Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members

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Little is known of the mechanisms controlling the G₀/G₁ transition of the cell cycle. The induction of immediate early gene expression, thought to be important for this process, suggests that the key factors controlling this transition preexist in quiescent cells. The E2F family of transcription factors likely play an important role in this process, because E2F DNA-binding activity exists in quiescent cells, and the induction of at least some immediate early genes requires intact E2F regulatory promoter sites. Here, we show that the major G₀ E2F activity of primary human T cells, E2F-4, is stably bound to the p130 pocket protein in association with a DP heterodimerization partner. p130–E2F-4 binding has functional implications because p130 effectively suppressed E2F-4-mediated *trans*-activation, and coexpression of E2F-4 overcame p130-mediated G₁ arrest more efficiently than RB-induced G₁ blockade. Conversely, E2F-1 overrode an RB-induced G₁ block more efficiently than E2F-4. Thus, p130 and RB appear to induce cell cycle arrest via biochemically distinct mechanisms that involve different E2F family members.

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The decision to enter into or to exit from the cell cycle can be mediated by signals from the environment and sensed by specific cell-surface receptors that subsequently initiate a complex series of signaling pathways. Some of these pathways ultimately are linked to key nuclear effectors that control the cell division system. The cell cycle regulatory machinery can be considered a focal point for the integration or convergence of the various signaling pathways mediating mitogenic and anti-mitogenic signals. Cell cycle effectors include cyclin-dependent kinases (cdks), their corresponding regulatory cyclin partners, a recently discovered array of cdk inhibitors, and members of the retinoblastoma family of "pocket proteins" including the retinoblastoma protein (RB), p107, and p130 (Sherr 1993; Ewen 1994; Peter and Herskowitz 1994). A complex interplay exists between these various effectors, one outcome of which is the timely transcription of genes encoding essential cell cycle progression factors.

Among the participants in this cell cycle-regulating gene activation program is the E2F family of transcription factors of which four members, E2F-1, E2F-2, E2F-3, and E2F-4 have been described so far (Helin et al. 1992;

Kaelin et al. 1992; Shan et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993; Beijersbergen et al. 1994; Ginsberg et al. 1994). All of them form stable complexes with at least one specific pocket protein. Moreover, E2F recognition sequences are present in the promoters of a number of cell cycle progression genes (for review, see Nevins 1992; La Thangue 1994).

E2F-1, the first E2F species cloned (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992), serves as a paradigm for what is known of the mechanisms involved in the regulation of E2F activity in general. It complexes with pRB and not with other known pocket proteins, and binding is primarily to underphosphorylated RB (pRB) (Chellappan et al. 1991; Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992). Its ability to bind both DNA and RB is dependent on an association with a heterodimeric partner, DP-1 (Bandara et al. 1993; Helin et al. 1993b; Krek et al. 1993).

When bound to pRB, E2F-1 retains its DNA-binding activity but loses its transcription activation function. In this setting, it likely functions as a sequence-specific transcriptional repressor, switching off the expression of certain E2F target genes, at least some of which, when active, promote cell growth (Dalton 1992; Hamel et al. 1992; Hiebert et al. 1992; Weintraub et al. 1992; Zamanian and La Thangue 1992). In response to certain growth-promoting signals, phosphorylation of pRB oc-

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curs, during mid-late G_1 , at the hands of specific cyclin/cdk complexes. This process results in the release of E2F-1 from the RB-containing complex and derepression of certain E2F-1 responsive promoters, which, in turn, activates genes needed for G_1 exit (Dalton 1992; Lam and Watson 1993; Hsiao et al. 1994). As cells progress through late G_1 early S phase, E2F-1 binds stably to cyclin A/cdk 2, and this enzyme catalyzes phosphorylation of the DP-1 subunit also bound to E2F-1, leading to a loss of E2F-1 DNA-binding function (Dymlacht et al. 1994; Krek et al. 1994). Thus, the temporal activity of E2F-1, which could be predicted to peak at the G_1 /S phase boundary is, at least in part, a result of its sequential interactions with RB during G_1 and cyclin A kinase during late G_1 and S phase.

Compared with the mechanisms contributing to continuous cell proliferation, the mechanisms controlling the G_0 / G_1 transition are poorly characterized. Some insight into the nature of the mechanisms involved can be gleaned from studies of genes whose expression at the G_0 / G_1 transition are important for cell cycle progression. For quiescent cells, the rapid, mitogen-induced stimulation of immediate early mRNA synthesis occurs in the absence of protein synthesis and suggests that key regulatory factors pre-exist in quiescent cells (for review, see Pardee 1989). The *c-myc* promoter (P2) contains an E2F recognition sequence, and this site is essential for *c-myc* expression in response to serum (Mudryj et al. 1990) as well as for its *trans*-activation in response to E1A (Thalmeier et al. 1989; Hiebert et al. 1991).

Consistent with a potential role for E2F in mediating the activation of certain G_0 exit genes, E2F DNA-binding activities exist in a variety of G_0 cells (Chittenden et al. 1993; Cobrinik et al. 1993; Schwarz et al. 1993). On the other hand, E2F-1 synthesis occurs primarily in late G_1 (Kaelin et al. 1992; Slansky et al. 1993; Ginsberg et al. 1994), and E2F-1 was not detected in extracts of G_0 human T lymphocytes (Chittenden et al. 1993). Furthermore, E2F-1 did not activate the *c-myc* promoter as efficiently as it activated other E2F site-containing elements (Li et al. 1994). When in the cell cycle E2F-2 and E2F-3 are synthesized remains undefined. However, these species, like E2F-1, predominantly associate with RB in vivo (Ivey-Hoyle et al. 1993; Lees et al. 1993); and RB/E2F complexes are, at most, a minor component of G_0 E2F DNA-binding activity (Chittenden et al. 1993; Cobrinik et al. 1993; Schwarz et al. 1993).

In unstimulated human T cells and growth-arrested immortal murine fibroblasts, there is a single, readily detectable, E2F complex designated X (Chittenden et al. 1993; Cobrinik et al. 1993). It consists of an E2F activity bound to p130 (Cobrinik et al. 1993). The sequence of p130 is more closely related to that of p107 than RB, with the greatest homology observed in the pocket region (Hannon et al. 1993; Li et al. 1993; Mayol et al. 1993). Unlike RB, p130 demonstrates significant homology in its "spacer" (a region in the pocket that separates the A and B pocket subdomains and is dispensable for viral oncoprotein binding) to that of p107, which mediates p107 interactions with cyclins A and E (Ewen et al.

