Purification and Characterization of a Functionally Homogeneous 60-kDa Species of the Retinoblastoma Gene Product*

(Received for publication, January 21, 1992)

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The retinoblastoma susceptibility gene (RB) encodes a 928-amino acid protein (pRB) that is hypothesized to function in a pathway that restricts cell proliferation. The immortalizing proteins from three distinct DNA tumor viruses (SV40 large T antigen, adenovirus E1a, and human papilloma virus Type 16 E7) have been shown to interact with RB protein through two non-contiguous regions comprised of amino acids 393-572 (domain A) and 646-772 (domain B). We constructed a truncated form of RB (RB p60) that retains these two domains but eliminates the N-terminal 386 amino acids of RB. RB p60 was expressed in Escherichia coli in inclusion bodies. After solubilization, it was refolded in the presence of magnesium chloride, and the active protein was isolated with an E7 peptide affinity column. The protein that elutes from this column is functionally homogenous in its ability to bind immobilized E7 protein. Thermal denaturation studies provide additional evidence for the conformational homogeneity of the isolated protein. This purification scheme allows the isolation of significant amounts of RB p60 protein that is suitable for structural and functional studies.

The retinoblastoma susceptibility gene is a member of a growing collection of genes that encode proteins that perform a growth suppressor function in the cell (for a recent review, see Ref. 1). Mutational inactivation of the retinoblastoma susceptibility gene has been linked to the development of pediatric retinoblastomas and has more recently been associated with other types of cancers, including small cell lung carcinoma and breast cancer (2, 3). In normal cells, pRB undergoes changes in its phosphorylation state that correlate to specific stages in the cell cycle (4–6). It has been hypothesized that the retinoblastoma gene product regulates growth of normal cells, since its inactivation by genetic changes leads to uncontrolled cellular proliferation (2, 3). An alternative mechanism for RB "inactivation" has been proposed to explain the immortalizing effects of proteins from three different viruses, SV40 large T antigen, adenovirus E1A, and HPV-16 E7. Immuno precipitation studies demonstrate that these viral proteins form complexes with pRB under physiological conditions (7–10). This interaction may be crucial to the immortalizing function of these proteins, since by binding pRB, the viral proteins may prevent pRB from interacting with normal cellular partners, thus effectively inactivating it. Several candidate cellular pRB-binding proteins have recently been identified, including the cellular transcription factor E2F (11–16). Antagonists of the E7/pRB interaction might be useful for the treatment of cervical cancer (17). An understanding of the molecular basis of this interaction would facilitate the design of these antagonists. Sequence homologies between E7, E1A, and SV40 large T antigen suggest a relatively small contact site on these proteins (9, 18). Peptides based on this sequence homology proved effective at blocking binding of E7 to pRB (19, 20). Mapping of the contact domain on pRB has proved more difficult. Deletion analysis indicates that the existence of two noncontiguous domains within pRB that are necessary for viral protein binding: amino acids 393–572 (domain A) and 646–772 (domain B) (21–23). The individual amino acids that make actual contact with the viral proteins during complex formation are currently unknown.

In order to isolate sufficient quantities of RB protein for structural and functional studies, we constructed a truncated form of RB protein that deletes the N-terminal 386 amino acids but retains domains A and B (RB p60). The protein was expressed in Escherichia coli and isolated from inclusion bodies. A novel refolding and affinity purification scheme afforded functionally homogeneous RB p60.

MATERIALS AND METHODS

Construction and Expression of RB p60—A human lung 1g11 cDNA library (Clontech, Palo Alto, CA) was screened for RB clones using end-labeled oligonucleotide-specific probes based on the published RB sequence (24). One of the clones identified contained a 1.9-kilobase EcoRI insert that encompassed amino acids 300–928 of the published RB sequence. This fragment was subcloned into M13, and single-stranded DNA was prepared. Using site-specific mutagenesis, a unique SpeI site was engineered in at amino acid position 386. Cutting with SpeI makes the methionine at amino acid position 387 the initiating methionine. The SpeI/EcoRI fragment was ligated into a PUC19 vector, and then the pTAC-RB196 plasmid was generated by digesting the PUC19 plasmid with HindIII and EcoRI and subcloning into the pTAC bacterial expression plasmid (25) (Fig. 1). The resultant clone was identified by hybridization to labeled oligonucleotide probes and by sequencing.

