this respect, even though cell death in our U2OS model is ARF-independent, it remains possible that p53, which is wild-type in U2OS cells, could contribute to it. In particular, the significant amount of endoreplication seen in U2OS-RbΔcdk/Myc cells (Santoni-Rugiu *et al*., 2000) as well as the fact that deregulated Myc may induce genomic instability in certain cell types (Felsher and Bishop, 1999; Li and Dang, 1999; Santoni-Rugiu *et al*., 2000), raise the possibility that p53 could be activated in response to potential DNA damage and be responsible for apoptosis in U2OS-RbΔcdk/Myc cells. To check this possibility, we transfected these cells with an expression vector encoding p53dd, a dominant-negative p53 mutant shown to reduce p53-dependent apoptosis (Bowman *et al*., 1996). However, even tough p53dd was functional, in that capable of silencing p53-responsive reporters such as mdm2-Luc and p21-Luc (Falck *et al*., 2001), it did not prevent cell death of derepressed U2OS-RbΔcdk/Myc cells. This result was consistent with the lack of p53 induction observed in these cells after transgene activation (ES-R, DD, JL and JB, unpublished observations). To further rule out a role for p53 in apoptosis caused by coexpression of Myc and RbΔcdk, p53-deficient human osteosarcoma SAOS-2 cells were transiently transfected with Myc and RbΔcdk expression plasmids and 4 days later, the sub-G1 population was analysed. Overexpression of either Myc or RbΔcdk alone did not show a significant increase of apoptotic cells compared with transfection of empty-vector, while this increase occurred when these transgenes were expressed together (Figure 6a).

Induction of apoptosis by inhibition of E2F activity occurs in Myc-overexpressing tumor but not normal cells

Contrary to what was observed in U2OS and SAOS-2 cells, the combination of Myc and RbΔcdk did not induce apoptosis in BJ human primary fibroblasts (Figure 6a), although the latter died effectively when exposed to Myc and serum-starved (data not shown). Thus, at least in cells of mesenchymal origin, the loss of viability due to the activation of Myc in conditions of E2F inhibition appears to occur specifically in tumor cells. As this observation may have important implications for designing strategies of selective tumor cell killing, HL-60 human promyelocytic leukemia cells, which are characterized by Myc gene amplification (Nowell *et al*., 1983; von Hoff *et al*., 1990; Mangano *et al*., 1998), deletion of p53 (Wolf and Rotter, 1985) and lack of ARF protein expression (Della Valle *et al*., 1997), were tested for their response to deprivation of E2F activity (Figure 6b). No toxic effect was observed by electroporation of either control vector or wild-type Rb that is promptly phosphorylated in these cells. In contrast, forced expression of RbΔcdk or dnDP-1 provoked considerable levels of apoptosis, which in turn was strongly reduced by inhibition of Myc activity via coexpression of MM. These results corroborate the concept that endogenous E2F activity may be crucial for survival of tumor cells with deregulated Myc activity, independently of ARF and p53.

