The EMBO Journal vol.9 no.4 pp.1147 – 1155, 1990

The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations

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Communicated by J.D.Watson

The protein product of the retinoblastoma (RB) gene is thought to function in a pathway that restricts cell proliferation. Recently, transforming proteins from three different classes of DNA tumor viruses have been shown to form complexes with the RB protein. Genetic studies suggest that these interactions with the RB protein are important steps in transformation by these viruses. In order to understand better the function of the RB – viral oncoprotein complexes, we have mapped the regions of the RB protein that are necessary for these associations. Two non-contiguous regions of RB were found to be essential for complex formation with adenovirus E1A or SV40 large T antigen. These two regions are found between amino acids 393 and 572 and 646 and 772. Interestingly, these binding sites on RB overlap with the positions of naturally occurring, inactivating mutations of the RB gene. These results strongly suggest that these viral oncoproteins are targeting a protein domain that is an important site in the normal function of the RB protein.

Key words: adenovirus E1A/ retinoblastoma protein/SV40 large T antigen

Introduction

The study of cancer has revealed two classes of genes that contribute to tumorigenesis. One class of genes includes the dominantly acting oncogenes, which are activated either by mutations in their coding regions that lead to an abnormal biochemical activity or by mutations that lead to inappropriate expression of the wild-type protein. Oncogenes activated by either type of mutation causes cells to proliferate or take on other characteristics of the transformed cell. The other class of genes involved in the genesis of cancer are the tumor suppressor genes. In contrast to the dominant oncogenes, tumor suppressor genes promote tumor formation through the loss or inactivation of their gene products, presumably removing a barrier to cell growth or differentiation. One of the best studied of the tumor suppressors is the retinoblastoma gene (RB-1). The RB-1 gene was first identified through its association with inherited predisposition to retinoblastomas (Knudson, 1971; reviewed in Murphree and Benedict, 1985; Albert, 1987; Benedict, 1987; Knudson, 1987; Weinberg, 1988). RB-1 maps to human chromosome 13q14 (Francke and Kung, 1976; Knudson *et al.*, 1976; Yunis and Ramsay, 1978; Strong *et al.*, 1981; Balaban *et al.*, 1982; Benedict *et al.*, 1983a,b; Godbout *et al.*, 1983), and the cDNA for this gene has been cloned in several laboratories (Friend *et al.*, 1986; Lee *et al.*, 1986; Fung *et al.*, 1987). It is now clear that retinoblastomas characteristically show a loss or mutation of both alleles of the RB-1 gene, resulting in the inability to synthesize the retinoblastoma protein (pBR) (Cavenee *et al.*, 1983; Dryja *et al.*, 1984, 1989; Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987; Whyte *et al.*, 1988; Dunn *et al.*, 1988; Horowitz *et al.*, 1989a; Xu *et al.*, 1989).

The RB-1 gene is also mutated or deleted in other types of human tumors and tumor cell lines, including osteosarcomas, small cell lung carcinomas, breast cancers, prostate carcinomas and bladder carcinomas (Friend *et al.*, 1986; Fung *et al.*, 1987; Harbour *et al.*, 1988; Lee *et al.*, 1988; T'Ang *et al.*, 1988; Yokota *et al.*, 1988; Horowitz *et al.*, 1989a,b; Toguchida *et al.*, 1989). It is believed that mutations of the RB-1 gene are responsible, at least in part, for some stage of oncogenesis in these cases. In addition to the genetic evidence, support for the hypothesis that the RB protein functions by inhibiting cellular proliferation also comes from the re-introduction of the cloned RB gene into retinoblastoma or osteosarcoma cell lines that do not produce normal RB protein (Huang *et al.*, 1988). Cells with the re-introduced wild-type RB protein have an altered phenotype; they grow less well than the parental cells and fail to produce colonies in soft agar or tumors in nude mice. Although the mechanisms by which the RB protein controls cell proliferation is unknown, the cell cycle-dependent phosphorylation of pRB may implicate RB in the regulation of the cell cycle (DeCaprio *et al.*, 1989; Buchkovich *et al.*, 1989; Chen *et al.*, 1989).

Recent studies from a number of laboratories have shown that the RB protein forms stable complexes with the adenovirus E1A proteins, polyoma-type virus large T antigens and human papilloma virus E7 proteins (Whyte *et al.*, 1988; DeCaprio *et al.*, 1988; Dyson *et al.*, 1989b,d; Munger *et al.*, 1989). Mutational analysis of these viral proteins has shown that the regions needed for interaction with pRB are also required for transformation (Kalderon and Smith, 1984; Moran *et al.*, 1986; Lillie *et al.*, 1987; Schneider *et al.*, 1987; Cherington *et al.*, 1988; DeCaprio *et al.*, 1988; Moran, 1988; Smith and Ziff, 1988; Whyte *et al.*, 1989; Munger *et al.*, 1989). This suggests that the RB protein is a cellular target for transformation by these viral oncoproteins. By binding to pRB the viral proteins might interfer with its normal function, thus mimicking the loss of pRB seen in tumor cell lines.

