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EBNA-5, an Epstein–Barr virus-encoded nuclear antigen, binds to the retinoblastoma and p53 proteins

(tumor suppressor proteins/viral strategy)

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ABSTRACT **Epstein–Barr virus (EBV) immortalized human lymphoblastoid cell lines express six virally encoded nuclear proteins, designated EBV nuclear antigens 1–6 (EBNA-1–6). We show that the EBNA-5 protein (alternatively designated EBNA-LP) that is required for B-cell transformation can form a molecular complex with the retinoblastoma (RB) and p53 tumor suppressor proteins. Using EBNA-5 deletion mutants, we have found that a 66-amino acid-long peptide, encoded by the W repeat of the EBV genome, is sufficient for binding. Point mutations of RB and p53 that inhibit their complexing with other DNA viral oncoproteins do not affect their binding to EBNA-5. p53 competes with RB for EBNA-5 binding. Our data suggest that the mechanisms involved in EBV transformation may include impairment of RB and p53 function.**

Simian virus 40 (SV40), adenoviruses, and human papilloma viruses (HPVs) encode transforming proteins that can form complexes with the two major tumor suppressor proteins, the retinoblastoma (RB) and p53 proteins (1–8). The transforming proteins of these viruses probably play an essential role in establishing viral latency. Their transforming and/or tumorigenic effect may be a biproduct of this normal viral strategy. The fact that the transforming domains of these viral proteins form complexes with the two most important tumor suppressor proteins, RB and p53, represents a remarkable case of convergent evolution and suggests a common functional requirement. The viruses may have to impair the function of these two cell cycle checkpoint controls, to stimulate host cell DNA synthesis, a common feature of all transforming interactions induced by the DNA tumor viruses.

Herpes viruses are the largest among the DNA tumor viruses. They encode a higher number of transformation-associated proteins than the medium-sized adenoviruses or the small papova viruses, and their transforming strategy must be fundamentally different. It therefore appeared important to investigate whether any of their products interact with RB and p53 as well. We have chosen Epstein–Barr virus (EBV) as the most highly transforming member of the herpes virus family to approach this question.

EBV expresses six nuclear proteins in immortalized lymphoblastoid cell lines (LCLs), designated EBV nuclear antigens 1–6 (EBNA-1–6). In a previous study, we have examined the intranuclear distribution of EBNA-1, -2, -3, and -5 and their ability to colocalize with the RB protein, stained with the aRB1C1 monoclonal antibody (9). We have found a striking colocalization between EBNA-5, alternatively called EBNA-LP, and RB in the LCL IB4 (10).

EBNA-5 is required for B-cell transformation (11). This protein is encoded by the first open reading frame of an extensively spliced giant primary transcript, originating from two alternative (W or C) promoters. All six EBNA proteins are generated from messages spliced from this transcript in EBV-transformed immunoblasts (12). The N-terminal half of EBNA-5 contains a variable number of 22- and 66-amino acid repeats, encoded by the W1 and W2 exons. The C-terminal product of the Y1 and Y2 exons is referred to as the unique region, due to the absence of repeats (13). In freshly infected B cells EBNA-5 appears as a protein ladder between 20 and 130 kDa, as a result of heterogenous splicing that generates transcripts with different numbers of W repeats (14). Monoclonal LCLs express only one or two EBNA-5 species, as a rule. Immunofluorescence reveals discrete large EBNA-5-positive foci, localized to the euchromatic areas of the cell nucleus (15).

The monoclonal anti-RB antibody aRB1C1 was found to stain similar nuclear foci in the LCL IB4. Using computer-assisted overlap analysis, we found complete overlapping in the RB and EBNA-5-positive foci (10). They differed from the equally distinct dots of the small nuclear ribonucleoprotein-rich splicing islands. Neither EBNA-1, -2, nor -3 showed any colocalization with RB (10, 15).

