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Transcriptional Repression and Growth Suppression by the p107 Pocket Protein

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p107 is a member of the pocket family of proteins that includes the retinoblastoma tumor suppressor. Overexpression of p107 arrests cells in G_1, suggesting that it is important for cell cycle control. This growth suppression is mediated at least in part through the interaction of p107 with a member of the E2F family of cell cycle transcription factors, and this interaction can be disrupted by oncoproteins from DNA tumor viruses such as adenovirus E1a that bind p107. Not only does the binding of p107 to E2F inactivate E2F, but also we show that when p107 is tethered to the promoter through binding to E2F it functions as a general transcriptional repressor. This general repressor activity was also evident when p107 was fused to the DNA binding domain of Gal4 so that it could be directly targeted to the promoter in an E2F-independent fashion. Using p107 mutants, we compared the regions of the protein required for transcriptional repression and cell growth suppression. We found that the pocket domain is sufficient for inactivation of E2F, general repressor activity, and most of the growth suppressor activity. Binding of conserved region 1 from E1a to p107 blocked interaction with E2F, but it did not affect general repressor activity, demonstrating that binding and inactivation of E2F and general repressor activity are distinguishable properties of p107. Within the pocket, two conserved domains, A and B, were sufficient for growth suppression and transcriptional repressor activity. Surprisingly, we found that these two domains were fully functional when they were coexpressed as separate proteins, and we present results suggesting that the domains may interact at the promoter to form an active pocket.

p107 belongs to a family of proteins (p107, p130, and the retinoblastoma tumor suppressor [Rb]) that share a common region known as a pocket (7, 10, 15, 24, 32, 48, 50). Overexpression of these pocket proteins arrests cells in the G_1 phase of the cell cycle (12, 29, 36, 55, 64, 65, 75), suggesting that they are important in regulating cell cycle progression. The pocket proteins appear to function at least in part by binding to and inactivating transcription factors that are important for cell cycling (for a review, see reference 23). One such family of factors is E2F, which forms DNA-binding heterodimers with a second family of proteins known as DP (for a review, see reference 45). Mutations in the Rb or p107 pocket disrupt binding to E2F/DP, preventing transcriptional repression and cell cycle arrest (11, 34, 55). E2F binding sites are present in the promoters of genes important for cell cycle progression such as those encoding c-myc, c-myb, DNA polymerase α, dihydrofolate reductase, thymidine kinase, cdc2, Rb, and E2F-1 (for a review, see reference 52), and these sites have been shown to be targets of repression by pocket proteins (14, 17, 31, 51). Oncoproteins from DNA tumor viruses (i.e., adenovirus E1A, simian virus 40 [SV40] T antigen, and human papillomavirus E7) bind to the pocket, thereby disrupting the interaction of pocket proteins with transcription factors (1, 3). This binding inactivates the pocket proteins, leading to a loss of cell cycle control. Mutations in the oncoproteins that disrupt pocket binding also inhibit their transforming activity (57), suggesting a link between inactivation of pocket proteins by oncoproteins from DNA tumor viruses and the ability of these oncoproteins to cause transformation. In support of such a link, Rb has been shown to be a tumor suppressor whose loss, mutation, or inactivation is linked to carcinogenesis (66).

Thus far, five different E2F genes have been cloned (4, 27, 28, 33, 35, 40, 42, 47, 60, 62). The pocket proteins differ in their interactions with the various E2F species. p107 forms complexes with E2F-4, whereas Rb appears to interact exclusively with E2F-1 to -3 in vivo (4, 19, 47, 70). The significance of the existence of multiple E2Fs is unclear. Conceivably, the different E2Fs each target a distinct set of genes; however, this has not yet been demonstrated. It is known that E2F-4 is expressed constitutively throughout the cell cycle, whereas E2F-1 is cell cycle specific, showing the highest levels in G_1 (27, 60). Therefore, different E2Fs may form complexes with genes at different phases of the cell cycle. p107 forms two complexes with E2F-4: one with cdk2 and cyclin E found in G_1 phase and one with cdk2 and cyclin A that is found predominantly in S phase (8, 16, 46, 53, 63). The role of these complexes is still unclear. However, the spacer within the p107 pocket is required for cyclin A-cdk2 binding, and this region has been shown to compete with the cdk inhibitor p21 for binding to the cyclin A-cdk2 complex (74).

The structural relationship between p107 and Rb, along with their abilities to inhibit E2F activity and arrest cells in G_1, indicates that the two proteins have similar biochemical activities. However, there are clear distinctions between p107 and Rb: (i) p107 forms complexes with cyclins and cdks that are more stable than those formed with Rb (16, 46); (ii) as discussed above, the pocket proteins interact differently with members of the E2F family; (iii) in addition to interacting with E2F, p107 interacts with c-myc in vivo, whereas Rb apparently does not (6, 30); (iv) the Rb-mediated arrest of cells in G_1 is reversible by overexpression of cyclins A and E, but these cyclins have little effect on growth suppression by p107 (36, 75); (v) p107 suppresses growth of the C33A cervical cancer cell line, whereas Rb does not (75); (vi) growth suppression by Rb

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results in cells with a characteristic flat morphology, whereas overexpression of p107 appears toxic to cells; (vii) the presence of p107 in tumor cell lines that have lost Rb is unable to compensate for the lack of Rb function; and (viii) in contrast to Rb, p107 has not yet been found to be mutated in tumors (23). Thus, it seems that p107 and Rb have related but distinct functions.

It has been demonstrated that distinct regions of p107 are required for binding cyclin A-cdk2 and E2F and that both of these regions can cause growth suppression (64, 73, 75). Further, it has been demonstrated in vitro that cyclin A-cdk2 bound to p107 can phosphorylate p107, resulting in the dissociation of E2F from p107 (74). However, in vivo studies suggest that p107 is phosphorylated and inactivated selectively by complexes of D cyclins and cdk4; cdk2 in combination with D, A, or E cyclins was ineffective (5).

Here, we demonstrate that binding of p107 to E2F not only inactivates E2F; p107 in this complex acts as a general transcriptional repressor. Also, we demonstrate that these two properties of p107 (binding and inactivation of E2F and general repressor activity) are distinguishable activities. The repressor activity is due entirely to the A and B domains within the p107 pocket. Additionally, we show that full repressor activity is retained when these domains are coexpressed on separate proteins and present evidence that these domains may interact at the promoter to form a repressor motif. Finally, we examine regions of p107 required for growth suppression and compare them with the region that mediates transcriptional repression.

MATERIALS AND METHODS

Cell culture and transfection. C33A, a human cervical carcinoma cell line, and Saos-2, a human osteosarcoma cell line, were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 15% heat-inactivated fetal bovine serum. Cell lines were transfected by the calcium phosphate method as described elsewhere (9). For transfections in C33A cells, vector DNA was added to bring the total amount of DNA transfected in each assay to 7 μg in a 6-cm-diameter plate. The DNA precipitate was left on C33A cells for 5 h and on Saos-2 cells for 18 h. Cells were then washed once with phosphate-buffered saline (PBS) and cultured in fresh media. Twenty four hours later, cells were harvested and chloramphenicol acetyltransferase (CAT) assays were performed as described elsewhere (68). As an internal control, 1 μg of pRSVβGal, which contains the β-galactosidase gene under the control of the Rous sarcoma virus long terminal repeat, was cotransfected and β-galactosidase assays were performed as described elsewhere (58). CMVp107 had little effect on pRSVβGal activity.

