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Analysis of p107-Associated Proteins: p107 Associates with a Form of E2F That Differs from pRB-Associated E2F-1

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The binding of viral oncogenes to cellular proteins is thought to modulate the activities of these cellular targets. The p107 protein is targeted by many viral proteins, including adenovirus E1A, simian virus 40 large T antigen, and human papillomavirus type 16 E7 protein. A panel of monoclonal antibodies against p107 was raised and used to identify cellular proteins that interact with the p107 protein in vivo. p107-associated proteins included cyclin A, cyclin E, and cdk2. In addition, p107 was found to associate with 62- to 65- and 50-kDa phosphoproteins in ML-1 cells, a human myeloid leukemia cell line. The 62- to 65-kDa proteins have many of the properties of the transcription factor E2F but were distinguished from pRB-associated E2F-1 by both immunologic and biochemical properties.

The transforming regions of adenovirus E1A, simian virus 40 large T antigen, and human papillomavirus type 16 E7 gene contain homologous sequences that are essential for their oncogenic properties. Mutational analysis suggests that the homologous sequences enable the viral products to form stable protein-protein complexes with a set of cellular proteins (7, 14, 16, 21, 22, 42, 51). This set includes the retinoblastoma protein (pRB), cyclin A, cdk2, p130, and the pRB-related protein p107. It is presently unclear exactly how the binding by viral oncoproteins alters the normal functions of these cellular proteins. In the case of pRB, it is widely thought that the viral oncogenes bind to pRB in order to inactivate this protein, producing an effect similar to the mutation of the *RB-1* gene observed during the genesis of retinoblastomas and other tumors (7, 13, 50).

It is anticipated that the identification of cellular proteins that interact with pRB and p107 will lead to a better understanding of their normal function. Many proteins have been suggested to bind to pRB (1–3, 5, 8, 10, 12, 17, 23–25, 29, 32, 33, 35–38, 43–45, 49), but it remains to be determined how many of these candidates are true targets when expressed at endogenous levels in human cells. Since p107 and pRB contain regions of amino acid sequence with greater than 40% amino acid identity (18), it is likely that some of the proteins that interact with pRB in vitro may also bind to p107. At present, the list of p107 binding proteins is shorter and comprises cyclin A, cyclin E, cdk2, and the transcription factor E2F (4, 9, 15, 19, 39, 47). The study of these interactions has been limited by the absence of good immunological reagents for p107.

We have prepared monoclonal antibodies to p107 in order to identify and study the proteins that associate with p107 in human cells. Mice were immunized with a glutathione *S*-transferase (GST)–p107 fusion protein, and monoclonal antibodies were prepared by fusing splenocytes to NS-1 myeloma cells 3 days after the final boost. Positive tissue culture supernatants were identified by immunoprecipitation of [^{35}S]methionine-labeled p107 polypeptides that were synthesized by in vitro translation of the cRNA in rabbit reticulocyte lysate. As shown in Fig. 1A, the anti-p107 monoclonal antibodies immunoprecipitated various subsets of p107 polypeptides that correspond to translation initiation at internal methionines. The subset of polypeptides immunoprecipitated provided an approximate location of the respective epitopes on p107. The epitopes recognized by SD6 and SD9 were in different portions of p107 than those recognized by SD2, SD4, and SD15. Competition experiments were performed with SD2, SD4, and SD15 to determine whether these antibodies recognized distinct or overlapping epitopes (data not shown). The epitope recognized by SD15 was distinct from SD2 and SD4. SD2 and SD4 competed with each other for binding to p107, suggesting that they recognized the same or overlapping epitopes.