In the present study, we have investigated the ability of EBNA-5 to form complexes with the RB and p53 proteins, using bacterially produced glutathione *S*-transferase (GST) fusion proteins. We demonstrate that EBNA-5 can bind RB and p53.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The LCL IARC139, the Burkitt lymphoma (BL) lines BL28, E95A-BL28, Namalwa, and Ramos, the promyelocytic leukemia cell line HL60, and the colon carcinoma cell line SW480 were used in this study. E95A-BL28, a subline of the originally EBV-negative BL28, carries a fragment of EBV DNA encoding EBNA-2 and EBNA-5 but no other members of the EBNA family (16). Cells were grown in Iscove's medium supplemented with 10% fetal calf serum and antibiotics.

Plasmids. The GST-RB plasmid, containing GST fused in frame to a fragment corresponding to amino acids 374–928 of wild-type (wt) RB, and the GST-RBm706 plasmid, containing GST fused in frame to a fragment corresponding to amino acids 379–928 of a mutant RB that carries a Cys → Phe mutation at position 706 (17), were provided by William G. Kaelin (Dana-Farber Cancer Institute). The pGEX-3X-Cyl1 plasmid, carrying GST fused in frame to the mouse cyclin D1 gene (18), was provided by Chuck J. Sherr (St. Jude Children's Research Hospital). The GST-abl and GST-src plasmids that contain GST fused in frame to the SH2 domains of c-*abl* and c-*src*, respectively (19), were gifts from Bruce

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Abbreviations: BL, Burkitt lymphoma; EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; GST, glutathione *S*-transferase; HPV, human papilloma virus; LCL, lymphoblastoid cell line; SV40, simian virus 40; wt, wild-type; RB, retinoblastoma.

Mayer (Rockefeller University). The plasmid p53-H-19, carrying human wt p53 driven by the T7 polymerase promoter, was provided by Varda Rotter (The Weizmann Institute, Israel). The EBNA-LP plasmid was a gift from Fred Wang and Elliott Kieff (Harvard University).

A GST-wt p53 plasmid was constructed by PCR amplification of wt human p53 cDNA from the plasmid pC53-SN3, provided by Bert Vogelstein (Johns Hopkins University), using appropriate primers to generate an open reading frame upon cloning in the *Bam*HI and *Eco*RI site 3′ of the GST coding sequence in the vector pGEX-2T (Pharmacia LKB). The EBNA-5 mutants were generated in the pUC18 vector and further subcloned into the pGEX-2T vector. The unique region was removed by *Sfi* I–*Sac* I double digestion, blunt ending, and recircularization. Digestion with *Apa* I or *Sty* I resulted in constructs containing one or two repeat regions, respectively. Combination of *Sfi* I, *Apa* I and *Sfi* I, and *Sty* I digestions generated fragments of only one or two repeats without the unique region of EBNA-5.

Monoclonal Antibodies. The anti-RB monoclonal antibody aRB1C1 was generated in our laboratory using a bacterially produced tryptophan E (trpE)-RB fusion protein as immunogen as described (9). The anti-EBNA-5 monoclonal antibody JF186, generated in this laboratory, has been described elsewhere (14). The anti-EBNA-2 monoclonal antibody PE2 was obtained from Martin Rowe (University of Birmingham, U.K.) (20). Anti-p53 monoclonal antibodies were purchased from Oncogene Sciences (Mineola, NY).

Peptides. The sequences of the competing peptides were DLQPETTDLYCYEQLNDSSEE (HPV 16 E7) and NVKHKSAIVTLTYDSEWQRDQC (HPV 16 E2).

Preparation of Bacterial Fusion Proteins. GST fusion proteins were produced in *Escherichia coli* and purified as described by Kaelin *et al.* (17). The proteins were immobilized on the surface of glutathione Sepharose 4B beads (50% slurry, Pharmacia LKB, Sweden) and blocked with 3% bovine serum albumin, 0.2% Tween 20, and 5% glycerol in phosphate-buffered saline (PBS) for 1 hr. The trpE-RB fusion protein and the p53 protein were produced and purified in the form of inclusion bodies from *E. coli* as described (9). The proteins were solubilized in the presence of 6 M urea and 2% SDS and subsequently purified by gel filtration and refolded by electrodialysis.

