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The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes

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Unregulated expression of either the retinoblastoma protein (pRB) or the related protein p107 can cause growth arrest of sensitive cells in the G_1 phase of the cell cycle. However, growth arrests mediated by p107 and pRB are not identical. Through structure–function and co-expression analyses we have dissected the p107 molecule into two domains that independently are able to block cell cycle progression. One domain corresponds to the sequences needed for interaction with the transcription factor E2F, and the other corresponds to the interaction domain for cyclin A or cyclin E complexes. In cervical carcinoma cell line C33A, which was previously shown to be sensitive to p107 but resistant to pRB growth suppression, only the cyclin binding domain is active as a growth suppressor. Furthermore, we show that these two independent domains are functional in untransformed mouse fibroblasts. Together, these results provide experimental evidence for the presence of two functional domains in p107 and pinpoint an important functional difference between p107 and pRB.

Key words: cell cycle/functional domains/growth suppression/p107

Introduction

Effective control of cell proliferation involves precise regulation of positive and negative signaling pathways. The cellular protein p107 is a member of a family of proliferation suppressors exemplified by the retinoblastoma protein pRB. The proteins of this family, pRB, p107 and p130, share a stretch of homologous sequences called the 'pocket' that serves as the binding domain for viral oncoproteins such as E1A of adenovirus, large T antigen of SV40 and E7 of human papillomavirus. Recently, it has become clear that these pRB-related proteins use the pocket domain to associate with transcription factors such as E2F (Nevins, 1992). The interaction of the pocket proteins with E2F inhibits transcription through promoters containing E2F sites either by repressing active promoters (Weintraub *et al.*, 1992; Lam and Watson, 1993; Hsiao *et al.*, 1994; Johnson *et al.*, 1994) or through blocking the transactivation by E2F (Hamel *et al.*, 1992; Hiebert *et al.*, 1992; Flemington *et al.*, 1993; Helin *et al.*, 1993a; Schwarz *et al.*, 1993; Zamanian and Thangue, 1993; Zhu *et al.*, 1993; Dynlacht *et al.*, 1994).

E2F is a class of heterodimeric transcription factors formed by the association of one member of the E2F-x subfamily and one of the DP-x subfamily (where x describes the order of their identification). In this manuscript the term 'E2F' is used to describe the dimeric transcription factor and its activity, while 'E2F-1', etc., is used to indicate the particular polypeptide that interacts with DP. pRB appears to bind exclusively to complexes containing E2F-1, E2F-2 or E2F-3, while p107 binds to E2F-4 (Lees *et al.*, 1993; Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994). The interaction of certain pocket proteins with various E2Fs at certain cell cycle stages (Lees *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993; Schwarz *et al.*, 1993) suggests that these interactions help to regulate the correct temporal expression of these genes. Since E2F binding sites are found in the promoter regions of many genes whose protein products are directly involved in cell proliferation, regulation of the E2F activities appears to be an important mechanism through which the pocket proteins regulate cell cycle progression.

Another indication that these pocket proteins play important roles in the regulation of the cell cycle is the interaction of the pocket proteins with cyclins and cyclin-dependent kinases (cdks). p107 and p130 both interact stably in independent complexes with cyclin A/cdk2 or cyclin E/cdk2 (Ewen *et al.*, 1992; Faha *et al.*, 1992; Lees *et al.*, 1992; Li *et al.*, 1993). The function of pRB can apparently be regulated by cyclin/cdk complexes such as cyclin A/cdk2, cyclin E/cdk2 and cyclin D/cdk4 (Hinds *et al.*, 1992; Dowdy *et al.*, 1993; Ewen *et al.*, 1993), but stable *in vivo* interactions between pRB and cyclin E/cdk or cyclin A/cdk complexes have not been seen. The interaction between cyclin D/cdk complexes and pRB has been detected following *in vitro* mixing, but *in vivo* associations either show low stoichiometry or are less stable than the p107/cyclin A/cdk2 or p107/cyclin E/cdk2 complexes. To date, the functional significance of the stable p107/cyclin A or cyclin E interaction has not been understood.

Transfection of pRB- or p107-expressing constructs into sensitive cells leads to growth arrest in G_1 (Hinds *et al.*, 1992; Zhu *et al.*, 1993). Although the exact mechanism involved in growth suppression is not understood, it has been postulated that repression of E2F transcriptional activity plays an important role. In the case of pRB mutagenesis, studies have demonstrated a good correlation between growth suppression and E2F binding (Hiebert *et al.*, 1992; Qian *et al.*, 1992). We have previously

established that growth suppression mediated by p107 and pRB are not identical, despite the similarities between the two proteins (Zhu *et al.*, 1993). These differences were detected by three experimental approaches. First, the C33A cervical carcinoma cell line could be arrested by unregulated p107 expression but not by pRB. Second, rescue experiments demonstrated that the growth arrest in Saos-2 cells by p107 differed from that by pRB. The pRB, but not the p107, block could be rescued efficiently by co-expression of cyclin A or cyclin E. Conversely, E1A rescued the p107 block more efficiently than the pRB-mediated arrest. Third, the E1A binding ability of p107 was not absolutely required for its growth suppression activity, whereas this interaction region of pRB is essential for pRB-mediated arrest. These results led us to postulate that pRB and p107 suppressed cell growth through different molecular mechanisms. We have now established the molecular basis for these differences. There are two functional domains of p107, one shared with pRB and the other unique to p107. These two functional domains of p107 mediate growth suppression through apparently different mechanisms. The shared function appears to be interaction with transcription factor E2F, and the growth-arresting domain unique to p107 correlates with the interaction with cyclin/cdk complexes.

Results

Two independent domains for growth suppression by p107

Unregulated expression of p107 in Saos-2 cells causes efficient growth arrest in the G_1 phase of the cell cycle (Zhu *et al.*, 1993). This p107-mediated growth arrest is similar but not identical to pRB-mediated growth arrest. To investigate these differences, detailed mutagenesis experiments of p107 were carried out to localize the functional domains for growth suppression. Previously we had shown that the large pocket (the pocket domain plus the C-terminus, amino acids 385–1068, mutant N385 in Figure 1A) was sufficient for growth suppression. In addition, the C-terminal truncation mutant C768 (Figure 1A, amino acids 1–781) also led to efficient growth arrest (Zhu *et al.*, 1993). Based on these results, it was expected that a domain of p107 containing amino acid residues 385–768 would contain a single growth suppression domain that represents a different mechanism from that of pRB. However, this was not the case in the initial set of mutagenesis experiments.

First, to confirm the activity of C768 with a similar, independent mutant we constructed mutant L19 covering the same amino acid sequences as in C768 but with an added sequence at the C-terminus [residues 1035–1068 from the p107 C-terminus followed by the hemagglutinin epitope (HA), Figure 1A]. Amino acid residues 1035–1068 facilitate efficient nuclear localization of mutant proteins that lack a complete C-terminus (L.Zhu unpublished results), and the HA tag was added to allow the recognition of mutant proteins separately from the endogenous p107. For these two reasons this segment was added to all the mutants described in this report. Second, mutant N19, which covers the region overlapped in mutants N385 and C768, was generated to determine its effectiveness.

