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The Rb-Related p107 Protein Can Suppress E2F Function Independently of Binding to Cyclin A/cdk2

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The interaction of the retinoblastoma susceptibility gene product (Rb)-related p107 protein with the E2F transcription factor in S-phase cells facilitates the formation of a multicomponent complex also containing cyclin A and the p33^{cdk2} kinase. We have created a series of p107 mutants to assess the ability of p107 to inhibit E2F function and the role of the cyclin A/cdk2 complex in this process. We find that p107 mutants that do not bind to E2F also fail to repress E2F-dependent transcription. Moreover, we find that the ability of p107 to suppress E2F-dependent transcription is not dependent on the ability of p107 to associate with cyclin A/cdk2. Finally, an analysis of the ability of the p107 mutant proteins to suppress cell growth suggests that both E2F-dependent and E2F-independent events correlate with this activity.

A variety of recent experiments have demonstrated that the E2F transcription factor is a target for the action of the retinoblastoma susceptibility gene product (Rb) family of proteins (25). While the full range of cellular functions of E2F undoubtedly remain to be defined, it does appear that the E2F-dependent activation of transcription is involved in some aspects of cellular growth control. E2F binding sites are found in the promoters of S-phase genes such as the dihydrofolate reductase, thymidine kinase, and DNA polymerase α (25) genes, and in the case of the dihydrofolate reductase gene, these sites have been shown to be critical to the activation of this gene in late G_1 (32). Additionally, overexpression of the product of the E2F1 gene, one member of the E2F family, has been shown to drive quiescent REF52 cells into S phase (20).

A group of proteins initially identified as adenovirus E1A-binding proteins on the basis of coimmunoprecipitation experiments (16, 34) have now been shown to interact with the E2F transcription factor (1–3, 5, 7–9, 24, 28, 31). The E1A protein has the capacity to dissociate these E2F complexes, releasing E2F and presumably leaving E1A bound to the proteins. Most attention has focused on one of these proteins, Rb, and its role in controlling the transcriptional activity of E2F. Two additional proteins of 130 and 107 kDa, originally identified in E1A immunoprecipitates and later identified as E2F-associated proteins, are clearly related to Rb (15, 22, 23). A detailed analysis of p107 revealed a homology with Rb in a region of the protein referred to as the pocket which appears to be the domain involved in interactions with the E1A protein as well as other viral oncoproteins (12). The pocket consists of two domains of sequence homology, termed A and B, that are separated by a spacer region of sequence not conserved among the Rb family members and which is not required for interaction with viral oncoproteins. In addition, Rb and p107 differ outside of the pocket sequences, suggesting that they perform similar but distinct roles in the cell. For instance, these two proteins have been shown to differ with respect to their interactions with E2F. Whereas the Rb protein appears to interact with the majority of E2F species in the cell, only a subset of E2F is in a complex with p107 (10).

Additional experiments have shown that the p107 spacer mediates an interaction with yet another cellular polypeptide, the cell cycle regulatory protein cyclin A (11, 13). The ability of p107 to interact with cyclin A would appear to mediate the formation of a multicomponent complex that also includes E2F and the cdk2 kinase (5, 9, 28, 31). This complex forms at the beginning of S phase, with kinetics that coincide with the appearance of the cyclin A protein (24). Moreover, since in many instances the majority of E2F activity within the S-phase cell is found in this complex, it seems unlikely that this is merely an intermediate in some process. The p107 protein also appears to facilitate the interaction of a cyclin E/cdk2 complex with E2F during the G_1 phase of the cell cycle, but unlike the case for cyclin A/cdk2 interaction, only a very small fraction of the E2F in a G_1 cell is in this complex (21).

Recent experiments have shown that overexpression of the p107 protein can repress E2F-dependent transcription in transient transfection assays (30, 36). Moreover, overexpression of p107 has been shown to bring about a G_1 arrest (37). These results suggest that binding of p107 to E2F is responsible for some of the cellular controls exercised upon E2F. We have now sought to determine whether the interaction of p107 with cyclin A/p33^{cdk2} is required for these observed functions.

MATERIALS AND METHODS

Cells. Saos-2 and C33A cell lines were obtained from the American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Transfection assays. The human cervical carcinoma cell line C33A, which lacks functional Rb, was employed for calcium phosphate transfection assays, using the reporter plasmid pE2WTx4CAT as described previously (27). The amounts indicated in Fig. 5 and 6B were brought to a total DNA concentration of 24 μg, using pCMV4 and sonicated salmon sperm DNA. The total micrograms of pCMV4 backbone in each transfection was kept at constant level. Additionally, 2 μg of a Rous sarcoma virus (RSV)-driven β-galactosidase (β-Gal) expression plasmid was included in each transfection as an internal control. β-Gal assays were performed on the lysed whole-cell extracts as described previously (17), and the resulting β-Gal activities were used to normalize the chloramphenicol acetyltransferase (CAT) activities of each sample. Values shown represent the averages of two independent transfection assays.

