The retinoblastoma gene product (pRB) participates in regulating mammalian cell replication. The mechanism responsible for pRB's growth regulatory activity is uncertain. However, pRB is known to bind viral transforming proteins including the papilloma virus E7 protein, cellular proteins, and DNA. pRB contains a critical domain termed the "binding pocket" which is required for binding activities. This binding pocket contains 8 cysteine residues. A naturally occurring mutation affecting one of these cysteines is known to eliminate pRB's protein and DNA binding activities. To investigate the cysteine residues in pRB's binding pocket, each residue was mutated to alanine, phenylalanine, or serine. These mutant genes were used to prepare pRBs harboring specific amino acid substitutions. Individual mutations at positions 407, 553, 666, and 706 depressed pRB binding to E7 protein, DNA, and a conformation-specific anti-pRB antibody, XZ133. Combinations of these inhibitory mutations exhibited additive inhibitory effects on pRB's binding properties. Mutations at positions 438, 489, 590, 712, and 853 did not affect pRB binding to E7 protein, DNA, or the XZ133 antibody. Combination of these five neutral mutations yielded a pRB species with full E7 protein, DNA, and XZ133 binding activities. These studies indicate that the cysteine residues at positions 407, 553, 666, and 706 contribute to the E7 protein and DNA binding properties of pRB and appear to do so by maintaining pRB's normal conformation.

The retinoblastoma suppressor gene product is a 105-kDa phosphoprotein (pRB105) (1, 2) which is thought to play a key role in regulating mammalian cell replication (3–5). The wild type retinoblastoma (RB)1 gene is normally expressed in all body tissues. However, mutated or partially deleted versions of the RB gene have been found in a variety of human tumors and tumor cell lines (6–9). Introduction of normal RB genes into these tumor cell lines retards their growth and inhibits their ability to form tumors in nude mice (10, 11). The association of tumors with the loss of normal RB alleles has given rise to the hypothesis that RB's normal function is to inhibit cell proliferation. The precise mechanism employed by the RB gene product (pRB) to block cell replication is unknown. However, two general biochemical properties of RB proteins have been described. First, pRB can form specific complexes with a variety of proteins including the viral transforming proteins of SV40, adenovirus, and human papilloma virus (HPV): large-T, E1A, and E7, respectively (12–14). pRB also binds normal cellular proteins (15–17) and has recently been shown to associate with the E2F/DRTF transcription factor complex (18–20). The segment of pRB that binds to these proteins consists of two discontinuous regions designated domains A and B, which encompass amino acids 394–571 and 649–772, respectively (21, 22). These regions have been proposed to form a "binding pocket" which is essential for binding of viral oncoproteins and normal cellular proteins to pRB. The second biochemical property associated with pRB is its ability to bind double-stranded DNA. RB protein binds to DNA in a non-sequence-specific manner which is commonly demonstrated using random fragments of calf thymus DNA (23–25). The functional significance of pRB's DNA binding activity is unknown.

Both full-length pRB105 and a smaller 60-kDa version of the RB protein (pRB60) contain the protein binding pocket and exhibit DNA binding activity. The critical amino acid residues within these pRB species that govern their biochemical properties are as yet undefined. Nonetheless, two observations call attention to the cysteine residues within the RB protein binding pocket. First, a naturally occurring mutant form of pRB has been found which contains a point mutation that converts Cys-706 to phenylalanine. This mutant protein fails to bind viral transforming proteins and lacks cell growth-inhibitory activity in vitro (26, 27). Second, examination of the amino acid sequence of the related adenovirus E1A and HPV-16 E7-binding protein, p107, suggests that these residues are important (28). Four of the 8 cysteine residues within the pRB60 binding pocket are conserved in p107. The biochemical mechanism underlying the importance of these residues is unclear. However, cysteines commonly contribute to structural interactions via hydrogen bonds, disulfide bonds, or coordinate metal ion binding. The presence of critical cysteine residues within the binding pocket of pRB suggests that one or more of these biochemical activities may be important for maintaining the functional integrity of RB proteins.