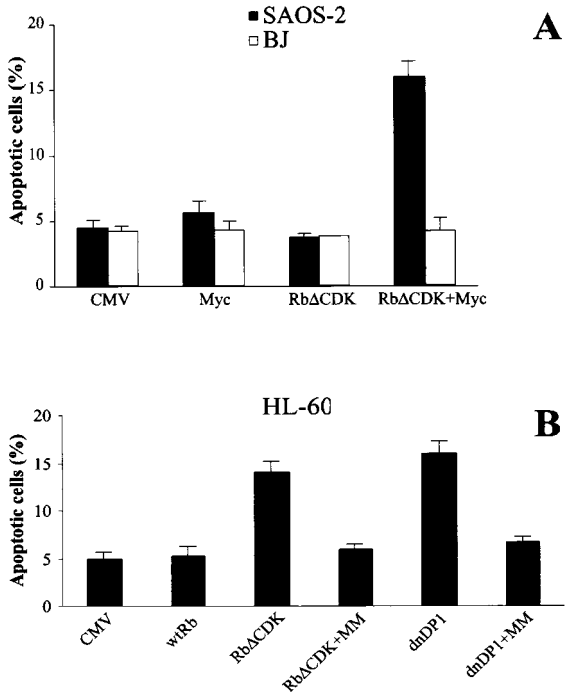

Figure 6 Apoptosis caused by inhibition of E2F activity in Myc-overexpressing tumor cells is p53-independent and tumor specific. (**a**) Apoptosis induced by coexpression of Myc and RbΔcdk is reproducible in the p53-deficient, SAOS-2 human osteosarcoma cells but not in BJ human primary fibroblasts. SAOS-2 and BJ cells were exposed for 4 days to the indicated plasmids (each transfected at 5 μg) and assayed for sub-G1 DNA content by flow cytometry as described in Materials and methods. (**b**) Inhibition of endogenous E2F activity in HL-60 cells results in Myc-dependent cell death. Sub-G1 DNA content of E-GFP-positive cells was analysed 4 days after cell electroporation with 0.3 μg E-GFP and 3 μg of the indicated plasmids. RbΔcdk and dnDP-1 but not wild-type Rb or empty vector increased the level of apoptosis, which in turn was inhibited by MM. In (**a**) empty vector was added to a total of 25 μg DNA/10-cm-diameter dish, in (**b**) to a total of 8.5 μg/dish. Results are means $\pm$ s.d. of three independent experiments

Discussion

Signals that stimulate or hinder growth and survival determine whether cells respond to abnormal Myc expression with proliferation or apoptosis (Prendergast,

1999). We have previously shown in a U2OS model that effective cell cycle completion requires both Myc and E2F activities (Lukas *et al*., 1999a; Santoni-Rugiu *et al*., 2000). However, whether these two activities interact to regulate cell survival remains to be tested. It is known that Myc sensitizes cells to apoptosis but requires additional signals to induce apoptosis (Juin *et al*., 1999). Here we show that an important event triggering apoptosis in human cancer cells overexpressing Myc is the inhibition of E2F activity. Together with our previous results, this suggests that Myc and E2F co-regulate fundamental cell behaviors, like proliferation and survival of neoplastic cells. Indeed, it is widely accepted that tumor cells possess high and/or unscheduled E2F activity, given the constant activation of mitogenic signaling pathways by oncogenes and growth factors as well as the variety of aberrations targeting the p16/cyclin D/CDK4/Rb/E2F pathway in virtually all types of human cancer cells (Bartek *et al*., 1996; Nevins, 2001). While in normal cells Rb-E2F may regulate gene expression chiefly by a mechanism of transrepression and derepression, the higher levels of free E2F in tumor cells may also enable and/or accentuate a mechanism of direct transactivation (Dyson, 1998; Harbour and Dean, 2000; Müller and Helin, 2000). In addition, many tumor cell types are characterized by high levels of Myc expression and activity (Nesbit *et al*., 1999), thereby facing the risk of being killed if Myc-mediated apoptotic signals are not tackled. Therefore, it is legitimate to propose from our data that human cancer cells overexpressing Myc need E2F activity not only for coordinated completion of cell cycles (Lukas *et al*., 1999a; Santoni-Rugiu *et al*., 2000) but also for a process of adaptive survival. This process possibly occurs via direct transactivation of E2F target genes implicated in survival, whose identification has started to emerge through the recent employment of DNA microarray analysis (Müller *et al*., 2001; Ishida *et al*., 2001; Ren *et al*., 2002). In essence according to the model we propose, the increased E2F activity present in human cancer cells subverts the normal transcriptional gene regulation, resulting somehow in the induction of factors that either directly or indirectly are capable of counteracting Myc-dependent apoptotic signals. Indeed, very few serum-starved U2OS-Myc cells die, presumably because they produce their own survival factors, while, regardless of the presence of serum, they undergo apoptosis upon inhibition of E2F activity, indicating that these factors are at least in part regulated by E2F. In contrast, in normal cells like BJ fibroblasts, the concomitant inhibition of E2F activity and Myc expression has no apparent impact on survival, perhaps because their E2F activity is not high enough to directly transactivate survival genes and regulate cell viability or alternatively but not mutually exclusive, because these cells may lack some factors important for the pro-apoptotic pathway modulated by Myc and E2F. However, BJ cells can be killed by deregulated Myc under condition of serum starvation, consistent with the notion that survival factors/cytokines in the serum are essential for tolerating upregulated Myc activity in normal fibroblasts (Harrington *et al*., 1994). It is interesting in this context that the DU-145 prostate adenocarcinoma cell line, which in addition to lacking pRb has mutant p53 (Carroll *et al*., 1993) and amplified *Myc* (Asadi and Sharifi, 1995), can be rendered sensitive to irradiation- and ceramide-induced cell death by reintroduction of pRb (Bowen *et al*., 1998). *Myc* amplification also characterizes the Rb-positive LNCaP prostate cancer cells (Nag and Smith, 1989), which undergo apoptosis upon expression of a constitutively active form of Rb or upon constitutive activation of pRb by different signaling pathways (Day *et al*., 1997, 1999; Zhao *et al*., 1997), apparently without involvement of p53 (Day *et al*., 1999). Collectively, these results suggest that some types of human epithelial tumor cells with deregulated Myc expression may be subject to E2F-dependent and p53-independent mechanisms regulating survival similar to those we have identified in osteosarcoma and leukemia cells.