Plasmid constructs. All p107 mutants were cloned into the pCMVneoBam vector (2) under the control of the cytomegalovirus (CMV) promoter-enhancer. This vector also contains the neomycin resistance gene under the control of the herpes simplex virus thymidine kinase promoter. pCMV107 and C768 were described previously (75). G107 was cloned by removing the sequence encoding the Gal4 DNA binding domain from pM3 (59) by digesting with *Bam*HI and *Bgl*II and cloning it into the *Bam*HI site of pCMV107. The open reading frame across the Gal4-p107 junction was confirmed by sequencing. GC768 was constructed in the same way: the Gal4 DNA binding domain from pM3 was inserted into the *Bam*HI site of C768. G107S was constructed by removing the spacer from GST-p107S (22) with *Bam*HI and cloning it into the *Bam*HI site of pM2 (59). The resulting plasmid was digested with *Bgl*II and *Bsab*BI and cloned into the *Bam*HI and blunt-ended *Xho*I sites of pCMV107. GN577 was constructed by cloning the *Msc*I-*Xho*I fragment of p107 from pCMV107 into G107S cut with *Xho*I and *Msc*I. The resulting plasmid was digested with *Bgl*II and *Xho*I, and the fragment encoding Gal4 and p107 was cloned into the *Bam*HI and *Xho*I sites of pCMV107. G107A was constructed by cloning the *Bam*HI-*Hin*dIII fragment from GST107A/B-RBS (22) into the *Bam*HI and *Hin*dIII (partial digestion) sites of G107S. GC576 was constructed by digesting G107A with *Xho*I and *Dra*III and replacing this fragment with the *Xho*I-*Dra*III fragment from p107. G107B was constructed by removing the B domain from G107AB by *Bgl*II and *Bam*HI digestion and cloning the domain into the *Bam*HI site of G107S (the spacer cloned into the *Bam*HI site was removed). G107P was cloned by removing the p107 spacer from G107S by digestion with *Bam*HI and replacing it with the p107 pocket removed from GST107P by digestion with *Bam*HI. GST107P was constructed from GST107A/B-RBS by replacing the *Dra*III-*Eco*RI fragment containing the Rb spacer with the original p107 *Dra*III-*Eco*RI cDNA fragment from pCMV107. G107PCH was constructed by removing the chimeric pocket from GST107A/B-RBS by *Bam*HI digestion and cloning the pocket into the corresponding site of G107S. G107AB was constructed by first removing the Rb spacer from GST107A/B-RBS by digestion with *Hin*dIII and *Sal*I and replacing it with the annealed oligonucleotides 5′-AGCTTAGATCTG-3′ and 5′-TCGAC AGATCTA-3′. The resulting plasmid, GST107AB, was digested with *Bam*HI, and the AB domain fragment was cloned into the *Bam*HI site of G107S. GN787 was constructed by cloning the *Dra*III-*Eco*RI fragment from G107AB in frame into the same sites of the original p107 coding sequence placed in the *Bam*HI and *Sal*I sites of the pM3 plasmid in reversed orientation. The *Bgl*II-*Xho*I fragment of this product was cloned into G107S cut with *Bam*HI and partially cut with *Sal*I. To create pSVEC, the SV40 enhancer was cloned into the *Bgl*II site immediately upstream of the SV40 early-gene promoter in pCAT-Promoter (Promega). The SV40 enhancer was obtained from pCAT-Control (Promega) by PCR. The 5′ primer was 5′-ACTAGAGATCTGATCA**TGTCTGGATCTGCTGTGGA**-3′, and the 3′ primer was 5′-ATGACAGATCTGCGCGCG**GAGGATCTGAACCATGGG GC**-3′; in addition, *Bgl*II and *Bss*HII sites were engineered into the primers for cloning purposes. Nucleotides in boldface type are derived from the SV40 enhancer; *Bgl*II sites are underlined with a solid line, and the *Bss*HI site is underlined with a dotted line. The PCR product was digested with *Bgl*II and cloned into the *Bgl*II site of pCAT-Promoter (Promega) in an orientation such that the *Bss*HII site is 5′ to the SV40 enhancer. We then cloned into the *Bss*HII site a *Bss*HII fragment containing the polylinker of Bluescript SKII (Stratagene); finally, the *Bgl*II fragment of the resulting plasmid (containing the SV40 enhancer along with the polylinker) was blunted into the blunted *Sal*I site of E1bCAT, which contains the adenovirus E1b TATA box driving the CAT gene (49). pSVEC-G is identical to pSVEC except that the Gal4 sites from pG_5E1bCAT (49) were cloned into the *Sma*I site of the polylinker. G-Rb was constructed by cloning an *Eco*RI fragment (amino acid [aa] 300 to 928) encoding the growth suppressor region of Rb into the *Eco*RI site of pM2. pGal4-ATF-CAT was cloned by removing the mutated E2F sites from pmE2F-ATF-CAT (68) with *Hin*dIII and replacing them with the annealed oligonucleotides 5′-AGCTTCGG AGGACTGTCCTCCGAGTCGACTCGGAGGACTGTCCTCCGT-3′ and 5′-A GCTACGGAGGACAGTCCTCCGAGTCGACTCGGAGGACAGTCCTCCG A-3′. All constructs made by PCR or involving blunt-end ligation were sequenced.

Expression of Gal4-p107 fusion proteins. For immunoprecipitation assays, 20 μg of constructs encoding Gal4-p107 fusion proteins was transfected into 10-cm-diameter plates of subconfluent C33A cells. Twenty-four hours after removal of DNA precipitates, the transfected cells were washed twice with PBS and incubated for 6 h with 0.2 μCi of [^{35}S]methionine per plate in methionine- and cysteine-free medium. Cells were lysed in 150 mM NaCl–50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.0)–1% Nonidet P-40–0.1% sodium dodecyl sulfate (SDS)–100 μg of phenylmethylsulfonyl fluoride per ml–1 μg of leupeptin per ml–1 μg of aprotinin per μl. The lysate was precleared by incubation with protein A for 1 h on ice and subjected to immunoprecipitation with anti-Gal4 antibody (diluted 1:500; Santa Cruz) for another hour on ice. The immune complexes were collected by protein A-Sepharose and separated on an SDS–10% polyacrylamide gel.

Colony formation assay. Saos-2 cells on 10-cm-diameter dishes were transfected for 18 h by the calcium phosphate method with 11 μg of G107 or equimolar amounts of other p107 expression plasmids; vector was added to bring the final amount of DNA to 20 μg. After 24 h, the cells were washed once in PBS and fresh medium containing 500 μg of G418 per ml was added. Selection was continued for 4 weeks, at which time the cells were stained with crystal violet (75). Colonies containing more than 20 cells were counted.

RESULTS

p107 is a general transcriptional repressor. It has been shown that p107 blocks transactivation by E2F (61, 72, 75). Previously, we found that not only does the interaction of Rb with E2F block transactivation by E2F (34, 71), but also the Rb-E2F complex is an active repressor that inhibits the activity of surrounding enhancers on the promoter, thereby efficiently blocking transcription and thus preventing the G_1/S transition (67, 68). To determine whether the p107-E2F complex is also an active repressor, we tested the effect of overexpressing p107 on the activity of a construct that contains an enhancer in addition to E2F sites (pE2F-ATF-CAT) (Fig. 1A). In this construct, an activating transcription factor (ATF) site is present between the TATA box and E2F sites (68). The addition of E2F sites caused an approximately twofold increase in promoter activity over that of the ATF site alone (Fig. 1B). Cotransfection of pCMV107 had little or no effect on pmE2F-ATF-CAT, in which the E2F sites are mutated; however, the activity of pE2F-ATF-CAT was reduced below the level of activity of pmE2F-ATF-CAT (Fig. 1B), indicating that p107 not only blocks E2F site activity but also has general repressor