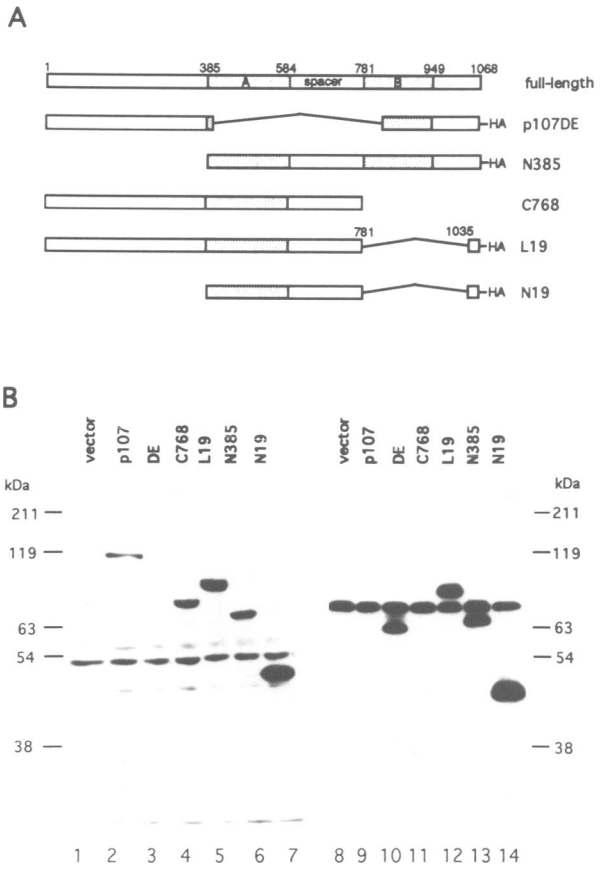

Fig. 1. Two growth suppression domains of p107. (**A**) Schematic drawing of p107 mutants. Amino acid residue numbers are noted above a deletion or other transition boundaries. HA represents the hemagglutinin epitope: YPYDVPDYASL (single amino acid code). (**B**) Mutant protein expression. Saos-2 cells were transfected with the indicated expression constructs and total cell extracts were made 40 h later. Twenty μg protein of the extract was separated on an 8% SDS–PAGE (lanes 1–7) or first immunoprecipitated with the anti-HA monoclonal antibody 12CA5 [lanes 8–14, the antibody was first covalently coupled to protein A–Sepharose beads (Harlow and Lane, 1988)] and then separated on SDS–PAGE. Protein gels were blotted and probed with anti-p107 monoclonal antibody SD9 (lanes 1–7) or 12CA5 (lanes 8–14, note the non-specific protein bands in all the lanes). Blots were developed with ECL according to the manufacturer's instructions (Amersham). Protein molecular weights in kDa are marked.

After transient transfection into Saos-2 cells, each mutant protein was shown to be expressed at a high level by immunoblotting with the anti-p107 monoclonal antibody (Figure 1B lanes 1–7). In Figure 1B lanes 8–14, whole cell extracts were first immunoprecipitated with the anti-HA epitope monoclonal antibody and then blotted with the same antibody, demonstrating efficient recognition of the HA-tagged proteins with the anti-HA antibody. When probed with the anti-p107 monoclonal antibody, the same blot did not reveal any endogenous p107, the transfected full length p107, or p107DE, the latter of which does not contain epitopes for this antibody (data not shown). Thus, the mutant proteins were expressed at similar levels and the HA-tagged versions were recognized separately from the endogenous p107 in immunoprecipitations.

To test for effects on cell cycle progression, these constructs were co-transfected with the cell surface marker

Table I. Two independent growth suppression domains of p107

Expression plasmid	G_1	S	G_2+M
Vector	42.4	19.9	37.7
p107	76.4	5.6	18.1
DE	47.8	20.7	31.5
N385	79.1	4.8	16.1
C768	72.0	10.0	18.0
L19	73.0	10.5	16.5
N19	45.5	22.8	31.7

Saos-2 cells were transfected with 23 μg of the indicated expression plasmids plus 2 μg of pCMVCD20. Two days later, cells were harvested for flow cytometry analysis to determine the cell cycle profiles (percentage of cells in each phase) of the transfected cells (at least 1000 CD20-positive cells were analyzed, as in Zhu *et al.*, 1993).

CD20 into Saos-2 cells. Transfected cells were identified by the anti-CD20 antibody, and their DNA contents determined by flow cytometry analysis. Consistent with our previous data, overexpression of the N385, C768, L19 or wild-type p107 significantly increased the G_1 cell population and decreased the S phase population (Table I). When nocodazole was included in the media for the last 24 h prior to harvest, cycling cells were able to progress to mitosis, while cells blocked in G_1 did not. In this setting, 50–70% of the vector-transfected cells or the untransfected cells in the same dish were in the G_2+M phase while 70–80% of the cells transfected with p107, N385, C768 or L19 were still in G_1 phase (data not shown), indicating again the presence of a cell cycle block before S phase.

Importantly, the mutant N19, which covers the region overlapped in mutants N385 and C768, was inactive in this assay, indicating that N19 is not sufficient for growth suppression. The simplest explanations for this observation were either that the N19 protein folded improperly or that there was more than one protein domain that contributed to growth arrest.

We reasoned that if mutants N385 and L19 represented independent growth suppression functional domains they may have different biochemical properties. The two biochemical properties that have been characterized for p107 are physical associations with E2F and cyclin/cdk2 complexes. The ability of the mutant proteins to associate with E2F was determined by the so-called DOC release assay as shown in Figure 2A. Equal amounts of protein extracts of transiently transfected cells were immunoprecipitated with either anti-p107 antibody or anti-HA antibody. The presence of E2F DNA binding activity in the immunoprecipitates was then detected by treating these complexes with DOC (sodium deoxycholate) and testing the released proteins for gel shift of E2F binding site-containing oligonucleotides. E2F activity was easily detected in anti-p107 immunoprecipitations (lanes 1–5). Anti-HA antibody, which recognized all these mutant proteins (Figure 1B), brought down E2F activity only from N385-transfected cells (lane 7). Mutants L19 and N19 did not have the ability to associate with E2F in the cell.

The experiment presented in Figure 2B was performed to study the ability of these mutant proteins to bind to various endogenous cyclins. Whole cell extracts of transfected cells were first immunoprecipitated with anti-p107 or anti-HA antibodies, the protein gel of the immunoprecipitations was blotted and probed with monoclonal antibodies against cyclin A, cyclin E or cyclin B, as indicated. It is clear that the full-length p107, as well as the mutant L19, bound to cyclins A and E efficiently, while mutants N385 and N19 did not. Binding to cyclin B1 was examined in parallel to demonstrate the specificity of cyclin binding. Although a trace amount of cyclin B1 was detected in anti-p107 immunoprecipitations, it was not specific since a similar amount of cyclin B1 could also be seen in control 12CA5 immunoprecipitations in many other experiments.

It was shown previously that the p107 spacer–glutathione S-transferease (GST) fusion protein was able to bind cyclin A *in vitro* (Ewen *et al.*, 1992). To test if the spacer region of p107 alone was sufficient for cyclin A binding *in vivo*, we overexpressed HA-tagged spacer protein in transfected cells. No cyclin A association with the spacer protein was detected (data not shown), consistent with the inability of mutants N385 and N19 to bind cyclin A (Figure 2B). Thus additional protein sequences N-terminal to the pocket region are required for association with cyclin A *in vivo*. We conclude that mutants N385 and L19 encompass independent domains that interact with different cellular proteins. The functional properties of these two domains were studied in further detail below.