Plasmid construction. The pCMV4/p107 (amino acids [aa] 254 to 1068) vector was constructed as described previously (30). Plasmid pc107 (aa 254 to 1068) was constructed by cloning a *Bgl*II-*Xba* fragment of pCMV4/p107 into plasmid pcDNA I/amp (Stratagene), using the *Bam*HI-*Xba* sites in the polylinker region. Standard site-directed mutagenesis protocols were used on the pc107 (aa 254 to 1068) construct to create point mutants which introduce *Afl*II restriction sites (underlined below) into the spacer region of the p107 coding sequence. The following DNA primers were used: pc107 591/592 (5′-CCTACCTGTGAA

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GAACTTAAGTTCCCAAATAACTTT-3′), pc107 628/629 (5′-AAGGAAGTTCGAACTCTTAAGGGGAGTCTTCGAAGA-3′), pc107 733/734 (5′-GATGCTGGAGAGATCCTTAAGATACCTCTTTCCATG-3′), and pc107 770/771 (5′-CAGACCAATCTGACTCTTAAGCAAGAGGTACATTCA-3′). These double-point mutants were then used to create the deletion mutants described in this report by ligating pc107 mutant *Afl*II-*Xba* backbones to pc107 mutant *Afl*II-*Xba* insert fragments in the desired combinations: pc107 591-628 (pc107 591/592 backbone plus pc107 628/629 insert), pc107 591-770 (pc107 591/592 backbone plus pc107 770/771 insert), pc107 628-733 (pc107 628/629 backbone plus pc107 733/734 insert), and pc107 733-770 (pc107 733/734 backbone plus pc107 770/771 insert).

The pCMV4 variants of these plasmids were created by ligating the *Bsm*I-*Cla*I fragment of the relevant mutant p107 sequence into the vector backbone of *Bsm*I-*Cla*I-cut pCMV4/p107. The pCMVcdk2 expression plasmid has been described elsewhere (19). The pCMVcyclin A vector was a gift of Jon Horowitz, and the p107 full-length, p107-C768, and p107-F846 vectors were kind gifts of E. Harlow.

Cell growth suppression assays. Asynchronously growing Saos-2 cells were plated on coverslips in 35-mm-diameter dishes. Calcium phosphate precipitates containing 5 μg of the plasmids indicated in Tables 1 and 2 were prepared as described above. The E1A transfection included 2 μg of the E1A expression plasmid but was otherwise identical to the other transfections. All transfections also included 2 μg of pCMV4 β-Gal as a control for transfected cells. The control experiment also included 5 μg of pCMV4 as a control for nonspecific effects of the plasmid backbone. The Saos-2 cells were then transfected with 0.2 ml of the desired precipitate solution for 18 h, after which the cells were washed and incubated in Dulbecco's modified Eagle's medium–10% fetal bovine serum for an additional 24 h. Bromodeoxyuridine (BrdU; final concentration, 10 mM) was then added to the cells, which were incubated for an additional 24 h before fixing. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature and then dehydrated with methanol-acetone (1:1) for 5 min. Cells were incubated in 1% bovine serum albumin (BSA) in PBS for 30 min and then stained for β-Gal, using a rabbit polyclonal antibody (1:500 in 1% BSA–PBS for 1 h at 37°C) and a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Sigma) (1:500 in 1% BSA–PBS for 30 min at 37°C). The cells were then fixed for 10 min with 4% paraformaldehyde in PBS, after which they were incubated in 2 M HCl for 45 min at 37°C. The coverslips were then washed with 0.1 M sodium borate (pH 8.5) and stained with an anti-BrdU monoclonal antibody (Boehringer-Mannheim) (1:40 in 0.1% BSA–PBS for 1 h at room temperature). A Texas red-conjugated goat anti-mouse immunoglobulin G (Sigma) was used as the secondary antibody (1:100 in 1% BSA–PBS for 30 min at 37°C), following which the coverslips examined by fluorescence microscopy.

Gel mobility shift assays. The gel mobility shift assays were performed as described previously (7, 18), with the following modifications. The gel in Fig. 2 was a 5% acrylamide (75:1 acrylamide/bisacrylamide) gel containing 5% glycerol. The gel in Fig. 3 was run by using a 20-μl (total volume) reaction mix. The glutathione *S*-transferase (GST) fusion proteins used in the complex reconstitution experiments were prepared as described previously (30) and were used at concentrations of 100 ng/ml for GST-cyclin A and 250 ng/ml for GST-cdk2. The Mono Q-purified E2F used in the gel shift assays was purified as described previously (33). p107 protein was produced via in vitro transcription-translation, using the pc107 and mutant pc107 vectors as templates. The reactions were performed by using the TnT coupled reticulocyte lysate system (Promega) according to the protocol provided.