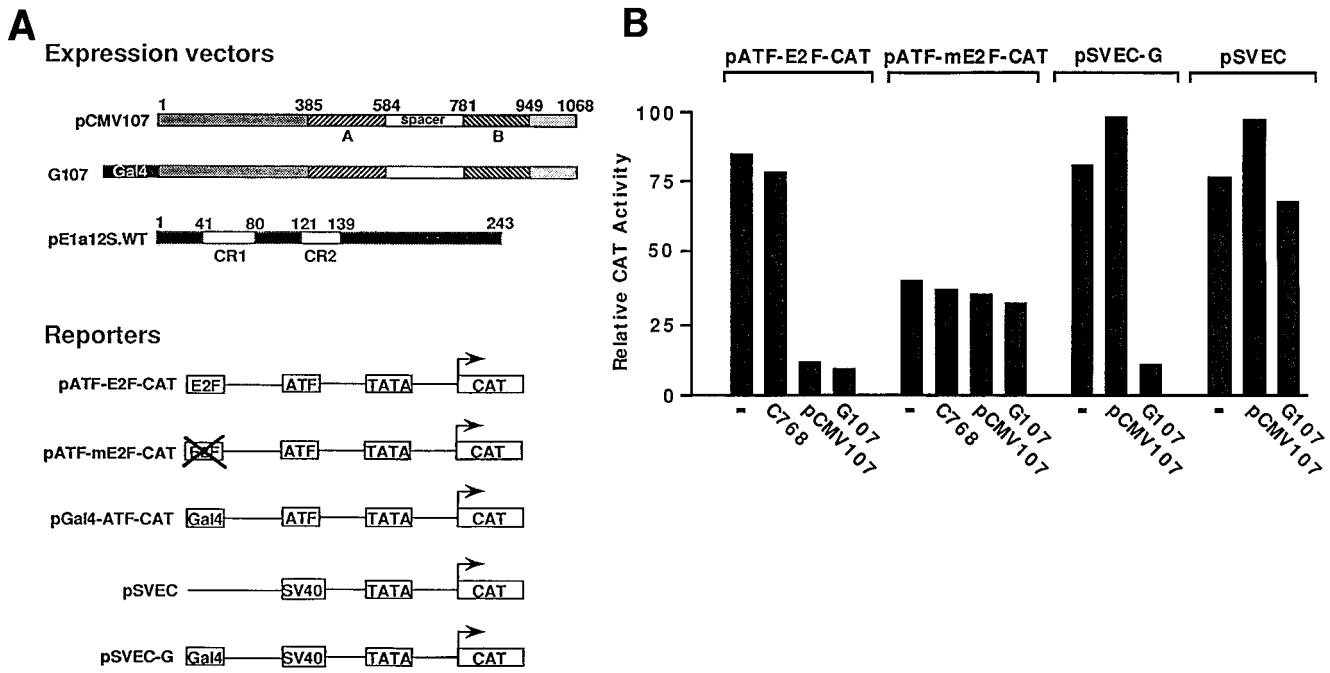

FIG. 1. p107 has intrinsic transcriptional repressor activity. (A) Diagram of expression vectors and reporter constructs. (B) p107 represses transcription when it is tethered to the promoter through E2F or directly through a Gal4 DNA binding domain. A 5-μg amount of pE2F-ATF-CAT, which contains an ATF site along with E2F sites driving the CAT gene, or pmE2F-ATF-CAT, in which the E2F sites are mutated (68), was cotransfected into C33A cells on 6-cm-diameter plates with 1 μg of pCMV107 or C768 or 0.5 μg of G107. Note that p107 inhibits the activity of pE2F-ATF-CAT to a level below that of pmE2F-ATF-CAT, indicating that it not only inactivates the E2F sites but also generally represses transcription. A 1-μg amount of pSVEC-G containing five Gal4-binding sites or the pSVEC parent vector without Gal4-binding sites was cotransfected with the same amounts of pCMV107 and G107 expression plasmids as described above; vector DNA was added to bring the total amount of DNA to 7 μg. Each plate was cotransfected with 1 μg of pRSVβGal, which was used as an internal control for transfection efficiency. CAT activity was determined and adjusted to the level of the internal control as described in Materials and Methods. Three times the amount of cell extract was used for the experiments with pE2F-ATF-CAT and pmE2F-ATF-CAT. Results are an average for two duplicate assays and are representative of more than five separate experiments. Relative CAT activity of 100 corresponds to 40% acetylation of CAT. −, no vector.

activity, resulting in the inhibition of ATF site activity. Similar results were obtained with other E2F-site-containing promoters (results not shown). As a negative control, C768 (expresses only the first 768 aa of p107 [see Fig. 4A] and therefore does not contain an intact pocket, which is required for interaction with E2F) had no activity in the transfection assays. The fact that p107 had no effect on transcription when E2F sites were mutated suggests that it inhibits transcription only when it is tethered to the promoter.

To further demonstrate this general repressor activity, p107 was fused to the DNA binding domain of the yeast transcription factor Gal4 (G107) so that it could be brought directly to the promoter in an E2F-independent fashion (Fig. 1A). As with wild-type p107, G107 repressed transcription through E2F sites (Fig. 1B). G107 was then cotransfected with pSVEC-G, which contains Gal4 sites upstream of the SV40 enhancer (Fig. 1A). pSVEC-G was repressed when G107 was cotransfected; however, there was no effect with pCMV107, which lacks the Gal4 DNA binding domain (Fig. 1B). Neither G107 nor pCMV107 affected expression from the control plasmid, pSVEC, which lacks Gal4 sites. Together, these results indicate that p107 is a general repressor when it is tethered to the promoter, either naturally through E2F or artificially through the Gal4 DNA binding domain. Similar results were obtained with other cell lines (results not shown).

CR1 of E1a blocks the inhibition of E2F activity by p107, but not Rb. Oncoproteins from DNA tumor viruses have been shown to interact with the pocket of Rb family proteins, blocking their interaction with E2F (1, 3). One of the best studied of these oncoproteins is adenovirus E1A. It has been shown that two domains in E1A (conserved region 1 [CR1] and CR2) which are conserved in different adenovirus serotypes are important for pocket interactions (20, 21, 37, 54, 57). These domains interact independently with the pocket (25, 39). CR2 contains the L-X-C-X-E motif that is also found in several other pocket-binding oncoproteins (26) but not in E2F. It binds with high affinity to the pocket, but it does not disrupt the interaction of the pocket with E2F. After CR2 targets E1A to the pocket, CR1 binds with lower affinity to a separate site and displaces E2F. Mutation of CR2 inhibits binding of E1A to Rb but not to p107 (13). These results suggested that CR1 may have a higher affinity for p107 than for Rb, and this has subsequently been shown to be the case (39).

We compared the effects of E1A with CR1 and CR2 deletions on p107- and Rb-mediated inactivation of E2F. Wild-type E1A blocked the inhibitory activity of both p107 and Rb, and mutation of CR1 eliminated this activity (Fig. 2 and results not shown). However, deletion of E1A aa 120 to 140 (pE1A12S.dl120-140), which removes CR2, differentially affected p107 and Rb: CR1 alone was sufficient to block the inactivation of E2F by p107, but it had no effect on the activity of Rb. These results further suggest functional differences in the interactions of E1A with the pockets of p107 and Rb.

CR1 does not block the general repressor activity of p107. As shown in Fig. 2, CR1 of E1A prevents p107 from inactivating E2F. We then asked whether CR1 could also block the general repressor activity of p107 when p107 was brought to a promoter independently of E2F, through the Gal4 DNA binding domain. E2F sites in pE2F-ATF-CAT were replaced with Gal4 sites to create pGal4-ATF-CAT (Fig. 1A). As expected,