To characterize the contribution of each cysteine residue in the pRB binding pocket to the biochemical function of RB protein, individual cysteine residues were mutated to alanine or other amino acids. A series of altered RB proteins were obtained from these mutated genes and analyzed for their abilities to bind HPV-16 E7 protein, DNA, and a conformation-specific anti-pRB antibody.

MATERIALS AND METHODS

Plasmids—The DNA sequence encoding human pRB was cloned from a human lung fibroblast library (Clontech, Palo Alto, CA) using

4 Cysteine Residues in the Protein Binding Pocket*

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1The abbreviations used are: RB, retinoblastoma protein; ELISA, enzyme-linked immunosorbent assay; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPV, human papilloma virus.
oligonucleotide probes derived from a published RB nucleic acid sequence (1, 29). The cloning of the 60-kDa fragment of pRB105 which begins at Met-387 has been described previously (30). The substitution mutations were made by site-directed mutagenesis using oligonucleotide probes and the pSelect mutagenesis system (Promega, Madison, WI). The substitution mutants were placed in pGemZ (Promega) for in vitro transcription-translation. Combinations of substitution mutants were made by either successive site-directed mutagenesis or by swapping restriction fragments directly containing the desired single mutation. All mutations were confirmed by DNA sequencing.

**Reticulocyte-translated pRB— EcoRI-linearized pGemZ-RB constructs were used to transcribe capped mRNAs using T7 DNA polymerase according to the protocols described by the manufacturer (Promega). The mG655;pppG555Gm cap analog was obtained from Pharmacia LKB Biotechnology Inc. Rabbit reticulocyte lysate translations were performed as described by the manufacturer (Promega) using [35S]methionine (Amersham Corp., 1100 Ci/mmol).

**Recombinant HPV-16 E7**—The HPV-16 E7 gene (P. M. Howley, National Cancer Institute, Bethesda, MD) was cloned in a Tac promoter bacterial expression plasmid and expressed as a fusion protein with the addition of 16 additional amino acids at the N-terminus of E7. Expression and purification have been described previously (31).

**Peptide Synthesis**—Peptides were prepared by solid-phase synthesis using a double coupling protocol on the model 430A Applied Biosystems automated peptide synthesizer. Deprotection, purification and identity testing were as described previously (30). The generating peptide sequence was TDLICYEQLN-amide. Lyophilized peptides were taken up in 0.1 M Tris-HCl, pH 7.5, and 1 mM dithiothreitol and passed over a 1.5-ml column of DNA-cellulose at 4°C (Pharmacia). The E7(20–29) peptide was synthesized, lyophilized, and diluted in 50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, 1 mM diethiothreitol, 0.1% Triton X-100, pH 7.2) and added to a 1:200 dilution in LB buffer at 23°C generating the desired single mutation. All mutations were confirmed by DNA sequencing.

**rppB** and **rppB:** **Immunoprecipitation**—Equivalent quantities of[^*]-labeled reticulocyte-translated pRB60 and pRB60 mutants were diluted 1:10 in LB buffer (final volume 250 μl) at 4, 23, or 30°C, and anti-pRB monoclonal antibody XZ133 (32) was added at a final dilution of 1:100,000. The reactions were incubated at the desired temperatures for 40 min at which time 3 μl of undiluted rabbit antimouse antibody (Organon Teknika, West Chester, PA) was added and incubation continued for 40 min. Antibody-pRB complexes were precipitated by adding 100 μl of a 1:10 slurry of protein A-Sepharose to the reactions and incubating at the respective temperatures for an additional 40 min. Sepharose pellets were collected by centrifugation and washed with LB buffer and used to elute E7-RB complexes.

