

Inactivation of oncoprotein binding by a single Cys⁷⁰⁶-to-Tyr substitution in the retinoblastoma protein

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Abstract

We previously found a new single amino acid substitution at codon 706 (Cys-to-Tyr) of the retinoblastoma (RB) gene in a sporadic retinoblastoma patient. The glutathione *S*-transferase–RB fused protein containing this mutation was here tested for binding to SV40 large T antigen and adenovirus E1A protein, and was shown to have lost its binding affinity. Thus, Tyr, as well as Phe, residues substituted for Cys⁷⁰⁶ were found to abolish the RB protein activity.

Key words: Retinoblastoma protein; Retinoblastoma; Tumor suppressor gene; E1A protein; SV40 large T antigen

1. Introduction

Inactivation of both alleles of the retinoblastoma (RB) gene appears to trigger a wide range of human tumors [1]. The RB gene product (pRB) is a 110 kDa nuclear phosphoprotein [2]. It has been demonstrated that the adenovirus E1A protein [3] and the SV40 large T antigen [4] as well as the human papillomavirus E7 protein [5] form a complex with pRB, presumably resulting in inactivation of pRB function. It was also shown that pRB is phosphorylated cell cycle-dependently [6–9]. This phosphorylation is likely to be performed by cyclin-dependent kinases [10–16] and appears to regulate the anti-proliferative activity of pRB [17]. It has been suggested that this activity of pRB is mediated through binding to several cellular proteins including E2F [17].

Several types of naturally occurring inactivated mutant pRB have been found in tumor cells [18–24]. Among

these, a 35-amino acid deletion in exon 21 was first identified in the bladder carcinoma cell line J82 [19] and the prostatic carcinoma cell line DU145 [20]. C-Terminal truncation lacking exons 21–27 was found in an osteosarcoma cell line Saos-2 [21]. Similarly, a 25-amino acid deletion around exon 16 was found in the small cell lung carcinoma cell line SCLC-SD1 [22]. In addition, a single amino acid substitution (Cys to Phe) at codon 706 which was derived from exon 21 was identified in a small cell lung cancer cell line H209 [23,24]. These mutant pRB are uniformly underphosphorylated, rendering them unable to bind to the adenovirus E1A protein or SV40 large T antigen. It is interesting to note that all of these mutations are located within two non-contiguous regions of pRB, amino acid residues 394–571 and 649–773, which are needed for binding to E1A protein or SV40 large T antigen [25,26].

We previously found a new single amino acid substitution at codon 706 (TGT-to-TAT: Cys-to-Tyr) of the RB gene in a sporadic bilateral retinoblastoma patient RB210 [27]. The mutation was heterozygous in the fibroblast of this patient and homozygous in the tumor. We have now demonstrated that this mutation results in the inability of pRB to bind to E1A protein or SV40 large T antigen.

2. Materials and methods

2.1. Materials

Plasmid p4.95BT which contains RB cDNA was generously provided by Dr. T.P. Dryja. Fibroblasts of the RB210 patient were obtained and cultured as described previously [28]. Purified SV40 large T antigen was

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Abbreviations: RB, retinoblastoma; pRB, retinoblastoma protein; GST, glutathione *S*-transferase; PMSF, phenylmethyl-sulfonyl fluoride; IPTG, isopropyl β -D-thiogalactopyranoside.

kindly supplied by Dr. T. Eki (RIKEN). Monoclonal antibodies against adenovirus E1A protein, SV40 large T antigen, and pRB were purchased from Oncogene Science.

2.2. Construction of pGEX-3XX

To generate in-frame glutathione *S*-transferase-RB fused proteins, we modified plasmid pGEX-3X [29]. For this purpose, pGEX-3X was linearized with *Bam*HI, blunt-ended by T4 DNA polymerase, and then ligated. The plasmid vector thus obtained was named pGEX-3XX.

2.3. Construction of plasmids to produce GST-RB fusion proteins

Plasmid p4.95BT was digested with *Vsp*I and the resulting 2.0 kb fragment, which contains a coding region (385–928) and the 3' non-coding region of the RB gene, was purified from an agarose gel. Termini of the fragment were blunt-ended by T4 DNA polymerase, and then ligated to the *Sma*I site of pGEX-3XX. This clone will be referred to as pGST-RB(385–928).