1992; Faha et al. 1992; Lees et al. 1992). In keeping with this observation, p130 also interacts with cyclins A and E (Li et al. 1993). Although there is no direct evidence that p130 functions as a tumor suppressor, the p130 gene maps to a region of the genome that is frequently deleted in a variety of human tumors (Hannon et al. 1993; Li et al. 1993; Yeung et al. 1993). Therefore, one might speculate that like pRB, p130 is also a growth-regulating protein and that its interactions with certain E2F species contribute to this function. In this report we describe results that strongly support this hypothesis and speak to a specific role of p130 complexed with E2F-4, a known oncogene (Ginsberg et al. 1994), in G_0 / G_1 exit control.

Results

The G_0 X complex is composed of p130, E2F-4, and a DP family member

Figures 1a and b depict the results of an electrophoretic mobility-shift assay (EMSA) analysis performed on extracts of resting (G_0) primary human T cells and cycling U937 human promonocytic leukemia cells. The E2F species of both of these cell populations have been characterized previously (Shirodkar et al. 1992; Chittenden et al. 1993). Asynchronous U937 cells contain three predominant E2F complexes designated A, B and C that are composed of free (unbound) E2F, RB-E2F, and p107-E2F-cyclin A/cdk2 complexes, respectively (Cao et al. 1992; Devoto et al. 1992; Shirodkar et al. 1992). In G_0 T cell extracts, a prominent E2F complex was detected (labeled X) that, as described previously, migrated a bit more slowly than B (Fig. 1a, cf. lanes 1 and 2). The DNA-binding specificity of these various E2F complexes was confirmed in competition experiments where addition of excess, unlabeled wild-type E2F oligonucleotide, but not an equal amount of an oligonucleotide containing a mutant E2F site, abolished binding to the radioactive probe (data not shown).

Addition of a polyclonal anti-E2F-4 mouse serum (which does not recognize any of the other known E2Fs—see below) resulted in an almost complete loss of X with a concomitant appearance of a supershifted band (Fig. 1a, cf. lanes 2 and 3), whereas a control mouse serum had no effect (Fig. 1a, cf. lanes 2 and 4). E2F-1 activity results from an E2F-1/DP-1 heterodimeric complex (Bandara et al. 1993; Helin et al. 1993b; Krek et al. 1993). Because E2F-4 can also heterodimerize with DP-1 in vivo (Beijersbergen et al. 1994; Ginsberg et al. 1994) and DP-1 protein is present in G_0 cells (Bandara et al. 1994), we asked whether DP-1, or a related protein, exists in X. Addition of a polyclonal anti-human DP-1 mouse serum suppressed X significantly (Fig. 1a, cf. lanes 2 and 5). A rabbit polyclonal anti-mouse DP-1 serum (Fig. 1a, lane 6) led to marked suppression of X and a supershift effect. The corresponding preimmune serum was inactive in this regard (Fig. 1a, lane 7). As described previously (Shirodkar et al. 1992; Chittenden et al. 1993), whole rabbit sera resulted in the appearance of a sharp band corresponding to a nonspecific DNA-binding activity that migrated just above C (Fig. 1a, lanes 6,7).

Figure 1. Analysis of E2F-4 DNA-binding activities in G_0 and cycling cells. (a) G_0 T cells. Extracts from resting T cells (lanes 2–15) or asynchronous U937 cells (lanes 1, 17–20) were incubated with the various antibodies as indicated and then subjected to EMSA analysis using a 32 P-labeled E2F probe derived from the *c-myc* promoter. The respective antibodies used are as follows: (α -E2F-4 MS) A mouse polyclonal antiserum raised against clonal E2F-4; (MS cont) an irrelevant control mouse serum; (α -hDP1 MS) a mouse polyclonal antiserum raised against human DP-1; (α -mDP1 RS) a polyclonal rabbit serum raised against murine DP-1; R12 preimmune serum; and (α -p130) an affinity-purified rabbit polyclonal p130 peptide antibody. SD2, SD6, and SD15 are p107 mAbs (Dyson et al. 1993). (X) The position of the predominant E2F complex in G_0 T cells; (A, B, C) the positions of the E2F complexes in the U937 extracts. The asterisk (*) indicates a nonspecific DNA-binding activity (Shirodkar et al. 1992; Chittenden et al. 1993). (b) Cycling U937 cells. Extracts from asynchronous cultures of U937 cells were incubated with the various antibodies as indicated and then subjected to EMSA analysis as in a. (c) E2F-4 antibody specificity. HA-tagged E2F-1, E2F-2, E2F-3, and E2F-4 were translated in vitro and subjected to immunoprecipitation with polyclonal HA peptide antibody (lane 1), polyclonal anti-E2F-4 mouse serum (lane 2), control mouse serum (lane 3), irrelevant monoclonal antibody YS140 (lane 4), and E2F-4 mAb, GG22 (lane 5). Aliquots (1 μ l) of the in vitro translation products (IVT) of the various E2Fs were analyzed directly as controls (lane 6). The resulting immunoprecipitates were analysed by SDS-gel electrophoresis. The positions of the proteins immunoprecipitated by HA antibodies are indicated at right. The gel mobilities of prestained markers and their native molecular masses (in kD) are indicated at left.

Addition of an affinity-purified anti-p130 peptide antiserum resulted in a clear supershift of X (Fig. 1a, lane 8) as well as the appearance of a band migrating slightly slower than X itself (Fig. 1a, cf. lanes 7 and 8). In other gels electrophoresed longer than the one depicted in Figure 1, this band was completely resolved from X and because no X remained, we concluded that all of X was supershifted by the p130 antibody (data not shown). The nature of the band that migrated more slowly than X remains unclear, although in other experiments it did appear to bind the E2F probe specifically (data not shown). To determine whether the various protein components of X were present in the same complexes, combinations of antibodies were added in search of sequential supershifting effects. Addition of both p130 and E2F-4 or p130 and DP-1 antibodies, together, resulted in a further mobility shift of the supershifted p130 band (Fig. 1a, cf. lanes 9 and 11, respectively, to lane 8), whereas control mouse serum had no such effect (Fig. 1a, lane 10). Addition of a combination of antibodies to p130, E2F-4, and DP-1 together resulted in the appearance of an even more slowly migrating species, suggesting that a triple supershift had occurred (Fig. 1a, cf. lane 12 with lanes 9 and 11). Consistent with a previous report (Chittenden et al. 1993), addition of three monoclonal p107 antibodies (SD2, SD6, or SD15), which recognize distinct epitopes of p107 (Dyson et al. 1993), had no effect on X (Fig. 1a, lanes 13–15), whereas they were effective in supershifting complex C from U937 cells in the same experiment (Fig. 1a, lanes 17–20). This suggests that, at a minimum, X contains p130 in the same complex with E2F-4 and a DP family member. It also is consistent with the view that X lacks p107.