It might seem difficult at a first glance to reconcile our results with the notion that excessive E2F activity can trigger apoptosis, as observed in certain cells from Rb-deficient mice or upon forced expression of E2F itself in several cell types (reviewed in Harbour and Dean, 2000; Nevins, 2001). However, our study is not in contrast with the current knowledge on E2F and apoptosis but rather it complements it by highlighting the importance of the endogenous E2F activity for the homeostasis of human tumor cells with deregulated Myc function. In this regard, it is worth mentioning that we could not rescue the apoptotic phenotype of U2OS-RbΔcdk/Myc cells by restoring E2F activity through overexpression of E2F-1, -2 or -3 in doses able to saturate RbΔcdk, or through expression of an Rb-binding deficient E2F-1 mutant. Indeed, all our attempts resulted in generation of E2F activity several fold higher than the endogenous one and marked toxicity in either TET-repressed or induced cells (ESR, DD, unpublished results). This toxic effect after forced expression of ectopic E2Fs is consistent with that previously observed in parental U2OS cells (Lukas *et al*., 1996) or other cell types (Harbour and Dean, 2000; Müller and Helin, 2000). Therefore, a delicate balance must exist in cancer cells between levels of E2F activity capable of protecting from apoptotic signals such as those induced by deregulated Myc and excessive levels of E2F resulting in activation of the apoptotic machinery and cell toxicity. This balance could, at least partly, explain why genetic alterations resulting in either loss or deregulated expression of E2F-1, -2, and -3, that is, the E2Fs implicated in cell proliferation and apoptosis, have not been reported in human tumors (Nevins, 2001). The factors and mechanisms regulating this balance remain elusive. A candidate could be the murine double minute 2 (MDM2) oncoprotein and its human homolog, HDM2. MDM2 can stimulate E2F activity by interacting with the E2F-1/DP-1 heterodimer and pRb (Martin *et al*., 1995; Xiao *et al*., 1995). Moreover, recent work has shown that exogenous

HDM2 can antagonize E2F-dependent apoptosis, possibly by down-regulating the levels of E2F and DP proteins, and can cooperate with E2F-1/DP-1 in promoting cell viability and cell cycle progression in a manner dependent on the DP subunit but independent of p53 (Loughran and La Thangue, 2000). Based on these results, a model has been proposed in which an increased expression of HDM2 might prevent the potentially toxic effects of excessive E2F activity in cancer cells and maintain E2F in a continuous state of growth stimulator rather than apoptosis inducer (Loughran and La Thangue, 2000). However, this hypothesis remains to be verified in human cancers that spontaneously overexpress HDM2. In addition, it renders a possible implication of HDM2 as major determinant in the phenomena described in our manuscript improbable, in that U2OS cells unlike some other types of osteosarcoma cells, do not bear *HDM2* amplification and express only moderate levels of HDM2 protein (Böttger *et al*., 1997). Similarly, neither SAOS-2 nor HL-60 are characterized by amplification and/or overexpression of *HDM2*.