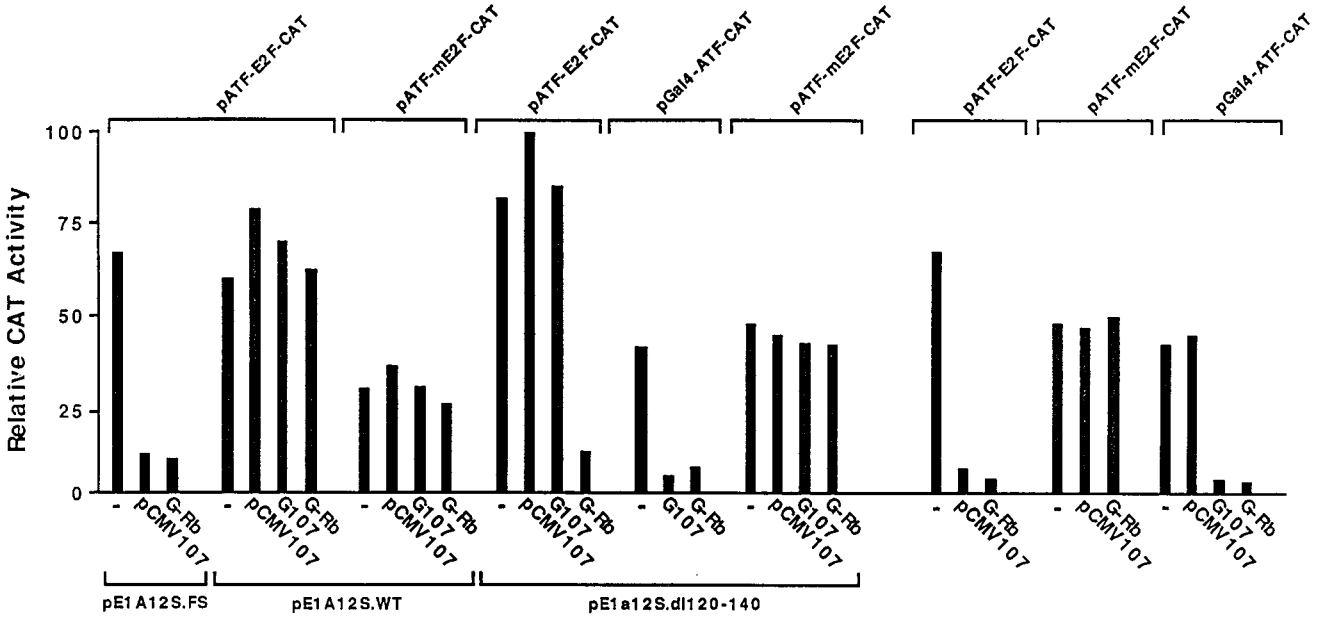

FIG. 2. CR1 of E1a blocks inactivation of E2F by p107 but does not affect the general transcriptional repressor activity of p107. A 5-μg amount of reporter plasmids was cotransfected into C33A cells with 1 μg of G-Rb or p107 expression vectors together with 5 μg of wild-type E1a12S or mutant E1a expression vectors. pE1A12S.WT encodes the 243-aa form of E1a, pE1A12S.dl120-140 encodes the 243-aa form of E1a with aa 120 to 140 (CR2) deleted, and pE1A12S.FS expresses only E1a aa 1 to 36 (43). G-Rb encodes the growth suppressor domain of Rb (aa 300 to 928) fused to the Gal4 DNA binding domain. E2F sites in pE2F-ATF-CAT were replaced with two Gal4 sites to create pGal4-ATF-CAT. Diagrams of reporter plasmids are shown in Fig. 1A. Mixtures for experiments with pE1A12S.WT and pE1A121S.FS contained only 70% of the protein extract used in experiments with pE1A12S.dl120-140. Relative CAT activity of 100 corresponds to 25% acetylation of CAT. −, no vector.

G107 and G-Rb (the growth suppressor domain of Rb linked to Gal4) repressed the activity of pGal4-ATF-CAT (Fig. 2).

Next, the effect of CR1 on repression of pGal4-ATF-CAT by G107 was tested. In contrast to the results with pE2F-ATF-CAT, where CR1 blocked the inactivation of E2F by p107, there was no detectable effect on p107-mediated repression of pGal4-ATF-CAT (Fig. 2). These results suggest that binding of CR1 to the p107 pocket displaces E2F, thereby preventing inactivation of E2F; however, this binding does not prevent general repressor activity when p107 is tethered to the promoter through Gal4. Thus, binding to and inactivation of E2F and general repressor activity are distinguishable properties of p107. The ATF-based Gal4 construct, pGal4-ATF-CAT, was used for these studies instead of pSVEC-G because the amino-terminal, p300-binding region of E1A inhibits the activity of the SV40 enhancer in pSVEC-G.

Pocket domains A and B are sufficient for general repressor activity. Mutations were made in G107 to determine which regions are required for repressor activity. These mutants were then examined in transfection assays with C33A cells for their ability to repress transcription from pSVEC-G. The results indicate that the central pocket is sufficient for full repressor activity; the N- and C-terminal regions have no detectable repressor activity (Fig. 3). Deletion of most of the spacer that separates domains A and B in the pocket or replacement of this spacer with the spacer from Rb, which shows no similarity to the p107 spacer, had little effect on repressor activity. These results are summarized in Fig. 4A. Immunoprecipitation assays of the Gal4-p107 proteins are shown in Fig. 4B.

Conserved domains A and B in the p107 pocket are sufficient for inactivation of E2F. The pocket domains of p107 and Rb are sufficient for binding to viral oncoproteins; however, the C-terminal region of Rb in addition to the pocket is required for high-affinity binding and inactivation of E2F (34, 56). Therefore, it was of interest to determine which region of p107 is sufficient for inactivation of E2F. The p107 fusion protein constructs were cotransfected into C33A cells with the pE2F-ATF-CAT reporter (Fig. 1A and 4A). In contrast to the situation with Rb, the pocket domain of p107 was sufficient for inactivation of E2F (Fig. 5). As when p107 was brought to the promoter through Gal4 binding sites, deletion of the spacer or

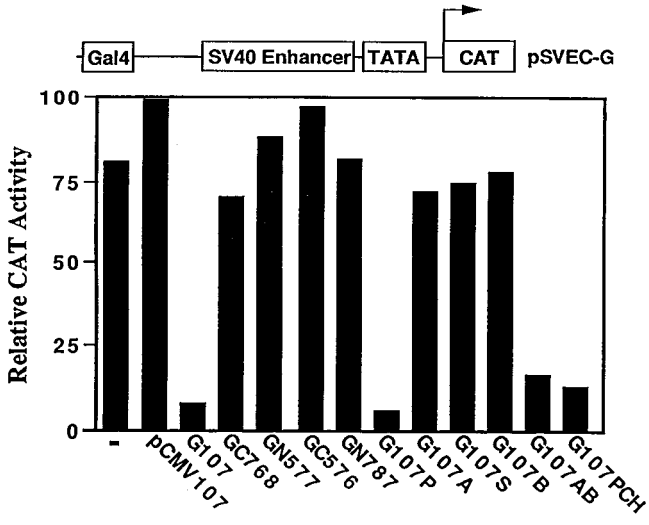

FIG. 3. Conserved domains A and B in the pocket are sufficient for the general repressor activity of p107. A 1-μg amount of pSVEC-G and 0.5 μg of G107 or equimolar concentrations of the indicated Gal4-p107 mutant expression vectors were cotransfected into C33A cells. Diagrams of Gal4-p107 constructs are shown in Fig. 4A. Relative CAT activity of 100 corresponds to 38% acetylation of CAT. −, no vector.