To understand the function of the RB – viral oncoprotein complexes better, we have mapped the regions of the RB protein that are necessary for these associations. There are two regions of the RB protein that are required for stable protein – protein interactions with either the adenovirus E1A protein or the SV40 large T antigen. Interestingly, these regions are also common sites of naturally occurring

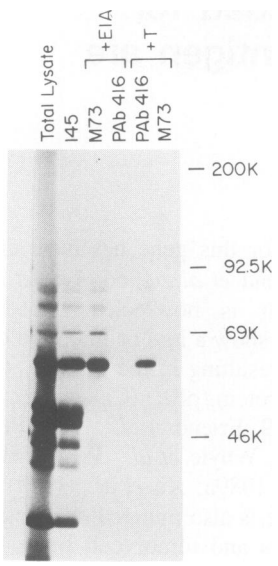

Fig. 1. Complex formation between the RB polypeptides and adenovirus E1A or SV40 large T antigen. [^{35}S]Methionine-labelled RB polypeptides were synthesized by *in vitro* transcription/translation from a T7 promoter driven RB cDNA construct. The synthesized RB polypeptides were immunoprecipitated with anti-RB carboxy-terminal antibodies, 145 (Whyte *et al.*, 1988); or mixed with either unlabelled 293 cell lysate (containing E1A) or purified SV40 large T antigen. The mixtures were immunoprecipitated with M73 (E1A-specific), or PAb416 (specific for SV40 large T antigen). Protein complexes were separated by electrophoresis on a 10% SDS−polyacrylamide gel and detected by fluorography.

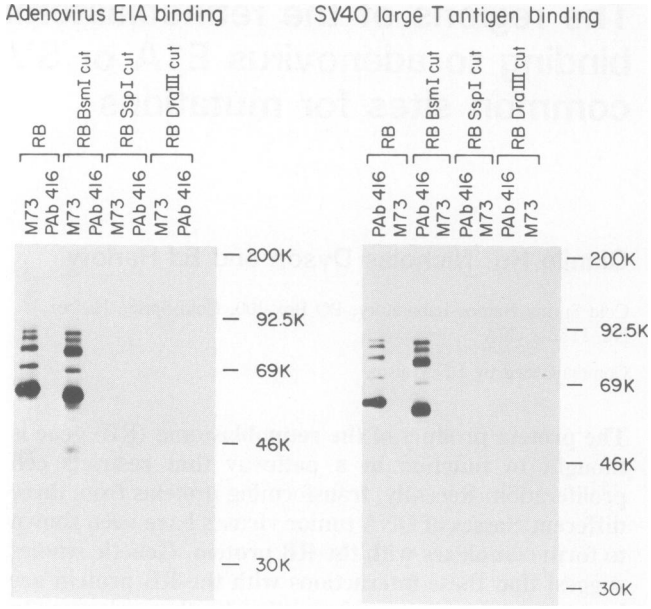

Fig. 2. The carboxy-terminal boundary of E1A and large T antigen binding sequence determined after linearizing the RB cDNA with restriction enzymes. The RB cDNA was digested with three different restriction enzymes (*Bsm*I, *Ssp*I and *Dra*III) that recognize unique sites in the coding sequence. After *in vitro* transcription/translation, the carboxy-terminal truncated RB polypeptides were mixed with either unlabelled 293 cell lysate (containing E1A) or purified SV40 large T antigen and protein complexes were immunoprecipitated with M73 or PAb416. Proteins were separated by SDS−polyacrylamide gel electrophoresis and detected by fluorography.

mutations in the RB-1 gene, suggesting that these viral proteins are targeting a protein domain that is an important site in the normal function of the RB protein.

Results

The binding of the RB protein to other polypeptides can be easily shown using an *in vitro* mixing assay that previously has been used to detect RB interactions with transforming proteins from a wide variety of small DNA tumor viruses (Dyson *et al.*, 1989a,b,c,d; Munger *et al.*, 1989). *In vitro* complex formation is highly sequence specific and, where known, requires regions of the viral proteins that have been shown to be necessary for complex formation *in vivo*. We have used a modified version of this assay to determine the portions of the RB polypeptide that are sufficient for stable interaction with the adenovirus 5 E1A protein or SV40 large T antigen. For these experiments the open reading frame of the RB cDNA was cloned downstream of the bacteriophage T7 promoter, allowing RB cRNA to be synthesized directly from the cDNA by *in vitro* transcription (Friend *et al.*, 1986; Whyte *et al.*, 1988; Dyson *et al.*, 1989b,c). cRNA was then used to program an *in vitro* translation reaction in rabbit reticulocyte lysates. By introducing deletion mutations into the cDNA or by cutting the cDNA clone at restriction sites within an open reading frame, mutant RB polypeptides were synthesized that contained only part of the normal RB sequence. Mutant or wild-type ^{35}S-labeled RB products were mixed with either E1A (added from an E1A-containing cell lysate) or purified SV40 large T antigen and assayed for binding activity. If complexes formed, the radiolabelled RB polypeptides were seen in immunoprecipitations with monoclonal antibodies specific for the viral proteins. The antibodies used in these experiments were M73, a monoclonal antibody specific for E1A (Harlow *et al.*, 1985) and PAb416, a monoclonal antibody specific for SV40 large T antigen (Harlow *et al.*, 1981). Precipitation of the RB polypeptides by PAb416 is only seen when large T antigen is included in the reaction. Similarly, a positive signal with M73 is conditional on the presence of E1A (see Figures 1, and 3−7).

Only a small amount of full length RB protein is produced by the *in vitro* translation reactions. Instead, a series of RB polypeptides are synthesized that arise from translational initiation at internal AUG codons, and all these products can be immunoprecipitated using an antibody that recognizes an epitope in the carboxy terminus of RB (Figure 1 and Whyte *et al.*, 1988; Dyson *et al.*, 1989b,c). Although for some experiments these internal initiation events would be undesirable, the appearance of these polypeptides is advantageous in mixing experiments. Initially, the nested set of peptides helped determine the general boundaries of the E1A protein and large T antigen binding sites. In subsequent mutagenesis the internal initiations served as useful internal controls, confirming the positions of deletions within the protein sequence and ensuring that samples were not mixed.