Growth suppression by the N385 domain correlates with its ability to bind E2F

Protein sequence comparison between pRB and p107 has revealed the strong homologies in the pocket region. As shown in Figure 2A, the mutant N385, representing the large pocket region of p107, has the ability to associate with E2F. Successive deletions of the C-terminal sequence (Figure 3A) were generated to localize the E2F association domain. Using the DOC release assay, we were able to show that the ability to associate with E2F requires the presence of almost all the C-terminus, as a deletion of only 17 amino acids residues near the C-terminus (mutant L26) significantly diminished E2F binding. Deletion of 94 amino acids (mutant L28) abolished this activity (Figure 3B). Protein expression levels of the mutants were similar, as determined by immunoprecipitation with the same anti-HA antibody in the same experiment (Figure 3C). These mutant proteins were tested for their growth suppression activities. As shown in Table II, the loss of growth suppression activity correlated with the loss of E2F binding ability.

Cys846 of p107 is within a stretch of amino acid sequences that are homologous with pRB, and this amino acid is the site of a naturally occurring point mutation (Cys→ Phe) of pRB in a small cell lung carcinoma cell line (Kaye *et al.*, 1990). This point mutation disrupts the pocket function of both pRB and p107, as demonstrated by the loss of E1A binding (Ewen *et al.*, 1991, and Figure 4A). When introduced into the N385 domain, this mutation significantly reduced the E2F binding ability (Figure 4B) as well as the growth suppression activity (Table II). This indicates that the pocket function of p107 is important for both E2F binding and growth suppression.

To provide further experimental evidence for the

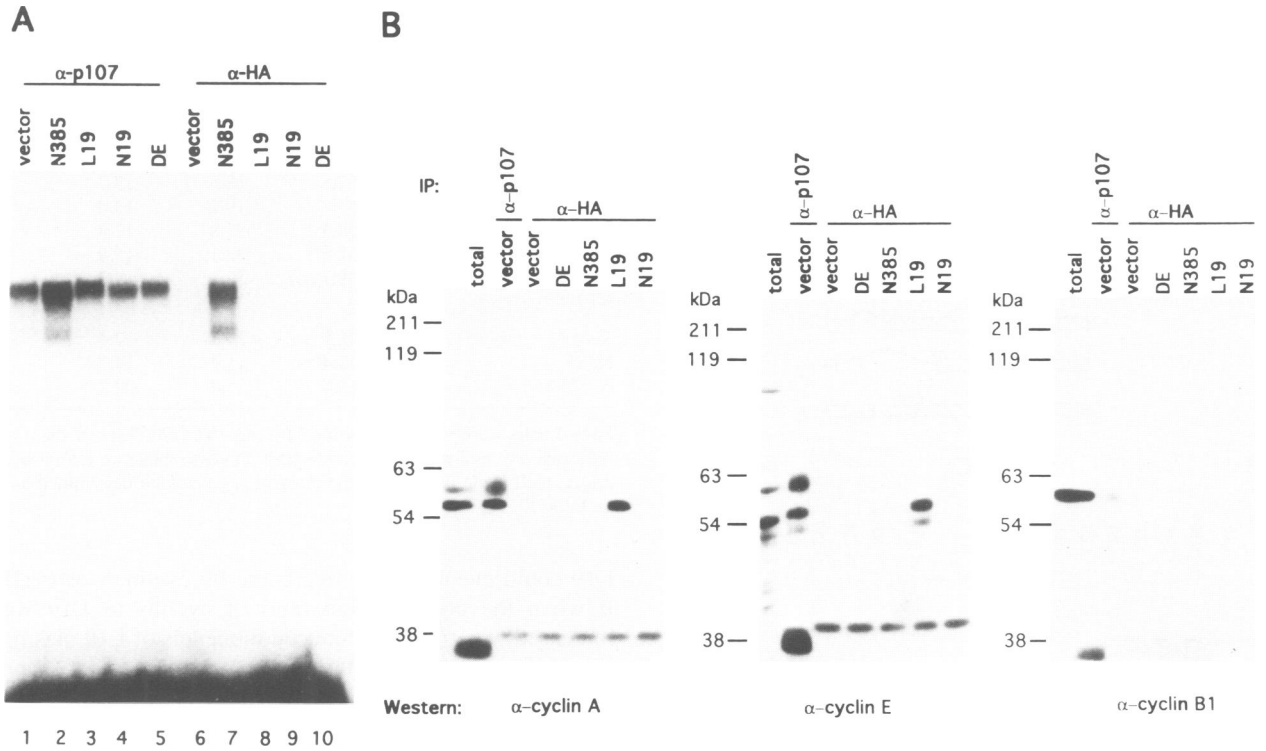

Fig. 2. E2F and cyclin binding abilities of the two growth suppression domains. (**A**) Ability of p107 proteins to associate with E2F. Extracts were prepared from Saos-2 cells transfected with the indicated constructs, and divided into two halves to be immunoprecipitated with either anti-p107 (SD4) or anti-HA (12CA5) antibodies. The immunoprecipitated proteins were treated with deoxycholate, and E2F activity in the supernatant was detected with ^{32}P-labeled E2F binding site-containing oligonucleotides (Zhu *et al.*, 1993). (**B**) Ability of p107 proteins to bind cyclins. Saos-2 cells were transfected with the indicated expression plasmids, and lysates were immunoprecipitated with monoclonal antibodies coupled to protein A beads, as indicated. Immunocomplexes were resolved on 8% SDS−PAGE and blotted. Three identical blots were probed with monoclonal antibodies against cyclin A, cyclin E or cyclin B1 and developed with ECL. In the first lane of each blot, 20 μg of protein of the total extract was loaded.

involvement of E2F in the N385-mediated growth suppression, we tested whether growth suppression by N385 could be rescued by co-expression of E2F. Since the E2F/DP dimer represents the active form of E2F activities (Bandara *et al.*, 1993; Helin *et al.*, 1993; Krek *et al.*, 1993) and an E2F that interacts with p107 *in vivo* has recently been cloned (E2F-4, Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994), we co-transfected E2F-4 and DP-1 constructs with various amounts of N385 into Saos-2 cells. Transfection of N385 or E2F-4 with DP-1 resulted in opposite cell cycle effects: growth suppression by N385 and stimulation by E2F-4/DP-1 (Beijersbergen *et al.*, 1994, and Table III). When equal amounts of plasmid were used in co-transfections, the suppressive effect of N385 was dominant, reflecting the inhibitory effect of N385 on the growth stimulating function of E2F-4/DP-1 dimer. However, as the amount of N385 was gradually reduced, the growth arrest was eventually overcome by the E2F-4/DP-1 dimer (Table III). These results are consistent with the notion that N385 suppresses cell growth through a stoichiometric interaction with E2F-4/DP-1 that resulted in functional antagonism.