Immunoprecipitation assays. C33A cells were transfected as described above with 15 μg of the indicated p107 pCDNA expression vector. Also included in the transfection were 5 μg of a pCMVcyclin A expression plasmid and 2 μg of a pCMVcdk2 expression vector. The transfection was brought to 24 μg of total DNA with pCMV4 and sonicated salmon sperm DNA. The total micrograms of pCMV in these experiments was kept constant. Cells were harvested 48 h post-transfection and lysed for 20 min on ice in lysis buffer (250 mM KCl, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 0.1% Nonidet P-40, 10% glycerol, 0.4 mM NaF, 0.4 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mg of pepstatin per ml, 1 mg of leupeptin per ml, 2 mg of aprotinin per ml). Lysates were then spun for 10 min at 13,000 × *g* in a cold room, and the pellet was discarded. The immunoprecipitation was performed overnight, using 800 μg of total protein from each sample in a total volume of 200 μl. The anti-cdk2 samples were precipitated with 5 μl of a commercial polyclonal anti-cdk2 antibody (100 mg/ml; Santa Cruz Biotechnology), while the anti-p107 samples were precipitated with 10 μl of a rabbit polyclonal anti-p107 antibody (30). The antibodies were precipitated with 30 μl of protein A-protein G agarose beads (50% slurry; Oncogene Science) for 2 h. Beads were washed four times in lysis buffer and resuspended in 30 μl of 1× sodium dodecyl sulfate (SDS) loading buffer. Samples were run on a 7% acrylamide gel and then transferred to nitrocellulose via semidry blotting (1.4 h at 10 V). Blots were blocked overnight in 5% lowfat milk–0.1% Tween 20 in PBS and then probed for 1 h with the polyclonal anti-p107 antibody (1:1,000 dilution in 0.5% lowfat milk–0.1% Tween 20 in PBS). The blots were then washed in PBS-Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000 dilution; in 0.5% lowfat milk–0.1% Tween 20 in PBS). Bands were detected via the use of an enhanced chemiluminescence detection system (Amersham), using the protocol provided.

RESULTS

p107 mutations that alter interaction with E2F and cyclin A/cdk2. To investigate the functional significance of the interaction of E2F with p107 and cyclin A/p33^{cdk2}, we sought to generate p107 mutant proteins that were altered in these interactions. To this end, site-directed mutagenesis was used to introduce a series of new restriction sites within the sequence encoding the spacer region of the p107 protein. *Afl*II restriction sites were created by altering six nucleotides in four locations, resulting in the creation of four double-point mutants. The newly created restriction sites were then used to construct the deletion mutants shown in Fig. 1. These mutants consist of two small 37-aa deletions at the beginning and end of the spacer region as well as two larger deletions intended to completely eliminate the function of the spacer. The p107 referred to as wild type in the experiments described below contains aa 254 to 1068.

To assay the ability of these mutant proteins to bind to E2F, a gel mobility shift assay was used to define E2F DNA binding activity and measure the formation of complexes involving the p107 protein. To clearly separate the free E2F band from the complex formed with some of the p107 deletion mutant proteins, a 5% acrylamide gel containing 5% glycerol was used. In vitro-transcribed and -translated p107 proteins were added to a partially purified E2F fraction and incubated for 1 h at room temperature. As can be seen in Fig. 2, all of the mutants except the *dl*591–628 mutant retained the ability to bind to E2F, as evidenced by the formation of specific complexes upon addition of the p107 proteins. The inability of the *dl*591–628 mutant to bind to E2F was surprising in light of the fact that the larger deletion, *dl*591–770, is capable of forming a complex with E2F. We presume that the loss of function with respect to E2F binding is not due to a simple loss of sequence but rather must be due to an altered conformation specific to this mutation.

Gel mobility shift assays were also used to measure the ability of the p107 mutants to reconstitute the S-phase-specific complex composed of E2F, p107, cyclin A, and cdk2. As can be seen in Fig. 3, both the wild-type p107 protein and the *dl*733–770 mutant allowed the reconstitution of a higher-mobility complex upon addition of GST-cyclin A and GST-p33^{cdk2}. This complex was not seen with the independent addition of GST-cyclin A or GST-p33^{cdk2} to reactions containing E2F and p107 (data not shown), consistent with the observation that cyclin A/p33^{cdk2} association is required for binding to p107 (29). In contrast, the larger spacer deletion mutants *dl*591–770 and *dl*628–733 were unable to form the larger complex upon addition of cyclin A and cdk2. Because the *dl*591–628 mutant was unable to bind free E2F, it was not assayed for reconstitution of the complex. Thus, through the *dl*591–770 mutation and the *dl*628–733 mutation, it is possible to separate the p107 function of E2F binding from the function of cyclin A/cdk2 interaction.

To confirm the cyclin A/cdk2 binding characteristics of these p107 proteins in an in vivo assay, cells were transfected with plasmids expressing the various p107 proteins together with plasmids expressing cyclin A and cdk2. Cell lysates were immunoprecipitated with an anti-cdk2 polyclonal antibody, and the immunoprecipitates were scored for the presence of p107 as an indication of an association. As a control for protein expression, these same lysates were also immunoprecipitated with a polyclonal anti-p107 antibody. The immunoprecipitates were analyzed in SDS-acrylamide gels, and Western blotting (immunoblotting) was then used to identify the presence of the