When RB polypeptides synthesized from the wild-type cDNA sequences were mixed with E1A-containing cell lysates or purified SV40 large T antigen, only RB polypeptides with mol. wts greater than or equal to ~60 000 daltons could be immunoprecipitated with the viral proteins (Figure 1). This indicates that the carboxy-terminal 60 K of the RB coding region contains all the sequences needed for binding to E1A and T antigen. There are three methionine

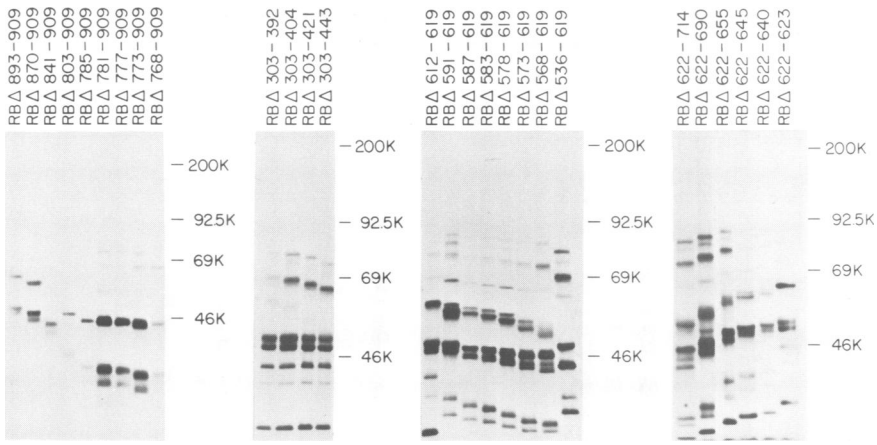

Fig. 3. Immunoprecipitation of RB deletion mutant polypeptides with anti-RB carboxy-terminal antibodies. [^{35}S]Methionine-labelled RB polypeptides, containing the deletions indicated, were synthesized in *in vitro* translation reactions and immunoprecipitated with 145, an anti-peptide polyclonal antibody that recognizes an epitope in a carboxy-terminal sequence present in all RB mutants generated by PCR. Labelled proteins were separated by SDS−polyacrylamide gel electrophoresis and detected by fluorography.

residues between amino acids 378 and 388 that are the initiating region for the 60 K bands that bind to E1A or T antigen. This assignment is confirmed by the more detailed mapping discussed below, but these internal initiation events provided the first indication that only a small region of the RB sequence was needed for interaction.

An approximate location of the carboxy-terminal boundary of the E1A or large T antigen binding region was determined by assaying the properties of RB polypeptides that were synthesized after linearizing the RB cDNA construct at different unique restriction enzyme sites within the coding sequence. Truncated RB polypeptides with carboxy termini at amino acids 912, 767 or 665 were synthesized after digestion of the cDNA with *Bsm*I, *Ssp*I or *Dra*III, respectively. The correct size of these truncated RB polypeptides was confirmed by immunoprecipitation of the translation products with the monoclonal antibody C36 (data not shown), an antibody that recognizes an epitope in the amino-terminal half of RB, probably between amino acids 300 and 380 (Whyte *et al.*, 1988). As shown in Figure 2, the RB polypeptides that terminated at amino acid 912 (*Bsm*I) were bound to and co-immunoprecipitated with E1A or large T antigen. RB polypeptides that had carboxy termini at either amino acid 767 (*Ssp*I) or amino acid 665 (*Dra*III) failed to bind to the viral proteins. Identical co-precipitation patterns were seen in experiments performed with E1A or large T antigen suggesting that RB sequences between amino acid 767 and amino acid 912 are needed for association in each case.

Generation of a series of deletion mutants in RB by PCR

In order to define more closely the regions of RB that are needed for association with E1A and large T antigen, a series of RB mutants that contained deletions in the RB coding region were produced using a variation on the polymerase chain reaction (PCR) technology (Ausubel *et al.*, 1988). The method used for mutagenesis was based on ideas suggested to us by Mike Wigler and John Colicelli. This method is detailed in Materials and methods and is described briefly here. A wide variety of deletions can be made between two unique restriction enzyme sites by incorporating one of the sites into the tail of a PCR primer. Taking the mutant RBΔ803-909 as an example, two primers were prepared in order to make this deletion within the RB cDNA sequence. The first primer was completely homologous to the RB sequence between amino acids 608 and 613 and was made in the sense strand. The second primer was complementary to the nucleotide sequence-encoding RB amino acids 797−802 and had the nucleotides needed for a *Bsm*I site added at the 5′ end. After polymerase amplification, the DNA was digested with *Mlu*I (which cuts at amino acid 620) and *Bsm*I. The mutated fragment was used to replace wild-type *Mlu*I−*Bsm*I RB sequences in the cDNA clone and a deletion was introduced between amino acids 803 and 909.

Importantly for these experiments, the observation that RB polypeptides that terminate at amino acid 912 were able to bind to both E1A and T antigen provided a convenient method to monitor the mutagenesis. The polyclonal antibody 145 was prepared against a peptide representing the carboxy-terminal 14 amino acids of RB (Whyte *et al.*, 1988), and this region is dispensible for binding. Therefore, we used the carboxy-terminal fragment from 912 to 928 as a immunological handle to tag mutants and allow the detection of the synthesized polypeptides. Thus, the validity of the reading frame after cloning and PCR can be assessed quickly by immunoprecipitating the resultant RB polypeptides with the anti-carboxy-terminal peptide antibodies.

