Large T Antigens of Many Polyomaviruses Are Able To Form Complexes with the Retinoblastoma Protein

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Stable protein complexes between the large T antigens of mouse, monkey, baboon, or human polyomaviruses and the retinoblastoma protein were detected by an in vitro coimmunoprecipitation assay. All of the large T antigens tested were able to bind to both human and mouse retinoblastoma polypeptides, showing that these interactions have been conserved during evolution.

The E1A proteins of adenovirus 5 (Ad5), the large T antigen of simian virus 40 (SV40), and the E7 protein of human papillomavirus type 16 all form stable complexes with the protein product of the retinoblastoma (RB) susceptibility gene, RB-1 (3, 8, 17, 38). In each case, genetic studies have shown that regions of the viral protein that are necessary for association with the RB protein (pRB) also contribute to transformation by the viral oncoproteins (2, 9, 18-20, 23, 24, 26-28, 32, 37, 39, 40, 42, 43). The correlation between the loss of pRB binding and the loss of transforming ability suggests that the association with pRB is an important component of virus-mediated transformation.

Regions of the human papillomavirus type 16 E7 protein, Ad5 E1A polypeptides, and SV40 large T antigen that are necessary for association with pRB can be aligned to show significant sequence homology (11, 30, 34). Similar sequences can also be found in the large T antigens of other polyomaviruses. The large T antigens of all these polyomaviruses are thought to perform similar roles during viral infections, and each has potent transforming or immortalizing properties when introduced into appropriate host cells. However, the oncogenic properties of these proteins vary considerably, both in tissue culture assays and in their association with naturally occurring malignancies. We were interested in determining the pRB-binding abilities of these viral proteins. The work described here dealt with several different genera of polyomaviruses; a comparison of the pRB-binding activities of several human papillomavirus E7 proteins can be found in a report by Münger et al. (27a).

Amino acid sequences in the large T antigens of human JC virus, human BK virus (Dunlop and MM strains), mouse polyomavirus, baboon SA12 virus, and monkey lymphotropic papovavirus that are homologous to the pRB-binding sequences in SV40 large T antigen are shown in Fig. 1. These sequences are aligned with the pRB-binding sequences of E1A. The sequences in E1A that are thought to be important for interaction with pRB are not contiguous (40) and here are designated regions 1 and 2. Region 2 of SV40 large T has been shown to form an actual contact site with pRB (4). This sequence is characterized by the sequence Asp or Asn-Leu-any amino acid-Cys-any amino acid-Glu (D or N-L-

X-C-X-E). This same sequence is found in all proteins that are known to bind to pRB. The contributions of region 1 sequences to interaction with pRB are less well understood. The analogous region in Ad5 E1A is thought to form a physical contact site with pRB that promotes high affinity interactions. It is not known whether similar binding occurs with the similar region of any large T antigen or E7 protein.

The ability of the large T antigens to associate with pRB was tested in an in vitro assay that has previously been shown to be a highly sensitive method for detecting pRB interactions (7, 8). In this assay, RB polypeptides were synthesized by in vitro transcription of the human or mouse cDNA clones and subsequent translation of the cRNA in rabbit reticulocyte lysates. The RB polypeptides were mixed with detergent lysates of unlabeled tissue culture cells that contained the appropriate viral proteins. After a suitable incubation, portions of the mixture were immunoprecipitated with test or control antibodies. The ability of the viral protein to associate with pRB was determined by coimmunoprecipitation of the labeled RB polypeptides with antibodies specific for the viral protein but not with control antibodies.

The fidelity of this coprecipitation assay, in which RB polypeptides were mixed with cell lysates prepared from HeLa cells previously infected with wild-type (Ad5) or mutant (*d1922-947*) adenovirus, is illustrated in Fig. 2. The mutant *d1922-947* contains a deletion of E1A amino acids 122 to 129 and produces E1A polypeptides that are transformation defective and which, although stably expressed, have been shown to be unable to coimmunoprecipitate ³⁵S-labeled pRB from infected HeLa cells (40). A subset of human or mouse RB polypeptides was coimmunoprecipitated with wild-type E1A, but no coprecipitation was seen with the mutant E1A or with an E1A-minus cell lysate. This result is consistent with published observations and shows that the appearance of a positive signal in the in vitro assay is highly sequence specific and requires sequences similar to those shown to be necessary for interactions in vivo.

The RB polypeptides that are produced by in vitro transcription and translation of the human cDNA clone are a nested set of polypeptides that share a common carboxy terminus (8, 40). Since the sequences of pRB that are necessary for complex formation reside in the carboxy-

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FIG. 1. Alignment of amino acid sequences from the large T antigens of human JC virus (JCV) (12), human BK virus (BKD and BKM; Dunlop [33] and MM [41] strains, respectively), mouse polyomavirus (PY) (14), baboon SA12 virus (T. P. Cunningham and J. M. Pipas, unpublished data), and monkey lymphotropic papovavirus (LPV) (29) that are homologous to the pRB-binding sequences in SV40 large T antigen (amino acids 105 to 114 [3]), Ad5 E1A (amino acids 30 to 60 and 120 to 127 [38]), and HPV-16 E7 (8, 27a). Boxed sequences indicate amino acid identities or highly conservative changes.

terminal portion of the protein (7, 8; Q. Hu, N. Dyson, and E. Harlow, unpublished observations), only a subset of the polypeptides synthesized was able to associate with E1A. A similar pattern of carboxy-terminal fragments was seen in the mouse RB translation products, producing a set of polypeptides whose sizes correspond to the positions of methionines in the mouse RB sequence. The subset of mouse RB polypeptides that complexed with E1A were similar in size to the human polypeptides, suggesting that

similar regions in mouse pRB are necessary for binding to E1A.