**Critical Cysteines in the Retinoblastoma Protein**

**RESULTS**

The RB protein binding pocket contains 8 cysteine residues (see Fig. 1). Four cysteines lie in domain A, 3 lie in domain B, and 1 cysteine is present in the linker region between domains A and B. Each of these cysteines was individually mutated to an alanine residue. The cysteine residue at position 853 in the C-terminal region of pRB60 lying outside the protein binding pocket was also changed to an alanine. Additionally, the cysteine residues at positions 666 and 706 in the binding pocket were changed to either serine or phenylalanine. To examine the effects of these substitutions on the binding properties of pRB60, each of the mutated RB genes was transcribed and translated in vitro to produce radiolabeled pRB60 proteins harboring specific cysteine substitutions (see "Materials and Methods").

The radiolabeled pRB60 wild type and mutant proteins were individually incubated with bacterially produced recombinant HPV-16 E7 protein (31) and immunoprecipitated using an anti-E7 antibody. As seen in Fig. 2, the translation products of each pRB60 species exhibited similar levels of radiolabel incorporation and product purity as evidenced by comparable SDS-PAGE gel analyses. In comparison to the wild type pRB60 immunoprecipitate, it is evident that alanine substitutions at cysteine residues 438, 489, 590, 712, and 853 had little or no effect on pRB binding to the E7 protein. By contrast, alanine substitutions at cysteine residues 407, 553, 666, and 706 produced pRB60 species with varying degrees of reduced binding to E7 protein. To better quantify the E7 binding activity of these mutated pRB60 species, a separate series of experiments were performed using a pRB-HPV-16 E7 protein ELISA-style binding assay. In this assay, recombinant pRB

**FIG. 1.** Cysteine residues in pRB105 and pRB60. The C-terminal pRB60 fragment of pRB contains the A and B domains, which form the HPV-16 E7 binding pocket, and 9 of the 15 cysteine residues present in full-length pRB.
Critical Cysteines in the Retinoblastoma Protein

**Fig. 2. Binding of HPV-16 E7 protein to pRB60 cysteine substitution mutants.** Wild type and mutant pRB60s were translated and 35S-labeled in reticulocyte lysates as described under "Materials and Methods." The in vitro translated products for each mutant are shown in panels A and B under the heading Translations. The slowest migrating band represents pRB60 with other bands precipitated with E7 in the presence of recombinant HPV-16 E7 protein species were added to the matrix in solution. The unbound pRB60 was washed away, and the bound pRB60 was quantitated using an anti-pRB monoclonal antibody. The results of these assays are reported in Table I. In general, these studies are in agreement with the immunoprecipitation analyses. Alanine substitution at position 706 was most deleterious to E7 binding followed by similar substitutions at positions 666, 407, and 553. Substitutions of serines for cysteines at positions 666 and 706 and substitution of phenylalanine for cysteine at position 706 also impaired E7 binding. The phenylalanine substitution at position 706 had the most deleterious effect on E7 binding activity, while the alanine substitution had the least effect on E7 binding. In contrast to the relative effects of serine versus alanine substitutions at position 706, the alanine substitution at position 666 reduced E7 binding more than the serine substitution at the same position.

Substitution mutations for cysteine residues involved in hydrogen bonding or coordinate metal ion bonding might be expected to exhibit additive deleterious effects on E7 binding when incorporated into the same mutant protein. Alternatively, substitution mutations for cysteine residues involved in disulfide bonds would not be expected to sum their effects on E7 binding once the possibility of forming a critical disulfide bond was eliminated by mutating either single residue from the cysteine pair comprising the disulfide bond. To determine whether multiple simultaneous cysteine substitutions would exhibit additive effects on pRB60 binding to E7 protein, mutant pRB60 species were created that combined two or more alanine substitutions. The 4 cysteine residues at positions 407, 553, 666, and 706 were initially chosen for substitutions in the same RB gene yielded pRB60 species with a greater loss of E7 binding activity than seen with either alanine substitution alone (see Table I). This result suggests that these residues do not participate in disulfide bonds in the pairwise combinations examined here. Lastly, a mutant pRB60 species was prepared combining all five cysteine to alanine substitutions within pRB60 that did not affect E7 binding activity when examined as single substitution mutants. The pRB60 proteins corresponding to these substitution mutants were prepared and analyzed in the E7 ELISA binding assay. In both cases, the combination of two alanine substitutions in the same RB gene yielded pRB60 species with a greater loss of E7 binding activity than seen with either alanine substitution alone (see Table I). This result suggests that these residues do not participate in disulfide bonds in the pairwise combinations examined here.