Using this strategy, deletion mutants were prepared to determine the regions needed for binding to E1A or large T antigen. In order to identify the boundaries of the regions, deletions were prepared and tested in groups. The initial deletions were chosen to fall at the boundary of local secondary structures as predicted by Chou/Fasman statistical predictions determined using the Intelligenetics secondary structure prediction calculations. After these mutations were tested and the general location of a boundary was determined, more detailed mapping was achieved by dividing the target region into three, four or five equal deletions. This process was repeated until all of the boundaries were known to within a few amino acid residues. All mutations were checked for the production of appropriately sized polypeptides by analysis of total translated proteins on SDS−polyacrylamide gels (data not shown) and by immunoprecipitation with the 145

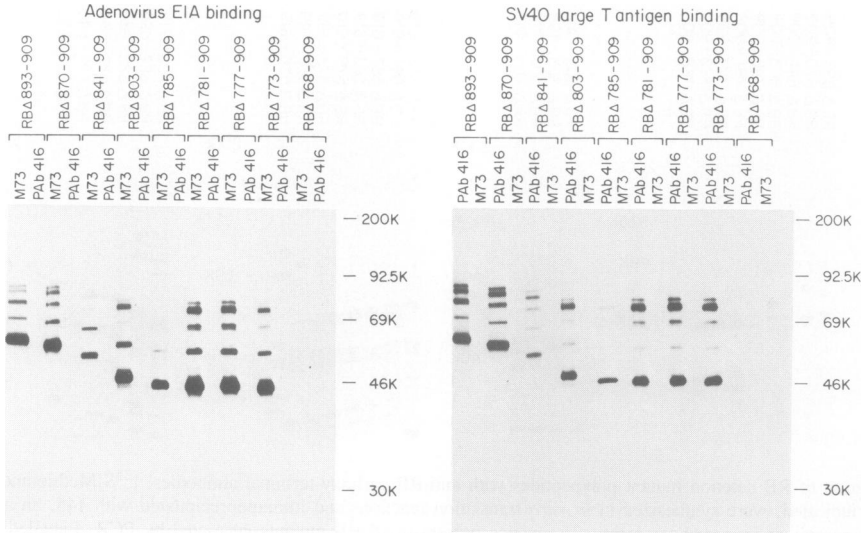

Fig. 4. Co-immunoprecipitation of RB carboxy-terminal deletion mutant polypeptides with E1A or T antigen. [^{35}S]Methionine-labelled polypeptides, containing the carboxy-terminal deletions indicated, were synthesized in *in vitro* translation reactions and mixed with either unlabelled 293 cell lysate (containing E1A) or purified SV40 large T antigen. Protein complexes were immunoprecipitated with the antibodies shown, separated by SDS–polyacrylamide gel electrophoresis and labelled proteins detected by fluorography.

carboxy-terminal antibodies (Figure 3). In addition, the precise structure of all mutations that were used to define boundaries for the binding regions were confirmed by nucleotide sequencing (data not shown).

The amino- and carboxy-terminal boundaries of E1A and large T antigen binding sequences

To begin the fine structure mapping of the sequences needed for binding to SV40 large T or adenovirus E1A, we first determined the carboxy-terminal boundary. Experiments using the *Bsm*I restricton sites had shown that sequences to the carboxy side of amino acid 912 were not required for association. Analysis of the mutants shown in Figure 4 demonstrated that sequences between 773 and 909 could be removed without affecting binding of either E1A or large T. Taken together with the *Bsm*I experiment, this indicated that the sequences amino-terminal to amino acid 772 were all that were required for binding. Deletion mutant RBΔ768-909 and other upstream deletion mutants (*Dra*III cut at amino acid 665) showed no detectable binding activity, indicating that the carboxy-terminal boundaries of the E1A and large T antigen binding sequences lies between residues 768 and 772 of the RB sequence.

The initial indication of the approximate amino-terminal boundary for the binding region was based on the ability of an RB fragment that initiated around amino acid 386 to bind E1A or large T. The mutations shown in Figure 5, in combination with the previous data, show that the amino-terminal boundary of the binding region lies between residues 392 and 404.

To ensure that the amino-terminal sequences upstream from residue 393 did not contribute to the interaction of E1A or large T antigen with RB polypeptides, we constructed a mutant that coded only for residues 393–772 and 912–928. As expected, this protein bound equally well to both E1A and large T antigen (data not shown). This demonstrates that all of the RB sequences needed for interaction are contained within residues 393–772 and excludes the possibility that homopolymers of pRB polypeptides might be responsible for the co-precipitation of the 60 K species seen in Figure 1.

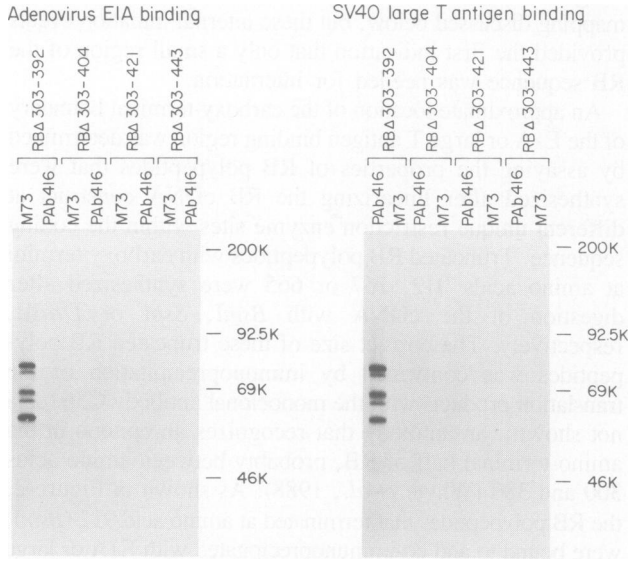

Fig. 5. Co-immunoprecipitation of RB amino-terminal deletion mutant polypeptides with E1A or T antigen. [^{35}S]Methionine-labelled polypeptides, containing the amino-terminal deletions indicated, were synthesized in *in vitro* translation reactions and mixed with either unlabelled 293 cell lysate (containing E1A) or purified SV40 large T antigen. Protein complexes were immunoprecipitated with the antibodies shown, separated by SDS–polyacrylamide gel electrophoresis and labelled proteins detected by fluorography.