When large-T-antigen-containing cell lines were used in this in vitro assay, the characteristic subset of RB polypeptides was coprecipitated with each of the large T antigens tested (Fig. 3A). The following cell lines were used: LCP726, mouse cells expressing monkey lymphotropic polyomavirus large T antigen (J. Chen and T. VanDyke, unpublished data); HJC, hamster cells expressing human JC virus large T antigen (25); PyT54, hamster cells expressing mouse polyomavirus large T antigen (35); SA12, mouse cells expressing baboon SA12 virus (31, 36) large T antigen; pBKD and

FIG. 2. Coimmunoprecipitation assays using wild-type or mutant Ad5 E1As. HeLa cells were infected with viruses at 100 PFU per cell as described in reference 40. Plates of cells that were infected in parallel either were labeled with [³⁵S]methionine, lysed, and immunoprecipitated or were mock treated, and the lysates were used in the coimmunoprecipitation assay. Equivalently labeled E1A polypeptides and E1A-associated 300K proteins were immunoprecipitated by E1A-specific monoclonal antibodies from the labeled cell lysates (data not shown). Mixing reactions were set up with 50 μ l of cell lysate and human or mouse RB polypeptides as described in references 7 and 8. A mix was also set up with a cell lysate prepared from uninfected HeLa cells as a negative control. Mouse RB polypeptides were synthesized by in vitro transcription and translation as described for the human RB clone in reference 40 with a clone of the mouse cDNA behind a T7 promoter. Portions of the mixture were immunoprecipitated with an E1A-specific monoclonal antibody, M73 (16), or with PAb416, a monoclonal antibody raised against SV40 large T antigen (15), as a negative control. Immune complexes and 1 μ l of the translation reactions were separated on a 10% polyacrylamide gel (21), and labeled proteins were detected by fluorography (1).

FIG. 3. Coimmunoprecipitation assays using cell lysates prepared from T-antigen-expressing cell lines. (A) Assays with human RB polypeptides; (B) assays with mouse RB polypeptides. Mixing reactions were prepared as previously described (7, 8, 27a) with lysates from 4×10^6 cells and 30 μ l of in vitro translation reaction. Portions of the mixture were immunoprecipitated with anti-T antigen or negative control antibodies. Immune complexes and 1 μ l of the translation reactions were separated on 10% polyacrylamide gels (21), and labeled proteins were detected by fluorography (1). The cell lines used are indicated and described in the text. The following anti-T antibodies were used: a hamster polyclonal antiserum raised against SV40 large T antigen as described in reference 22 that cross-reacts to monkey lymphotropic papovavirus large T antigen (LCP), PAb416 and PAb419 (15) (HJC and COS), PAb416 and PAb430 (15) (SA12, pBKM, and pBKD), and LT1 (5) (PyT54). Control antibodies were normal mouse sera and normal rabbit sera (LCP and PyT 54) or M73 (HJC, SA12, BKM, BKD, and COS) plus the appropriate secondary antibodies. None of these antibodies recognized the RB polypeptides directly (data not shown).

pBKM, mouse cells expressing human BK virus (Dunlop and MM strains, respectively) large T antigens (L. R. Gooding and J. M. Pipas, unpublished data); and COS, monkey cells expressing monkey SV40 large T antigen (13). Similar results were obtained with both human and mouse RB polypeptides (Fig. 3B). In these experiments, the levels of RB polypeptides coprecipitated with these large T antigens were similar, but slight differences were also apparent. The cell lines used for these assays were chosen because they express the corresponding large T antigens at relatively high levels (data not shown), and cell lysate from 4×10^6 cells was added to each reaction to ensure that the large T antigens were in excess. However, since the amount of large T antigen added was not normalized and since it was necessary to use a range of antibodies that vary in their large-T-antigen affinities, it is difficult to gauge the significance of differences in levels of coprecipitated RB polypeptides. Nevertheless, several important conclusions can be drawn from these results. First, the ability to bind to pRB is not restricted to SV40 large T antigen but is conserved in the large T antigens of polyomaviruses that infect human, monkey, baboon, and mouse cells. Second, the amino acid differences between these large T antigens and the pRB binding site of SV40 large T antigen do not remove the capability of the large T antigens to bind to pRB. Third, since all the large T antigens can bind to human or mouse RB polypeptides, the ability of the large T antigens to bind to pRB per se does not restrict the host range or transforming potential of these viruses, although there may be qualitative differences in the associations that were not detected in these assays.

In vitro coimmunoprecipitation assays have proven a highly sensitive method for detecting specific pRB associations. More work will be needed to show that all of these interactions occur in vivo. Recent data show that region 2 of SV40 large T antigen and its analogous region in Ad5 E1A

are also necessary for association with a second cellular protein (6, 10, 40) with a relative molecular weight of approximately 107,000. Because this region has homology with the other polyomavirus large T antigens, it is likely that these viral oncoproteins will also bind to the 107K protein. Little is known about the biochemical significance of either the pRB or 107K interactions. However, where known, mutations that lie in these regions drastically affect the transforming ability of these viral oncoproteins, suggesting that these viruses use this small region of the large T antigen to interact with important cellular regulatory proteins. Here we show that the ability to target pRB may be conserved in the large T antigens of a variety of polyomavirus-type viruses. This adds to an emerging view in which pRB is a common cellular target for the transforming proteins of several families of small DNA tumor viruses.

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