We also decided to examine the effect of E2F-4 antibody on the various E2F activities observed in extracts of cycling cells. In Figure 1b it can be seen that addition of E2F-4 antibody (Fig. 1b, lane 3), but not control mouse serum (Fig. 1b, lane 4), to U937 extracts clearly (albeit not completely) supershifted complexes A and C and, to a lesser extent, complex B. In both the U937 cell and resting T-cell extracts, these complexes were disrupted by a second mouse anti-E2F-4 serum (data not shown), again indicating the presence of E2F-4. The identities of these various E2F activities as free E2F, RB-E2F, and p107-E2F complexes (see above) were confirmed in independent supershift assays performed with specific antibodies to RB and p107 (for B and C, respectively, in Fig. 1) and by the ability of wild type, but not a suitable mutant, E1A-GST fusion protein, to disrupt B and C, but not A (data not shown). As expected, p107 monoclonal antibody supershifted C specifically (Fig. 1b, lane 5) with a subsequent further supershift of this band when both E2F-4 and p107 antibodies were added together (Fig. 1b, lane 6), but not when anti-p107 was added with control mouse serum (Fig. 1b, lane 7). These results not only confirm that in cycling U937 cells, E2F-4 is predominantly associated with p107 and, to a lesser extent with RB, but also suggest that E2F-4 is a component of the major free E2F species (i.e., complex A). This conclusion is consistent with previous results obtained with human

T-cell extracts, demonstrating that disruption of X with E1A results in increased amounts of A (Chittenden et al. 1993).

In the above-noted experiment, a polyclonal antiserum to E2F-4 was used. Because E2F is a family of structurally related gene products (Helin et al. 1992; Kaelin et al. 1992; Shan et al. 1992; Ivey-Hoyle et al. 1993; Lees et al. 1993; Beijersbergen et al. 1994; Ginsberg et al. 1994), we asked whether this antiserum displayed cross-reactivity with any of the hitherto cloned E2Fs. Figure 1c demonstrates that it did not immunoprecipitate *in vitro*-translated E2F-1, E2F-2, or E2F-3. Similarly, an E2F-4 monoclonal antibody (mAb GG22), described previously (Ginsberg et al. 1994) (which does not supershift in EMSA, data not shown) was also specific for E2F-4 in this assay. Neither of these antibodies immunoprecipitated *in vitro*-translated DP-1 (a frequent component of cellular E2F complexes) (Girling et al. 1993) or E2F-5, an E2F family member identified more recently that is structurally more homologous to E2F-4 than the other known E2Fs (Sardet et al. 1995; data not shown). Nevertheless, we cannot exclude the possibility that these E2F-4 antibodies may recognize other, as yet unidentified, E2F species.

Interestingly, immunoprecipitation of *in vitro*-translated, hemagglutinin (HA)-tagged E2F-4 with the various E2F-4 antibodies, as well as with HA antibody, gave rise to a series of gel electrophoretic bands. Because the HA epitope was present on the amino terminus of E2F-4, this result depicts only products bearing an intact amino terminus. The heterogeneity of the E2F-4 banding pattern has been described previously and is most likely a result of post-translational modification of the E2F-4 polypeptide (Beijersbergen et al. 1994; Ginsberg et al. 1994; see below) and demonstrates that such modifications can arise both in reticulocyte lysates and *in vivo*.

E2F-4 protein is present in resting G₀ T cells

To confirm that E2F-4 exists in G₀ T cells, Western blot analysis was performed with the E2F-4 mAb GG22 (Ginsberg et al. 1994). Figure 2 depicts steady-state E2F-4 levels in extracts of unstimulated human T cells, T cells stimulated with phytohemagglutinin (PHA) for 70 hr, and exponentially growing U937 cells. As mentioned previously, E2F-4 was discernible in SDS gels as a series of bands of varying mobility that most likely reflect differential phosphorylation (Beijersbergen et al. 1994; Ginsberg et al. 1994). Interestingly, although E2F-4 was readily detected in all of the samples, the relative abundance of the different forms changed after T-cell stimulation. The poststimulation pattern and that in cycling U937 cells were nearly identical (Fig. 2, cf. center and right lanes). The G₀ T-cell population displayed prominent levels of the two most slowly migrating forms, whereas after stimulation, the faster migrating forms predominated. The slowest migrating band present in the stimulated T-cell preparation was absent in the U937 cell extract, possibly reflecting the presence of a greater fraction of G₀/G₁ cells in the T-cell culture (possibly

Figure 2. Steady-state E2F-4 protein levels in G_0 and cycling cells. Whole-cell extracts were prepared from resting G_0 T cells, T cells stimulated with PHA for 70 hr, or asynchronous cultures of U937 cells. Steady-state E2F-4 levels were determined by Western blotting using anti-E2F-4 mAb GG22, as described in Materials and methods. The percentages of cells of the different cell populations in the various cell cycle stages are shown (*bottom*). The gel mobilities of prestained markers and their native molecular masses (in kD) are indicated at *left*.

residual nonresponding T cells) as shown by cell cycle analysis (Fig. 2, bottom). All extracts were prepared simultaneously, and equivalent amounts of cellular protein were analyzed in all cases. In addition, there were no detectable signs of E2F-4 proteolysis in electrophoretic mobility shift assay (EMSAs); and similar Western blotting results were obtained in multiple, independent cell extracts (data not shown). Hence, it is unlikely that the differences in E2F-4 banding pattern among the three samples tested simply reflect protein degradation during their preparation. These results suggest that there are cell cycle-specific modifications of E2F-4, possibly phosphorylation, that occur when cells exit G_0 and enter the cell cycle.