Two regions of the RB coding regions are needed for binding with E1A or T antigen

As amino acids 392–772 still represented a significant portion of the RB sequence, we looked at the effects of deletions within this region on binding activity. Perhaps surprisingly, when sequences surrounding the *Mlu*I site at amino acid 621 were deleted, efficient binding could still be detected. Initially the two amino acids around the *Mlu*I site were removed and no decrease in binding was detected (RBΔ622–623). The *Mlu*I site was used as an initial site for preparing two or other sets of mutations, one set that removed sequences toward the amino terminus (Figure 6)

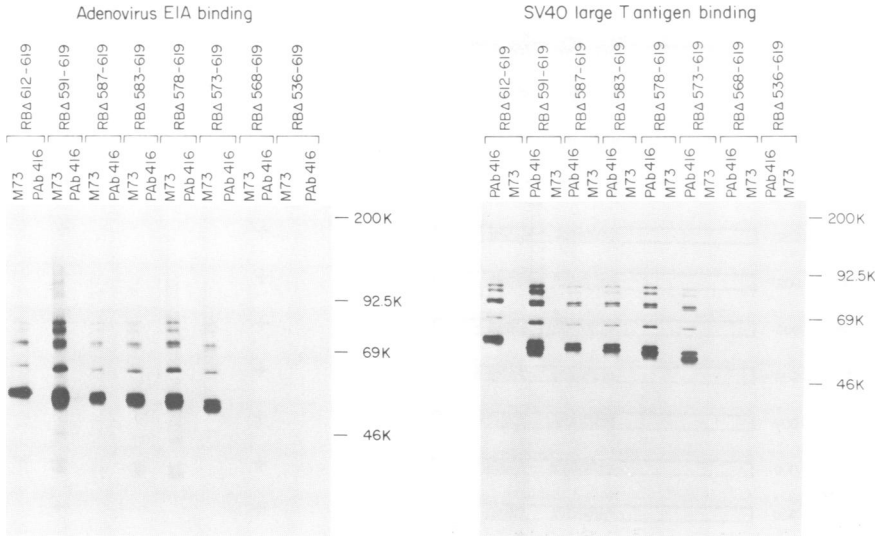

Fig. 6. Co-immunoprecipitation of RB internal deletion mutant polypeptides with E1A or T antigen. [^{35}S]Methionine-labelled polypeptides, containing the internal deletions indicated (towards the amino terminus), were synthesized in *in vitro* translation reactions and mixed with either unlabelled 293 cell lysate (containing E1A) or purified SV40 large T antigen. Protein complexes were immunoprecipitated with the antibodies shown, separated by SDS–polyacrylamide gel electrophoresis and labelled proteins detected by fluorography.

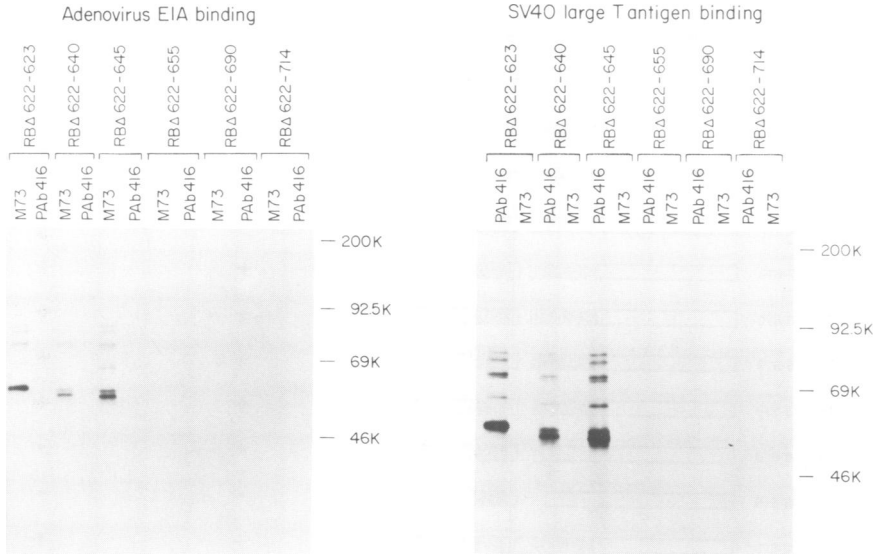

Fig. 7. Co-immunoprecipitation of RB internal deletion mutant polypeptides with E1A or T antigen. [^{35}S]Methionine-labelled polypeptide, containing the internal deletions indicated (towards the carboxy terminus), were synthesized in *in vitro* translation reactions and mixed with either unlabelled 293 cell lysate (containing E1A) or purified SV40 large T antigen. Protein complexes were immunoprecipitated with the antibodies shown, separated by SDS–polyacrylamide gel electrophoresis and labelled proteins detected by fluorography.

and one set that removed sequences toward the carboxy terminus (Figure 7). Using this strategy amino acids between 572 and 646 could be removed without affecting binding activity. However, when the entire region between 572 and 646 was removed in a single mutation, binding was not seen (data not shown). Although the exact structural reason for this result is not known, we assume that a spacer of some length is needed to allow the correct folding at this location. Possibly there is a requirement for a β-turn or random coil sequence to separate the two adjacent regions. Nevertheless, the boundaries are precise as long as an intervening sequence is present.