Fermentation—Fermentation of E. coli containing the plasmid pTAC-RB196 was performed from frozen vials of cells. The cells were grown at 37 °C in 3 × LS medium (15 g/liter yeast extract (Difco), 30 g/liter soya peptone (Sherfield), 10 g/liter sodium chloride; plus 100 mg/liter ampicillin, until they reached midlog growth (A600 = 5, 6 h). Isopropylthiogalactoside was added at a final concentration of 1 mM, and 2 h later (A600 = 10), the cells were harvested by membrane filtration and subsequent centrifugation. The cell paste, about 2 kg from 200 liters, was then frozen and stored at –70 °C.

Purification and Refolding—All steps were carried out at 4 °C

1 The abbreviations used are: RB, retinoblastoma gene; PBS, phosphate-buffered saline; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPV, human papilloma virus.

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Fig. 1. Plasmid construct for RB p60. The plasmid used to express RB p60 in *E. coli* pTAC-RB195 contains a pTAC promoter followed by an open reading frame that begins with 12 amino acids resulting from cloning procedures, followed by RB-(387–928).

unless otherwise noted. Cell paste (approximate 200 g) was resuspended in phosphate-buffered saline (PBS, 6 mM sodium phosphate, 150 mM NaCl, pH 7.2) containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl₂, 0.01 mg/ml DNase I, and 0.1 mg/ml lysozyme at 2.5 ml/g cell paste. The mixture was stirred for 30 min, and the cells were then lysed by sonication. An additional 0.5 volume of PBS was added, and the mixture was centrifuged for 20 min at 13,000 × g. PBS and solid urea were added to the pellet to give a final urea concentration of 6 M and a volume of 3 ml/g cell paste. Triton X-100 was added to 1%, and the mixture was stirred for 1 h. After dilution with 1 volume of PBS, the solution was centrifuged for 20 min at 13,000 × g. The pellet was washed with PBS and then resuspended in 50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1 mM EDTA. Solid guanidine hydrochloride was added to give a final concentration of 6 M guanidine hydrochloride and approximately 2 ml/g cell paste. Following a brief incubation, the solution was centrifuged for 20 min at 13,000 × g. The supernatant was then frozen.

Subsequent purification steps were typically performed on a 10-ml aliquot of the guanidine extract (resulting from approximately 7 g of cell paste). Solid dithiothreitol was added to 0.1 M, and the mixture was stirred at 30 °C for 30 min. Refolding was achieved by a dropwise 100-fold dilution of the extract into 1 liter of buffer containing 0.1 M Tris-Cl, pH 8.0, 0.4 M MgCl₂, 0.6 M guanidine hydrochloride, 0.1 mM EDTA, 0.1 mM DTT, and a protease inhibitor mixture (10 µg/ml benzamidine and 5 µg/ml leupeptin, pepstatin A, and aprotinin). The refolding mixture was stirred for at least 5 h, and then centrifuged for 60 min at 10,000 × g. The buffer was changed to 50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% Nonidet P-40, and 1 mM DTT by cross-flow filtration using an EraLab cross-flow cartridge (Microdyne, Raleigh, NC). The dialysate was centrifuged for 1 h at 11,000 × g and was then sequentially filtered through 0.45- and 0.2-µm filters.

The filtrate (approximately 1 liter) was sequentially passed through a 2-ml ethanolamine-capped Affi-Gel 15 (Bio-Rad) guard column and a 2-ml E7-(20–29) peptide amide Affi-Gel 15 column at a flow rate of approximately 1 ml/min. The columns were washed with 50 ml of dialysis buffer, and the guard column was then disconnected. The affinity column was eluted with unbuffered 50 mM Na₂CO₃, 1 mM DTT. Three-ml fractions were collected in tubes containing 0.3 ml of 1 M HEPES, pH 7.0, to neutralize the elution buffer. The active RB p60 typically eluted in fractions 2–4. Protein was quantitated by the Bradford assay (26) with standardization by amino acid composition analysis.

**RESULTS**

Upon induction of the pTAC-195 *E. coli* expression system with isopropyl-β-D-thiogalactoside, RB p60 was produced at approximately 2% of the total cell protein (Fig. 2A). After lysing the cells by sonication, virtually all the induced protein was recovered in the centrifugation pellet. The RB p60 in the lysate pellet remained insoluble when resuspended in 6 M urea, 1% Triton-X100, whereas other contaminating materials dissolved and were removed by centrifugation. Sedimentation of RB p60 protein was achieved in 6 M guanidine hydrochloride, 0.1 M DTT.