Each of the anti-p107 antibodies specifically recognized proteins of approximately 107 kDa (Fig. 1B) from a cell lysate prepared from [^{35}S]methionine-labeled human ML-1 cells, and three of these (SD6, SD9, and SD15) immunoprecipitated similarly sized proteins from mouse and monkey cells (data not shown). None of the antibodies cross-reacted to the approximately 105-kDa retinoblastoma protein that shares sequence similarity with p107.

p107 was originally defined as an E1A-associated protein that was detected in extracts of 293 cells (28, 52). To demonstrate that the 107-kDa protein immunoprecipitated by the SD antibodies was the same as the E1A-associated p107, these preparations were compared by partial proteolytic mapping. Under three different digestion conditions the proteolytic patterns generated from the E1A-associated p107 and from the 107-kDa proteins immunoprecipitated by either SD4 or a cocktail of SD2, SD4, SD6, SD9, and SD15 were identical (Fig. 1C). In a complementary approach, E1A immunoprecipitations were Western blotted (immunoblotted) and the filters were probed with the SD9 antibody. As expected, SD9 specifically recognized the E1A-associated protein (Fig. 1D). Taken together, these data demonstrate that the SD series of antibodies specifically recognize the E1A-associated p107 protein.

Anti-p107 immune complexes prepared with a cocktail of monoclonal antibodies from ^{35}S-labeled cell extracts contained two predominate proteins of 107 and 58 kDa (Fig. 2A). The identities of these proteins were confirmed by a double-immunoprecipitation procedure. The anti-p107 immunoprecipitates were denatured by heating the sample in 2% sodium

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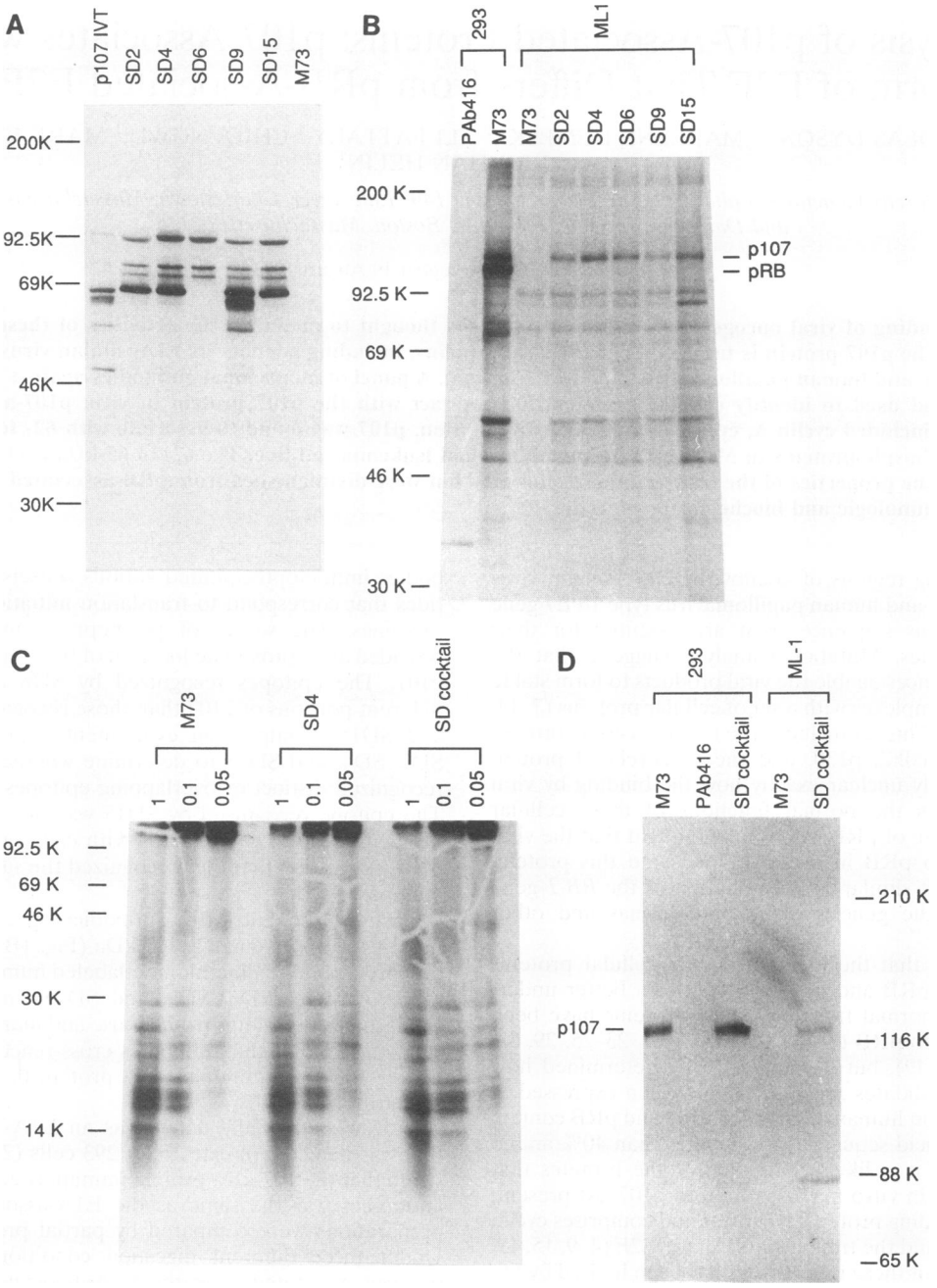