The structures of all the mutants described in this study are shown diagramatically in Figure 8 together with a summary of their binding properties. From these data we conclude that there are two non-contiguous regions of RB that are needed for complex formation with E1A or large T antigen and that these regions must be non-contiguous to achieve binding. The two regions include a fragment of 180 amino acid residues (393–572) and one of 127 residues (646–772). While these regions are larger than might be expected *a priori*, particularly when compared with the pRB-binding sequences of E1A, all of the small deletion mutations within these regions that we have tested to date disrupt binding activity completely (data not shown). At the level of detection provided by this assay, these regions of pRB appear to be identical in their binding properties for either E1A or large T antigen. A notable feature, apparent in

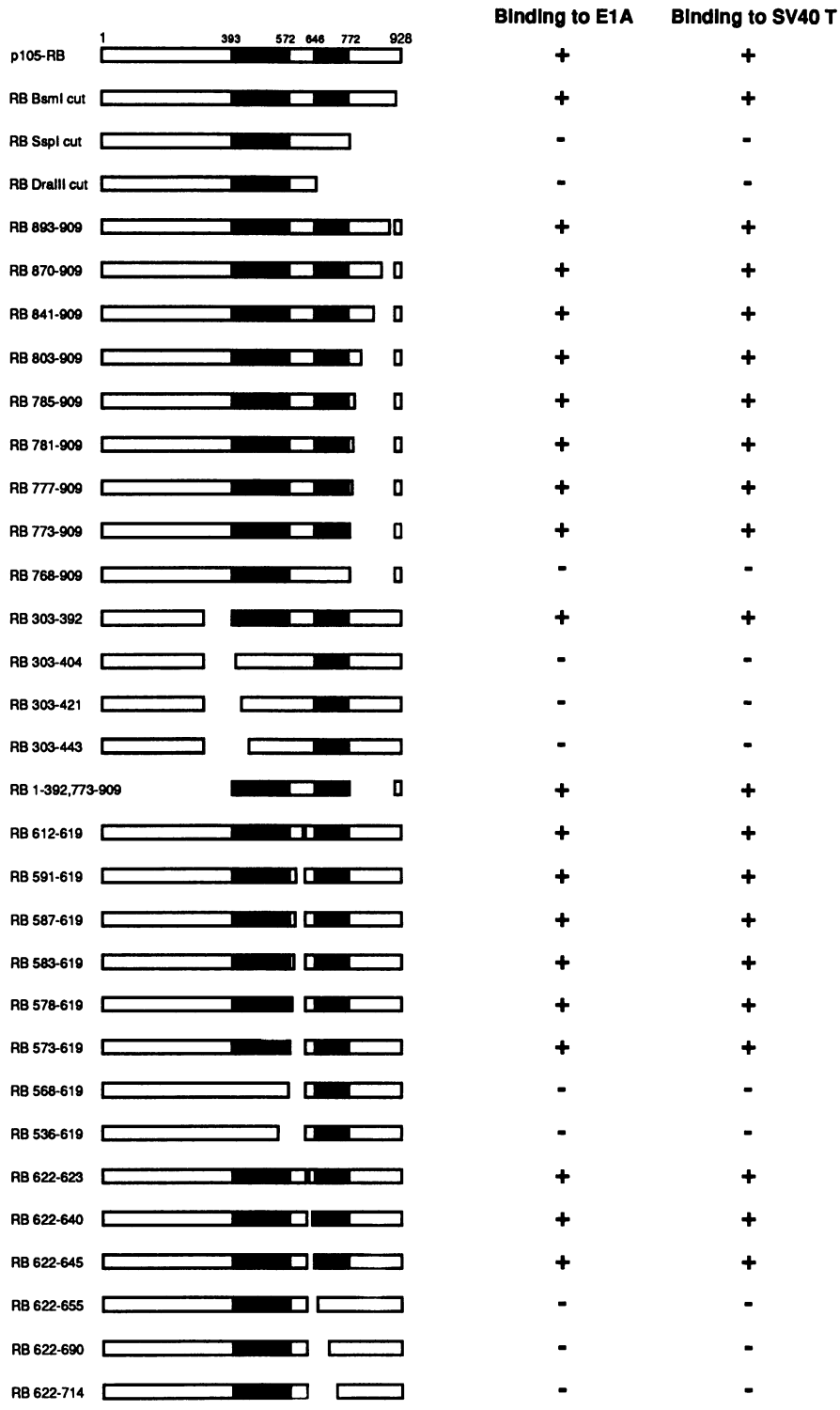

Fig. 8. Summary showing the structure of all the deletion mutants and their E1A- or T antigen-binding properties. The left column represents the physical map of all RB deletion mutants and the amino acids deleted in each mutant are shown. Two regions of RB that are essential for binding to E1A or large T antigen (amino acids 393–572 and 646–772) are indicated as solid boxes. In the mutants which fail to bind to E1A or T antigen, the binding site sequences that are present but insufficient for binding are open boxes. The right columns show the individual binding activities of each mutant.