In vivo interaction of E2F-4 with p130 in quiescent and cycling cells

We next searched for complexes of E2F-4 bound to p130 and/or p107 in quiescent and cycling cell populations by immune coprecipitation. Resting and stimulated T cells, as well as asynchronous U937 cells, were labeled with [35 S]methionine and lysed, and the resulting extracts were immunoprecipitated, in parallel, with antibodies to E2F-4, p130, and p107. Equivalent amounts of radiolabeled cellular protein were assayed. After washing, the presence of pocket protein coprecipitating E2F-4 was determined by reimmunoprecipitation with the E2F-4

mAb GG22 and analyzed by SDS-gel electrophoresis (Fig. 3a). Significant quantities of E2F-4 were discernible in the p130 immunoprecipitates from both resting and stimulated T cells. A less intense signal was detected in the U937 extract. E2F-4 coprecipitated with p107 in the stimulated T-cell and U937 cell extracts, with little association detected in G_0 T cells. This may reflect the lower abundance of p107 in quiescent cells (Cobrinik et al. 1993; and Fig. 3b). The band that coprecipitated with p107 from unstimulated T-cell extracts (lane 3) was a background band, because it was also observed when control monoclonal antibody was used (Fig. 3a, lane 1). In summary, these results reveal an *in vivo* interaction of E2F-4 and p130 in both quiescent and cycling cell populations, with a significant interaction of p107 with E2F-4 occurring only in the proliferating cells tested. In all three cell populations assayed, and in agreement with data published previously (Ginsberg et al. 1994), a weak interaction of E2F-4 with RB could be detected (data not shown). Interestingly, direct precipitation of E2F-4 resulted in the appearance of significant levels of E2F-4 in both quiescent and stimulated T-cell extracts. In contrast, little, if any, was detected in U937 cells. The reason for this difference is unclear at present.

Consistent with the results shown in Figure 2 and, as reported previously (Beijersbergen et al. 1994; Ginsberg et al. 1994), the results of Figure 3a also show that E2F-4, isolated from both G_0 and cycling cell populations, migrated as a heterogeneous series of bands ranging from ~57 to 64 kD. The distribution of the E2F-4 species isolated by direct E2F-4 immunoprecipitation differed, in part, from that of the E2F-4 bound to p130 and p107. These results suggest that p130 and p107 (Ginsberg et al. 1994) associate with a specific subset of the available E2F-4 species.

To compare the levels of labeled p130 and p107 in the immunoprecipitates from the various cell lysates analyzed above, fractions of the initial immunoprecipitates used in Figure 3a were analyzed by gel electrophoresis (Fig. 3b). p130 was readily detected in both resting and stimulated T cells, as well as in U937 cells. Notably, there were differences in the migration of p130 in the various samples. In G_0 T cells, most immunoreactive p130 migrated as a band of ~126 kD. After 60 hr of mitogenic stimulation, more slowly migrating species appeared. In asynchronous U937 cells, a single major band of ~130 kD was detected. These findings are analogous to the cell cycle-dependent changes in RB mobility that depend on its time-dependent, differential phosphorylation (Buchkovich et al. 1989; Chen et al. 1989; DeCaprio et al. 1989; Mihara et al. 1989). Consistent with this proposal and in keeping with results published previously (Cobrinik et al. 1993), we have found that the slower migrating p130 band can be converted to the faster species by phosphatase treatment of p130 immunoprecipitates (data not shown). This implies that the electrophoretic heterogeneity of p130 is attributable to phosphorylation.

In contrast to p130, the levels of labeled p107 isolated in Figure 3a were low in unstimulated T cells and in-

To exclude the possibility that coprecipitation of E2F-4 with the polyclonal p130 antibody from cycling cells was attributable to cross-reactivity with p107, we performed the experiment depicted in Figure 3c. Lysates

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tained with lysates of stimulated T cells (data not shown).

To further establish the specificity of the interaction of E2F-4 with p130 in cycling cells, an expression plasmid encoding p130 carrying an amino-terminal HA epitope was employed (Fig. 3d). U2OS cells transfected with expression plasmids encoding HAp130 or untagged p107 were metabolically labeled with [³⁵S]methionine, and the association of these exogenously introduced proteins with endogenous E2F-4 or E2F-1 was tested by coimmunoprecipitation. The results show that endogenous E2F-4 could be detected in immune complexes from cells overproducing HAp130 or p107. These complexes were precipitated with anti-HA mAb 12CA5 and p107 mAbs SD9 and SD15, respectively (Fig. 3d, lanes 5,7). Interestingly, weak coprecipitation of E2F-1 with p107 occurred in cells overproducing p107 (Fig. 3d, lane 8), but the analogous E2F-1/HA-p130 interaction was not evident in this experiment. These results suggest that although p107 does not bind E2F-1 under physiological conditions *in vivo* (Chittenden et al. 1993; Dyson et al. 1993), overproduction of p107, but not p130, can result in a detectable interaction with endogenous E2F-1.

The electrophoretic heterogeneity of the E2F-4 that coprecipitated with p130 is attributable, at least in part, to phosphorylation. Lambda phosphatase treatment of the E2F-4 coprecipitating with p130 from [³⁵S]Methionine-labeled C33A cells resulted in the appearance of a single, more intense, faster migrating band of ~57 kD (Fig. 3e, lane 3), when compared with parallel samples prepared in the absence of phosphatase (Fig. 3e, lane 1), or with phosphatase in the presence of phosphatase inhibitors (Fig. 3e, lane 2).

E2F-4 and a DP family member are substrates of p130-associated kinases in vitro

p130/E2F complexes have been demonstrated, *in vivo*, to interact with cyclins and their associated kinases (Cobrinik et al. 1993; Li et al. 1993). Therefore, we tested whether E2F-4 may be phosphorylated by p130-associated kinases. To address this, *in vitro* kinase assays were performed on p130 or p107 immunoprecipitates derived from lysates prepared from resting and mitogen-stimulated T cells. In these tests any resulting E2F-4 phosphorylation was measured after this target protein was reprecipitated by E2F-4 monoclonal antibody (Fig. 4). The results indicate that similarly migrating species of phosphorylated E2F-4 could be detected in both the p130 and p107 complexes from stimulated, but not resting, T cells. That similar levels of p130 were immunoprecipitated from both of these resting and stimulated populations was assured by immunoprecipitating lysates of cultures metabolically labeled in parallel with [³⁵S]methionine (data not shown). The phosphorylation of E2F-4 was catalyzed by kinases specifically bound to p130 or p107, because phosphorylated E2F-4 was not detected when the p130 antibody was preblocked with antigenic peptide or when an irrelevant monoclonal antibody was used (Fig. 4, lanes 6,9). Because we found that a DP-related