It is also necessary to clarify which are the pro- and antiapoptotic genes regulated by E2F that may be orchestrating the net effect of E2F activity on cell survival. Interestingly, in this respect, recent work has shown that increased expression of E2Fs induces not only proapoptotic genes (Moroni *et al*., 2001; Müller *et al*., 2001) but also antiapoptotic genes and survival/growth factors, such as Bcl2 and TGF-α (Müller *et al*., 2001), which can inhibit Myc-induced apoptosis and cooperate with Myc in tumorigenesis of certain tissues (Strasser *et al*., 1990; Bissonnette *et al*., 1992; Fanidi *et al*., 1992; Amundadottir *et al*., 1996; Santoni-Rugiu *et al*., 1998).

Since RbΔcdk and the other E2F inhibitors used in our study are capable of functionally repressing all endogenous E2Fs (Lukas *et al*., 1997, 1999a,b) and given the above-mentioned toxicity caused by overexpression of different E2Fs in U2OS-RbΔcdk/Myc cells, the present work is not able to clarify the contribution of different E2F-family members to the antiapoptotic effect in Myc-overexpressing cancer cells nor whether this effect is due to a specific E2F or to a cooperative effect of different E2Fs. This deserves future investigations, as certain laboratories have indicated that in tissue culture models, E2F-1 to -3 have similar proapoptotic effects when overexpressed (Müller and Helin, 2000), while others have implicated only E2F-1 in the induction of apoptosis (Nevins, 2001) and suggested that Myc requires distinct E2F activities to induce S phase and apoptosis in MEFs (Leone *et al*., 2001).

The type of cell death elicited by concomitant overexpression of Myc and inhibition of endogenous E2F activity in cancer cells occurs via a caspase-mediated apoptotic pathway, as in other models of Myc-dependent apoptosis (Kangas *et al*., 1998; Kagaya *et al*., 1997; Hotti *et al*., 2000). Moreover, it appears to be independent of cell cycle progression, consistent with previous investigations on Myc-induced apoptosis in other systems (Packham *et al*., 1996; Rudolph *et al*., 1996). Likewise, cytokine-mediated protection from Myc-induced apoptosis is not linked to the cytokines' abilities to promote growth and can occur in cells whose cell cycle has been arrested (Harrington *et al*., 1994) and Bcl2 can inhibit Myc-induced apoptosis but not proliferation (reviewed in Hoffman and Lieberman, 1998; Prendergast, 1999). Nevertheless, our results using MM indicate that Myc transcriptional activity is necessary for the apoptotic effect described in this report, suggesting a key role for a Myc transcriptional target in eliciting it. However, unlike Myc-induced apoptosis in MEFs, it does not require ARF and/or p53, as it is reproducible in U2OS (ARF−/−; p53+/+) and SAOS-2 (ARF+/+; p53−/−) cells, in agreement with the fact that Myc-mediated apoptosis is p53-dependent in certain cell types but -independent in others (Hoffman and Liebermann, 1998; Prendergast, 1999; Blyth *et al*., 2000). We also show that reconstitution of the ARF/p53 axis in osteosarcoma cells deficient for ARF expression and expressing deregulated Myc, does not result in cell death, even when the cells are serum-starved. This may conceivably point toward possible alterations of the pathway downstream of ARF and/or p53 (Soengas *et al*., 2001). Alternatively but not mutually exclusive, U2OS cells might possess survival mechanisms interfering and blocking the induction of apoptosis by this pathway. Regardless of the mechanism, caution should be exercised in designing tumor gene therapy with ARF or with drugs activating pathways that induce ARF, in that ARF expression may not be enough to kill human tumor cells that overexpress certain classes of oncogenes.

Finally, we show that while human primary fibroblasts do not show loss of viability upon concomitant overexpression of Myc and inhibition of E2F activity, human HL-60 leukemia cells which have amplified *Myc* gene (Nowell *et al*., 1983; Von Hoff *et al*., 1990; Mangano *et al*., 1998) and deleted p53 (Wolf and Rotter, 1985) and lack ARF protein expression (Della Valle *et al*., 1997), can be killed by blocking their endogenous E2F activity. Thus, cell death by inhibition of E2F activity seems to selectively occur in human neoplastic cells with deregulated Myc and is independent of their ARF and p53 status. Our observations suggest that the protection from Myc-elicited apoptotic signals provided by increased, nontoxic levels of E2F activity in cancer cells may represent a novel mechanism by which the disruption of the Rb pathway may cooperate with deregulated Myc in oncogenesis. They may also have important implications for therapeutic strategies aimed at specifically eliminating Myc-overexpressing tumor cells, in particular those with defects of the tumor suppressor pathways regulated by ARF and p53.