FIG. 1. Immunoprecipitations with the anti-p107 monoclonal antibodies. (A) Immunoprecipitation of p107 polypeptides synthesized by in vitro transcription and translation from the partial human cDNA clone. SD2, SD4, SD6, SD9, and SD15 are anti-p107 monoclonal antibodies. A total of 1 μl of total translation product was run as a marker (p107 IVT). M73 is an anti-E1A monoclonal antibody (27) and is used here as a negative control. (B) Immunoprecipitates prepared from lysates of human ML-1 cells labeled with [^{35}S]methionine. The cell lysate was either immunoprecipitated directly (ML1) with the antibodies indicated or was mixed with unlabeled cell extract prepared from 293 cells and immunoprecipitated (293). p107 is not recognized directly by M73 but is coprecipitated after being mixed with the E1A-containing lysate. PAb416 is monoclonal antibody to simian virus 40 large T antigen (26) and is used as a negative control. (C) Comparison of 107-kDa proteins immunoprecipitated by the anti-p107 antibodies with E1A-associated p107. The 107-kDa proteins were immunoprecipitated from 293 cells by M73 (E1A monoclonal antibody), by SD4, or by a cocktail of SD2, SD4, SD6, SD9, and SD15. The 107-kDa proteins were excised and digested with *Staphylococcus aureus* V8 protease (6) at three different concentrations (indicated in micrograms). (D) SD9 recognizes E1A-associated p107 on a Western blot. Immune complexes were prepared with anti-p107 antibodies (SD cocktail) or with a monoclonal antibody specific for E1A from 293 cell lysate (containing E1A) or ML-1 cell lysate (lacking E1A). The proteins were separated on a 6% gel, transferred to nitrocellulose, and probed with SD9. PAb416 is an additional negative control for the 293 cell lysate. The identity of the 88-kDa protein detected in ML-1 cells is unclear.