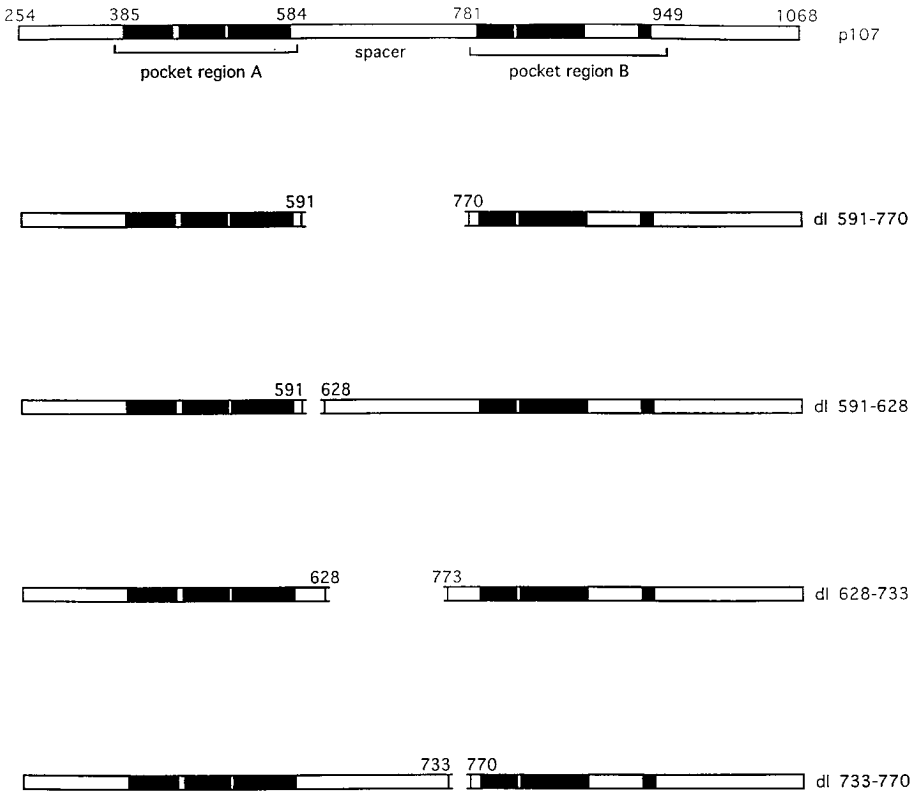

FIG. 1. Schematic representation of the p107 proteins used in these assays. The 254–1068 construct is also referred to as the wild-type form of p107 in these experiments. The spacer deletion mutants shown were constructed as described in Materials and Methods. Deletions are indicated by the gaps. The blackened areas indicate regions of sequence homology between Rb and p107.

p107 proteins. As can be seen from Fig. 4, the wild-type p107 protein and the *dl*733–770 mutant were precipitated by the anti-cdk2 antibody, whereas the *dl*591–628, *dl*591–770, and *dl*628–733 mutant proteins were not. Each of the mutant proteins was detected in the direct p107 immunoprecipitation, thus indicating that the lack of association with cdk2 was not a result of instability. This result coincides with those of the in vitro assays and provides further evidence of the inability of the *dl*591–770 and *dl*628–733 mutants to associate with cyclin A/cdk2. The *dl*591–628 mutant was also found not to associate with cdk2 despite the fact that the protein was readily detected in the transfected cells. Thus, in addition to the fact that this mutation impairs the interaction of p107 with E2F as shown in the assays of Fig. 2, the mutation also prevents an interaction with cyclin A/cdk2.

Repression of E2F-dependent transcriptional activation. The isolation of the p107 mutants described above provided a means to test if the interaction of cyclin A and cdk2 was important for p107 function. Previous experiments have shown that transfection of the p107 cDNA inhibits E2F-dependent transcription in transient transfection assays (30, 35). C33A cells (a cervical carcinoma cell line lacking a functional Rb gene) were transfected with vectors expressing the various p107 proteins along with a reporter plasmid which expresses CAT under the control of a promoter containing four copies of the E2F element. To control for transfection efficiency and nonspecific effects of p107, an internal control was included in each transfection in the form of a β-Gal expression plasmid under the control of the RSV long terminal repeat. Levels of β-Gal expression were used to normalize the CAT activities for

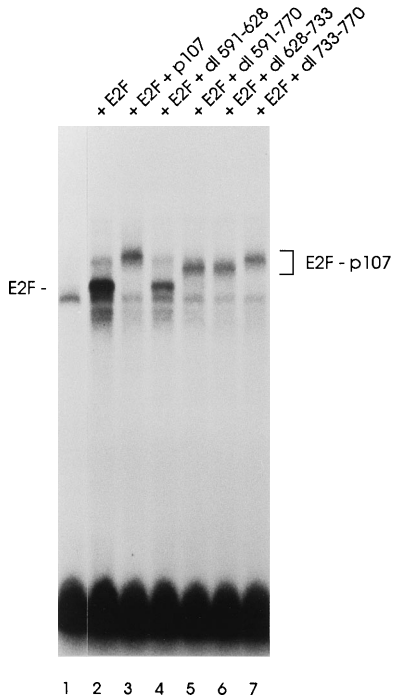

FIG. 2. Interaction of p107 mutant proteins with E2F. One microliter of a Mono Q-purified E2F fraction (see Materials and Methods) was incubated for 40 min at room temperature with 1 μl of rabbit reticulocyte lysate programmed with the indicated plasmid under the conditions described in Materials and Methods. Lane 1, unprogrammed reticulocyte lysate; lane 2, Mono Q-purified E2F and unprogrammed reticulocyte lysate. The mobilities of the uncomplexed E2F and the E2F-p107 complexes are indicated.