Figure 4. E2F-4 and a DP family member are substrates for p130- and p107-associated kinases *in vitro*. Equivalent amounts of total cellular protein from lysates of resting T cells or T cells stimulated with PHA for ~60 hr were subjected to immunoprecipitation with p130 peptide antibody, C20 (p130), or a mixture of p107 mAbs SD6 and SD15 (p107). Where indicated, immunoprecipitations were carried out in parallel with p130 antibody preblocked with antigenic peptide (p130 + pep) or with an irrelevant mAb YS140 (MAb cont) as specificity controls. The resulting precipitates were then subjected to *in vitro* kinase reactions as described in Materials and methods and boiled in SDS release buffer, and the resulting soluble fractions reimmunoprecipitated with YS140 control mAb (MAb cont), anti E2F-4 mAb GG22 (E2F-4), anti-mouse DP-1 rabbit serum, R12 (DP-1), or the corresponding R12 preimmune serum (DP-1 p.i.) as indicated. The gel mobilities of prestained markers and their native molecular masses in kD are indicated at left.

protein was also present in p130/E2F-4 complexes (Fig. 1) and because DP-1 associated with E2F-1 is a substrate for cyclin A kinase (Dynlacht et al. 1994; Krek et al. 1994), we also asked whether phosphorylation of DP-related proteins occurs in p130 immune complexes. Phosphorylation of an ~45-kD protein specifically recognized by anti-DP-1 immune serum, present in both p130 and p107 immune complexes from proliferating T cells, was detected (Fig. 4, lanes 13,15). These data therefore suggest that both E2F-4 and a DP subunit are *in vitro* substrates of p130- and p107-associated kinases present in proliferating, but not quiescent, T cells.

p130 represses E2F-4-dependent trans-activation

To determine the functional significance of the p130/E2F-4 interaction, we examined the effect of introducing exogenous p130 upon the ability of E2F-4 to *trans*-activate an E2F-responsive promoter. Whereas E2F-4 efficiently *trans*-activated an adenovirus E2-chloramphenicol acetyltransferase (CAT) reporter plasmid when cotransfected into U2OS osteosarcoma cells (Fig. 5), addition of increasing quantities of a p130 expression plas-

Figure 5. p130 represses E2F-4-mediated *trans*-activation. U2OS cells were cotransfected, as indicated, with 5 μ g of an adenovirus E2-CAT E2F reporter plasmid (pE2wtCAT), pCMV β -GAL (2 μ g) as an internal control, pCMV-E2F-4 (500 ng), plus 50, 100, or 500 ng of a pCMV-driven p130 expression plasmid (pCDNA1-HAp130), using backbone pCMV vector as carrier. A total of 8 μ g of DNA was transfected in each case. CAT activities were determined for the various extracts, and the values were normalized for the corresponding β -galactosidase activity.

mid led to a progressive reduction in CAT activity (Fig. 5). *Trans*-activation of this reporter by E2F-4 has been demonstrated previously to require an intact E2F site (Ginsberg et al. 1994). p130 also suppressed an E2F-4-dependent *trans*-activation of a B-myb promoter luciferase reporter when cotransfected into C33A cervical carcinoma cells. This effect occurred under conditions where p130 cotransfection resulted in no detectable change in cell cycle distribution of successfully transfected cells (assayed by CD19 coexpression; see below). Hence, *trans*-activation suppression is not attributable to an indirect cell cycle effect (data not shown).

E2F-4 cotransfection overrides state of p130-induced G_1 arrest

To determine whether overproduction of p130 can interfere with cell cycle progression, flow cytometry analysis of transfected SAOS-2 cells was performed. Cells were cotransfected with a p130 expression plasmid and a plasmid encoding the cell surface protein, CD19. The CD19 protein was employed as a marker for separation of the successfully transfected cells from the untransfected ones, using an anti-CD19 monoclonal antibody, as in the past (Qin et al. 1992). Introduction of the CD19 plasmid, alone, did not affect cell cycle distribution of an acutely transfected culture (data not shown), whereas the p130

expression plasmid reproducibly led to a significant increase in the fraction of cells with a G_0/G_1 DNA content (Fig. 6). Cotransfection of p130 with E2F-4 led to a marked decrease in the percentage of CD19-positive cells in G_0/G_1 compared with that observed in cultures transfected with p130 alone. Cotransfection of p130 with another member of the E2F family, E2F-1, resulted in a less prominent reduction in the G_0/G_1 fraction, suggesting that E2F-4 is the more potent of the two E2Fs in overcoming a p130-induced cell cycle arrest. This result has been reproduced in three independent experiments, and it is also consistent with the finding that p130 clearly associates with E2F-4 in preference to E2F-1 when overproduced in vivo (Fig 3d).

In keeping with results published previously (Goodrich et al. 1991; Hinds et al. 1992; Qin et al. 1992), introduction of RB into SAOS-2 cells, in parallel with the p130 transfection of aliquots of the same culture, also resulted in an increase in the fraction of cells in G_0/G_1 . However, unlike what was observed when p130 was analyzed as a growth-blocking agent, E2F-1 was active in overcoming growth suppression, whereas E2F-4 was inactive. In other experiments, however, E2F-4 had a weak override effect which was, nevertheless, consistently much less potent than that of E2F-1. In all cases, equal amounts of E2F-4 and E2F-1 plasmids were used, and in the experiment depicted in Fig-

Figure 6. E2F-4 overrides p130-induced cell cycle arrest. SAOS-2 cells were cotransfected, as indicated, with expression plasmids encoding CD19 (2 μ g), HAp130 (8 μ g), or RB (8 μ g), with or without 10 μ g of HAE2F-4 or E2F-1 expression plasmid, as indicated. Three days after transfection, cells were collected and stained for the presence of CD19. The cell cycle distribution of these cells was also determined, as described in Materials and methods. In cells solely transfected with the plasmid-encoding CD19, 50.9% of the cells were in G_0/G_1 . The results shown represent the relative increases at each data point above this level. For example, p130 cotransfection with CD19 resulted in an absolute value of 68.1% G_0/G_1 cells. This translates into a 33.8% relative increase in G_0/G_1 cells over the CD-19 control.

ure 6, appropriate expression of the two E2Fs was detected by Western blotting analysis of extracts of the transfected cultures (data not shown). These results suggest that like RB, p130 can also induce cell cycle arrest with an increased fraction of cells exhibiting a G₀/G₁ DNA content. However, the molecular mechanisms by which these two pocket proteins interfere with cell cycle progression are, most likely, not identical.