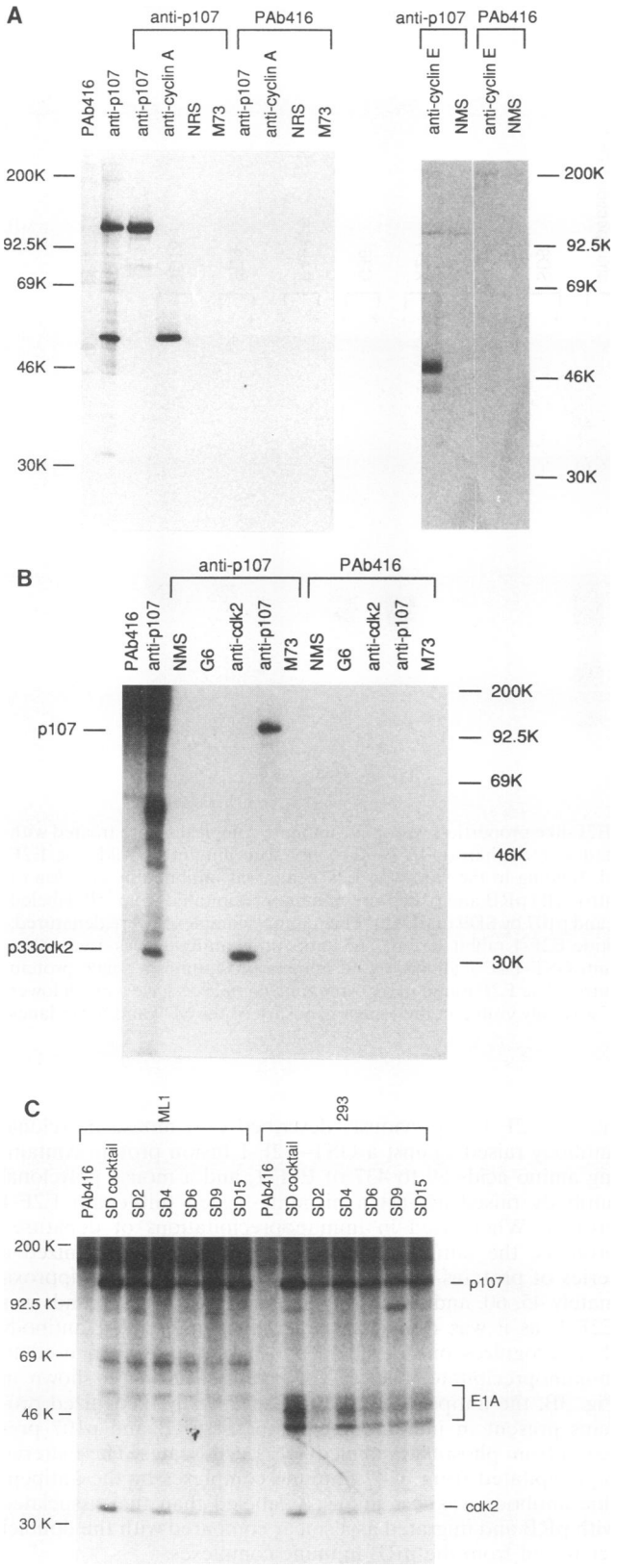

FIG. 2. Analysis of immune complexes prepared with anti-p107 antibodies. (A) Immune complexes prepared with a cocktail of anti-p107 antibodies (anti-p107) or control antibody (PAb416) from [^{35}S]methionine-labeled Ml-1 cells were denatured and reimmunoprecipitated with the antibodies shown. Normal rabbit serum (NRS) and normal mouse serum (NMS) were used as controls. The two leftmost lanes contain 5% of the initial immunoprecipitations and twice as much material was used for each reimmunoprecipitation. The signal in the anti-cyclin E lane was approximately 10-fold less than the cyclin A lane, and the four lanes on the right show a longer exposure. (B) Analysis of immunoprecipitates from $^{32}P_i$-labeled cells. The two leftmost lanes show the initial immunoprecipitation; the remaining lanes are reprecipitations as indicated. G6 is an antipeptide antibody specific for human p34^{cdc2} (11). M73 is used as a control antibody (28). (C) Immunoprecipitates prepared with each anti-p107 monoclonal antibody from $^{32}P_i$-labeled ML-1 cells or 293 cells contain phosphoproteins in addition to p107. The position of E1A coprecipitated from 293 cells is indicated. Additional proteins of approximately 50 and 62 to 65 kDa were present in p107 immunoprecipitations from Ml-1 cells but not 293 cells.

dodecyl sulfate, and the presence of associated proteins was detected by reimmunoprecipitation with antibodies specific for proteins previously reported to bind to p107. As expected, the 107-kDa band was specifically reprecipitated by the p107 antibodies. The other protein was specifically reprecipitated by a polyclonal antibody to cyclin A (20) and at lower levels by a monoclonal antibody to cyclin A (BF683 [19]) (data not shown). Cyclin E was also present in these immunoprecipitates and was detected by using a polyclonal mouse antiserum specific for cyclin E (39) (Fig. 2A). However, the level of cyclin E associated with p107 appeared to be low, as these polypeptides were not readily discernible in the initial immunoprecipitation. These observations are consistent with and complement previous studies that detected p107 in immunoprecipitates prepared by using antibodies to cyclin A or cyclin E (19, 39).