In order to obtain the RB cDNA with a point mutation at position 706, 10 mg of total RNA from cultured RB210 fibroblasts was reverse-transcribed and amplified using GCGAATTCCAGACTGATTCTA-TAGACAG, which is derived from the exon 10 sequence, as the upstream primer and ATGTCGACTCCAGAGGTGTACACAGTG, which is derived from the sequence of the 3' non-coding region of exon 27, as the downstream primer, according to the method of Kato et al. [27]. The PCR product was digested with *Nhe*I and *Bsm*I and purified. A *Nhe*I-*Bsm*I fragment of the wild-type RB cDNA was excised from pGST-RB(385–928) and replaced by the corresponding fragment of PCR product. A clone was obtained which was confirmed to have a mutation at codon 706 by DNA sequencing using double-stranded DNA as a template. This clone was designated pGST-RB(385–928; 706Y).

2.4. Expression and purification of GST fusion proteins

GST-RB fusion proteins were expressed and purified essentially as described by Smith and Johnson [29]. *E. coli* cells transformed with pGST-RB(385–928) or pGST-RB(385–928; 706Y) were grown to A_{600} 0.5, and IPTG was added to a final concentration of 1 mM. After 3 h of additional growth, *E. coli* cells were pelleted, washed once with PBS, and stored at -80°C . Cells were resuspended in 1/30–1/50 culture volume of NETN (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.25 mM PMSF, 10 $\mu\text{g}/\text{ml}$ each of antipain, chymostatin, leupeptin and pepstatin) and lysed on ice by sonication. Lysates were centrifuged at $28,000 \times g$ for 15 min, and cleared lysates were incubated with glutathione-Sepharose (Pharmacia) for 30 min at 4°C . The beads were washed three times with NETN. Elution of the GST-RB fusion proteins was performed with a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 5 mM glutathione.

2.5. Assay for binding of pRB to DNA tumor virus oncoproteins

293 cells, which produce adenovirus E1A protein, were used for the source of E1A. 293 cells (3×10^6) were lysed by a lysis buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% NP-40, 0.25 mM PMSF, and 5 $\mu\text{g}/\text{ml}$ each of antipain, chymostatin, leupeptin, and pepstatin) on ice for 30 min, and then centrifuged at 15,000 rpm for 5 min by a microcentrifuge. The resultant supernatant was used for the binding assay.

GST-RB fused proteins (500 ng) adsorbed to glutathione-Sepharose beads (10 μl) were incubated with purified SV40 large T antigen (100 ng) or 300 μl of whole-cell lysate of 293 cells in a final volume of 1 ml of lysis buffer for 2 h at 4°C , and then washed six times with NETN. The beads were boiled in SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M DTT, and 0.01% Bromphenol blue) and bound proteins were separated by 7.5% SDS-polyacrylamide gels. SV40 large T antigen and E1A protein were identified by immunoblotting with the corresponding monoclonal antibody using the ECL Western blotting detection system (Amersham).

To confirm the specificity of the binding, reciprocal binding experiments were also carried out. Purified T antigen (400 ng) were immunoprecipitated with anti-T antigen monoclonal antibody, then the immunoprecipitates were mixed with GST-RB fusion proteins (500 ng) in a final volume of 1 ml of lysis buffer for 2 h at 4°C . The bound GST-RB proteins were detected as described above with anti-pRB monoclonal antibody.

3. Results and discussion

To test the binding affinity for E1A protein or SV40 large T antigen, GST-RB fused proteins containing the E1A-binding domain (Fig. 1A) were expressed using the wild-type RB gene as well as the RB gene of RB210 mutated at codon 706 (Cys-to-Tyr) as described in section 2. These two clones were named pGST-RB(385–928) and pGST-RB(385–928; 706Y), respectively. Nucleotide sequences around codon 706 of these two plasmids, are shown in Fig. 1B.