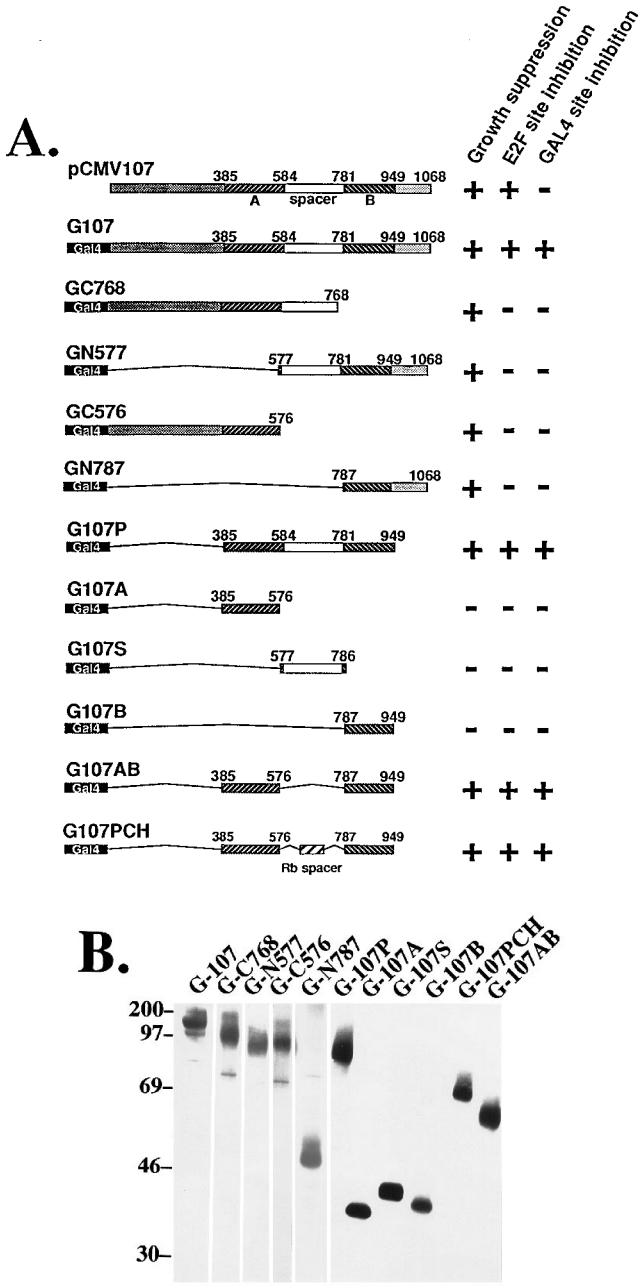

FIG. 4. Structure, expression, and summary of the activity of p107 mutants. (A) Diagram of p107 mutants, showing a summary of their activities. (B) Expression of p107 mutants in C33A cells. C33A cells were transfected with 20 μg of the indicated p107 expression vectors and labeled with [^{35}S]methionine, and extracts from the cells were prepared 24 h after the transfection. Proteins were subjected to immunoprecipitation with anti-Gal4 antibody and separated on an SDS–10% polyacrylamide gel. Size markers in kilodaltons are shown.

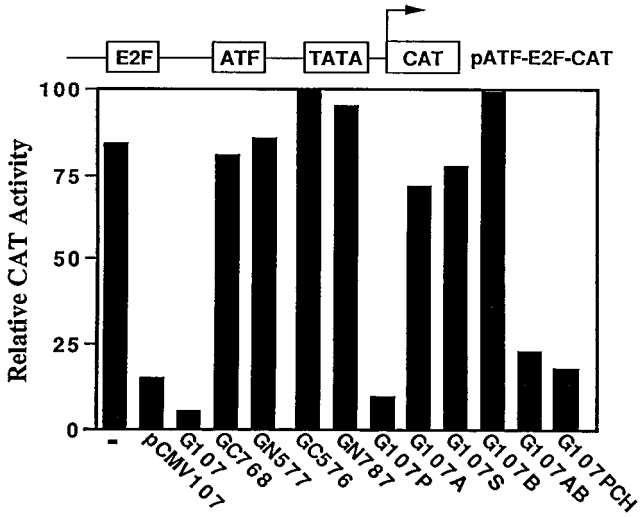

FIG. 5. Domains A and B in the p107 pocket are sufficient for repression by p107 through E2F sites. A 5-μg amount of pE2F-ATF-CAT (Fig. 1A) was cotransfected into C33A cells along with 0.5 μg of G107 or an equimolar concentration of the indicated Gal4-p107 mutant expression vectors. Diagrams of Gal4-p107 expression vectors are shown in Fig. 4A. Relative CAT activity of 100 corresponds to 23% acetylation of CAT. −, no vector.

replacement with the Rb spacer in the context of full-length p107 or in the context of the pocket alone did not prevent inactivation of E2F. Deletions in conserved domain A or B blocked the inactivation of E2F, indicating that both domains are required for activity.

Domains A and B repress transcription when they are coexpressed on separate proteins. Domains A and B repressed transcription from pSVEC-G when they were coexpressed as separate Gal4 fusion proteins (Fig. 6). Likewise, cotransfection of increasing amounts of separate domain A and B expression vectors resulted in concentration-dependent inactivation of E2F; neither domain alone affected E2F activity (Fig. 6). Together, these results suggest that domains A and B can interact to form a pocket capable of inactivating E2F and generally repressing transcription. Domain B was then coexpressed with C768, which encodes the N terminus, domain A, and a portion of the spacer, and E2F activity was inhibited. Since C768 lacks a Gal4 domain, any interaction between domains A and B would not be dependent upon Gal4 dimerization.

Domains A and B are sufficient for most of the growth suppression activity of p107. As with C33A cells, the p107 pocket was sufficient for transcriptional repressor activity in the Rb$^-$ osteosarcoma cell line, Saos-2 (results not shown). Growth of Saos-2 cells is suppressed when wild-type Rb is expressed or when p107 is overexpressed (12, 29, 36, 55, 64, 65, 75). For growth suppression assays, p107 constructs, which also contain the neomycin resistance gene, were transfected into Saos-2 cells, the cells were treated for 4 weeks with G418; and the number of resistant colonies was determined. It has been shown previously that C768, which expresses only the N terminus, domain A, and the spacer, is sufficient for growth suppression and for cyclin A-cdk2 binding (75). Likewise, we found that C768 and GC768 have some growth suppressor activity (Table 1). Additionally, the same level of growth suppression was observed with GN577, which expresses the spacer, domain B, and the C terminus. The common region between C768 and GN577 is the spacer. However, there was no effect on growth suppression with the spacer alone, and deletion of the spacer from GC768 (GC576) and GN577 (GN787) had no effect on growth suppression (Table 1). These results indicate that separate regions of p107, domain A plus the N terminus and domain B plus the C terminus, have equal growth suppressor activities. Neither region was sufficient for transcriptional repressor activity, indicating that growth suppression by these regions is independent from transcriptional repressor activity in our assays. Additionally, since the spacer is required for cyclin-cdk binding, the growth suppression associated with domain A plus the N terminus and domain B plus