Cell Lysis and Analyses of Protein Complexes. Cells were washed twice with ice-cold PBS and lysed (3×10^6 cells per sample) in 200 μl of lysis buffer containing 1 M KCl, 1% Nonidet P-40, 10 mM Tris·HCl (pH 7.6), 5 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 units of aprotinin per ml for 5 min. The cell lysates were diluted 1:5 with potassium-free lysis buffer and cell debris was pelleted at 12,000 × *g* for 15 min at 4°C. Cell lysates were precleared with blocked beads loaded with GST alone for 1 hr at 4°C. The precleared lysates were precipitated either with 2 μg of the JF186 monoclonal antibody together with 50 μl of protein G-Sepharose beads (Pharmacia LKB, Sweden) or with 20 μg of GST fusion protein immobilized on 50 μl of blocked glutathione beads overnight at 4°C. The beads were washed three times with ice-cold lysis buffer containing 300 mM KCl. Bound proteins were released by boiling in SDS sample buffer, separated on 9% SDS/PAGE, and transferred to polyvinylidene difluoride membranes. The filters were blocked with 3% bovine serum albumin, 0.2% Tween 20, and 5% glycerol in PBS overnight and probed with the JF186 monoclonal antibody for 2 hr at room temperature. The bound immunoglobulins were detected using alkaline phosphatase-conjugated goat anti-mouse IgG serum (Bio-Rad) and developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride substrate (Promega).

The purified trpE-RB fusion protein or bacterially produced p53 was incubated with blocked beads loaded with GST fusion proteins in 20 mM Tris·HCl, pH 7.6/1% Triton X-100/5% glycerol/200 mM KCl/0.34% ovalbumin/1 mM EDTA/5 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/100 units of aprotinin per ml for 1 hr at 4°C and washed four times with the complexing buffer. For the p53 competition experiments, 0.3% bovine serum albumin and 0.5 M KCl were used in the reaction buffer. The precipitates were separated by SDS/PAGE and probed with the aRB1C1 or PAb1801 monoclonal antibodies on Western blot.

Immunoprecipitation. Metabolic labeling and immunoprecipitation of p53 were carried out as described (21). Radiolabeled proteins were visualized by 2,5-diphenyloxazole fluorography.

RESULTS

Complex Formation Between the GST-RB and EBNA-5 Proteins. To test the ability of EBNA-5 to complex with RB, lysates of the EBV-negative BL28 cells that do not express any EBNA protein and lysates of E95A-BL28 cells that express EBNA-2 and a single 60-kDa EBNA-5 species at a high level (16) were prepared. After preclearing with glutathione Sepharose loaded with GST alone, aliquots were incubated either with the anti-EBNA-5 monoclonal antibody JF186 immobilized on protein G-Sepharose or with the glutathione Sepharose-immobilized fusion proteins GST-RB, GST-RBm706, GST-cyclin D1, and GST alone.

After washing the Sepharose beads extensively, bound proteins were eluted as described and separated by SDS/PAGE. EBNA-5 was detected by Western blotting. As shown in Fig. 1, intact GST-RB and the mutant GST-RB carrying a Cys → Phe change at codon 706 (22) could precipitate EBNA-5 from the E95A-BL28 cell lysate, whereas the control GST or GST-cyclin D1 could not. No