GST-RB fused proteins thus expressed were adsorbed to glutathione-Sepharose beads and tested for binding of purified SV40 large T antigen (Fig. 2A). Mutant GST-RB protein did not bind SV40 large T antigen (lane 5), while wild-type GST-RB protein clearly bound to it (lane 2). Binding of SV40 large T antigen to wild-type GST-RB protein was completely abolished by the addition of T-peptide [30] which contains the consensus LXCXE sequence for binding to pRB, but not by K-peptide [30] which is changed at this sequence (lanes 3, 4), indicating that binding of GST-RB protein to SV40 large T antigen shown here is specific. GST alone did not show binding (lane 1).

These GST-RB fused proteins were also tested for binding to adenovirus E1A proteins (Fig. 2B). Lysates of 293 cells which express E1A protein were used for this experiment. E1A protein strongly bound to wild-type GST-RB protein (lane 2), and this binding was strongly inhibited by T-peptide (lane 3) but not by K-peptide (lane 4). In contrast, no binding was observed in the case of mutant GST-RB protein (lane 5). GST alone did not bind E1A protein, either. In Fig. 2A and B, the amounts

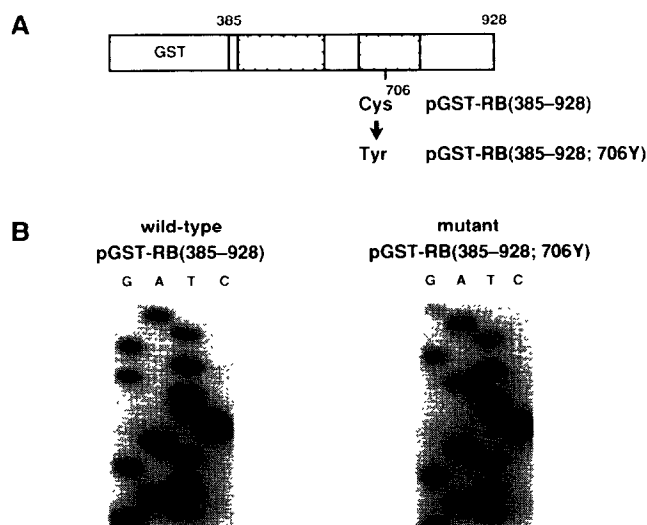


Fig. 1. pGST-RB(385–928) and pGST-RB(385–928; 706Y). (A) Schematic representation of GST-RB proteins. The hatched regions indicate the two domains needed for E1A or SV40 large T-binding [25,26]. (B) Nucleotide sequences around codon 706 of the two pGST-RB plasmids.

of wild-type and mutant GST-RB proteins used were almost the same, as shown in Fig. 3, lanes 5 and 6.

Reciprocal binding experiments were carried out using immunoprecipitated SV40 large T antigen and purified soluble GST-RB proteins (Fig. 3). It is clear that wild-type GST-RB protein but not mutant GST-RB protein bound SV40 large T antigen (lane 3 and 4).