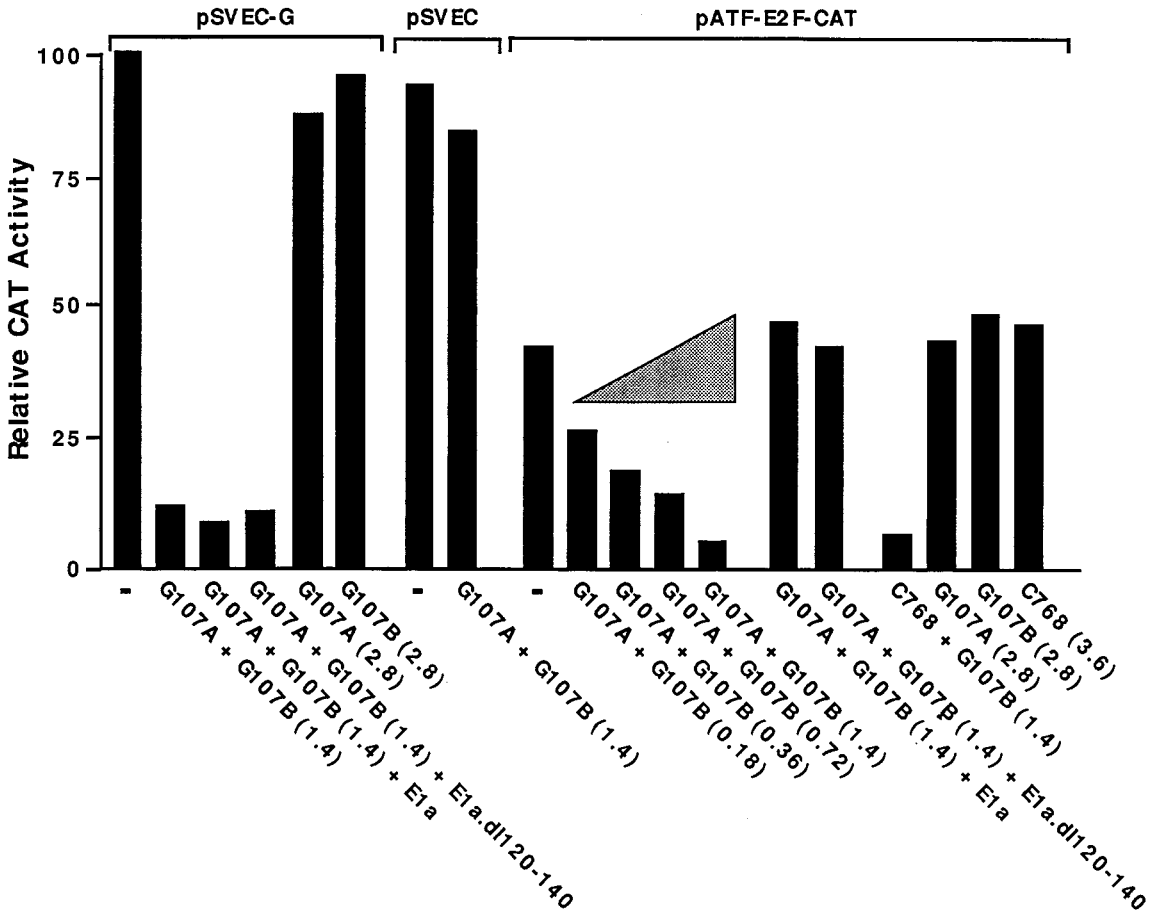

FIG. 6. Conserved domains A and B of the p107 pocket interact to form a repressor motif. A 1-μg amount of pSVEC-G or pSVEC or 5 μg of pE2F-ATF-CAT was cotransfected into C33A cells along with the indicated amount (micrograms; in parentheses) of p107 expression vectors. A diagram of expression vectors is shown in Fig. 4A. Relative CAT activity of 100 corresponds to 45% acetylation of CAT. −, no vector.

the C terminus does not appear to be linked to cyclin-cdk binding. Attempts to further dissect these growth suppressor regions were not successful: removal of the N- and C-terminal regions from the constructs, leaving only domain A or domain B, prevented growth suppression, and the N- and C-terminal regions alone had no growth suppressor activity (Table 1 and results not shown).

Since domain A plus the N terminus or domain B plus the C terminus caused only limited growth suppression (Table 1), we tested the central pocket domain for growth suppressor activity. The pocket was a more effective growth suppressor than was either domain A plus the N terminus or domain B plus the C terminus (Table 1). Deletion of the spacer had little effect on growth suppression by the pocket; there was more of an effect when the Rb spacer replaced the p107 spacer, but this could be due to conformational changes induced by the insertion of the Rb sequence. The growth suppressor activity of the pocket appears to be distinct from that observed with domain A plus the N terminus or domain B plus the C terminus, since the N- and C-terminal regions are absolutely required for growth suppression with the individual domains, but there is no such requirement with the pocket. Also, the pocket is associated with transcriptional repressor activity, whereas domain A plus the N terminus and domain B plus the C terminus are not. Our results suggest that full growth suppression is a product of three separate regions in p107; however, the pocket is responsible for most of this growth suppressor activity.

As with transcriptional repression, coexpression of domains A and B on separate proteins led to growth suppression similar to that observed when the domains were present on a single proteins (Table 1).

DISCUSSION

E2F sites in the context of several different promoters have been shown to be capable of acting as negative elements that can inhibit promoter activity (18, 44, 56, 67). Here we demonstrate that, as with Rb (67, 68), p107 not only inhibits E2F activity but also acts as a general repressor that blocks transcription when it is tethered to a promoter through E2F. In simple promoters where transactivation through E2F sites is responsible for the bulk of the promoter activity, inactivation of E2F by pocket proteins may be sufficient to inhibit transcription. However, in more complex promoters containing enhancers in addition to E2F sites, this general repressor activity may be required to effectively silence transcription. Thus, the context of E2F sites in a promoter may determine the significance of general repression versus simple inactivation of E2F.

The pocket domain was first characterized as the region of Rb that is sufficient for binding to oncoproteins from DNA tumor viruses (38, 41). Subsequently, it was found to be required for interaction with E2F; however, in Rb efficient binding to E2F and inhibition of E2F activity require the C-termi-

TABLE 1. Growth suppression by p107 and p107 mutants[a]

Construct(s)	Result for[b]:			
	Expt 1	Expt 2	Expt 3	Avg % ± SD[c]
Untransfected	0	0	0	0
Vector	541 (100)	753 (100)	323 (100)	100
pCMV107	101 (19)	133 (18)	87 (27)	21 ± 5
G107	30 (6)	81 (11)	79 (25)	14 ± 9
GN577	326 (60)	427 (57)	154 (48)	55 ± 6
GC576	290 (54)	513 (68)	150 (47)	56 ± 11
GN787	325 (60)	415 (55)	186 (58)	58 ± 2
GC768	301 (56)	436 (58)	170 (53)	56 ± 2
G107P	173 (32)	173 (23)	142 (44)	33 ± 11
G107A	556 (103)	687 (92)	229 (71)	87 ± 16
G107S	641 (119)	726 (97)	337 (105)	107 ± 11
G107B	612 (113)	662 (88)	328 (100)	100 ± 12
G107AB	207 (38)	202 (27)	152 (47)	37 ± 10
G107PCH	212 (39)	372 (50)	154 (48)	46 ± 6
G107A + G107B	117 (22)	187 (25)		23
G107A + GN577	92 (17)	203 (27)		22

[a] Saos-2 cells on 10-cm-diameter dishes were transfected with 11 μg of G107, which contains a neomycin resistance gene, or equimolar amounts of the other p107 expression plasmids; vector DNA was added to bring the final amount of DNA to 20 μg. Cells were treated with 500 μg of G418 per ml for 4 weeks and then stained with crystal violet. Colonies containing more than 20 cells were counted.

[b] The number of colonies per 10^5 cells transfected is presented; numbers in parentheses are percentages.

[c] The results are averages for three separate experiments, each in duplicate, and are presented with standard deviations. Diagrams of p107 constructs and a summary of the results are shown in Fig. 4A.

nal region in addition to the pocket (34, 55). In contrast to the situation with Rb, we found that the p107 pocket alone (domains A and B in the pocket, but not the spacer) was sufficient to inhibit E2F activity. These results are in apparent contrast to those obtained in a previous study in which it was found that, in addition to the pocket, much of the region of p107 C terminal to the pocket is also required for efficient E2F binding (73). However, the two studies are not directly comparable—we examined inhibition of E2F activity, and Zhu et al. measured E2F binding. Also, it is unclear how much E2F binding activity is actually required for inhibition in vivo.

E1A interacts differently with the pocket in p107 and with that in Rb: both CR1 and CR2 of E1A are required to block the inhibition of E2F by Rb, whereas CR1 alone was sufficient to block the inhibitory effect of p107 on E2F. However, CR1 did not affect repressor activity when p107 was brought to the promoter through the Gal4 DNA binding domain. It thus appears that binding of CR1 to p107 selectively disrupts the interaction between p107 and E2F without affecting the general repressor activity of the p107 pocket, implying fundamental differences between interaction of the p107 pocket with E2F and pocket interactions that are responsible for general repressor activity. The above results, together with the finding that Rb and p107 interact with different E2F family members, suggest functional and structural differences between the pockets in p107 and Rb.