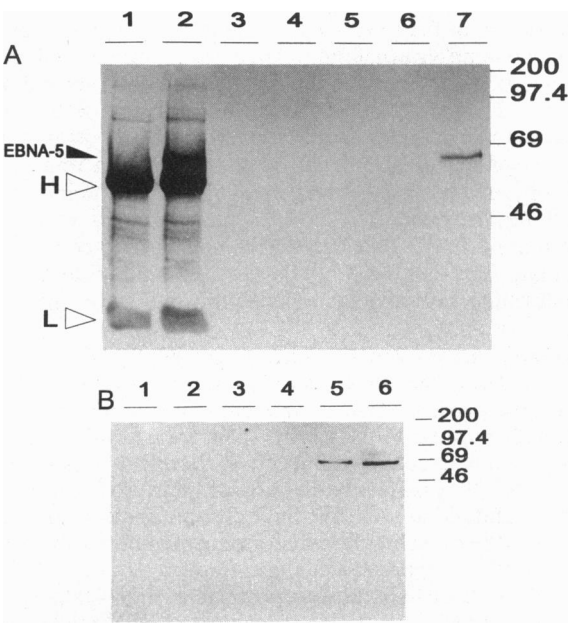

FIG. 1. Western blot analysis of EBNA-5 protein precipitated from E95A-BL28 cell lysates with GST-RB fusion proteins or with the JF186 anti-EBNA-5 monoclonal antibody. EBV-negative BL28 cells were used as negative control. (*A*) BL28 lysates precipitated with JF186 (lane 1), GST alone (lane 3), or GST-RB (lane 4); E95A-BL28 lysates precipitated with JF186 (lane 2), GST alone (lane 5), GST-cyclin D1 (lane 6), or GST-RB (lane 7). The solid arrowhead indicates the EBNA-5 band; open arrowheads point to the immunoglobulin heavy (H) and light (L) chains. (*B*) EBNA-5 binding to a mutant (amino acid 706 Cys → Phe) RB protein. Cell lysates from BL28 (lanes 1–3) or E95A-BL28 (lanes 4–6) were precipitated with GST alone (lanes 1 and 4), GST-RBm706 (lanes 2 and 5), or GST-RB (lanes 3 and 6). Molecular weights are indicated as $M_r \times 10^{-3}$.

EBNA-5 band was detected in any of the precipitates from the EBV-negative BL28 cells. In control experiments, GST-RB but not GST-RBm706 could bind the SV40 large tumor protein (data not shown). The GST-RB/EBNA-5 complex was stable in 1 M NaCl or KCl and was not affected by the presence or absence of divalent cations (5 mM $MgCl_2$, $CaCl_2$, or EDTA) but was disrupted by 1 M urea, 1% deoxycholate, or 0.2% SDS. EBNA-2 could not be precipitated by the GST-RB fusion protein, as evidenced by Western blotting using the PE2 monoclonal anti-EBNA-2 antibody (data not shown).

Complex Formation Between the GST-EBNA-5 and trpE-RB Proteins. We have also detected a direct interaction between RB and EBNA-5 using a bacterially produced trpE-RB fusion protein, containing amino acids 300–928 of RB (9), and a GST-EBNA-5 fusion protein. The trpE-RB protein was solubilized with urea and SDS, purified by gel filtration, and renatured by electrodialysis. Full-length EBNA-5 cDNA was subcloned into the GST expression vector pGEX-2T. In frame fusion was confirmed by DNA sequencing. GST-EBNA-5 was produced in bacteria and verified by Western blotting. The purified trpE-RB was incubated with Sepharose beads loaded with GST-EBNA-5, GST-p53, GST-cyclin D1, or GST alone in the presence of 3×10^3-fold excesses of bovine serum albumin as nonspecific competitor. After washing, the precipitate was resolved on SDS/PAGE and examined by Western blotting using the aRB1C1 monoclonal antibody. GST-EBNA-5 precipitated the RB fusion protein, whereas control proteins did not (Fig. 2*A*). Additional control proteins—i.e., GST fused to the SH2 domains of c-*src* or c-*abl* (19)—did not bind trpE-RB either (data not shown). The complex was not disrupted by 1 M urea, indicating that it was more stable than the GST-RB/EBNA-5 complex.