Discussion

In this paper E2F-4 was shown to be a partner for the RB family member, p130. Given the earlier results (Beijersbergen et al. 1994; Ginsberg et al. 1994), E2F-4, therefore, binds to both p130 and p107 in vivo (Ginsberg et al. 1994) and is thus the first member of the E2F family to display affinity for multiple pocket proteins under physiological conditions. The interaction of E2F-4 with p130 has functional implications, because p130 suppressed E2F-4-mediated *trans*-activation and overproduction of E2F-4 prevented the growth arrest mediated by p130. The G₁ block effected by p130 indicates that this protein can suppress growth. Moreover, this effect correlates with negative regulation of a defined E2F species. The differential ability of E2F-4 and E2F-1 to override the p130- and RB-mediated G₁ blocks also reveals, for the first time, a functional difference between different E2F family members and suggests that p130 and RB may function to induce growth arrest via distinct molecular mechanisms. One might also speculate, in this regard, that E2F-1 and E2F-4 modulate the transcription, at least in part, of different promoters.

Unlike E2F-1, E2F-4 is synthesized throughout the cell cycle, with significant levels detected in G₀ in both resting human T cells (Fig. 2) and serum-starved mouse fibroblasts (Ginsberg et al. 1994). Moreover, results presented here show that E2F-4 exists in complex with one pocket protein during G₀ and at least part of G₁ and another (p107) in late G₁ and S (see also Cao et al. 1992; Lees et al. 1992; Shirodkar et al. 1992). Whether the same E2F-4 molecule is handed off from one pocket protein to another remains the subject of future inquiry.

In keeping with the notion that p130/E2F-4 complexes exist largely in G₀ and/or G₁, p107 was the predominant E2F-4 partner in cycling U937 leukemic cells (Figs. 1b and 3a), although small quantities of p130/E2F-4 complexes were still detected at those times (Fig. 3a). The finding that E2F-4 associated with p130 in a continually cycling cell population demonstrates that this interaction is not exclusive to G₀ cells or to cells entering the cell cycle from G₀, and, to some extent, probably occurs in part of G₁ (see also Cobrinik et al. 1993).

We also observed that in cycling U937 cells, the predominant free E2F activity corresponding to complex A [which is also evident in other cell types, including human T cells (Chittenden et al. 1993; data not shown)], was supershifted by E2F-4 antiserum. This suggests that E2F-4 is also a component of the major free E2F activity in cycling cells. It should be noted that A is not evident in extracts prepared from resting human T cells (Fig. 1a;

Chittenden et al. 1993), suggesting that free E2F-4 may arise after release from p130 in the G₁ phase of cycling cells. Such a result is also consistent with the ability of overproduced E2F-4 to induce S-phase entry (Beijersbergen et al. 1994).

Several observations point to phosphorylation of E2F-4 as a potentially important regulatory mechanism for this transcription factor. First, as shown by its mobility in SDS gels, E2F-4 displayed dramatic heterogeneity because of differential phosphorylation in vivo that was, to some extent, regulated during entry of G₀ T cells into the cell cycle. Second, p130 and p107 interacted specifically with a more slowly migrating subset of phosphorylated E2F-4 than was detected in the total E2F-4 pool in vivo, suggesting that specific elements of the E2F-4 phosphorylation program can direct its interaction with these negative regulators. Implicit in this model is the assumption that such specific phosphorylation of E2F-4 facilitates its interaction with two negative regulators of its function. Notably, the fact that p130/ and p107/E2F-4 complexes scored positively in EMSAs implies that neither its specific phosphorylation nor its binding to pocket proteins leads to an inhibition of its DNA-binding function.

The in vivo mechanisms regulating E2F-4 phosphorylation remain to be determined. However, some clues to their nature exist. Endogenous E2F-4 was an in vitro substrate for p130-associated kinases in extracts of proliferating cells. Conceivably, this activity corresponds to one or both of the cyclin-dependent kinases known to interact with p130 in vivo (Fig. 4; see also Cobrinik et al. 1993; Li et al. 1993). In quiescent cells, however, in vitro kinase activity of this type was not detected. Nevertheless, p130-associated E2F-4 from G₀ cells and E2F-4 bound to p130 in proliferating cells displayed similar electrophoretic heterogeneity, suggesting that they were similarly phosphorylated. Because phosphorylated E2F-4 bound to p130 exists both in G₀ and in cycling cells, but known p130- cyclin/kinase complexes do not exist in G₀, E2F-4, phosphorylated *de novo* in G₀, cannot be phosphorylated by known p130-bound kinases like cyclin E or A kinase (Cobrinik et al. 1993; Hannon et al. 1993; Li et al. 1993). Conceivably, there are other kinases, yet to be identified, which bind to p130 in G₀ cells. However, we failed to detect them in the in vitro kinase assays described in Figure 4.

A number of possible mechanisms could be invoked to account for the increased mobility of E2F-4 observed following mitogenic stimulation of T cells (Fig. 2), including E2F-4 dephosphorylation by cell cycle-dependent phosphatase activation or failure of phosphorylation of newly synthesized E2F-4 attributable to inactivation of a G₀-specific kinase coupled with degradation of preexisting phospho-E2F-4. The identification of a novel phosphatase that associates with RB in vivo provides a precedent for the concept of pocket protein-associated phosphatases (Durfee et al. 1993).

With the data presented here, one can now propose the outlines of a generic model for p130 and E2F-4 function in cell cycle control. We have shown that E2F-4 and p130 are components of a G₀ complex in quiescent primary

human T cells and that p130 has a growth-suppressive function that can be overcome, in a relatively specific manner, by overproduction of E2F-4 that is, itself, capable of promoting G₁/S-phase transit (Beijersbergen et al. 1994). Thus, E2F-4 can function downstream of p130, and p130 growth arrest may involve suppression of E2F-4 activity. Overproduction of E2F-1 was not as potent as overproduction of E2F-4 in overriding p130-induced growth arrest. This was the case, despite the fact that E2F-1 does not interact with p130 *in vivo* and is, therefore, presumed to be present largely as free E2F-1 in p130-cotransfected cells. These results suggest that E2F-1 and E2F-4 target different, although perhaps overlapping, sets of E2F-regulated promoters. By analogy with what is known of the effect of pRB on E2F-1 (Weintraub et al. 1992; Helin et al. 1993a; Flemington et al. 1993; Qin et al. 1995; W.R. Sellers, J. Rodgers, and W.G. Kaelin, in prep.), binding of p130 to E2F-4 in G₀ might result in the suppression of E2F-4 target genes during quiescence. By specifically repressing certain E2F target genes that can initiate and/or perpetuate cell cycle progression, it is possible that formation of a p130/E2F-4 complex plays an active role in inducing and/or maintaining cellular quiescence. Such a model would speak to at least one aspect of the control mechanisms operating at the G₀/G₁ transition.