Many attempts were made to refold RB p60 by dilution or dialysis from the guanidine/DTT solution into a wide variety of buffers, but this generally resulted in precipitation of the protein, with no soluble RB p60 capable of binding E7. Under certain conditions, the presence of 0.4 M MgCl₂ in the diluting buffer appeared to facilitate proper folding of RB p60, as determined by the E7 binding assay (Table I). The role of the MgCl₂ in promoting refolding of the protein is unclear, since bound Mg²⁺ does not appear essential to RB p60's E7-binding activity (data not shown).

Although the majority of the RB p60 was soluble after the refolding step, it was assumed to be conformationally hetero-
TABLE I

| Fraction                  | Protein (mg) | Total activity (units/mg) | Specific activity (units/μg) |
|--------------------------|--------------|---------------------------|-----------------------------|
| Lysate                   | 1500         | <1                        | <0.001                       |
| Lysate pellet            | 120          | ND                        | ND                           |
| Guanidine extract        | 100          | 1.5                       | 0.015                        |
| Refolded RB p60          | 40           | 36                        | 0.9                          |
| Affinity column pool     | 3            | 25                        | 8.3                          |

* As determined by the Bradford assay (26).
* As determined in the ELISA assay using immobilized E7 protein.

Fig. 3. Binding of HPV-16 E7 to immobilized RB p60. Binding of purified recombinant HPV-16 E7 to immobilized RB p60 was monitored in an ELISA assay using an anti-E7 polyclonal antibody to detect complex formation. Replotting the binding data according to the method of Scatchard (29) (inset) generates a straight line, consistent with a single class of binding sites with an apparent affinity of approximately 0.6 nM. Because the ELISA format does not allow rigorous quantitation of [E7]bound, the binding data are represented by Y, the fractional occupancy of RB p60 by ligand. Only data points in which the amount of E7 exceeded the amount of RB p60 are included in the replot.

Fig. 4. Circular dichroism spectrum of RB p60. The circular dichroism spectrum of affinity-purified RB p60 was measured as described under "Materials and Methods." The data (solid line) are closely approximated by a model that contains 39% α-helix, 16% β-sheet, 22% turn, and 30% unordered conformations (dashed line) (27).

**Fig. 4.** Circular dichroism spectrum of RB p60. The circular dichroism spectrum of affinity-purified RB p60 was measured as described under "Materials and Methods." The data (solid line) are closely approximated by a model that contains 39% α-helix, 16% β-sheet, 22% turn, and 30% unordered conformations (dashed line) (27).
sites (Fig. 3) (29). These results suggest that the RB p60 eluted from the E7 affinity column is functionally homogeneous.

As a first step toward the elucidation of the structure of RB p60, we performed a series of circular dichroism studies on the affinity-purified protein. The ultraviolet circular dichroism spectrum of affinity-purified RB p60 is shown in Fig. 4. Analysis of this spectrum by the variable selection method (see "Materials and Methods") suggests that this protein contains approximately 39% a-helix, 16% b-sheet, 22% turn, and 30% unordered conformations. The addition of E7-(20-29) peptide amide to RB p60 at a 10-fold molar excess (17 μM) had no effect on the spectrum (data not shown).

The thermal stability of the secondary structure of RB p60 was examined by monitoring the ellipticity at 222 nm as a function of temperature. This wavelength was selected because of the relatively large percentage of helical structure in RB p60. A sharp melting transition was observed, suggestive of a cooperative thermal denaturation process (Fig. 5A). The midpoint of this transition (T_m) was estimated to be at 48°C from the peak of the first derivative plot of the denaturation data (Fig. 5B). The addition of a 20-fold molar excess of E7-(20-29) peptide amide (30 μM) caused a 12-degree shift in the T_m, to 58°C. At the same concentration, a scrambled E7 peptide (for sequence, see "Materials and Methods") that does not bind RB p60 had no effect on the transition temperature. The same results were obtained whether the experiment was performed in PBS or 6 mM sodium phosphate, pH 7.4. These results demonstrate that E7-(20-29) peptide amide specifically binds to RB p60 and significantly stabilizes it to thermal denaturation. A single melting transition is observed in the presence or absence of E7 peptide, consistent with the hypothesis that the affinity-purified RB p60 contains a single conformational population.