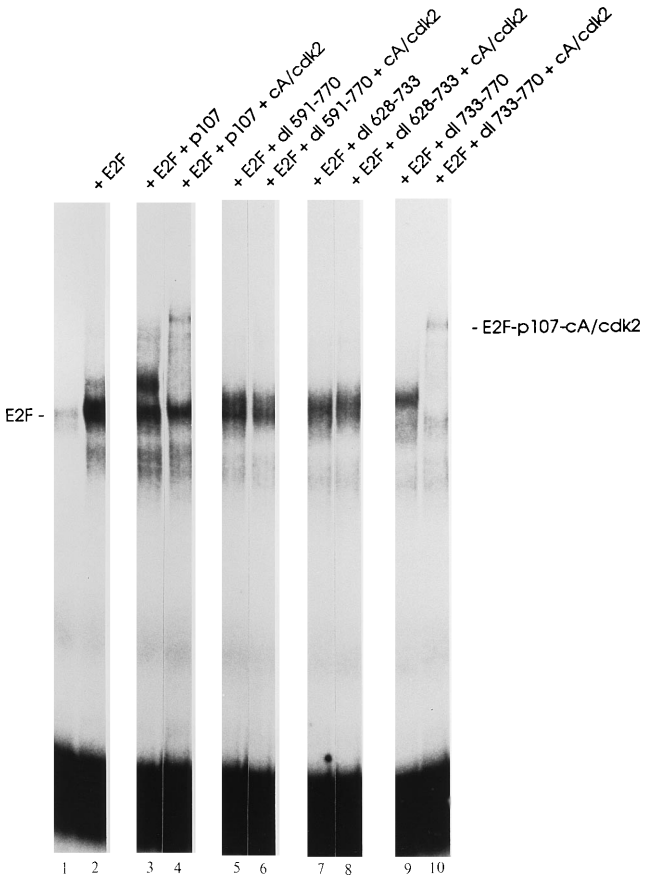

FIG. 3. Formation of E2F-cyclin A-cdk2 complexes with mutant p107 proteins. One microliter of a Mono Q-purified E2F fraction (see Materials and Methods) was incubated with the indicated components for 40 min on ice followed by 20 min at room temperature and then assayed for E2F binding activity. Lane 1, assay of unprogrammed reticulocyte lysate; lane 2, assay of E2F with unprogrammed reticulocyte lysate; lanes 3 to 10, assay of E2F with reticulocyte lysate program with the indicated components.

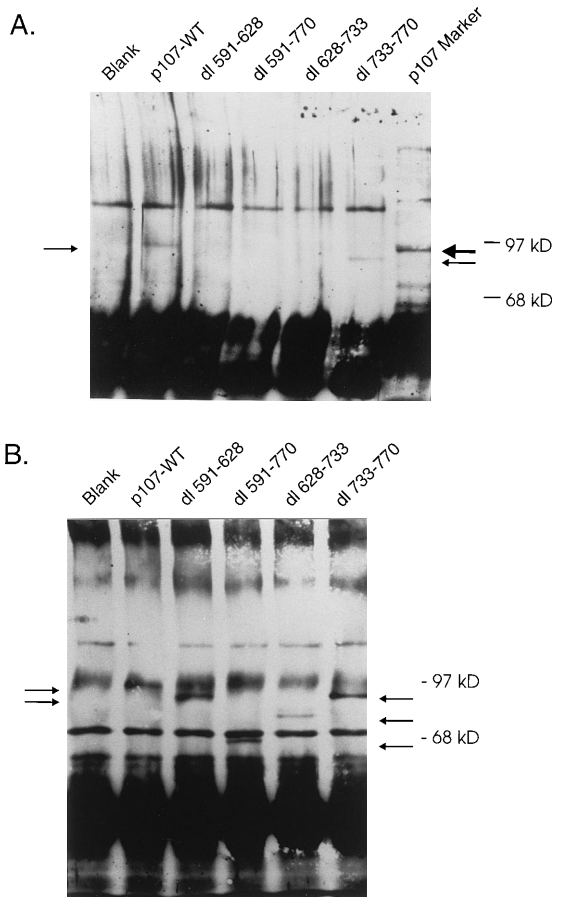

FIG. 4. In vivo interactions involving p107 and cdk2. C33A cells were transfected with the indicated p107 constructs as well as expression vectors encoding cyclin A and cdk2. Cell lysates were immunoprecipitated with either a cdk2 antibody (A) or a p107 antibody (B). Western blots of the immunoprecipitated proteins were probed with a polyclonal anti-p107 antibody. Bands corresponding to the expressed mutant p107 proteins are indicated by arrows. The band of wild-type 107 protein (p107-WT) in panel B (lane 2) is comigrating with the nonspecific material.

each sample. As can be seen in Fig. 5, each of the p107 mutants that retained E2F binding capacity was also capable of repressing E2F-dependent transcription equivalent to that seen with the wild-type protein. In contrast, transfection of the *dl*591–628 p107 mutant, which does not bind to E2F in vitro, resulted in notably less efficient repression than that shown by all of the other proteins. From these results, we conclude that the ability of p107 to mediate an E2F-dependent repression coincides with the ability of p107 to bind to E2F. However, the repression of E2F-dependent transcription activation function is independent of the ability of p107 to form the E2F-cyclin A-cdk2 complex.

p107-mediated growth inhibition. It has previously been shown that overexpression of the p107 protein in Saos-2 cells leads to an arrest of the cell cycle in G_1, similar to the effects of Rb (37). Given the set of p107 mutants described above, we tested whether the ability of p107 to arrest cell growth was dependent on its ability to bind to E2F and to form the cyclin A/p33^{cdk2} complex.