The protein kinases most likely to be a partner for these cyclins and associated with p107 were p33^{cdk2} and p34^{cdc2}. However, only a small quantity of ^{35}S-labeled protein could be detected in the p107 immunoprecipitates in the size range of these kinases. Since both kinases are phosphorylated on multiple sites in vivo, p107 immunoprecipitates were prepared from cells labeled with $^{32}P_i$ (Fig. 2B). In addition to p107, these immune complexes contained several phosphoproteins including 88, 62 to 65, 50, 45, 35, and 33 kDa that appeared to be associated with p107, since they were not recognized by the p107 antibodies after denaturation. The 33-kDa band was immunoprecipitated specifically by anti-cdk2 peptide antibodies (48) but was not recognized by the G6 anti-cdc2 peptide antisera (11). p33^{cdk2} was the only p107-associated protein that was recognized by anti-PSTAIRE antibodies (data not shown).

To exclude the possibility that the 88-, 62- to 65-, 50-, 45-, and 35-kDa and p33^{cdk2} phosphoproteins were present in the p107 immunoprecipitates as a result of antibody cross-reaction, each of the p107 antibodies was used to prepare immune complexes separately. Each of the monoclonal antibodies immunoprecipitated p107 and the 62- to 65- and 50-kDa and p33^{cdk2} proteins (Fig. 2C). Since these antibodies recognize at least four different epitopes on p107, the possibility that they all cross-react to the same proteins was essentially eliminated.

The 62- to 65-kDa and p33^{cdk2} p107-associated proteins were detected in several different cells lines, but the 62- to 65-kDa proteins were absent from p107 immunoprecipitates prepared from E1A-containing 293 cells (Fig. 2C). This suggested that 62- to 65-kDa proteins might bind to the pocket domain (E1A-binding domain) of p107 and be dissociated from p107 by the E1A present in 293 cells. These features seemed similar to the properties of the p107-E2F interaction, and as the product of the E2F-1 cDNA is approximately 60