Materials and methods

Plasmids and chemicals

The hemagglutinin (HA)-tagged pBI-HA-RbΔcdk and pECE-HA-RbΔcdk vectors, expressing the phosphorylation-deficient

murine pRb mutant RbΔcdk in a tetracycline (TET)-repressible or constitutive manner, respectively, the PECE-pRbΔB/X expressing wild-type pRb, and the pBI-Myc or pCMV-Myc vectors for TET-repressed or constitutive c-Myc expression, as well as pCMV-CD20, pCMV-HA-dnDP-1 (Δ103-126), and pX-p16, were described before (Lukas *et al*., 1997, 1999b; Santoni-Rugiu *et al*., 2000). The pCMV-HA-ARF plasmid for expression of human ARF was generated as previously published (Della Valle *et al*., 1997). The vectors pCMV-MadMyc (MM), pCMV-dnCDK2 (dnK2), pCMV-p27T187A, and pCMV-p53dd were as reported (van den Heuvel and Harlow, 1993; Bowman *et al*., 1996; Berns *et al*., 1997; Nguyen *et al*., 1999).

Z-Val-Ala-$_{D,L}$-Asp-fluoromethylketone (ZVAD-fmk; Bachem) was diluted to 10 mM in methanol and added to the cells at final concentrations from 1 to 100 μM. Acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO; Neosystems) was diluted to 10 mM in 10% DMSO and used at final concentrations of 1-100 μM.

Cell culture and gene transfer

Human osteosarcoma U2OS cells stably expressing human c-Myc (U2OS-Myc) or c-Myc and RbΔcdk (U2OS-RbΔcdk/Myc) in a tetracycline (TET)-repressible manner were as described (Santoni-Rugiu *et al*., 2000). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (10 U/ml), streptomycin (10 U/ml), geneticin (G418; 400 μg/ml), puromycin (1 μg/ml) and TET (2 μg/ml). Transgene derepression was performed by removal of TET according to procedures previously published (Lukas *et al*., 1999b). SAOS-2 and BJ cells were cultured in DMEM containing 10% FCS, penicillin and streptomycin. Calcium-phosphate transfection of U2OS-derived cell lines and SAOS-2 cells were carried out as reported previously (Lukas *et al*., 1996, 1997). Gene transfer into BJ cells was performed with DOTAP (Sigma) according to the manufacturer's instructions. Expression of the ectopic proteins after transfection was confirmed by immunocytochemical staining as described (Lukas *et al*., 1996, 1999b).

HL-60 cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin and streptomycin, keeping cell density lower than 1 million/ml, and were electroporated as reported (Chen *et al*., 2000) with minor modifications. Briefly, cells were collected, washed once with serum- and antibiotic-free RPMI 1640 and resuspended in this medium at working cell density of 2.5×10^6/100 μl. Approximately 4.5×10^6 cells/cuvette were electroporated at room temperature with the indicated DNAs and 0.3 μg of E-GFP DNA (Clontech), 350 V and 960 μFD by using a Bio-Rad Gene Pulser. Empty vector was added to a total of 8.5 μg of DNA. After electroporation the cells were incubated on ice for 15 min and then diluted with 15 ml antibiotic-free RPMI 1640 containing 10% FCS. After 12 h the cells were washed with fresh medium and then incubated for other 3 days before being harvested and assayed for viability. Gene transfer efficiency was verified by immunostaining of electroporated cells grown on coverslips, as described (Santoni-Rugiu *et al*., 2000).