The mechanism of the general repressor activity of p107 is unknown. There are several possible models that could explain this activity. First, p107 could, once tethered to the promoter through E2F, disrupt the basal transcription complex. Alternatively, transcription factors that bind enhancers are thought to function through an interaction with components of the basal transcription complex, and p107 could disrupt these transcription factor interactions once it is tethered to the promoter through E2F. The pocket appears capable of the multiple protein-protein interactions that both of these mechanisms would require on the basis of the fact that it can bind simultaneously to E2F and CR2 of E1a. In support of the second mechanism, we have found that Rb can bind simultaneously to E2F and other Rb-binding transcription factors (i.e., PU.1, c-myc, and Elf-1) and that this interaction blocks their binding to TFIID (67). Taken together, our studies suggest that the pocket is a general transcriptional repressor motif utilized by a family of Rb-related proteins.

In vitro, the p107 spacer is sufficient for binding to cyclin A-cdk2 (22); however, in vivo the N-terminal region of the protein appears to be required in addition to the spacer (64, 73, 75). Recently, it has been demonstrated that p107 can compete with the p21 inhibitor for binding of the cyclin A-cdk2 complex (74). Those authors show that a construct containing the first ~75% of p107 (N terminus, domain A, and the spacer) is sufficient for cyclin A-cdk2 binding, and they identify a p21-related sequence within the spacer. This region also suppressed growth. Deletion of the N-terminal region blocked both binding and growth suppression, indicating that this N-terminal region is important for cyclin-cdk binding and further suggesting a link between cyclin-cdk binding and the growth suppression activity of this region. However, our results demonstrate that the spacer, which is critical for cyclin-cdk binding, can be deleted from this construct with no apparent loss of growth suppression activity. Moreover, we found equal growth suppressor activity with a construct containing domain B plus the C terminus. This lack of a requirement for the spacer in the growth suppression by these two regions suggests that their activity may not be linked to cyclin-cdk binding. Deletion of the N-terminal or C-terminal regions from domain A plus the N terminus and domain B plus the C terminus, leaving domains A and B, respectively, eliminated growth suppressor activity, demonstrating that the individual pocket domains alone were not sufficient for growth suppression.

If these regions (domain A plus N terminus and domain B plus C terminus) do not suppress growth through an interaction with cyclin-cdk (it would appear that they do not because of the lack of the spacer), then how might they function? It is possible that these regions of p107 serve as binding sites that sequester other cellular proteins that do not require an intact pocket for interaction. By analogy to Rb, this could be a protein such as c-abl, which binds to the C-terminal region of Rb (69), or another p107-binding protein such as c-myc (6), whose binding site on p107 has not yet been mapped.

Although we observed reproducible growth suppression with domain A plus the N terminus and domain B plus the C terminus, the most potent growth suppressor region consisted of conserved domains A and B from the p107 pocket. Thus, we found most of the growth suppression to be linked with the transcriptional repressor activity of this region.

The fact that p107 has not yet been shown to be a tumor suppressor leaves its role in growth suppression unclear. However, by analogy to the situation with Rb the growth suppression by the E2F binding-transcriptional repressor region (pocket) of p107 is likely to be significant. The significance of growth suppression by the other domains (domain A plus the N terminus and domain B plus the C terminus), though, is less clear.

We conclude that the pocket is a general transcriptional repressor motif shared by the family of Rb-related proteins. Also, the finding that domains A and B have pocket activity when they are coexpressed on separate proteins suggests that these domains may interact to form the pocket.

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REFERENCES

1. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1990. Adenovirus E1A proteins can dissociate heteromeric complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell **62:**659–669.
2. **Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Wilson, and B. Vogelstein.** 1990. Suppression of human colorectal carcinoma cell growth by wild-type p53. Science **249:**912–915.
3. **Bandara, L. R., and N. B. La Thangue.** 1991. Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature (London) **351:**494–497.
4. **Beijersbergen, R., R. M. Kerkhoven, L. Zhu, L. Carlee, P. M. Voorhoeve, and R. Bernards.** 1994. E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. Genes Dev. **8:**2680–2690.
5. **Beijersbergen, R. L., L. Carlee, R. M. Kerkhoven, and R. Bernards.** 1995. Regulation of the retinoblastoma protein-p107 by G1 cyclin complexes. Genes Dev. **9:**1340–1353.
6. **Beijersbergen, R. L., E. M. Hijmans, L. Zhu, and R. Bernards.** 1994. Interaction of c-myc with the pRb-related protein p107 results in inhibition of c-myc mediated transactivation. EMBO J. **13:**4080–4086.
7. **Buchkovich, K., L. Duffy, and E. Harlow.** 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell **58:**1097–1105.
8. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) **355:**176–179.
9. **Chen, C. A., and H. Okayama.** 1987. High efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
10. **Chen, P. L., P. Scully, J. Y. Shew, J. Y. J. Wang, and W. H. Lee.** 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell **58:**1193–1198.
11. **Chittenden, T., D. M. Livingston, and W. G. Kaelin, Jr.** 1991. The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell **65:**1073–1082.
12. **Claudio, P. P., C. M. Howard, A. Baldi, A. De Luca, Y. Fu, G. Condorelli, Y. Sun, N. Colburn, B. Calabretta, and A. Giordano.** 1994. p130/pRb2 has growth suppressive properties similar to yet distinctive from those of retinoblastoma family members pRb and p107. Cancer Res. **54:**5556–5560.
13. **Corbeil, H. B., and P. E. Branton.** 1994. Functional importance of complex formation between retinoblastoma tumor suppressor family and adenovirus E1A proteins as determined by mutational analysis of E1A conserved region 2. J. Virol. **68:**6697–6709.
14. **Dalton, S.** 1992. Cell cycle regulation of the human cdc2 gene. EMBO J. **11:**1797–1804.
15. **DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, M. C. Huang, and D. M. Livingston.** 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell **58:**1085–1095.
16. **Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins.** 1992. A cyclin A-protein kinase complex possesses sequence-specific DNA binding activity: p33cdk2 is a component of the E2F-cyclin A complex. Cell **68:**167–176.
17. **Dou, Q. P., P. J. Markell, and A. B. Pardee.** 1992. Thymidine kinase transcription is regulated at G1-S phase by a complex that contains retinoblastoma-like protein and a cdc2 kinase. Proc. Natl. Acad. Sci. USA **89:**3256–3260.
18. **Dynlacht, B. D., O. Flores, J. A. Lees, and E. Harlow.** 1994. Differential regulation of E2F transactivation by cyclin/cdk2 complexes. Genes Dev. **8:**1772–1786.
19. **Dyson, N., M. Dembski, A. Fattaey, C. Ngwu, M. Ewen, and K. Helin.** 1993. Analysis of p107-associated proteins: p107 associates with a form of E2F that differs from pRB-associated E2F-2. J. Virol. **67:**7641–7647.
20. **Dyson, N., P. Guida, C. McCall, and E. Harlow.** 1992. Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J. Virol. **66:**4606–4611.
21. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–937.
22. **Ewen, M., B. Faha, E. Harlow, and D. M. Livingston.** 1992. Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. Science **255:**85–87.
23. **Ewen, M. E.** 1994. The cell cycle and the retinoblastoma protein family. Cancer Metast. Rev. **13:**45–66.
24. **Ewen, M. E., Y. Xing, J. B. Lawrence, and D. Livingston.** 1991. Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. Cell **66:**1155–1164.
25. **Fattaey, A. R., E. Harlow, and K. Helin.** 1993. Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. Mol. Cell. Biol. **13:**7267–7277.
26. **Figge, J., T. Webster, T. F. Smith, and E. Paucha.** 1988. Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and myc oncoproteins. J. Virol. **62:**1814–1818.
27. **Ginsberg, D., G. Vairo, T. Chittenden, Z. Xiao, G. Xu, K. L. Wydner, J. A. DeCaprio, J. B. Lawrence, and D. M. Livingston.** 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. **8:**2665–2679.
28. **Girling, R., J. F. Partridge, L. R. Bandara, N. Burden, N. F. Totty, J. J. Hsuan, and N. B. La Thangue.** 1993. A new component of the transcription factor DRTF1/E2F. Nature (London) **362:**83–87.
29. **Goodrich, D. W., N. P. Wang, Y. W. Qian, E. Y. H. Lee, and W. H. Lee.** 1991. The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle. Cell **67:**297–302.
30. **Gu, W., K. Bhatia, I. T. Magrath, C. V. Dang, and R. Dalla-Favera.** 1994. Binding and suppression of the myc transcriptional activation domain by p107. Science **264:**251–254.
31. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Transcriptional repression of the E2-containing promoters EIIaE, c-myc, and RB1 by the product of the RB1 gene. Mol. Cell. Biol. **12:**3431–3438.
32. **Hannon, G. J., D. Demerick, and D. Beach.** 1993. Isolation of the Rb-related p130 through its interaction with cdk2 and cyclins. Genes Dev. **7:**2378–2391.
33. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell **70:**337–350.
34. **Hiebert, S. W., S. P. Chellappan, and J. M. Horowitz.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. **6:**177–185.
35. **Hijmans, E. M., P. M. Voorhoeve, R. L. Beijersbergen, L. J. van't Veer, and R. Bernards.** 1995. E2F-5, a new E2F family member that interacts with p130 in vivo. Mol. Cell. Biol. **15:**3082–3089.
36. **Hinds, P. W., S. Mittnacht, V. Dulic, A. Arnold, S. I. Reed, and R. A. Weinberg.** 1992. Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell **70:**993–1006.
37. **Horowitz, J. M., D. W. Yandell, S. Park, S. Canning, P. Whyte, K. Buchkovich, E. Harlow, R. A. Weinberg, and T. P. Dryja.** 1989. Point mutational inactivation of the retinoblastoma antioncogene. Science **243:**937–940.
38. **Hu, Q., N. Dyson, and E. Harlow.** 1990. The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J. **9:**1147–1155.
39. **Ikeda, M., and J. R. Nevins.** 1993. Identification of distinct roles for separate E1A domains in disruption of E2F complexes. Mol. Cell. Biol. **13:**7029–7035.
40. **Ivey-Hoyle, M., R. Conroy, H. Huber, P. Goodhart, A. Oliff, and D. C. Heinbrook.** 1993. Cloning and characterization of E2F-2, a novel protein with biochemical properties of transcription factor E2F. Mol. Cell. Biol. **13:**7802–7812.
41. **Kaelin, W. G., M. E. Ewen, and D. M. Livingston.** 1990. Definition of the minimal simian virus 40 large T antigen- and adenovirus E1A-binding domain in the retinoblastoma gene product. Mol. Cell. Biol. **10:**3761–3769.
42. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell **70:**351–364.
43. **Kannabiran, C., G. F. Morris, C. Labrie, and M. B. Mathews.** 1993. The adenovirus E1A 12S product displays functional redundancy in activating the human proliferating cell nuclear antigen promoter. J. Virol. **67:**507–515.
44. **Lamm, E. W. F., and R. J. Watson.** 1993. An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. **12:**2705–2713.
45. **La Thangue, N. B.** 1994. DRTF1/E2F: an expanding family of heterodimeric transcription factors implicated in cell-cycle control. Trends Biochem. Sci. **19:**108–114.
46. **Lees, E., B. Faha, V. Dulic, S. I. Reed, and E. Harlow.** 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes Dev. **6:**1874–1885.
47. **Lees, J. A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin.** 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. **13:**7813–7825.
48. **Li, Y., C. Graham, S. Lacy, A. M. V. Duncan, and P. Whyte.** 1993. The adenovirus E1A-associated 130-kd protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E in a temporally distinct manner. Genes Dev. **7:**2366–2377.
49. **Martin, K. J., J. W. Lillie, and M. R. Green.** 1990. Evidence for interaction of different eukaryotic transcriptional activators with distinct cellular targets. Nature (London) **346:**147–152.
50. **Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu, and A. Giordano.** 1993. Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene **8:**2561–2566.
51. **Means, A. L., J. E. Slansky, S. L. McMahon, M. W. Knuth, and P. J.**