Growth suppression by the L19 domain requires its association with cyclin A or cyclin E

To determine if the ability of mutant L19 to bind cyclin A or cyclin E is linked with its growth suppression ability, we successively deleted amino acid residues from both ends of L19. Because mutant proteins (three out of three) with truncations between the N-terminus and the pocket boundary (amino acid residue 385) were not stable, we focused on the spacer region of p107 (Figure 5A). As shown in Figure 5B, mutant L20, which contains only the N-terminal half of the spacer, retained full binding capability. A further deletion of only 11 amino acid residues (mutant L30) abolished the ability to bind both cyclin A and cyclin E, establishing a boundary for the cyclin binding domain at the carboxy-terminus. Further deletion mutants L24 and L23 were also inactive, as expected. Mutant protein expression levels were checked in the same experiment and were comparable among all the mutants (Figure 5C). When these mutants were tested for their growth suppression activities, a good correlation between cyclin binding and growth suppression was observed. As shown in Table IV, mutant L20 was as active as L19 in growth suppression, but L30, which was 11 amino acids shorter but expressed at slightly higher levels than L20 and L19, was inactive. Therefore, the boundaries of the L19 growth suppressing domain correlate with its interaction with cyclin A or cyclin E, suggesting that a key aspect of the growth suppressing function of this domain is binding to the cyclins.

Next, we followed an analogous rescue strategy to the one described above for the E2F binding domain. Transient transfection of cyclin expression constructs into Saos-2 cells significantly increased cyclin protein levels (Figure 6). Effects of cyclin overexpression on cell proliferation were first determined after transient transfection.

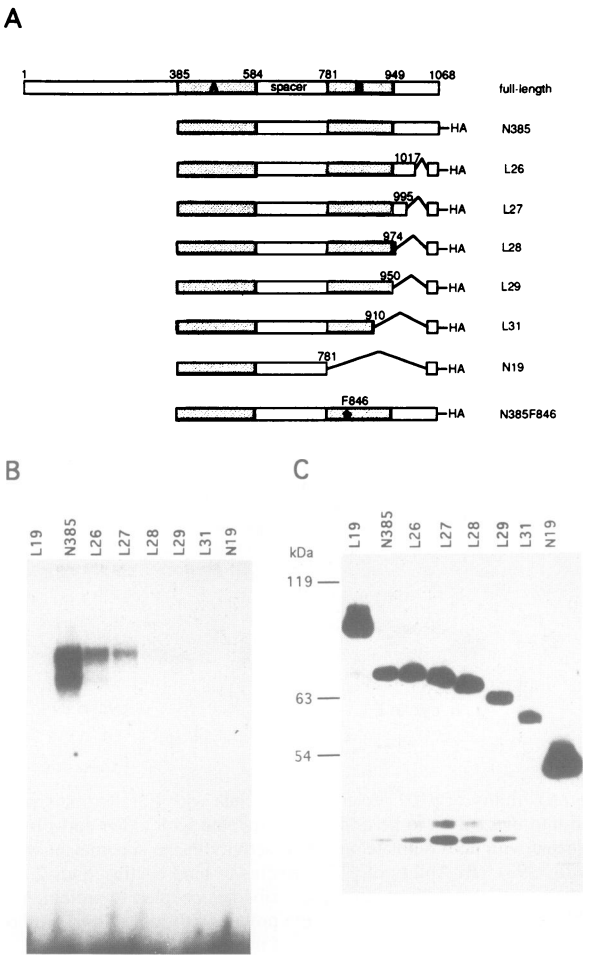

Fig. 3. Mutagenesis of the N385 domain. (**A**) Schematic drawings of further deletions of the N385 domain. Amino acid residue numbers at the deletion boundaries are indicated. (**B**) The abilities of the mutant proteins to associate with E2F after transfection into Saos-2 cells determined as in Figure 2A. (**C**) Expression levels of the mutant proteins. From the same transfection, half of the cells were used for the DOC release experiment presented in A and the other half were lysed in detergent and immunoprecipitated with 12CA5 coupled to protein A beads. Immunoprecipitates were separated on 8% SDS–PAGE and blotted. The blot was probed with anti-p107 monoclonal antibody SD9 and developed with ECL.

Comparisons of cell cycle profiles of the transfected cells with the untransfected cells in the same tissue culture plate showed that cells transfected with cyclins A or E exhibited a significantly more active proliferation profile (Table V). These results are consistent with previous work from other laboratories that suggest that cyclin E is rate limiting for G_1 progression (Ohtsubo and Roberts, 1993; Resnitzky *et al.*, 1994) and cyclin A is functioning to promote S phase (Girard *et al.*, 1991; Guadagno *et al.*, 1993).

Various cyclins (12 μg) were co-transfected with the L19 domain (10 μg) into Saos-2 cells. The growth suppressive activity of the cyclin binding domain L19 was dominant over the growth promoting activities of cyclin A or cyclin E at these plasmid input concentrations (Table V). Mutant L30, which did not bind cyclins A or E, did not have any effect on the growth promoting phenotype of the cyclins (data not shown). To test if the growth suppression by

Table II. Growth suppression assay for N385 and its derivatives

Expression plasmid	G_1	S	G_2+M
Vector	46.8	30.2	22.9
N385	73.5	10.2	16.3
L26	67.8	18.5	13.7
L27	67.8	19.2	13.0
L28	58.4	23.8	17.9
L29	52.5	32.1	15.4
L31	49.7	32.1	18.2
N19	47.9	31.1	21.1
Vector	48.5	29.1	22.4
N385	73.4	11.9	14.7
N385F846	52.4	25.4	22.2

Saos-2 cells were transfected with 2 μg pCMVCD20, 7 μg of the indicated expression plasmids and pBSK DNA to make up a total of 25 μg. Cell cycle profiles of the transfected cells were determined as in Table I.

L19 could eventually be overcome by cyclin A or cyclin E when the concentration ratio of cyclins to L19 was increased, we gradually reduced the levels of L19 plasmid in transfection. As shown in Table V, at 6 μg of L19 input plasmid, cyclin A largely and cyclin E completely rescued the growth suppression. Thus the data are consistent with the simple model in which L19 neutralizes the effects of cyclins A and E through stoichiometric binding. The fact that cyclins A and E were able to rescue when L19 concentration was gradually lowered also provides further evidence that the ability of L19 to bind cyclins A and E is likely to be responsible for its growth suppression.

Growth inhibition of C33A cells by p107 through its L19 domain but not the N385 domain

Previous work has shown that the cervical carcinoma cell line C33A is sensitive to p107-mediated growth inhibition but resistant to pRB-mediated growth suppression (Zhu *et al.*, 1993). To investigate which functional domain of p107 was responsible for the growth suppression, we introduced full-length p107 as well as the two functional domains into the cells (Figure 7). Full-length p107 and the L19 domain caused a significant increase in G_1 phase population and a corresponding decrease in S phase population. N385 and N19 were inactive (Figure 7A). Figure 7B shows the results obtained from a duplicate set of plates which were treated with nocodazole for a further 12 h period before flow cytometry analysis. The cell cycle arrest of the full-length p107 and L19 became more dramatic, since cells that were not blocked in G_1 had progressed into the M phase and were blocked there by nocodazole. These results further confirm that the two domains of p107 inhibit cell growth through different mechanisms and suggest that C33A cells are different from Saos-2 cells in regulation by the E2Fs but similar to Saos-2 cells in responsiveness to the cyclins.