Figures 4–7, is that each boundary of the binding regions shows an abrupt decline from 100% binding activity to no detectable binding with deletion of only a few amino acids. While we cannot exclude the possibility that this rapid decrease in signal is a feature of the assay system, we believe that these observations suggest that the binding regions represent discretely folded protein domains.

The E1A and T antigen binding regions on the retinoblastoma protein are common sites of naturally occurring RB mutations

Homozygous inactivation of the RB-1 gene has been detected in many tumors. In order to compare the relationship between DNA tumor viral oncoprotein binding and naturally occurring RB mutations, we compiled maps of all the RB

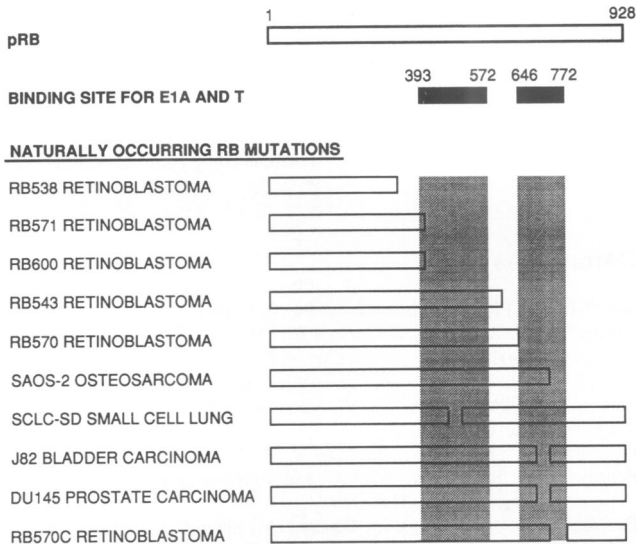

Fig. 9. Comparison of binding sites of RB to E1A/SV40 T antigen and naturally occurring RB mutations. The structures of naturally occurring RB mutants, which have been identified by DNA sequence, were collected. This comparison includes RB-1 mutations characterized in retinoblastoma, osteosarcoma, bladder carcinoma and prostate carcinoma cells (data from Dunn *et al.*, 1989; Horowitz *et al.*, 1989b; Shew *et al.*, 1989; R.Bookstein, J.-Y.Shew, P.Scully and W.H.Lee, personal communication). The open box at the top of the figure represents the RB coding region and solid boxes represent the regions of RB essential for binding to E1A and SV40 T antigen. The stippled regions indicate the positions of the binding regions relative to the RB sequences present in the naturally occurring mutants (open boxes). In each mutant amino acid sequences that are essential for binding to E1A and SV40 T antigen are absent.

mutations that have been determined to date by several different laboratories (Figure 9). These include RB mutations in cell lines derived from retinoblastomas, osteosarcomas, bladder carcinomas and prostate carcinomas. Each of these mutants fails to synthesize at least part of the regions of RB that are essential for binding to E1A or large T antigen. The most revealing data comes from comparison with the J82 bladder carcinoma, DU145 prostate carcinoma and the SCLC-SD small cell lung carcinoma. Both the J82 and DU145 tumor cell lines have been shown to contain mutations that cause the loss of exon 21 during mRNA processing (Horowitz *et al.*, 1989a; R.Bookstein, J.-Y.Shew, P.Scully and W.-H.Lee, personal communication). As a result, a mutant RB protein is synthesized in which exon 20 is fused directly to exon 22 and the correct reading frame is maintained. The missing region (amino acids 703−737) is located within the second binding region for complex formation with E1A and large T antigen. Similar types of mutations have been detected in the retinoblastoma cell line SCLC-SD (Shew *et al.*, 1989). Here a deletion mutation in the splice donor site causes the loss of exon 16 (amino acids 475−500) and damage part of exon 15 (amino acids 464−475). Again, the reading frame is maintained after splicing producing an internal mutation that is located within the first binding region that is essential for interaction with large T antigen and E1A.

From the results reported here we would predict that the RB proteins from J82, DU145 or SCLC-SD cells could not interact with either E1A or large T antigen. This is precisely the result previously reported by Horowitz *et al.* (1989a) for J82 cells, R.Bookstein, Y.-Y.Shew, P.Scully and W.-H.Lee (personal communication) for DU145 cells and Shew *et al.* (1989) for SCLC-SD cells. Similar predictions for the binding activities of other RB mutations have not been tested.

Discussion

We have generated a series of deletion mutants of the RB cDNA sequence that have been tested for their ability to interact with two viral oncoproteins, the adenovirus E1A and SV40 large T antigen. Two separate regions of RB are essential for association with E1A or large T antigen. The two regions include a fragment of 180 amino acid residues (393−572), and one of 127 residues (646−772).

A comparison of these binding sites on RB with the sites of naturally occurring mutations reveals that all of the RB mutations identified to date contain deletions in either one or both of the binding regions. The smallest of these deletions are perhaps the most interesting. Deletion of amino acids 703−737 (exon 21) or 738−775 (exon 22) or exon 16 plus part of exon 15 is sufficient to produce RB proteins that appear to be unable to function normally to restrict cell proliferation. The pRB with the 703−737 deletion (J82 or Du145 cell lines) or pRB from the SCLC-SD cell line have been shown to be unable to form stable complexes with E1A or SV40 T antigen. This correlation between functional inactivation and the loss of viral protein binding suggests that the binding regions for E1A and SV40 T antigen overlap with a functional domain of RB, and that by forming a complex with this domain the viral proteins are able to block a biologically important function of RB.