Measurement of cell death

Loss of viability in untransfected U2OS-Myc and U2OS-RbΔcdk/Myc was monitored by flow cytometric analysis of the sub-G1 content of DNA characteristic of apoptotic cells. Cells were harvested, fixed in 70% methanol, stained with propidium iodide and analysed with a Becton-Dickinson FACScalibur flow cytometer by using the CellQuest software as previously described (Santoni-Rugiu *et al*., 2000). In addition, the analysis of cell integrity via exclusion of the vital dye, trypan blue, was used to further assess cell viability. Cells were cultured in 10-cm dishes in TET-containing or -free DMEM, supplemented or deprived of 10% FCS as indicated. At the indicated time-points, cells were trypsinized, washed in DMEM and the number of trypan blue-positive cells evaluated on a hemocytometer by counting a minimum of 500 cells. To assess the viability of U2OS or SAOS-2 cells transfected with the indicated plasmids, the pCMV-CD20 vector encoding the CD-20 cell surface antigen was co-transfected at 1 μg and the sub-G1 DNA content of CD20-positive cells was analysed by immunostaining with fluorescein isothiocyanate-labeled anti-CD20 monoclonal antibody (mAb; Becton Dickinson) followed by flow cytometry analysis according to published procedures (Lukas *et al*., 1999b; Santoni-Rugiu *et al*., 2000). Analogous flow cytometric procedures were used to evaluate the viability of transfected BJ cells and electroporated HL-60 cells, except that cells were gated according to their expression of membrane-bound E-GFP (transferred at 0.5 and 0.3 μg, respectively) following the indications of commercially available methods (Clontech).

Immunoblot analysis

Western blot analyses were performed as described previously (Lukas *et al*., 1996) using the following primary mAbs: anti-Myc 9E10 (a kind gift of G Evan) as a hybridoma supernatant diluted 1 : 3 in a 5% skimmed milk solution in PBS; anti-HA 12CA5 (Lukas *et al*., 1997), DCS-24.1 anti-human ARF (NeoMarkers) and DO-1 for p53 (Vojtesek *et al*., 1992), all diluted 1 : 500 and anti-PARP C2-10 at a dilution of 1 : 15 000 (Lazebnik *et al*., 1994; Mathiasen *et al*., 1999). The MO-1 mAb to detect CDK7 (Tassan *et al*., 1994) and DCS-141 for MCM7 (Sørensen *et al*., 2000) as well as Ponceau-S staining were used as loading controls. After incubation with peroxidase-conjugated anti-mouse IgG (Vector), the immunodetection of proteins was achieved by using the enhanced chemiluminescence method (Pierce).

Caspase-3-like activity

DEVDase activity was assessed in U2OS-RbΔcdk/Myc cell lysates prepared at day 0 and 4 of transgene induction. Equal amounts of protein ($\sim5\times10^5$ cells) measured by Bio-Rad protein assay (Bio-Rad) were used in a colorimetric assay for the detection of DEVD-specific caspase-3-like activity as described previously (Mathiasen *et al*., 1999), using acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (DEVD-pNA) as a probe (Biomol), DEVD-CHO as reaction inhibitor and a microtiter plate reader (Molecular Devices).

E2F binding sites oligonucleotides

The DNA oligonucleotides (DNA Technology A/S) representing a wild-type E2F binding site (E2Fbswt) and a mutant binding site (E2FbsMut) unable to bind E2Fs (Helin *et al*., 1992; Lees *et al*., 1993) were: 5′-ATTTAAGTTTCGCGCCCTTTCTCAA-3′ (E2Fbswt sense); 5′-TTGAGAAAGGGCGCGAAACTTAAAT-3′ (E2Fbswt antisense); 5′-ATTTAAGTTTCGATCCCTTTCTCAA-3′ (E2FbsMut sense); 5′-TTGAGAAAGGGATCGAAACTTAAAT-3′ (E2FbsMut antisense). The sequences of the E2F unrelated SP1RBF oligonucleotides were: 5′-CCTCGCGGACGTGACGCCG-

CGGGCGGAAGT-3′ (sense); 5′-ACTTCCGCCCGCGG-CGTCACGTCCGCGAGG-3′ (antisense). For transfer into U2OS-Myc cells, 10 μM oligonucleotides were heated at 95°C for 5 min followed by incubation at RT for 1 h and then electroporated into 1.5×10^6 cells together with 1 μg E-GFP and with or without pCMVMadMyc (MM, 5 μg), at 4°C, 125 V and 960 μFD. Empty vector was added up to a total of 8.5 μg plasmid DNA. After electroporation cells were kept 10 min on ice, and then plated onto 10 cm culture dishes in TET-containing or -free DMEM and 10% FCS. Medium was changed 12 h later and cells harvested 3 days later. Cell viability of E-GFP-positive cells was assessed by flow cytometry analysis as described above.

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