The two growth inhibitory mechanisms of p107 can be extended to mouse fibroblasts

Thus far, we have studied the growth suppression activities of p107, as well as pRB, in transformed tumor cell lines. To determine if these growth suppressive mechanisms are also applicable in other cells, we performed microinjection experiments using mouse NIH-3T3 cells. Cells were serum

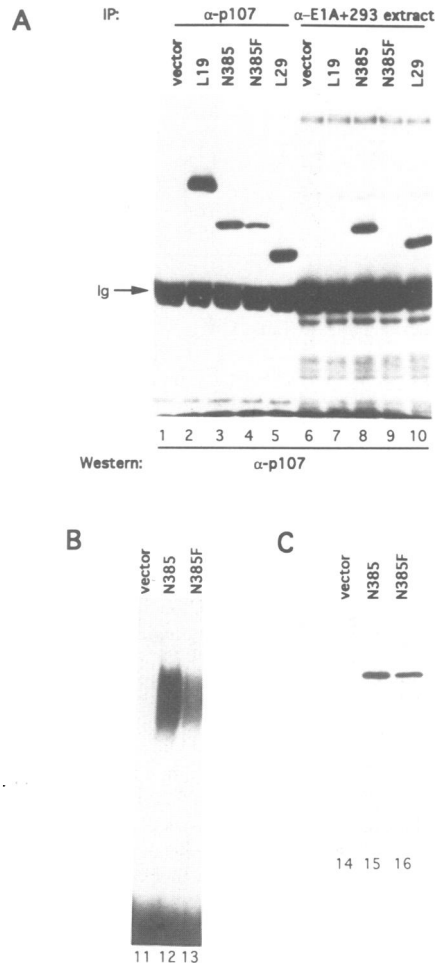

Fig. 4. Functional characteristics of mutant N385F846. (**A**) Abilities of the mutant proteins to bind E1A. Saos-2 cells were transfected with the indicated expression constructs (N385F for N385F846) and lysed. The lysate was incubated with 293 cell extract, which is abundant in E1A protein, and then immunoprecipitated with the anti-E1A antibody M73. Immunoprecipitates were resolved on 8% SDS−PAGE, blotted and probed with anti-p107 antibody SD9. The blot was developed with ECL. Since the antibodies for immunoprecipitation in this experiment were not coupled to protein A−Sepharose beads, immunoglobulin was also detected as marked. (**B**) Ability of the mutant N385F846 to associate with E2F determined as in Figure 2A. (**C**) Protein expression levels after transient transfection into Saos-2 cells as detected by immunoprecipitation with 12CA5 and Western blot with SD9.

starved and then restimulated with serum to exit G_0 and enter the cell cycle. Expression plasmids were injected into cells within 1–4 h after serum stimulation, and BrdU incorporation was used as a measure for cell proliferation. Protein expression was observed by immunofluorescence staining with the anti-HA monoclonal antibody (data not shown). However, to perform double labeling with the anti-BrdU monoclonal antibody, a β-galactosidase (β-gal) expression plasmid was co-injected to permit detection of the injected cells with a rabbit anti-β-gal antiserum, and species-specific fluorescent-labeled secondary antibodies. The results are expressed in Figure 8 as percent inhibition of BrdU incorporation, normalized to BrdU incorporation seen when the β-gal expression vector is co-injected with an empty vector. As shown, those cells co-injected with plasmids expressing pRB, p107, L19 or N385 all showed an inhibition of BrdU incorporation from 50 to 62%.

Table III. Rescue of N385-mediated growth arrest with the E2F-4/Dp-1 dimer

	+Vector			+E2F-4/DP-1		
	G_1	S	G_2 + M	G_1	S	G_2 + M
Vector	44.5	33.0	22.5	26.7	39.1	34.2
N385 (8 μg)	77.7	3.2	19.1	78.8	3.7	17.5
N385 (2 μg)	77.0	5.1	17.9	62.5	13.8	23.6
N385 (0.5 μg)	70.2	9.8	19.9	35.2	27.8	36.9

Saos-2 cells were cotransfected with 2 μg pCMVCD20, N385 (DNA amounts as indicated) together with either vector or pCMVE2F4 (8 μg) and pCMVDP1 (8 μg). Total amount of DNA for transfection was adjusted to 25 μg with pBSK. Cell cycle profiles of the transfected cells were determined as in Table I.

Mutant N19, as expected, had no significant inhibitory activity. From these results, we conclude that the growth suppressing effects of pRB or p107 (induced by either mechanism) are not limited to transformed cell lines.

Discussion

The family of pRB-related proteins has grown to include three members, pRB as well as p107, and p130. pRB is the product of the prototype tumor suppressor gene, which is mutated or deleted in all retinoblastomas and a wide variety of other tumors (for review see Weinberg 1991, 1992). Overexpression of wild-type pRB in certain tumor cell lines that lack functional pRB causes growth arrest in G_1 phase of the cell cycle (Goodrich *et al.*, 1991; Hinds *et al.*, 1992). From these observations it appears that the normal role of pRB is as a negative regulator of cell proliferation. The biological functions of p107 and p130, on the other hand, remain less clear, mostly because both genes have not been shown to be mutated in human tumors. However, several lines of experimental evidence have suggested that p107 and p130 may also function as negative growth regulators. First, when the sequences are compared these molecules share homology throughout their primary sequence, providing a structural basis for similar functions. Second, a common biochemical property has been established for pRB, p107 and p130. All three proteins form complexes with the E2F transcription factor (Bandara *et al.*, 1991; Chellappan *et al.*, 1991; Cao *et al.*, 1992; Devoto *et al.*, 1992; Shirodkar *et al.*, 1992; Cobrinik *et al.*, 1993). To date, the interactions of E2F with pRB and p107 with E2F have been studied in most detail. pRB and p107 repress the activity of E2F through physical interactions which are disrupted by E1A (Hiebert *et al.*, 1992; Qian *et al.*, 1992; Helin *et al.*, 1993; Schwarz *et al.*, 1993; Zamanian and Thangue, 1993; Zhu *et al.*, 1993). There are multiple lines of evidence suggesting that the release of E2F may lead to inappropriate cell proliferation. E2F binding sites are found in promoter regions of many genes whose products are required for DNA synthesis (reviewed in Nevins, 1992; Helin and Harlow, 1993). The overexpression of E2F-1 is sufficient to drive quiescent cells into S phase (Johnson *et al.*, 1994), can prevent cycling cells from exiting the cell cycle (Johnson *et al.*, 1994) and can transform rat embryo fibroblasts (Singh *et al.*, 1994). Thus it is proposed that the repression of E2F activity by pRB and p107 is an important component