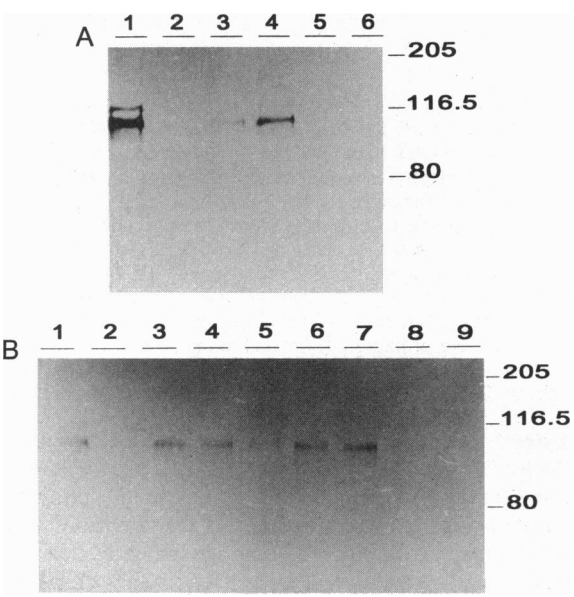

FIG. 2. Binding of bacterially produced trpE-RB to GST-EBNA-5. (*A*) One microgram of purified RB fusion protein was incubated with 50 μl of blocked glutathione beads loaded with GST alone (lane 2), 5 and 10 μg of GST-EBNA-5 (lanes 3 and 4), GST-p53 (lane 5), and GST-cyclin D1 (lane 6). Twenty nanograms of purified trpE-RB protein was loaded as a positive control (lane 1). The bound protein was immunoblotted with the aRB1C1 monoclonal anti-RB antibody. (*B*) Effect of the RB binding HPV 16 E7 peptide on the EBNA-5/RB interaction. Fifty and 200 μg of E7 peptide was included in the complexing mixture (lanes 4 and 5). Fifty and 200 μg of peptide from HPV 16 E2 was used as a control (lanes 6 and 7). Positive controls: 10 ng of purified trpE-RB (lane 1) or trpE-RB precipitated with GST-EBNA-5 in the absence of peptide (lane 3). Beads loaded with GST alone (lane 2), GST-p53 (lane 8), and GST-cyclin D1 (lane 9) were additional controls. Molecular weights are indicated as $M_r \times 10^{-3}$.

Inhibition of GST-EBNA-5/trpE-RB Binding by a HPV E7-Derived Peptide. Complex formation between RB and EBNA-5 was inhibited by a 21-amino acid-long peptide (see *Materials and Methods*) corresponding to the RB binding region of the HPV 16 E7 protein (Fig. 2*B*). This peptide comprises the 9-amino acid segment that was previously shown to inhibit E7/RB binding in peptide competition experiments (23). A significant reduction of trpE-RB binding to GST-EBNA-5 was evident when the GST-EBNA-5 and trpE-RB proteins were incubated in the presence of 200 μg of E7 peptide (lane 5). In contrast, the same amount of a control peptide derived from the E2 protein of HPV had no effect on the interaction between GST-EBNA-5 and trpE-RB (Fig. 2*B*, lane 7).

Complex Formation Between the GST-p53 and EBNA-5 Proteins. Next, the ability of EBNA-5 to bind the p53 protein was investigated. The entire coding region of wt human p53 was cloned into the pGEX-2T vector. The GST-p53 fusion protein could precipitate EBNA-5 from E95A-BL28 cell lysates, as shown in Fig. 3*A*, lane 4. No EBNA-5 was precipitated by the GST control protein (lane 5). GST-p53 did not precipitate any detectable EBNA-2, as assessed by Western blotting, using the PE2 anti-EBNA-2 antibody (data not shown).

GST-EBNA-5 Binds wt and Mutant p53 Proteins. The LCL IARC 139, expressing high levels of p53 that does not react with the mutant p53 specific PAb240 monoclonal antibody, was selected to examine complex formation between GST-EBNA-5 and wt p53 within a cell lysate. The cells were labeled with [^{35}S]methionine and the lysate was precipitated with the GST-EBNA-5 fusion protein and separated on SDS/PAGE. As demonstrated in Fig. 3*B*, lane 7, one of the major bands brought down by GST-EBNA-5 comigrated with p53 that was immunoprecipitated with the monoclonal antibody PAb1801 (lanes 1 and 9). No band of the corresponding size was precipitated by GST-EBNA-5 from HL60 cells that completely lack p53 (24). Fig. 3*C* shows that the same p53 species was also detected in the precipitate from the BL line Namalwa, known to carry a missense p53 mutation at amino acid codon 248 (25, 26). Additionally, GST-EBNA-5 could precipitate p53 from the BL line Ramos that has a missense p53 mutation at amino acid codon 254 (25, 26) and from the colon carcinoma line SW480, carrying missense p53 mutations at amino acid codons 273 and 309 (27) (data not shown).