This proposal is supported by the fact that the E2F sites present in the promoters of certain E2F-regulated genes, such as *B-myc*, *cdc2*, or even E2F-1 itself, are required for the repression of these genes during G₀/G₁ (Dalton 1992; Lam et al. 1993; Hsiao et al. 1994; Neuman et al. 1994). Again, by analogy with what happens to pRB/E2F-1 complexes during G₁ exit, a signal leading to G₀ exit may result in E2F-4 being freed from p130 (perhaps via phosphorylation mediated by one or more cyclin/cdk species) with subsequent derepression/activation of E2F-4 target genes. Such a proposal is consistent with detection of free E2F-4 in cycling, but not quiescent T cells and the ability of E2F-4 overexpression to induce S-phase entry in the face of p130 cotransfection. Conceivably, the E2F-4 interaction with p107, which clearly occurs later in G₁ or at G₁/S (Cao et al. 1992; Lees et al. 1992; Shirodkar et al. 1992), serves to regulate negatively E2F-4 at later times in the cycle.

Whatever the case, the fact that two related pocket proteins interact with the same transcription factor sequentially in the cell cycle implies that the functional consequences of these two sets of interactions are not identical. Among the potential effects of the processive binding of E2F-4 by p130 and p107 is the serial activation and repression of certain E2F-dependent genes during the period of progression out of G₀ and into S. This and other possibilities must be the subject of future investigations.

Materials and methods

Cell culture

C33A cervical carcinoma cells and U2OS and SAOS2 osteosarcoma cell lines were grown in Dulbecco's modified Eagle me-

dium (DMEM) with 10% fetal calf serum (FCS, Hyclone) at 37°C in a 10% CO₂-containing atmosphere. The U937 human promonocytic leukemic cell line was cultured in RPMI-1640–10% FCS at 37°C and 5% CO₂. Primary human T cells from peripheral blood of healthy donors were isolated as described previously (Chittenden et al. 1993). Briefly, T cells were prepared by Ficoll/Hypaque (Pharmacia) gradient centrifugation, and adherent cells were removed by incubation on plastic for 2 hr. Where indicated, T cells in RPMI-1640–10% FCS at 37°C and 5% CO₂ were stimulated by the addition of 1 µg/ml of PHA for the indicated times prior to use.

Whole cell extracts and EMSAs

Whole-cell extracts from primary human T cells or U937 cells were prepared as described previously (Raychaudhuri et al. 1987; Shirodkar et al. 1992). Extract (10 µg) was used for EMSA and electrophoresed in 4% nondenaturing polyacrylamide gels, as described previously (Shirodkar et al. 1992; Chittenden et al. 1993). The probe used was a ³²P-end labeled, double-stranded oligonucleotide corresponding to the –82 to –40 sequence of the human *c-myc* P2 promoter (Cobrinik et al. 1993). For competition studies, a 100-fold molar excess of unlabeled probe was included. For these studies, in addition to the *c-myc* probe, oligonucleotides corresponding to the E2F site derived from the dihydrofolate reductase promoter, (DHFR; wild type) or an E2F site mutant of this probe, DHFR (mutant), were also used as competitors (Shirodkar et al. 1992).

Western analysis

Whole-cell extracts, prepared as described above, were subjected to electrophoresis (200 µg of cellular protein per lane) using 8% polyacrylamide–SDS gels. After transfer to Hybond-C extra (Amersham), the membranes were blocked for 4–6 hr with 5% nonfat milk in Tris-buffered saline (pH 8.0) (TBS), washed, and incubated overnight at 4°C with a 1:10 dilution of E2F-4 mAb GG22, hybridoma tissue culture supernatant in TBS/3% BSA. After washing with 0.05% Tween 20 in TBS, immunoblots were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG and the bands were visualized by enhanced chemiluminescence, according to manufacturer's instructions (Amersham).

Metabolic labeling and immunoprecipitations

Cells were incubated in methionine-free DMEM (GIBCO) with 10% dialyzed FCS (GIBCO) for ~30 min prior to labeling for 4 hr in the same medium containing [³⁵S]methionine (2–3 mCi/4 ml in a 10-cm dish for adherent cells or 1 mCi/ml for suspension cultures at 10⁷ cells/ml). After washing with cold phosphate-buffered saline (PBS), cells were lysed in TNN buffer (50 mM Tris-HCl at pH 7.4, 120 mM NaCl, 5 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.2 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 20 µg/ml of aprotinin) using 1 ml/10-cm dish for adherent cells or 1 ml/10⁷ suspension cells. The lysate was centrifuged at 4°C for 10 min at 10,000g. Following preclearing for 30 min with protein A–Sepharose (Pharmacia), supernatants were incubated on a rocker for 1 hr at 4°C with the indicated antibodies [for immunoprecipitations using mouse monoclonal antibodies, rabbit anti-mouse IgG secondary antibody (Cappel) was added for the final 30 min]. Fifty microliters of a 50% (wt/vol) slurry of protein A–Sepharose was then added, and the incubation continued for 30 min. Immune complexes were collected by centrifugation and washed four times with TNN buffer. For reprecipitations, washed immunobeads were boiled

for 10 min in 100 μ l of 1 $\times$ SDS release buffer (50 mM Tris HCl at pH 7.5, 1% SDS, and 5 mM DTT), and 10 μ l of this was set aside as a control for the first immunoprecipitation. The remainder was diluted to 1.4 ml with TNN buffer and the indicated antibodies, and the resulting mixture was incubated overnight at 4°C. Immune complexes were then collected and treated, as described above. Samples were resuspended in SDS sample buffer, boiled, separated by electrophoresis in 8% SDS-polyacrylamide gels, and visualized by fluorography. The calculated molecular masses, noted in the text, were determined using the apparent molecular masses of the prestained markers.

For immunoprecipitations of in vitro-translated (IVT) HA-tagged E2F species, [³⁵S]Methionine-labeled translation products were prepared with the TNT lysate system (Promega) according to the manufacturer's instructions. Translated proteins (5 μ l) were immunoprecipitated, as above, except that TNN containing 250 mM NaCl was used. The final two washes were with TNN containing 1M NaCl.