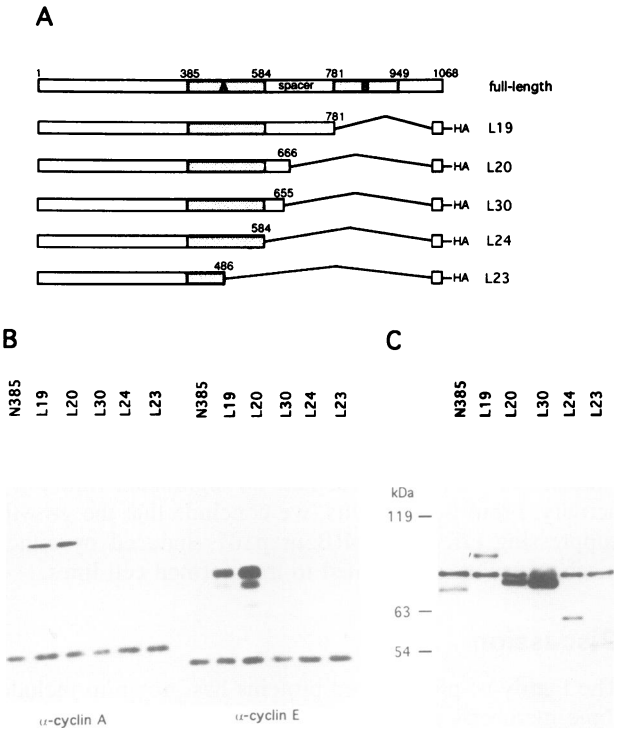

Fig. 5. Mutagenesis of the L19 domain. (**A**) Schematic drawings of further deletions of the L19 domain. (**B**) The abilities of the mutant proteins to bind cyclins A or E. Saos-2 cells were transfected with the indicated expression plasmids, and total cell extracts were immunoprecipitated with the anti-HA monoclonal antibody 12CA5 that was covalently coupled to protein A–Sepharose beads. Immunoprecipitates were resolved on 8% SDS–PAGE and blotted. Two identical protein blots were probed with either anti-cyclin A or anti-cyclin E monoclonal antibodies as indicated and developed with ECL. (**C**) Protein expression levels of the mutants. Samples for this gel were the same samples used in gels of B at one tenth the amount. The protein blot was probed with anti-HA monoclonal antibody 12CA5 (note the non-specific protein bands in all the lanes).

Table IV. Growth suppression assay for L19 and its derivatives

Expression plasmid	G_1	S	G_2 + M
Vector	51.1	31.7	17.2
L19	72.2	13.6	14.2
L20	70.3	17.8	11.9
L30	54.0	27.0	19.0
L24	50.7	31.4	17.9
L23	45.2	35.6	19.2

Saos-2 cells were transfected with 23 μg of the indicated expression plasmids and 2 μg pCMVCD20. Cell cycle profiles of the transfected cells were determined as in Table I.

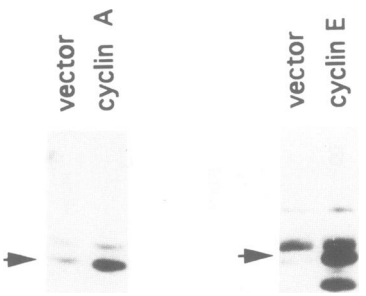

Fig. 6. Overexpression of cyclin A and cyclin E. Saos-2 cells were transfected with the indicated cyclin expression constructs or empty vector, and 20 μg of total cell extract protein was resolved on 8% SDS–PAGE and blotted. The cyclin A blot was probed with BF683 and the cyclin E blot with HE12. Blots were developed with ECL.

of the mechanisms by which these proteins negatively regulate cell proliferation. Third, our previous work has documented that p107, like pRB, is a cell type- and cell cycle stage-specific growth suppressor when overexpressed in sensitive cells (Zhu *et al.*, 1993). In pRB−/− muscle cells the level of the endogenous p107 protein increases upon myogenic differentiation and decreases upon serum-induced re-entry into the cell cycle (Schneider *et al.*, 1994), suggesting that p107 may function as a growth suppressor in this experimental system. Importantly, p107-mediated growth arrest differs from the pRB-mediated growth arrest in cell type specificity, rescue by cyclins and structural requirements for the intact pocket (Zhu *et al.*, 1993).

In this study we have investigated the relationship between the abilities of p107 to associate with E2Fs and cyclins A or E, two functional properties that can be readily detected *in vivo* (Cao *et al.*, 1992; Devoto *et al.*, 1992; Faha *et al.*, 1992; Lees *et al.*, 1992; Shirodkar *et al.*, 1992), and its growth suppression activity. Employing systematic mutagenesis, protein co-expression and cell type specificity studies, we have dissected the p107 molecule into two functional domains. One contains the intact pocket domain allowing interaction with E2F and the other contains the cyclin/cdk binding domain. We have examined each domain separately and are able to demonstrate that two different mechanisms are involved in growth arrest induced by elevated p107 expression. Interestingly, these two inhibitory functions of p107 are not limited to particular transformed tumor cell lines since we are able to demonstrate similar effects of the two p107 functional domains in untransformed mouse fibroblasts.

Mutagenesis studies of the pRB molecule have revealed that structural requirements for binding to E1A are not the same as those for binding to E2F (Hiebert *et al.*, 1992; Qian *et al.*, 1992). Protein sequences C-terminal to the 'pocket', which are not required for E1A binding, are necessary for interaction with E2F. Growth suppression activity of pRB correlated with its binding to E2F, while the E1A binding pocket region was necessary but not sufficient. In previous work, we showed that this correlation did not hold for p107, as the pocket region of p107 was not absolutely required for growth suppression. In this present work we have shown that the reason for this difference between p107 and pRB is that p107 contains two independent domains capable of suppressing growth. One of these domains, N385, appears to be the counterpart of the pRB growth suppression domain. For this domain, growth suppression required an intact pocket and most of the C-terminal sequences. Growth suppression correlated with E2F binding, while E1A binding ability was necessary but not sufficient. Furthermore, in co-expression experiments at similar protein levels, N385 efficiently inhibited

Table V. Rescue of L19-mediated growth arrest with cyclins A and E

	+Vector			+pRcCMVcyclin A			+pRcCMVcyclin E		
	G_1	S	G_2 + M	G_1	S	G_2 + M	G_1	S	G_2 + M
Vector (10 μg)				29.3	34.9	35.8	32.3	33.0	34.8
				(55.0)	(19.2)	(25.8)	(55.6)	(20.1)	(24.3)
L19 (10 μg)	72.3	12.2	15.5	67.4	18.1	14.5	71.6	18.1	10.3
	(53.6)	(20.6)	(25.8)	(52.7)	(23.7)	(23.6)	(56.3)	(25.9)	(17.8)
L19 (6 μg)	75.1	12.7	12.2	63.2	20.2	16.7	48.8	35.7	15.5
	(51.3)	(23.1)	(25.6)	(52.9)	(20.5)	(26.6)	(56.4)	(23.4)	(20.2)

Saos-2 cells were cotransfected with 2 μg pCMVCD20, L19 (DNA amounts as indicated) together with 12 μg of either vector, pRcCMVcyclin A or pRcCMV cyclin E. Total amount of DNA was made up to 25 μg with pBSK. Cell cycle profiles of the transfected cells were determined as in Table I. Numbers in parentheses indicate cell cycle profiles of the untransfected cells (CD20 negative) in the same dish as the transfected cells (CD20 positive).