GST-EBNA-5 could also bind bacterially produced wt human p53 expressed from the plasmid p53-H-19 (Fig. 4*A*, lane 3). This complex was not dissociated by 1 M NaCl, 1 M urea, or 0.5% deoxycholate. The GST or GST-cyclin D1 control proteins did not bind bacterially produced p53 (Fig. 4*A*, lanes 1 and 2).

Mapping the RB and p53 Binding Region of EBNA-5. Deletion mutants of EBNA-5, containing one, two, or four W repeats, with or without the C-terminal unique region, were expressed as GST fusion proteins, and their ability to bind bacterially produced trpE-RB and p53 was assessed. As shown in Fig. 4*A*, removal of the unique region did not inhibit the binding of RB and p53 to EBNA-5. One single EBNA-5 repeat, containing 66 amino acids, was sufficient for binding both proteins (Fig. 4*A*, lane 8).

Competition Between RB and p53 for EBNA-5 Binding. The ability of the short EBNA-5 W repeat to bind RB and p53 suggested that the binding sites for the two proteins are closely located or even overlapping. To test whether RB and p53 can compete for EBNA-5 binding, GST-EBNA-5 was incubated with trpE-RB in the presence of increasing amounts of bacterially produced purified wt p53. Fig. 4*B* demonstrates that p53 inhibited binding of the trpE-RB fusion protein to GST-EBNA-5. trpE-RB was less efficient as a