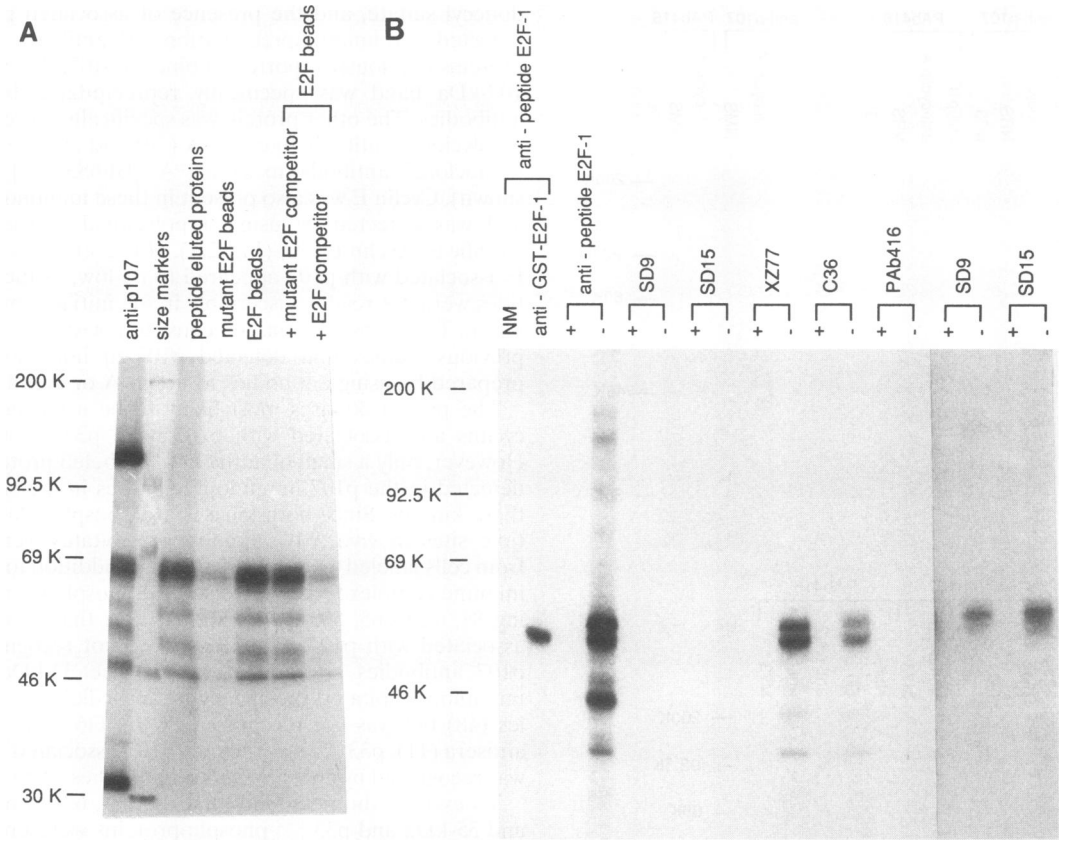

FIG. 3. The 62- to 65-kDa proteins coimmunoprecipitated with p107 have E2F-like properties. Anti-p107 immune complexes were treated with E1A peptide to elute associated proteins. The eluted proteins were incubated with Sepharose 4B beads coupled to mutant or wild-type E2F oligonucleotides. Proteins that bound to the beads were separated on the gel. Binding to the wild-type E2F beads was inhibited by addition of excess unlabeled wild-type E2F oligonucleotide but not by a mutated E2F control. (B) pRB and p107 were immunoprecipitated from $^{32}P_i$-labeled ML-1 cells with specific monoclonal antibodies (pRB by C36 [50] or XZ77 [31] and p107 by SD9 or SD15). The immune complexes were denatured, and associated E2F was detected by reimmunoprecipitation with an anti-peptide E2F-1 rabbit serum. The antipeptide antibody was used either in the absence (−) or presence (+) of excess peptide antigen. The mouse anti-GST–E2F-1 antibody (29) immunoprecipitates a single protein under these conditions. Normal mouse serum (NM) was used as a negative control. The E2F found in p107 immune complexes gave a much lower signal than the E2F detected in pRB complexes, and the p107-associated E2F was only visible in the longer exposure of the SD9 and SD15 lanes (shown on the right).

kDa in size (29, 35), we investigated whether 62- to 65-kDa proteins were indeed forms of the E2F-1 protein. Incubation of the p107 immune complexes with E1A peptides released the 62- to 65-kDa proteins from p107 (Fig. 3A) and enabled a direct test of their DNA-binding properties. The proteins eluted from the p107 complexes were incubated with Sepharose beads carrying oligonucleotides that contained either wild-type E2F sites or mutated E2F sites. All of the eluted 62- to 65-kDa phosphoproteins bound efficiently to wild-type E2F sites but had little affinity for the mutant sequences (Fig. 3A). Binding to E2F sites was specific, as it was inhibited by an excess of wild-type E2F oligonucleotide but not by mutated E2F oligonucleotide. We infer from this observation that at least one of the 62- to 65-kDa phosphoproteins that coprecipitated with p107 has DNA-binding properties similar to those of E2F.

In order to investigate whether the p107-associated proteins were the products of the E2F-1 gene, we tested whether the p107-associated proteins were recognized by antibodies to the E2F-1 protein. Three different antibodies were used: an antipeptide antibody raised by immunizing rabbits with a synthetic 18-amino-acid peptide that corresponds to the pRB-binding site of E2F-1, a previously described (29) mouse polyclonal antibody raised against a GST–E2F-1 fusion protein containing amino acids 89 to 437 of E2F-1, and a mouse polyclonal antibody raised against a histidine-tagged full-length E2F-1 protein. When used in immunoprecipitations of denatured proteins, the antipeptide antibody specifically recognized a series of proteins that included prominent bands of approximately 45, 60, and 64 kDa (Fig. 3B). One of these bands was E2F-1, as it was reprecipitated by the GST–E2F-1 antibody that recognizes only a single phosphoprotein when used to immunoprecipitate labeled ML-1 cell extracts. As shown in Fig. 3B, the antipeptide antibody specifically recognized proteins present in immunoprecipitates of pRB and p107 prepared from phosphate-labeled ML-1 cell lysates. The material reprecipitated from p107 immune complexes by the antipeptide antibody was less intensely labeled than that associated with pRB and migrated as a smear compared with the doublet recovered from the pRB immune complexes.