This suggestion fits well with previous models in which the DNA tumor virus transforming proteins were predicted to bind to and inactivate a normal RB function, thus removing a barrier to cell proliferation. While perturbation of RB function may facilitate tumor formation, it is clear that additional events are also necessary either for tumorigenesis or for transformation by these viral proteins. Strong genetic data support the conclusion that association with pRB is only part of the mechanism through which E1A- and SV40-mediated transformation occurs. In neither case is pRB association sufficient for transforming activity.

It is interesting to note that the regions of E1A and SV40 large T antigen involved in binding pRB also interact with a second cellular protein, 107 K, although the relative significance of associations with pRB and 107 K are unclear (Dyson *et al.*, 1989b; Ewen *et al.*, 1989). Since similar sequences are involved in binding to both pRB and 107 K, it is possible that these cellular proteins contain similar structures at their binding sites and may have functional similarities. The careful definition of the binding regions on pRB will allow more rational strategies to look for other polypeptides that can interact with viral transforming proteins.

Very short amino acid sequences within E1A and SV40 large T antigen are sufficient for association with pRB, and stable complexes can be formed readily between the RB protein and short synthetic peptides (N.Dyson, C.McCall and E.Harlow, in preparation). In contrast, the portions of pRB that are needed for association are much larger. This disparity in the sizes of the amino acid regions needed for binding is reminiscent of the interactions between antibody and antigen or receptor and ligand. Since it is impossible

that all of the sequences within pRB needed for association are actual contact sites for the viral proteins, we presume that the pRB sequences are also required to form a larger structure that defines the specificity and affinity of the interaction. This larger domain might provide a framework on which shorter regions are arranged to allow direct interaction. In view of its size it is possible that this region may form an enzymatic domain. These data could suggest many models for action; however, the most appealing is that the viral proteins act as competitive inhibitors, blocking the interaction between pRB and an important substrate or between pRB and an important ligand.

If viral proteins are able to modulate the activity of a functional domain of the RB protein, is the activity of pRB regulated in an analogous manner by a cellular protein? It is intriguing that transforming proteins from diverse DNA tumor viruses have evolved to interact with pRB, and it is possible that the pRB binding sequences have been acquired from cellular proteins. The overlapping positions of the E1A binding regions and the sites of naturally occurring mutation in pRB further suggest that such a protein might exist. However, this remains a provocative speculation.

Materials and methods

Oligonucleotide primer design and polymerase chain reaction (PCR)

Taking the mutant RBΔ803-903 as an example, two primers were prepared in order to make this deletion which is located between two unique restriction enzyme sites *MluI* (621) and *BsmI* (911). The first primer was an 18-mer oligonucleotide, 5′-TCTCCTGTAAGATCTCCA-3′, which is identical to the RB sequence coding for amino acids 608–613. The second primer was a 30-mer oligonucleotide, 3′-AATGCCTAAGGACCTCCCGCTTACGT-CTGA-5′. The first 18 nucleotides from the 3′ end of this primer are complementary to the RB sequence coding for amino acids 797–802. To these sequences a *BsmI* recognition sequence and four more extra nucleotides are added at the 5′ end.

The PCR reaction mixture included: 0.8 mM neutralized dNTP (Pharmacia), 1 μM each of two oligonucleotide primers, 100 ng of double strand RB cDNA, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 1 unit of *Thermus aquaticus* DNA polymerase (NEB) in a final volume of 50 μl. After covering with 50 μl of mineral oil, the reactions were carried out in a DNA thermal cycler (Perkin Elmer) at three different temperatures (94°C for 1 min; 55°C for 1 min and 72°C for 1.5 min) for 30 cycles.

The PCR amplified DNA fragments were examined by agarose gel electrophoresis. The appropriate sized fragments were cut from the gel and purified by Geneclean Kit (Bio 101). After digestion with *MluI* and *BsmI*, the fragment was cloned into the RB cDNA construct in place of the wild-type *MluI/BsmI* sequence.

Co-immunoprecipitation of the RB polypeptides with E1A or SV40 large T antigen

The procedures have been described previously (Dyson *et al.*, 1989b,c). A brief description is as follows: the RB cRNA was synthesized by *in vitro* transcription of the human cDNA with T7 RNA polymerase (Promega). The RB polypeptides were synthesized by *in vitro* translation of the cRNA in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S]methionine (Amersham). After mixing with cold detergent lysate of human 293 cells (E1A containing cells), or purified SV40 large T antigen, the proteins were immunoprecipitated with M73, a monoclonal antibody specific for adenovirus E1A (Harlow *et al.*, 1985), or PAb416, an monoclonal antibody specific for SV40 large T antigen (Harlow *et al.*, 1981). The protein complexes were resolved on a 10% SDS–polyacrylamide gels (Laemmli, 1970; Harlow and Lane, 1988) and detected by fluorography (Bonner and Laskey, 1974; Harlow and Lane, 1988).

Acknowledgements

We thank our colleagues at Cold Spring Harbor Laboratory for advice and discussions, M.Gilman, E.Moran and K.Buchkovich for careful reading of the manuscript, L.Duffy for excellent technical assistance, S.H.Friend, J.M.Horowitz and R.A. Weinberg for the RB cDNA construct and the 145 antibodies, D.McVey and I.Mohr for the gift of purified SV40 large antigen, R.McGuirk for providing the 293 cells, M.Iacovacci for synthesizing the oligonucleotides, J.Duffy, D.Green and P.Renna for art and photography work and M.Goodwin for typing the manuscript. N.D. is supported by Amersham International. This work was supported by Public Health Service grant CA 13106.

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Received December 27, 1989