DISCUSSION

As a member of the growing family of growth suppressor genes that have been linked to cancer, there is great interest in understanding the mechanism of action of the retinoblastoma gene product. Mutagenesis studies of RB have provided a great deal of structural information, including the elucidation of two discontinuous domains that appear critical for the high affinity binding of pRB to several virally encoded proteins (21-23). Alternative methods of probing these interactions may be utilized if sufficient quantities of functionally active protein are available. While pRB and fragments of pRB have been previously expressed in baculovirus (30), and as a fusion protein in E. coli (11), none of these expression/purification schemes were capable of providing sufficient quantities of chemically and functionally homogeneous protein for structural and biophysical characterization.

In the current study, we describe a system for the expression and purification of a functionally homogenous, truncated form of pRB from E. coli. Binding studies with recombinant HPV E7 protein are consistent with a single, high affinity binding site, and thermal denaturation studies exhibit a single thermal transition. In addition, the affinity-purified RB p60 exhibits DNA-binding properties that are virtually identical with those of rabbit reticulocyte transcribed/translated RB p60 (31). Taken together, these studies provide evidence for the functional and conformational integrity of our affinity-purified protein.

Circular dichroism spectroscopy on purified RB p60 provides the first estimate of the secondary structure of functional RB protein. In solution, RB p60 appears to contain a relatively high percentage of a-helix, with smaller amounts of b-sheet and turns (27). It is interesting to note that binding of E7-(20-29) peptide amide to RB p60 causes no significant change in the circular dichroism spectrum of the protein, which implies that the overall conformation of RB p60 is unaffected by the binding of this E7 fragment. Nevertheless, the interaction of RB p60 with the peptide affords significant stabilization of the protein to thermal denaturation, with a shift in the a-helix melting temperature of approximately 12°C.

In conclusion, we have demonstrated a method for the isolation of active, functionally homogeneous RB p60 in sufficient quantities for structural studies. The availability of this protein should allow the initiation of a number of studies that will help clarify, at the molecular level, the mechanism by which the retinoblastoma gene product contributes to the regulation of cell growth.

Acknowledgments—We wish to express our gratitude to Sam Thornton for atomic emission results, to Vic Garsky and Nancy Balashin for peptides, and to Jim Bailey for cell paste.

REFERENCES

1. Weinberg, R. A. (1991) Science 246, 1138-1146
2. Gallegos, W. K., Dyjaa, T. P., Phillips, R. A., Benedict, W. F., Godbout, R., Galle, R. L., Stroobant, V., Streep, D. R., Murphy, L., and White, R. L. (1983) Nature 305, 779-784
3. Lee, Y.-H., To, S., and Yew, J.-Y. (1988) Science 241, 218-221
4. Buczkowicz, K. J., Duffy, L., and Harlow, E. (1989) Cell 58, 1097-1105
5. Chen, P. L., Scully, P. J., Shaw, J. Y., Wang, J. J., and Lee, W.-H. (1989) Nature 343, 1193-1198
6. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C.-M., and Livingston, D. M. (1989) Cell 58, 1085-1095
7. DeCaprio, J. A., Ludlow, J. W., Figg, J., Shaw, J., Huang, C.-M., Lee, W.-H., Marsiellie, E., Paucha, E., and Livingston, D. M. (1988) Cell 54, 275-285
8. Whyte, P., Buczkowicz, K. J., Horowitz, J. M., Friend, S. H., Rayburn, M., Weinberg, R. A., and Harlow, E. (1986) Nature 324, 124-129
9. Dyson, N., Howley, P. M., Munker, R., and Harlow, E. (1989) Science 243, 934-937
10. Munger, K., Werness, B. A., Dyson, N., Phillips, W. C., Harlow, E., and Howley, P. M. (1988) EMBO J. 7, 4099-4105
11. Kaelin, W. G., Pallas, D. C., DeCaprio, J. A., Kaye, F. J., and Livingston, D. M. (1991) Cell 64, 521-532
12. Bagchi, S., Weinmann, R., and Rayachoudhuri, P. (1991) Cell 65, 1063-1072
13. Liao, W. K., and La Thangue, N. B. (1991) Nature 351, 449-497
14. Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevin, J. R. (1991) Cell 65, 1053-1061
15. Chittenden, T., Livingston, D. M., and Kaelin, W. G., Jr. (1991) Cell 65, 1073-1082
16. DeFeo-Jones, D., Huang, S. P., Jones, R. E., Haskell, K. M., Vucollo, G., Bandara, L. R., and La Thangue, N. B. (1991) Nature 352, 251-254
17. Rawn, J. S., and Schneider, A. (1987) in The Papillomaviruses (Saltman, N. P., and Howley, P. M., eds) Vol. 2, pp. 245-263, Plenum Publishing Corp., New York
18. Phillips, W. C., Yue, C. L., Munger, K., and Howley, P. M. (1990) Cell 63, 539-547
19. Jones, R. E., Wiegryn, R. J., Patrick, D. R., Balashin, L. N., Vucollo, G. A., Rieniun, M. W., DeFeo-Jones, D., Garisky, V. M., Heimreich, D. C., and Offit, A. (1990) J. Biol. Chem. 265, 12782-12785
20. Jones, R. E., Heimreich, D. C., Huber, H. E., Wiegryn, R. J., Kothberg, N. S., Stauffer, K. J., Lumma, P. K., Garisky, V. M., and Offit, A. (1991) J. Biol. Chem. 266, 908-912
21. Hu, Q., Dyson, N., and Harlow, E. (1990) EMBO J. 9, 1147-1155
22. Huang, H.-J., Wang, N.-P., Tseng, B. Y., Lee, W.-H., and Lee, Y.-H. P. (1990) EMBO J. 9, 1815-1822
23. Kaelin, W. G., Jr., Ewen, M. E., and Livingston, D. M. (1990) Mol. Cell. Biol. 10, 3761-3767
24. Lee, Y.-H., To, S., and Yew, J.-Y. (1987) BioTechnol. 5, 900-905
25. Rafalowicz, J. P., and Thomas, K. A. (1987) BioTechnology 9, 960-965
26. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254
27. Grell, L. A., and Johannsson, W. G. (1991) Anal. Biochem. 195, 155-157
28. Stewart, J. M., and Young, J. D. (1984) Solid Phase Peptide Synthesis, pp. 71-95, Pierce Chemical Co., Rockford, IL
29. DeFeo-Jones, D. (1985) Gene Synthesis: A Short Course on Theory and Methods, pp. 51-96, Martinus Nijhoff, Boston, MA
30. Wang, N. F., Qian, Y., Chung, A. E., Lee, W.-H., and Lee, Y.-H. P. (1990) Cell Growth Diff. 1, 429-437
31. Stirling, S. M., Patrick, D. R., Hufer, H. E., DeFeo-Jones, D., Garisky, V., Offit, A., and Heimreich, D. C. (1991) Mol. Cell. Biol., in press
32. Laemmli, U. K. (1970) Nature 227, 680-685