Farnham. 1992. The HIP1 binding site is required for growth regulation of the dihydrofolate reductase gene promoter. Mol. Cell. Biol. **12:**1054–1063.
52. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
53. **Pagano, M., R. Pepperkokok, F. Verde, W. Ansorge, and G. Draetta.** 1992. Cyclin A is required at two points in the human cell cycle. EMBO J. **11:**961–971.
54. **Phelps, W. C., C. L. Yee, K. Munger, and P. M. Howley.** 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell **53:**539–547.
55. **Qin, X. Q., T. Chittenden, D. Livingston, and W. G. Kaelin.** 1992. Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev. **6:**953–964.
56. **Qin, X. Q., D. M. Livingston, M. Ewen, W. R. Sellers, Z. Arany, and W. G. Kaelin.** 1995. The transcription factor E2F is a downstream target of RB action. Mol. Cell. Biol. **15:**742–755.
57. **Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins.** 1991. Domains of adenovirus E1A protein that are required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes Dev. **5:**1200–1211.
58. **Rosenthal, N.** 1987. Identification of regulatory elements of cloned genes with functional assays. Methods Enzymol. **152:**704–720.
59. **Sadowski, I., B. Bell, P. Broad, and M. Hollis.** 1992. GAL4 fusion vectors for expression in yeast or mammalian cells. Gene **118:**137–141.
60. **Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R. A. Weinberg.** 1995. E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA **92:**2403–2407.
61. **Schwartz, J. K., S. H. Devoto, E. J. Smith, S. P. Chellappan, L. Jakoi, and J. R. Nevins.** 1993. Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. EMBO J. **12:**1013–1020.
62. **Shan, B., X. Zhu, P. L. Chen, T. Durfee, Y. Yang, D. Sharp, and W. H. Lee.** 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. **12:**5620–5631.
63. **Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Morgan, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell **68:**157–166.
64. **Smith, E. J., and J. R. Nevins.** 1995. The Rb-related p107 protein can suppress E2F function independently of binding to cyclin A/cdk2. Mol. Cell. Biol. **15:**338–344.
65. **Templeton, D. J., S. H. Park, L. Lanier, and R. A. Weinberg.** 1991. Nonfunctional mutants of the retinoblastoma protein are characterized by defects in phosphorylation, viral oncoprotein association, and nuclear tethering. Proc. Natl. Acad. Sci. USA **88:**3033–3037.
66. **Weinberg, R. A.** 1991. Tumor suppressor genes. Science **254:**1138–1146.
67. **Weintraub, S. J., K. N. B. Chow, R. X. Luo, S. Zhang, S. He, and D. C. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature (London) **375:**812–815.
68. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (London) **358:**259–261.
69. **Welch, P. J., and J. Y. Wang.** 1993. A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. Cell **75:**779–790.
70. **Wu, C. L., L. Zukerberg, C. Ngwu, E. Harlow, and J. A. Lees.** 1995. In vivo association of E2F and DP family proteins. Mol. Cell. Biol. **15:**2536–2546.
71. **Zamanian, M., and N. B. La Thangue.** 1992. Adenovirus E1a prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. EMBO J. **11:**2603–2610.
72. **Zamanian, M., and N. B. La Thangue.** 1993. Transcriptional repression by the Rb-related protein p107. Mol. Biol. Cell **4:**389–396.
73. **Zhu, L., G. Enders, J. A. Lees, R. L. Beijersbergen, R. Bernards, and E. Harlow.** 1995. The pRB-related protein p107 contains two growth suppression domains: independent interactions with E2F and cyclin/cdk complexes. EMBO J. **14:**1904–1913.
74. **Zhu, L., E. Harlow, and B. D. Dynlacht.** 1995. p107 uses a $p21^{CIP1}$-related domain to bind cyclin/cdk2 and regulate interactions with E2F. Genes Dev. **9:**1740–1752.
75. **Zhu, L., S. van den Heuvel, K. Helin, A. Fattaey, M. Ewen, D. Livingston, N. Dyson, and E. Harlow.** 1993. Inhibition of cell proliferation by p107, a relative of the retinoblastoma protein. Genes Dev. **7:**1111–1125.