Further differences between the pRB-associated E2F and the p107-associated E2F were apparent when other E2F antisera were used. Both the anti-GST-E2F and the anti-His-E2F antisera immunoprecipitated proteins that were associ-

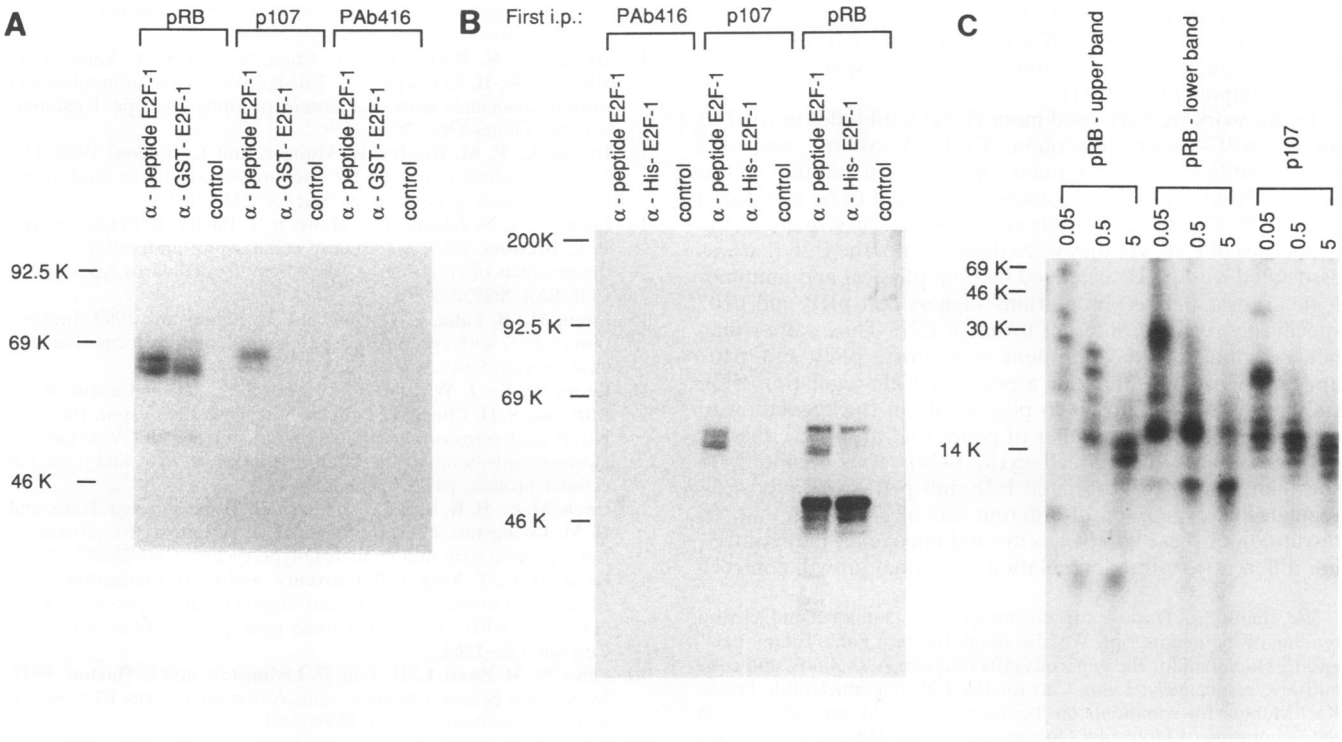

FIG. 4. The p107-associated E2F is not recognized by all E2F-1 antibodies. (A) Immune complexes were prepared from P_i-labeled ML-1 cells with C36-XZ77 (pRB), SD2, SD4, SD6, SD9, and SD15 (p107), or with a control antibody (PAb416) and denatured. E2F proteins were reprecipitated with anti-peptide E2F-1 rabbit serum (α-peptide E2F-1), with the mouse anti-GST–E2F-1 antibody (α-GST–E2F-1), or with normal mouse serum and normal rabbit serum (control). (B) Conditions were as for panel A, except that the cells were labeled with [^{35}S]methionine. His–E2F-1 is a mouse polyclonal antiserum raised against full-length E2F-1 with a poly-histidine tag. (C) p107- and pRB-associated E2F yield different partial proteolytic cleavage patterns. E2F proteins were reimmunoprecipitated from pRB and p107 immune complexes by the anti-peptide E2F-1 rabbit serum and separated on an 8% gel. Prominent E2F bands were excised from the p107-E2F and pRB-E2F lanes and were digested with V8 protease. The products were separated on a 20% polyacrylamide gel.