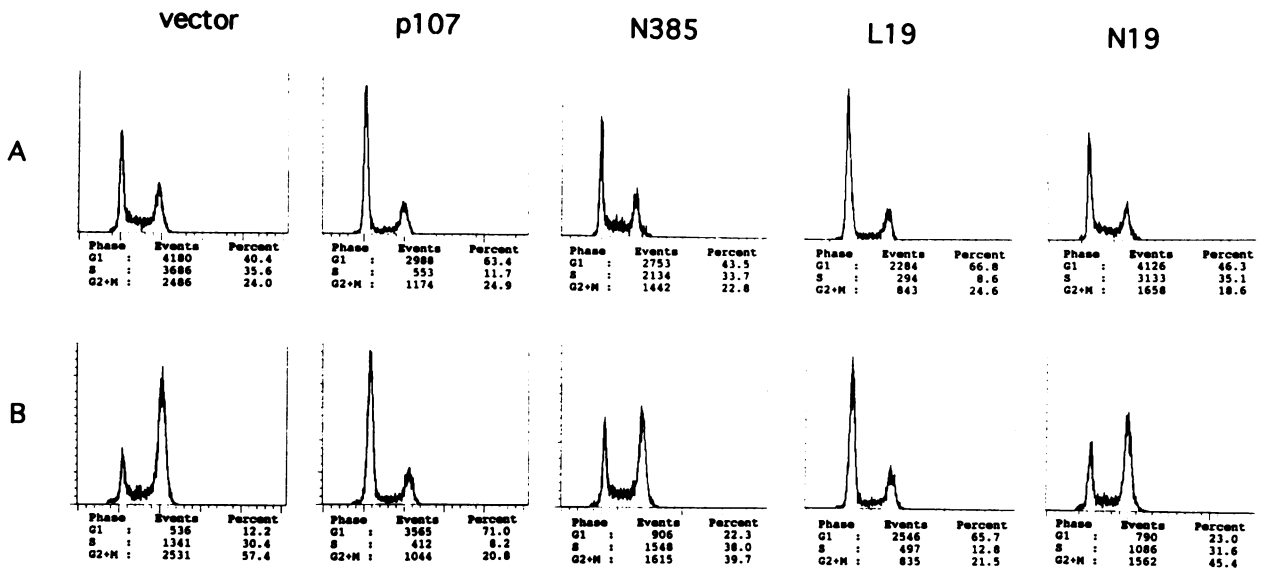

Fig. 7. Flow cytometry analysis of transfected C33A cells. C33A cells were transfected with 23 μg of the indicated expression plasmids and 2 μg of pCMVCD20. Cells were harvested for flow cytometry analysis 24 h later (**A**) or treated with 40 ng/ml nocodazole from 24 to 36 h after transfection and then harvested (**B**). Events are numbers of cells (CD20 positive) counted for each cell cycle phase, and percentages of cells in each phase are listed under Percent.

the growth stimulating activity of E2F-4/DP-1 dimer (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994), while at lower N385 concentrations the growth stimulating effect of E2F-4/DP-1 dimer became dominant. Together with previous work showing that the consequence of p107/E2F interaction was the repression of E2F-mediated transactivation (Schwarz *et al.*, 1993; Zamanian and Thangue, 1993; Zhu *et al.*, 1993), these results provide experimental evidence that p107 can suppress growth through its interaction with E2F.

The stable interaction between p107 and cyclin A/cdk2 and/or cyclin E/cdk2 has now been mapped to a domain that is functionally independent from the E2F binding domain of p107. Although we do not know the physiological consequence of these interactions, we can imagine three possible scenarios. A first possibility is a poised enzyme–substrate interaction. The cyclin/cdk complexes may associate with p107, and, through p107, with E2F, in order to allow rapid and focused phosphorylations of E2F immediately following their activation through some other signal(s). Recent studies have shown that cyclin A/cdk2 binds directly the pRB-associated E2F-1 (Krek *et al.*, 1994) and inactivates its DNA binding function through phosphorylation (Dynlacht *et al.*, 1994; Krek *et al.*, 1994). An analogous cyclin binding site is not found on E2F-4, the p107 interactor (Beijersbergen *et al.*, 1994; Ginsberg *et al.*, 1994). Thus it is possible that p107 provides a link between cyclin/cdk cell cycle regulation and certain E2Fs. A second potential role for p107 in these interactions might be as a transport mechanism for the cyclin/cdk complexes, targeting, for example, the cyclin/cdk complexes to the DNA through E2Fs to influence transcription. Third, since we have shown here that this interaction is sufficient for growth arrest, p107 may normally act as a negative regulator of the functions of cyclin A/cdk2 and cyclin E/cdk2 complexes. This negative regulation might be achieved either by preventing low levels of cyclin/cdk2 from phosphorylating their desired targets too early in the cycle or by keeping the cyclin/cdk complexes apart from their substrates until an appropriate time. *In vitro* biochemical studies are now being carried out to further address these possibilities.

The dissection of p107 into two separable functional domains has further pointed out a significant difference with pRB. The E2F binding properties of pRB and p107 are remarkably similar; however, the stable association

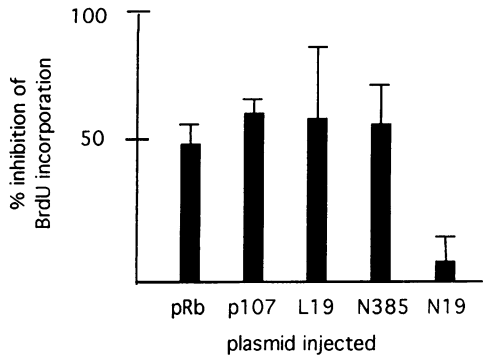

Fig. 8. Inhibition of DNA synthesis in NIH-3T3 cells by microinjection of plasmids expressing p107 and related proteins. Quiescent NIH-3T3 cells were restimulated and microinjected with plasmids expressing each of the noted proteins, together with a marker plasmid expressing β-galactosidase (see Materials and methods). The results are expressed as the mean percentage of inhibition of BrdU incorporation, normalized to the percentage of BrdU-positive cells seen when the β-gal expression vector was co-injected with an empty vector (50%). The error bars represent the standard deviation from three independent experiments. A total of 589 β-gal positive cells were scored.

between p107 and the cyclin/cdk complexes is not duplicated in pRB. No high affinity interactions between pRB and cyclin/cdk complexes can be demonstrated *in vivo*, and only the E2F binding domain of pRB has been shown to elicit growth arrest following overexpression. This difference is most clearly demonstrated in the cervical carcinoma cell line C33A. Here, overexpression of full-length pRB does not cause G_1 arrest, yet p107 is fully capable of blocking the cells. The ability of p107 to arrest in G_1 maps to the cyclin/cdk binding domain; overexpression of the E2F binding domain of p107 has no effect. This sensitivity of C33A cells to overexpression of the cyclin/cdk binding domain is not due to some general anti-cdk activity, as similar overexpression in other cell types does not affect cell cycle progression (Zhu *et al.*, 1993). The cells insensitive to arrest by the cyclin/cdk binding domain of p107 are capable of being blocked by other agents such as cdk2-dominant negative mutants (van den Heuvel and Harlow, 1993). These experiments suggest that the block in C33A cells is due to a specific property of the C33A cells. The different susceptibilities to p107-mediated inhibition between various tumor cell lines may allow identification of changes in the E2F/pRB and E2F/p107/cyclin/cdk pathways that are selected during tumorigenesis.

Finally, the dissection of p107 into two separable functional domains has presented opportunities to study the relationships between cyclin/cdk function and the regulation of E2F by p107. When the p107 E2F binding domain N385 was co-expressed with cyclin A or cyclin E at equimolar plasmid input, the growth suppression activity of N385 was readily released and the N385 protein became hyperphosphorylated (L.Zhu, unpublished observation), suggesting that in this case cyclin A- or E-activated kinases induced phosphorylation of N385 and inactivated its growth suppression function. These results are reminiscent of the rescue of pRB-mediated growth suppression by cyclin A or cyclin E (Hinds *et al.*, 1992; Zhu *et al.*, 1993). As we reported before (Zhu *et al.*, 1993), growth suppression mediated by the full-length p107 protein was not rescued by cyclin A or cyclin E. Thus, removal of the cyclin binding domain of p107 from the protein allows the co-transfected cyclin A or E to induce phosphorylation and inactivation of the E2F binding domain of p107. This result provides further evidence that these two domains of p107 are functionally distinct and suggests that E2F binding by p107 may be, like pRB, regulated by phosphorylation. Other cyclin/cdk complexes are now being tested for their ability to regulate the function of N385, as well as the full-length p107. We have also begun to investigate the functional relationship between the cyclin binding activity of L19 and E2F-4/DP-1 effects on cell proliferation. Our preliminary results show that the growth suppression activity of L19 could be rescued by the E2F-4/DP-1 dimer at equimolar plasmid input, different from the N385−E2F-4/DP-1 co-expression (Table III). This raises the interesting possibility that E2F-4/DP-1 may be downstream from cyclins A and E in the pathway leading to proliferation. Detailed analysis with these reagents will provide further insight into these important regulatory networks.