Asynchronously growing Saos-2 cells were transfected with either the wild-type p107 plasmid or one of the mutant p107 plasmids. Additionally, each transfection included the pCMV β-Gal plasmid to serve as a marker to identify the transfected cells. As a positive control, Saos-2 cells were transfected with a plasmid expressing the Rb protein. BrdU was added to the media 48 h after transfection, incubation was continued for an additional 24 h, and the cells were then fixed and stained for both β-Gal expression and BrdU incorporation as a measure of progression into S phase. Expression of the wild-type p107 protein prevented Saos-2 cells from progressing to S phase, with an efficiency similar to that of the Rb protein (Table 1). In addition, the p107-mediated growth suppression was reversed by cotransfection of a plasmid expressing the $E1A_{12S}$ product. As seen by the results of the *dl*591–770 and *dl*628–733 mutants, the ability of p107 to block progression into S phase was not dependent upon its ability to form the E2F-cyclin A-p33^{cdk2} complex. In contrast, the mutant that was unable to bind to E2F and to inhibit E2F-dependent transcription (*dl*591–628) was also deficient in blocking S-phase entry.

Two additional p107 mutants were assayed on the basis of a previous report that suggested that part of the capacity for p107 to repress growth may lie in the ability of p107 to interact with cyclin A, independent of E2F association (37). This interpretation was based on the properties of two mutant proteins, p107-F846 and p107-C768 (Fig. 6A), that retained growth-suppressing activity but lacked the ability to bind E1A. These mutants were not assayed, however, for E2F association. As

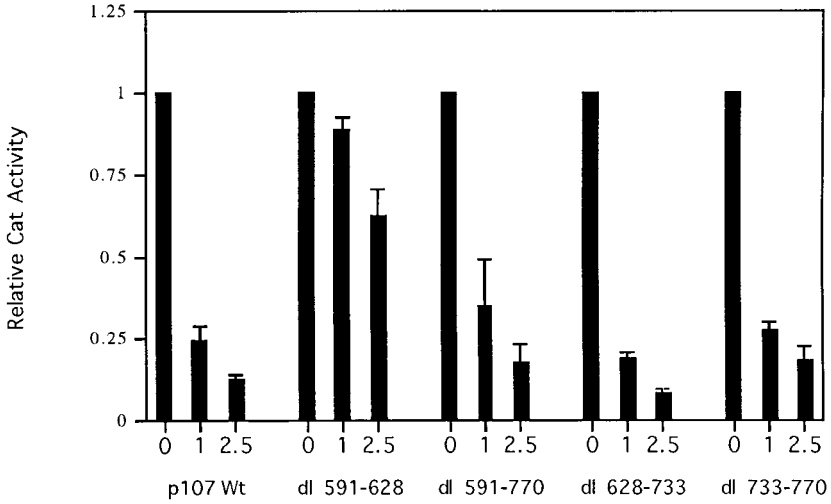

FIG. 5. Repression of E2F-dependent transcription activation. C33A cells were transfected with the indicated amounts (in micrograms) of a pCMV expression vector together with 5 μg of the reporter plasmid and 2 μg of an RSV β-Gal plasmid as an internal control. CAT activity was adjusted for the β-Gal activity and is expressed as a relative percentage of the unrepressed activity of the pE2WTx4CAT reporter construct. Each value represents the average of two independent transfection assays. Wt, wild type.

TABLE 1. p107-mediated growth arrest[a]

Plasmid	Expt	No. of β-Gal-positive cells	No. of BrdU-positive cells	Fraction BrdU positive
Control	1	331	202	0.61
	2	790	501	0.63
	3	150	101	0.67
	4	528	354	0.67
	5	346	261	0.75
p107wt	1	340	70	0.21
	2	723	184	0.25
	3	294	63	0.21
	4	312	73	0.23
	5	239	56	0.23
p107*dl*591–770	1	278	47	0.17
	2	823	243	0.30
	3	156	27	0.17
	4	203	49	0.24
	5	106	28	0.26
p107*dl*628–733	1	406	43	0.11
	2	893	139	0.16
	3	409	82	0.20
	4	272	48	0.18
	5	138	31	0.22
p107*dl*591–628	1	330	127	0.38
	2	513	280	0.55
	3	304	178	0.59
	4	168	92	0.55
	5	271	198	0.73
Rb	1	253	30	0.12
	2	357	61	0.17
	3	23	5	0.22
	4	81	16	0.20
	5	94	16	0.17
p107 + E1A	1	56	38	0.68
	2	24	19	0.79