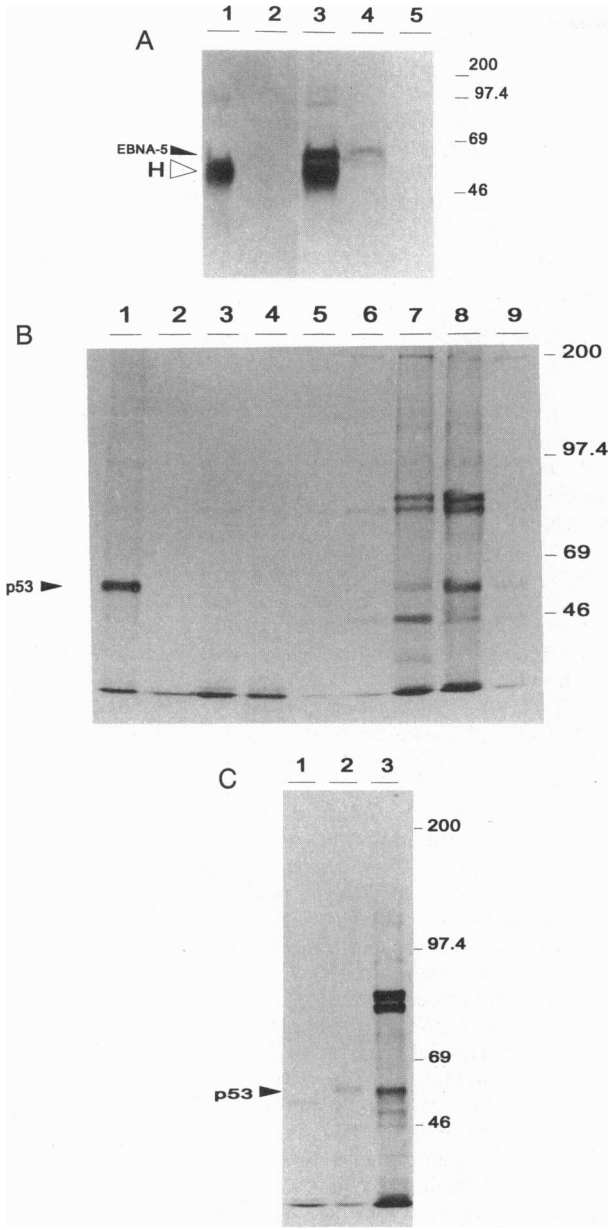

FIG. 3. Complex formation between p53 and EBNA-5. (*A*) Precipitation of EBNA-5 from E95A-BL28 (lanes 3–5) but not from BL28 (lanes 1 and 2) with the JF186 anti-EBNA-5 monoclonal antibody (lanes 1 and 3) and the GST-p53 fusion protein (lanes 2 and 4). No EBNA-5 was brought down with GST alone (lane 5). EBNA-5 was visualized by staining with JF186. Open arrowhead as in Fig. 1. (*B*) [^{35}S]Methionine-labeled cell lysates of the positive control Namalva (lane 1), negative control HL60 (lanes 2–5), and IARC139 (lanes 6–9) were precipitated with the PAb1801 anti-p53 antibody (lanes 1, 5, and 9), GST alone (lanes 2 and 6), GST-EBNA-5 (lanes 3 and 7), or GST-EBNA-5dSfiI deletion mutant, lacking the C-terminal unique region (lane 8). (*C*) Point mutant p53 from Namalva can bind GST-EBNA-5 (lane 3) but not GST alone (lane 1). The PAb1801 immunoprecipitate served as a positive control (lane 2). Molecular weights are indicated as $M_r \times 10^{-3}$.

competitor in reciprocal experiments (data not shown), indicating that the p53/GST-EBNA-5 complex was more stable than the trpE-RB/GST-EBNA-5 complex.

DISCUSSION

EBNA-5 does not contain any LXCXE RB interaction motif, in contrast to the RB complexing SV40 large tumor antigen, adenovirus E1A, and HPV E7 proteins. This suggests that EBNA-5 binds RB by a different mechanism. This is also