![Diagram](image-url)

**TABLE I**

| E7 protein species | Relative affinity for pRB60 |
|-------------------|---------------------------|
| Wild type         | 100                       |
| Ala-407           | 35                        |
| Ala-438           | 100                       |
| Ala-489           | 90                        |
| Ala-553           | 25                        |
| Ala-590           | 100                       |
| Ala-666           | 20                        |
| Ser-666           | 40                        |
| Ala-706           | 5                         |
| Phe-706           | <1                        |
| Ser-706           | 1                         |
| Ala-712           | 90                        |
| Ala-853           | 90                        |
| Ala-407 + Ala-553 | 5                         |
| Ala-666 + Ala-706 | 1                         |
| Cys-4             | 100                       |

Reticulocyte translated radiolabeled pRB60 and pRB60 mutants were analyzed for their ability to bind recombinant HPV-16 E7 protein as described under "Materials and Methods." The data presented represent the average of at least two experiments. All assays were run in triplicate. The variation between triplicates was less than 10%.
positions 407, 553, 666, and 706 yielded pRB60 species with reduced DNA binding activity. Serine substitutions for cysteines at positions 666 and 706 and a phenylalanine substitution at position 706 also reduced pRB60's DNA binding activity. Additional DNA binding studies were performed using pRB60 species harboring multiple alanine substitutions. The combination of alanine substitutions at positions 666 and 706 decreased DNA binding activity to a greater extent than occurred with either substitution alone. By contrast, the combination of alanine substitutions for cysteines at positions 438, 489, 590, 712, and 853 in the Cys-4 mutant had no apparent effect on pRB60's ability to bind DNA. These results were similar to the effects of substitution mutations on the E7 binding activity of pRB60 described above. The same cysteine residues that were important for maintaining pRB60's ability to bind E7 protein were also important for maintaining pRB60's ability to bind DNA. Similarly, those substitution mutants which did not affect pRB60 binding to E7 protein also did not affect pRB60 binding to DNA.

The tight correlation between the E7 and DNA binding properties of the pRB60 substitution mutants suggests that the protein domains within pRB60 that are responsible for both of these biochemical properties are identical or exist in close proximity to one another. Alternatively, the correlation between protein and DNA binding properties of pRB60 may reflect a common response to conformational changes in pRB60 caused by these substitution mutations. To explore how these mutations might affect both the E7 protein and DNA binding properties of pRB60, a new series of immunoprecipitation studies was performed. These experiments were carried out using a conformation dependent anti-pRB monoclonal antibody XZ133 (32) and our series of mutated pRB60 proteins produced in reticulocyte translation reactions. The XZ133 antibody recognizes native RB proteins that are capable of binding viral transforming proteins, but does not bind to denatured RB proteins. As seen in Fig. 5, at 4 °C the XZ133 antibody effectively immunoprecipitated wild type pRB60 and the Ala-407, Ala-489, Ala-553, Ala-666, Ser-666, Ala-706, and the Cys-4 combination mutants. In contrast, the Ala-407 plus Ala-553, Ala-666 plus Ala-706, and the Phe-706 mutant proteins showed a reduced efficiency of precipitation relative to wild type pRB60.