[a] The control transfection experiments contained only the pCMV β-Gal plasmid. All other transfections contained 5 μg of the indicated plasmid in addition to the pCMV β-Gal plasmid except that in the case of E1A, 2 μg of pCMV E1A was used. In all cases, the expression plasmid was the pCMV4/p107 form of the vector (see Materials and Methods) except where noted below. Experiments 1 to 3 used the pC591-628 vector.

can be seen in Fig. 6B, the p107-F846 protein could repress E2F-dependent transcription as efficiently as the wild-type protein, whereas the p107-C768 mutant was devoid of such activity. This result suggests that the p107-F846 mutant can associate in vivo with E2F, whereas the p107-C768 protein is clearly impaired in its ability to associate with E2F.

The ability of each of these two mutants to suppress growth was also examined. These assays were conducted similarly to those described above, and the results of three independent experiments are shown in Table 2. As can be seen from these data, the F846 mutant was able to suppress cell growth as efficiently as the wild-type protein. In contrast, the C768 mutant, although somewhat less efficient than the wild-type protein in growth suppression, did suppress more efficiently than the *dl*591–628 mutant, thus suggesting an E2F-independent event. The ability to bind to cyclins is one obvious choice for the origin of this capacity, since the C768 mutant retains the spacer region and, as can be seen in Fig. 6C, also retains the ability to associate with cdk2 in vivo. This is an activity deficient in the *dl*591–628 mutant, as was shown in Fig. 4. From these results, we conclude that the ability of p107 to arrest cell growth that coincides with a control of E2F is not dependent on the formation of an E2F-cyclin A-cdk2 kinase complex. However, it would also appear that p107 can suppress cell growth independent of E2F, possibly through a sequestration of cyclin A/cdk2.

DISCUSSION

Various experiments have shown that the p107 protein can regulate the transcriptional activation function of E2F, similar to the effects of Rb (30, 35). Unlike Rb, however, p107 also facilitates the formation of multimeric E2F complexes containing cyclin-dependent kinases (3, 5, 9, 21, 24, 28, 31). The experiments described here demonstrate that the ability of the p107 protein to suppress E2F transcriptional function does not require an interaction with the cyclin A/p33^{cdk2} complex. Transient transfection assays show that mutant p107 proteins that are deficient in interaction with cyclin A/cdk2 retain the ability to repress E2F-dependent transcription with an efficiency equivalent to that of the wild-type protein. In contrast, these