We showed that this newly found, naturally occurring

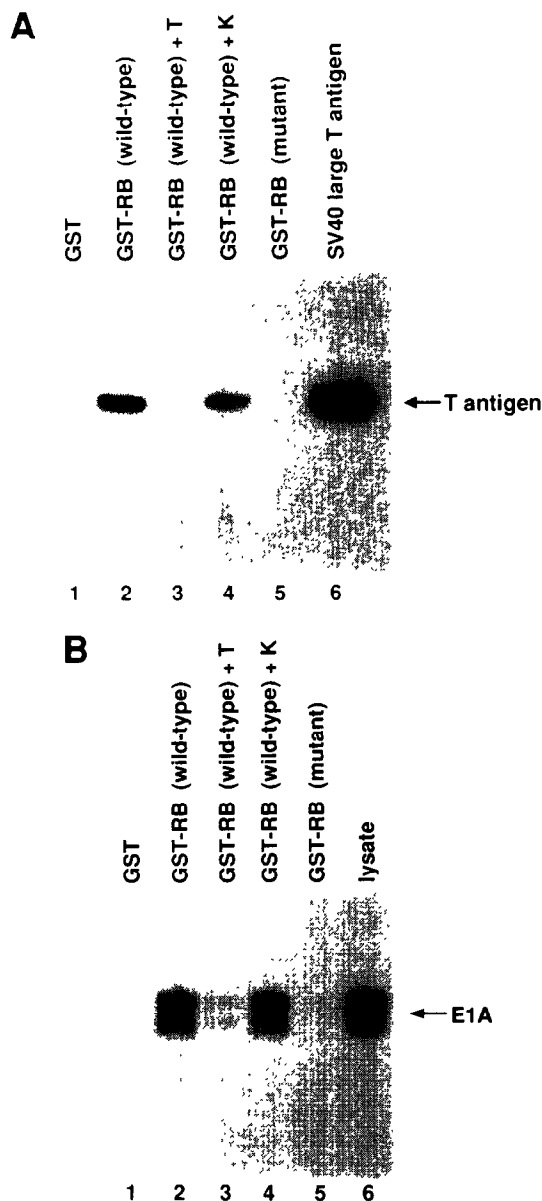


Fig. 2. Precipitation of SV40 large T antigen or E1A protein by wild-type GST-RB protein. (A) GST-RB proteins (lanes 2–5) or control GST (lane 1) bound to glutathione-Sepharose beads were incubated with SV40 large T antigens in the presence or absence of T- or K-peptides (100 ng each). SV40 large T antigens bound to the beads were detected by Western blotting with anti-SV40 large T antibody. In lane 6, 100 ng of SV40 large T antigen was loaded on SDS-polyacrylamide gels. (B) Lanes 1–5, experiments were carried out as in (A); however, 293 cell lysates were used in place of SV40 large T antigen and E1A proteins bound to the beads were detected by Western blotting with anti-E1A antibody. Lane 6, 10 μ l of 293 cell lysates were loaded for Western blotting.

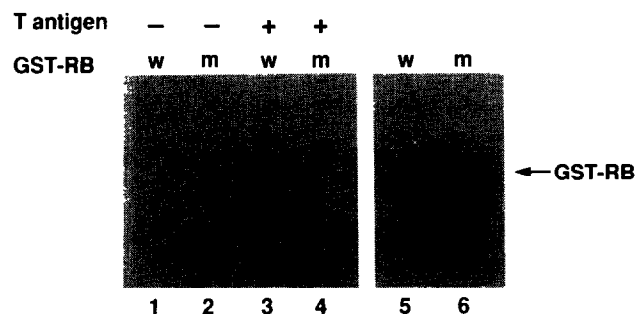


Fig. 3. Precipitation of wild-type GST-RB protein by SV40 large T antigen. Lanes 1–4, wild (w) or mutant (m) GST-RB proteins were mixed with SV40 large T antigen which was immunoprecipitated by anti-T antigen antibody and bound GST-RB proteins were detected by Western blotting using anti-pRB antibody. Lanes 5 and 6, 40 ng each of wild-type or mutant GST-RB proteins were directly loaded onto the SDS-polyacrylamide gels.

single amino acid substitution at codon 706 (Cys to Tyr) of pRB causes the loss of binding affinity to SV40 large T antigen and E1A protein. The only other example of a single pRB amino acid substitution, which was shown to inactivate pRB, is the Cys to Phe mutation at codon 706 in a small cell cancer cell line (H209). Thus, this Cys residue appears to be very important for the integrity of pRB structure and is a frequent target for mutation of the RB gene. Kratzke et al. [31] recently reported functional analysis at this Cys residue of the pRB. They constructed in vitro a series of missense mutants at this codon and found substitution to Tyr as well as Phe caused the affinity to bind SV40 large T antigen or E1A protein to be lost. This result is consistent with our results which were obtained from the naturally occurring mutant RB gene. Moreover, they found that substitution of Cys-to-Ala or -Ser retained this binding affinity. Thus, this Cys⁷⁰⁶ residue is not involved in disulfide pairing and a large R-group chain at this position disrupts, by steric hindrance, an important pRB folding conformation.

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