Materials and methods

Plasmid constructs

Plasmids pCMVp107, pCMVDE, pCMVN385 and pCMVC768 were described previously (Zhu *et al.*, 1993). Other mutant constructs were obtained by PCR amplification and confirmed by DNA sequencing. The L19 series of mutants were constructed by PCR with a common 5′ primer (L15: 5′-CCG-GGA-TCC-GCC-ACC-ATG-GTT-ACT-CCT-GTT-GCA-TCA-GCC-3′) and different 3′ primers: mutant L19: 5′-GGG-GTA-CCC-TAA-TCG-ATG-TTT-ATT-CCA-GTT-GAA-TGT-A-3′; mutant L20: 5′-GGG-GTA-CCC-TAA-TCG-ATT-GGG-GGG-TCC-TCT-CCA-AAG-A-3′; mutant L30: 5′-CCT-AAT-CGA-TAC-TCC-CTG-CGG-TAG-GAG-AAC-3′; mutant L24: 5′-GGG-GTA-CCC-TAA-TCG-ATT-TTG-TTT-GCA-GAA-ACC-TGG-A-3′; mutant L23: 5′-GGG-GTA-CCC-TAA-TCG-ATC-ATT-CCA-TGA-AGT-CTT-CGT-3′. PCR-amplified products were digested by restriction enzymes *Dra*III and *Cla*I and used to replace the original fragment in the p107 sequence. The HA tag was first added to the natural C-terminus of the p107 sequence (Zhu *et al.*, 1993). For the N385 series of mutants, the common 5′ primer was 5′-GAT-CGG-TAC-CTT-CGC-TAG-CAC-TAT-TTT-ACA-GAA-AG-3′. The 3′ primers were as follows: mutant L26: 5′-CCT-AAT-CGA-TAT-CTT-TCA-AAC-TCT-TAG-3′; mutant L27: 5′-CCT-AAT-CGA-TGC-CTG-ACC-CAT-TCT-TGT-GCG-3′; mutant L28: 5′-CCT-AAT-CGA-TGC-CTG-GCT-GTT-GTT-TAA-TAT-3′; mutant L29: 5′-CCT-AAT-CGA-TGT-CGT-ATT-TCA-GTG-CAA-ATG-3′; mutant L31: 5′-CCT-AAT-CGA-TAG-GTG-TTT-TTG-TAG-CAT-CTT-C-3′. The amplified DNA fragments were digested with *Eco*RI and *Cla*I and were used to replace the original fragment from N385. Mutant N19 was constructed by fusing the N-terminal part of N385 to the C-terminal part of L19 at the *Dra*III site. In a similar way, the Cys846→Phe mutation was transferred from p107F846 (Zhu *et al.*, 1993) to N385 resulting in N385F846.

Expression constructs for cyclins A and E were kindly provided by Dr P.Hinds (Hinds *et al.*, 1992). pCMVDP-1 and pCMVE2F-4 were previously described (Helin *et al.*, 1993; Beijersbergen *et al.*, 1994).

Cell culture and transfection

Human osteosarcoma cell line Saos-2 and cervical carcinoma cell line C33A were obtained from American Type Culture Collection, and NIH-3T3 cells were a gift from Dr C.Sherr (HHMI, St Jude Children's Research Hospital). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Transient transfection was performed as previously described (Zhu *et al.*, 1993).

Immunoprecipitation, E1A binding, immunoblot, E2F gel shift assay and growth suppression assay

These assays were performed based on protocols previously described (Zhu *et al.*, 1993), with certain particular details noted in the relevant figure legends.

Antibodies

Anti-p107 monoclonal antibodies SD4 and SD9 (Zhu *et al.*, 1993), anti-cyclin A monoclonal antibody BF683 (Faha *et al.*, 1992), anti-cyclin E monoclonal antibody HE12 (Dulic *et al.*, 1993) and anti-E1A monoclonal antibody M73 (Harlow *et al.*, 1985) have all been described. Anti-cyclin B1 monoclonal antibody GNS3 was kindly provided by Dr S.Shiff.

Microinjection, BrdU incorporation and immunofluorescence

Microinjection was performed using an Eppendorf micromanipulator and the Zeiss Automated Injection System video display and software. Injection capillaries (GC120TF-10 1.2 mm o.d.×0.94 mm i.d.) were obtained from Clark Electromedical Instruments (Reading, UK) and pulled to fine tips on a Sutter Instruments P-87 puller. Near confluent NIH-3T3 cells were treated with 0.1% fetal calf serum (FCS) for ~ 40 h, then trypsinized and replated in Corning 35 mm tissue culture dishes at 30–50% of confluence in media containing 10% FCS. Cesium-purified plasmids were injected perinuclearly within 1–4 h of stimulation. Injected fields of cells were identified by circles marked on the bottom of the dish.

To identify successfully expressing cells within the injected field, each test plasmid was injected at 25 μg/ml together with 25 μg/ml of a plasmid expressing β-galactosidase from the cytomegalovirus (CMV) promoter. BrdU (1:200 dilution, Amersham cell proliferation labeling kit) was added immediately following injection. Cells were fixed 21–23 h after stimulation with methanol/acetone (1:1) at −20°C for 30 min. All immunohistochemistry steps, except where indicated, were performed for 1 h each at room temperature in the dark on a rocking stage in phosphate-buffered saline (PBS)/0.1% Tween/10% normal goat serum. β-Galactosidase was detected with a rabbit antibody (1:300, 5 Prime 3 Prime), followed by a biotinylated donkey anti-rabbit antibody (1:50, Amersham) and a Texas Red-conjugated streptavidin (1:50, Amersham). BrdU was detected with a mouse monoclonal antibody plus nuclease (undiluted, Amersham) and a fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse antibody (1:25, Amersham), simultaneously with the last two steps of β-gal detection. All cells that stained red over background and all nuclei with a detectable outline of green staining were counted and only fields showing at least 15 red-stained cells were included in the results. Under these conditions, 70–80% (76% on average) of uninjected cells were BrdU-positive. At a concentration of 25 μg/ml, each cell receives many plasmids (Deuschle *et al.*, 1990; G.H.Enders, unpublished data), so that most cells co-express the proteins from co-injected plasmids. The ability to detect this depends on the specificity and sensitivity of the staining for each protein. Detection of β-gal is very sensitive and specific; detection of HA-tagged proteins is less so. Hence, under these conditions, 72% of the β-gal-positive cells that were co-injected with a plasmid expressing L19 clearly stained positive for the latter protein over background, whereas 100% of L19-positive cells were β-gal-positive.

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