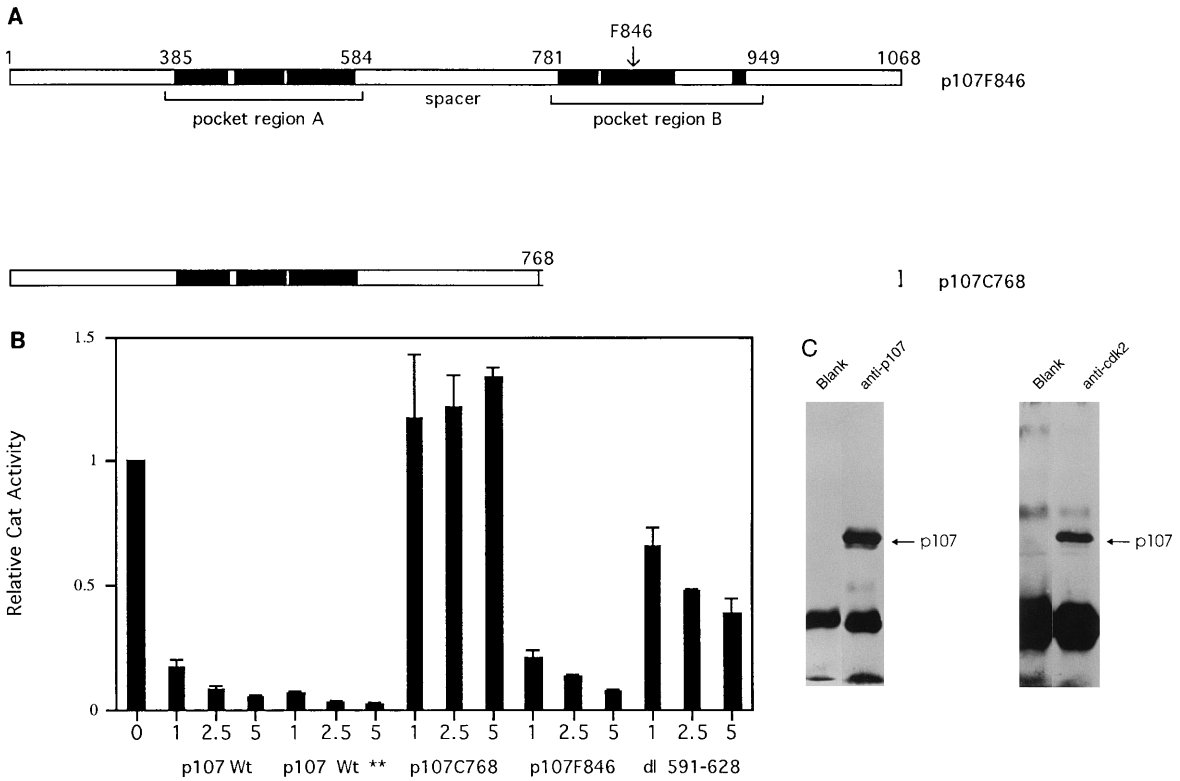

FIG. 6. Assays of additional p107 mutants for repression activity and association with cyclin A/cdk2. (A) Structure of the p107-C768 and p107-F846 mutants as described in a previous report (26). (B) C33A cells were transfected with the indicated amounts (in micrograms) of a pCMV expression vector together with 3 μg of the pE2WTx4CAT reporter plasmid and 2 μg of an RSV β-Gal plasmid as an internal control. p107 Wt represents the p107 aa 254 to 1068 construct, while p107 Wt** indicates the pCMV full-length p107 construct. CAT activity was normalized to the β-Gal expression level of each sample and is expressed as a relative percentage of the unrepressed reporter plasmid construct. Each value represents the average of two independent transfection assays. (C) C33A cells were transfected with 10 μg of pCMVp107-C768, 5 μg of pCMVcyclin A, and 2 μg of pCMVcdk2. Cell lysates were then immunoprecipitated with either anti-cdk2 or anti-p107 as indicated. The presence of p107 in the immunoprecipitate was measured by Western blotting using a polyclonal anti-p107 antibody. Extracts of cells transfected with the cyclin A and cdk2 plasmids but no p107 (Blank) were also analyzed.

p107 functions are abolished by a mutation that disrupts the ability of p107 to interact with E2F. These results therefore suggest that the critical event with respect to p107-mediated transcription inhibition is the association with E2F rather than its ability to recruit cyclin A/p33^{cdk2} to a complex.

The ability of p107 to function as a growth suppressor is clear from these assays as well as previous work, although the precise mechanism and the context for this activity have yet to be defined. Analysis of several of the mutants presented in this report has shown that this growth suppression can be accomplished by at least two distinct mechanisms. In one case, the ability to bind E2F has been shown to be important, as mutants which retain this ability yet fail to interact with cyclin A/cdk2 arrest cell growth at wild-type levels. However, the binding of p107 to cyclins also results in growth suppression, as a mutant which is able to bind cyclin A but has lost the ability to interact with E2F still represses growth at near-wild-type levels. Thus, the ability to interact with either E2F or cyclins can lead to the observed cell growth arrest. It must be noted, however, that other potential targets for the p107 protein may exist. For instance, recent experiments suggest a role for p107 in the regulation of the c-Myc transcription factor, and growth suppression by p107 can be partially reversed by the overexpression of c-Myc (4, 14). However, it should be emphasized that the interaction with c-Myc has been shown only in in vitro assays or under in vivo conditions in which both proteins are overexpressed. Currently, the only evidence for an in vivo in-

TABLE 2. p107-mediated growth arrest[a]

Plasmid	Expt	No. of β-Gal-positive cells	No. of BrdU-positive cells	Fraction BrdU positive
Control	1	107	69	0.64
	2	1,521	1,100	0.72
	3	467	333	0.71
p107wt	1	125	27	0.22
	2	868	199	0.23
	3	301	67	0.22
p107wt**	1	101	16	0.16
	2	646	117	0.18
	3	382	67	0.18
p107-C768	1	89	30	0.34
	2	515	86	0.17
	3	431	117	0.27
p107-F846	1	104	16	0.15
	2	577	118	0.20
	3	586	129	0.22
*dl*591–628	1	242	122	0.50
	2	521	363	0.70
	3	249	187	0.75
Rb	1	177	35	0.20
	2	633	107	0.17
	3	894	164	0.18

[a] Details of the assays are described in footnote *a* of Table 1. All transfections contained 5 μg of the indicated plasmid along with 2 μg of the pCMV β-Gal plasmid. p107 wt** represents a full-length p107 construct. The total levels of pCMV vector were kept constant in all transfections. The values represent the combined counts from two transfected plates for each sample.

teraction involving p107 under normal cellular conditions is the association of p107 with E2F and cyclin A/cdk2. Thus, the binding of p107 to these target proteins is the most reasonable explanation of the observed growth suppression phenotype.

It is possible that the Rb-mediated repression of E2F activity is the major physiological function of this interaction, since this complex does not appear to involve any other components. The p107 protein can also be found alone in a complex with E2F, again suggesting a role in the control of E2F transcription function. Nevertheless, it would appear that the majority of the E2F complexes involving p107 contain cyclin/cdk2 kinase components. In particular, previous work has shown that the majority of the E2F activity within an S-phase cell is found in association with p107, cyclin A, and cdk2 (9, 24). Although the experiments presented here indicate that this complex is not important for the ability of p107 to affect the E2F transcription-activating function, it surely must play a function in relation to S-phase events. It remains possible that the complex does affect E2F transcription function but in a promoter- and context-dependent manner. Alternatively, it is also possible that this complex plays a role in DNA replication events, particularly given the localization of both cyclin A and cdk2 to putative origins of replication (6).

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