Phosphatase treatment

Immunoprecipitates, prepared as described above, were washed once and resuspended in 60 μ l of phosphatase buffer (see NEB specifications). Lambda phosphatase (500 units) (NEB) was added to the appropriate tubes and incubated at 30°C for 1 hr. Where indicated, the phosphatase inhibitors vanadate (10 mM) and NaF (5 mM) were included as specificity controls.

In vitro kinase assay

Lysates from resting and stimulated T cells were prepared in TNN as above and total cellular protein concentrations determined (Bio-Rad). Equivalent amounts of total cellular protein were immunoprecipitated as above. After washing with lysis buffer, beads were washed once more with kinase buffer (50 mM HEPES at pH 7.0, 10 mM MgCl₂, 5 mM MnCl₂, and 1 mM DTT) and then incubated with 30 μ l of kinase buffer containing 10 μ Ci [γ -³²P]ATP (NEN) for 20 min at 37°C. The reaction was terminated by addition of 30 μ l of 2 $\times$ SDS release buffer (see above), boiled, diluted, reimmunoprecipitated for 1–2 hr as described above and subjected to electrophoresis in 8% polyacrylamide-SDS gels and visualized by autoradiography.

Plasmids

pCDNA1-HApl30, the first 96 nucleotides of a p130 clone [pBS(S) F-130 1-4853] [Li et al. 1993] were replaced with a PCR product spanning this region containing an in-frame *Bam*HI site and a consensus Kozak sequence introduced immediately 5' of the initiating ATG codon. The DNA sequence of the PCR-derived region was verified by sequencing. A 3488-bp *Bam*HI-*Xba*I fragment containing the full-length p130 open reading frame was cloned into pCDNA1(HAE2F-1) [Krek et al. 1993] from which the E2F-1 cDNA was excised by *Bam*HI-*Xba*I digestion to generate an expression plasmid encoding an amino-terminal HA-tagged full-length p130-encoding plasmid.

Other plasmids used here have been described previously: pCDNA1-HAE2F-1, pCDNA1-HAE2F-2, pCDNA1-HAE2F-3, pCMV-E2F-1 [Krek et al. 1994]; pCMV-p107 [Zhu et al. 1993]; pE2wtCAT (wtE2) [Helin et al. 1992]; pCMV-Rb [Qin et al. 1992]; pCMV-HAE2F-4 and pCMV-E2F-4 [Ginsberg et al. 1994].

Transient transfections

Cells were transfected by a modified calcium-phosphate protocol [Qin et al. 1992]. For E2F *trans*-activation assays, cells were

harvested 40 hr after transfection and lysed by three cycles of freeze-thawing in 100 μ l of 250 mM Tris-HCl (pH 8.0). Extracts were assayed for β -galactosidase and CAT activities, as described previously [Krek et al. 1993; Ginsberg et al. 1994].

Flow cytometric analysis

The effects of pocket protein or E2F expression on cell cycle distribution were determined as described previously [Qin et al. 1992]. Briefly, SAOS2 cells were cotransfected with 2 μ g of CD19 expression plasmid [pCMV-CD19] (kindly provided by T. Tedder, Duke University, Durham, NC) together with the various combinations of cytomegalovirus (CMV)-driven expression plasmids encoding p130, Rb, E2F-4, E2F-1. Backbone CMV vector was added such that all cultures were treated with equal total amounts of plasmid DNA. Eighteen hours after transfection, the cells were washed and incubated at 37°C for 2 more days prior to trypsinization and FITC staining for surface expression of CD19 and propidium iodide DNA staining. The cell cycle distribution of CD19-positive cells was determined by FACs analysis using SOBR cell cycle analysis software.

Antibodies

Affinity-purified rabbit p130 antibody raised against a peptide corresponding to the carboxy-terminal 20 amino acids of p130 (C20) (catalog no. sc-317) was obtained from Santa Cruz Biotechnology. One microgram of this was added per immunoprecipitation reaction or per 25 μ l EMSA reaction. Where indicated, the antibody was preblocked by mixing with antigenic peptide (Santa Cruz) (1 μ g of peptide/ μ g of antibody) at 4°C for 15 min prior to use. p107 monoclonal antibody (SD2, SD6, SD9, and SD15) hybridoma tissue culture supernatants, kindly provided by N. Dyson (Massachusetts General Hospital, Boston, MA), were used for immunoprecipitations as indicated at 100 μ l per immunoprecipitation reaction or at 2 μ l per EMSA reaction. The E2F-4 mAbs, GG22 and GG21, generated as described previously [Ginsberg et al. 1994], were used at 100 μ l of hybridoma tissue culture supernatant per immunoprecipitation reaction. Mouse polyclonal antiserum to human E2F-4, obtained from the immunized BALB/c mouse whose spleen was used to generate the monoclonal antibodies above, was used at 0.8 μ l per gel shift reaction or 1 μ l per immunoprecipitation reaction. Rabbit polyclonal antiserum to murine DP-1 (R12), kindly provided by S. Shirodkar (Dana-Farber Cancer Institute, Boston, MA), was raised against full-length [Girling et al. 1993] glutathione S-transferase (GST)-fusion protein and was used at 4 μ l per immunoprecipitation reaction or 1 μ l per EMSA reaction. Mouse polyclonal antiserum to human DP-1, raised in BALB/c mice against a GST-fusion protein encompassing amino acids 1–400 of the human DP-1 gene product, was used at 0.8 μ l per gel shift reaction. Affinity-purified anti-human E2F-1 mAb SQ41, kindly provided by W. Kaelin and J. DeCaprio (Dana-Farber Cancer Institute, Boston, MA), was used at 1.4 μ g per immunoprecipitation reaction. Control monoclonal antibody (anti-cdc10) YS140 and YS146 hybridoma tissue culture supernatants were kindly provided by J. Ayte (Dana-Farber Cancer Institute, Boston, MA) and J. DeCaprio. Affinity-purified anti-CD19 mAb CD19.15 (kindly provided by T. Tedder) was used at 1:500 dilution. FITC-conjugated goat anti-mouse secondary antibody (used at 15 μ g/ml) was purchased from Boehringer Mannheim. Affinity-purified anti-HA antibody consisted of either a rabbit polyclonal antibody or mAb 12CA5, from BAbCo.

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