Since conformational flexibility can change with increasing temperature and altered flexibility might be expected to affect the conformation of pRB60, the immune precipitation reactions were also run at 23 and 30 °C. The lower panels in Fig. 5 show that several of the substitution mutants which behaved similarly to wild type pRB60 at 4 °C exhibited progressively poorer binding to the antibody with increasing temperature. At 30 °C all of the mutants which showed altered E7 protein or DNA binding activity also bound less well to the conformation-dependent antibody. Again, the combinations of alanine substitutions at positions 407 plus 553 and 666 plus 706 yielded pRB60 species less capable of immunoprecipitation by XZ133 than found with the corresponding single-residue substitution mutants. As in the E7 protein and DNA binding assays the Phe-706 mutation was the most deleterious to antibody binding. The Ala-489 mutation and the Cys-4 combination mutations bound the antibody with the same efficiency as wild type pRB60 at all temperatures. A second conformation-sensitive monoclonal antibody, XZ104, was examined at 4 °C and yielded the same results as XZ133 (data not shown). In addition, a polyclonal rabbit antiserum to pRB60 was examined. As expected, the polyclonal antiserum, which presumably does not depend on pRB60's conformation for its binding activity, precipitated all the mutant pRB60 species equally well. Taken together, these studies argue that substitution mutations at positions 407, 553, 666, and 706 in the pRB60 protein binding pocket alter the conformation of pRB60. The progressive deleterious effect of increasing temperature on the binding of XZ133 to the four critical cysteine mutants suggests deterioration of pRB60's conformational stability at higher temperatures. This result supports the hypothesis that the 4 critical cysteines at positions 407, 553, 666, and 706 participate in maintaining the conformational integrity of the pRB60 protein binding pocket.

**DISCUSSION**

Substitution mutations at 4 of the 9 cysteine positions in pRB60 affected both the HPV-16 E7 protein and DNA-cellulose binding activities of pRB60. Alanine substitutions at positions 407 and 553, alanine or serine substitutions at

![Fig. 3. Co-immune precipitation of wild type pRB60 and Cys-4 pRB60.](image)

A co-immune precipitation of radiolabeled wild type (WT) pRB60 or the Cys-4 pRB60 mutant was performed using recombinant HPV-16 E7 protein and an anti-E7 antibody. The experiment was conducted as described in Fig. 2. The reticulocyte lysate translation products of the WT pRB60 and Cys-4 pRB60 species are shown on the left. The relative levels of pRB60 coprecipitation in the presence or absence of E7 protein and E7 peptides are shown on the right. Lanes are labeled as follows: A, pRB60 plus E7; B, pRB60 minus E7; C, pRB60 plus E7 and E7(20–29) peptide; D, pRB60 plus E7 and scrambled E7(20–29) peptide.

![Fig. 4. Binding of pRB60 and pRB60 mutants to DNA.](image)

Reticulocyte translated 35S-labeled pRB60 and substitution mutants were passed over DNA-cellulose columns in HNMDT buffer at 4 °C and eluted as described under "Materials and Methods." The fractions collected were analyzed by SDS-PAGE and fluorography. The impaired binding ability of the mutants at this salt concentration is illustrated by increased amounts of full-length pRB60 in the flow-through. Gel loads were normalized for collected total volume of the individual fractions to give a semi-quantitative comparison of pRB60 distribution between flow-through and DNA-bound fractions.
position 666, and alanine, serine, or phenylalanine substitutions at position 706 all adversely affected pRB60's protein and DNA binding activities. Amino acid changes at position 706 exhibited the strongest deleterious effects on RB60's ability to bind E7 protein or DNA. Interestingly, position 706 is also the site of a naturally occurring phenylalanine substitution mutation associated with a small cell lung cancer (26). Single-residue mutations at position 666 exhibited the next most deleterious effects on binding activities, while substitutions at positions 407 and 553 showed lesser degrees of impairment. By contrast, alanine substitutions at positions 438, 489, 590, and 712, and 853 caused no detectable change in RB60's binding properties. It should be noted that these neutral mutations were scattered throughout pRB60 while the four deleterious mutations were all found in the A and B domains (see Fig. 1). These observations are consistent with the hypothesis that the RB binding pocket is a critical region that contributes to both the protein and DNA binding properties of RB proteins. Moreover, these studies suggest that the cysteine residues at positions 407, 553, 666, and 706 play an essential role in maintaining these biochemical properties.