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J. Biol. Chem. 1992, 267:7971-7974.

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Vol. 267 (1992) 9430–9436

Removal of the amino-terminal acidic residues of yeast actin. Studies in vitro and in vivo.

R. Kimberley Cook, William T. Blake, and Peter A. Rubenstein

Page 9434, Fig. 5: The quality of the reproduction of this figure was poor. A clearer representation is shown below:

![Figure 5](image)

**FIG. 5.** Electron micrographs of wild type and mutant actin filaments. A, wild type; B and C, DNEQ actin; D–F, ΔDSE actin; G, a 1:1 copolymer of wild type and ΔDSE actin. Arrows mark individual filaments. Open arrowheads indicate areas where filaments appear in register. Triangles point to filaments that appear woven. Bar equals 0.1 μm.

Vol. 265 (1990) 20061–20064

Molecular cloning of the fMet-Leu-Phe receptor from neutrophils.

Kathleen M. Thomas, Hae Yung Pyun, and Javier Navarro

Errors have been discovered in the sequence published in the above paper. Please contact GenBank/EMBL or the authors for the correct sequence. The accession number is J05705.

The F3R cDNA encodes the IL-8 receptor as recently described in a published paper (*J. Biol. Chem.* 266, 14839–14841).

Vol. 267 (1992) 6183–6187

Inhibition of membrane fusion in vitro via cyclin B but not cyclin A.

Leo Thomas, Paul R. Clarke, Michele Pagano, and Jean Gruenberg

Page 6183, “Materials and Methods,” first paragraph: The addresses of the scientists who supplied reagents should appear as follows: “M. Glotzer and M. W. Kirschner (both University of California, San Francisco) (28); “T. Hunt (Imperial Cancer Research Fund, Hertfordshire, United Kingdom);” and “M. Dorée (Centre National de la Recherche Scientifique, Montpellier, France)”.

Vol. 267 (1992) 7971–7974

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Page 7973, Fig. 5: The legend to this figure is incorrect. The concentrations of RB p60 and the two peptides cited should read “μM,” not “mM.” The concentrations stated in the text of the manuscript are correct as described. These changes in no way affect the substance or the conclusions of this study.

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