ated with pRB but failed to recognize the p107-associated proteins. These distinctions were seen with immune complexes prepared from ML-1 cells that were labeled either with $^{32}P_i$ (Fig. 4A) or with [^{35}S]methionine (Fig. 4B; data not shown), suggesting that the failure of the antibodies to recognize p107-associated E2F was not simply the failure of the antisera to recognize certain phosphorylated forms.

The most direct method to compare these proteins further is to compare them by partial proteolytic digestion. However, the low signal that can be obtained by using [^{35}S]methionine-labeled proteins has precluded this type of analysis. As the E2F polypeptides are readily labeled with phosphate, partial proteolytic mapping has been possible by using phosphate-labeled proteins, and a comparison of the V8 digestion patterns of the p107-associated 65- to 66-kDa E2F with the pRB-associated 60- and 64-kDa polypeptides is shown in Fig. 4C. The partial proteolytic patterns generated from all three of these phosphoproteins are clearly different. This analysis suggests that the p107- and pRB-associated proteins are different phosphoproteins, but since the analysis uses ^{32}P-labeled polypeptides, this result cannot determine whether these forms of E2F are different modifications of the same gene product or are the products of different genes.

Homologous sequences of adenovirus E1A, simian virus 40 large T antigen, and human papillomavirus E7 protein enable these viral oncogenes to form stable protein-protein complexes with pRB and with several additional polypeptides, including the pRB-related protein p107. Genetic studies suggest that interactions with this set of proteins are an essential component of the mechanism used by viral oncoproteins to transform cells. The structural relationship between pRB and p107 suggests that these two proteins might have similar biochemical activities; however, pRB and p107 are clearly functionally distinct. Most significantly, the presence of p107 in tumor cell lines that have lost pRB is unable to compensate for the lack of pRB function. Furthermore, the p107 gene has not yet been found to be mutated in any tumor cell despite extensive searches, and in this respect, it is unlike the RB-1 gene that is often mutated during tumorigenesis. It is clearly important to determine the biochemical differences between pRB and p107 that are the basis for their apparently different biological properties.

One difference between pRB and p107 is that p107 forms complexes with cyclin-dependent kinases that are considerably more stable than pRB-containing cyclin complexes. A second, although more subtle, difference between pRB and p107 may lie in their interactions with the transcription factor E2F. Multiple lines of evidence indicate that the transcription factor E2F is the composite activity of a complex series of polypeptides. cDNAs for two components of E2F, E2F-1 and DP1, have been cloned (23, 29, 35, 46). These bind to DNA most effectively as a heterodimer and cooperate in transcriptional activation (30, 34). Recent work suggests the existence of a large family of E2F-related genes (40). We and others have previously shown that E2F-p107 complexes, unlike E2F-pRB complexes, contain stoichiometric amounts of cdk2 and either

cyclin A or cyclin E (4, 9, 39, 41, 47). The timing of the appearance of the E2F-p107 complexes differs from those of E2F-pRB and appears to coincide with the synthesis of the cyclin components (39, 41).

In this work we have used monoclonal antibodies to p107 to identify p107-associated proteins. Cyclin A, cyclin E, and cdk2 were identified in p107 immune complexes. In addition, 62- to 65- and 50-kDa phosphoproteins were found to be associated with p107. The 62- to 65-kDa polypeptides have many of the properties of E2F, yet they were distinct from the E2F that was associated with pRB, as judged by both physical and immunologic criteria. These observations suggest that pRB and p107 specifically regulate different forms of E2F. Thus, rather than being redundant or equivalent regulators, pRB and p107 appear to provide different aspects of E2F regulation. E2F sites have been proposed to play a role in the regulation of expression of a large number of genes that are transcribed at different points in the cell cycle (42a). It is tempting to speculate that pRB-associated E2F and p107-associated E2F regulate the expression of different sets of genes and that the disruption of these complexes by viral oncogenes may contribute differently to the deregulation of normal growth control.

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