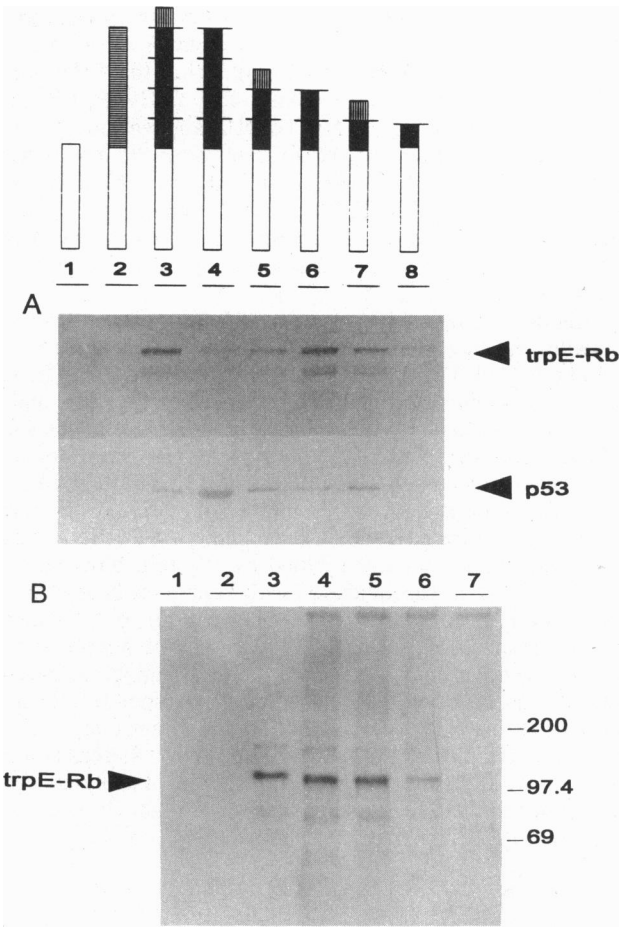

FIG. 4. Mapping the RB and p53 binding sites on EBNA-5. (*A*) *In vitro* binding of trpE-RB and p53 to various EBNA-5 deletion mutants, fused to GST (lanes 3–8). GST is represented by the open segments, and black segments denote EBNA-5 W repeats. The unique C-terminal region of EBNA-5 is indicated by vertical stripes. Note that the unique C-terminal region is not required for binding (lanes 4, 6, and 8) and that a single W repeat is sufficient (lane 8). Lane 1, GST alone; lane 2, GST-cyclin D1. (*B*) RB and p53 compete for EBNA-5 binding *in vitro*. One-half microgram of trpE-RB was incubated with GST-EBNA-5dSfiI-loaded beads in the presence of 0, 0.5, 1, and 2.5 μg of bacterially produced p53 (lanes 4–7). Positive control: 50 ng of trpE-RB (lane 3). Lane 1, GST alone; lane 2, GST-cyclin D1. Molecular weights are indicated as $M_r \times 10^{-3}$.

consistent with the finding that EBNA-5 could bind the GST-RBm706 protein, derived from a naturally occurring mutant RB that was shown to be deficient in phosphorylation and viral oncoprotein binding (22). The competition between EBNA-5 and the RB binding E7 peptide indicates that the RB protein "pocket," responsible for the binding of HPV E7, SV40 large tumor antigen, and adenovirus E1A (28), may be involved in complex formation with EBNA-5. Huen *et al.* (29) have shown that an octamer sequence in the unique region of EBNA-5 is homologous to the conserved region I (CRI) of the adenovirus E1A protein and the N-terminal part of the HPV E7 protein. The CRI of E1A is required for efficient complexing with the RB protein (30). We have found that the unique C-terminal region of EBNA-5 is not necessary for complexing with either RB or p53. The same region is required for transformation, however (11). It may be speculated that the C-terminal region of EBNA-5 is involved in segregation of the EBNA-5-RB/p53 complexes from active nuclear sites by mediating binding to other cellular proteins.

RB is known to complex with several cellular proteins (17, 23, 31–34). At least one of them, the E2F transcription factor,

lacks the LXCXE motif (35, 36). The RB/E2F complex can be disrupted by adenoviral E1A, leading to the release of free E2F. The RB binding domain of E2F has been mapped to a 16-amino acid region located near the C terminus. This region does not show significant homology with the EBNA-5 repeats.

The p53 binding site for viral transforming proteins is less well defined. Adenoviruses and papilloma viruses have evolved two separate proteins for RB and p53 binding, respectively, whereas SV40 large tumor antigen can bind both, but with different domains. Thus, the common or closely adjacent binding site of EBNA-5 for RB and p53, suggested by the competition experiments, is unprecedented.

The addition of yet another DNA virus to the already impressive list of tumor viruses whose transforming proteins complex with the RB and p53 proteins is of more than trivial interest. EBV is a large herpes virus. Its strategy of latent persistence differs from the strategy of the papova viruses and adenoviruses. The fact that it has nevertheless targeted the same two tumor suppressor proteins indicates strong selection for a common function in all four DNA virus systems. Mitotic activation of latently infected cells may represent such a common requirement in these viruses. In the case of EBV, the impairment of RB and p53 function may facilitate the entry of the target B cells into the mitotic cycle. This scenario is consistent with our observation that RB and p53 expression increases in normal B cells within 15 hr after EBV infection, as in mitogen-stimulated B cells (L.S., unpublished data). This occurs in parallel with the rapid increase in the frequency of EBNA-2- and EBNA-5-positive cells, as shown by immunostaining.

The location of RB and p53 interacting sites on the repetitive EBNA-5 peptide may serve the viral strategy by amplifying the complexing ability of EBNA-5. Conceivably, multimolecular complexes that contain a variable number of RB and p53 molecules may be present in EBNA-5-expressing cells.

The functional significance of the interaction of EBNA-5 with RB and p53 remains to be determined. Experiments designed to test whether complex formation between EBNA-5 and RB or p53 interferes with the known functional interactions of RB and p53 in the cell, such as E2F binding (RB) and specific DNA binding (p53), should clarify this issue.

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