It is important to note that the E7 protein and DNA binding properties of pRB60 were affected similarly by the substitution mutants examined in this study. The five neutral mutations failed to alter either E7 protein binding or DNA-cellulose binding while the four deleterious mutations impaired both of these activities. Moreover, those mutations which most severely affected pRB60's E7 protein binding activity, substitutions at positions 666 and 706, also exhibited maximal impairment of DNA binding. This apparent linkage of the E7 protein and DNA binding properties of pRB60 was not entirely unexpected. We previously showed that E7 protein binding to pRB60 impaired the ability of pRB60 to bind DNA (25). Those experiments suggested that pRB60's E7 protein and DNA binding properties were influenced by the same or adjacent regions of the RB protein. The current study indicates that changes in specific cysteine residues in the binding pocket of pRB60 affect both of these biochemical properties in parallel. Two mechanisms can be hypothesized to explain the parallel fall in pRB60's binding activities associated with the substitution mutations at positions 407, 553, 666, and 706. First, the cysteine residues normally present at these positions may serve as direct contact points between pRB60 and both E7 protein and DNA molecules. Second, these cysteine residues may help to form or maintain the proper conformation of pRB60 needed to bind E7 protein and DNA. Our data supports the latter hypothesis since it appears unlikely that the pRB60 cysteines at positions 407, 553, 666, and 706 would form contacts with both E7 protein and DNA while the nearby cysteines at positions 438, 489, 590, and 712 failed to make contact with either E7 protein or DNA. Moreover, the decrease in conformation-specific antibody recognition of pRB60 associated with the substitution mutations at positions 407, 553, 666, and 706 and the temperature sensitivity of this recognition suggests that these mutations led to a change in pRB60's conformational structure. This interpretation of our immunoprecipitation analyses is supported by the fact that the antibody used in these studies, XZ133, is dependent upon the conformation of pRB60 in order to recognize and immunoprecipitate RB proteins (32). Moreover, the differential effect of temperature on antibody binding to the critical substitution mutants suggests that the native conformation of pRB60 becomes labile at higher temperatures. The epitope recognition site for this antibody has been mapped to RB protein residues 444–535 or 620–665 (32). These segments of the RB protein do not contain any of the critical cysteine residues identified in this study. Therefore, the antibody's impaired ability to bind the critical cysteine mutants cannot be attributed to the loss of contact site residues within the A and B domains of the binding pocket.

The current study clearly demonstrates that the cysteine residues at positions 407, 553, 666, and 706 are important amino acids in maintaining pRB60's biochemical functions. Examination of the amino acid sequence of the related adenovirus E1A and HPV-16 E7 binding protein, p107 (28), also suggests that these residues are important. While the overall amino acid conservation between pRB105 and p107 is 34%, only 1 out of 8 cysteine residues—407, 489, 666, and 706—within the pRB binding pocket are conserved in p107. Three of these 4 residues (407, 666, and 706) are important in maintaining pRB60's biochemical binding properties. By contrast, only 1 of the 4 cysteine residues that are not conserved between the pRB binding pocket and p107 (553, 438, 489, 590, and 712) proved to be important for maintaining binding activity. Substitution of alanine for cysteine at position 553 reduced pRB60's binding properties. This correlation between the conservation of cysteine residues in pRB and p107 and the requirement of these residues for maintaining pRB60's binding properties reinforces the hypothesis that cysteine residues play a critical role in cellular proteins that bind the E1A and E7 viral oncoproteins.

Cysteine residues are known to serve at least three structural functions in proteins: formation of disulfide bonds, hydrogen bonding, and coordinate bond formation with metal ions. The results presented here do not conclusively implicate or eliminate any of these functions as possible mechanisms underlying the function of the cysteines at positions 407, 553, 666, and 706. However, disulfide formation appears to be an unlikely mechanism involving these residues both because the internal milieu surrounding intracellular proteins does not favor disulfide bond formation, and because the pairwise substitution mutations examined in this study did not support this hypothesis. Additional biophysical studies will be needed to determine whether the critical cysteine residues in pRB60's protein binding pocket are simply involved in maintaining
the proper conformation of RB proteins or participate in other biochemical functions.

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