Epstein–Barr virus EBNA-LP and transcription regulation properties of pRB, p107 and p53 in transfection assays

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The EBNA-LP protein (also known as EBNA-5) of Epstein–Barr virus (EBV) has been reported previously to colocalize in the nuclei of cells with the pRb protein and to bind in vitro to pRB and to the p53 protein, suggesting a role for EBNA-LP in modulation of the function of these proteins. Here we test in transfection assays whether EBNA-LP expression has any functional consequence for repression of E2F-1 activity by pRb or p107 or for activation of transcription by the p53 protein. No significant effect could be found, although the assay systems were sensitive to the established effects of simian virus 40 large T antigen and human papillomavirus type 16 E6 protein. There was very effective repression of GAL4/E2F-1 transactivation by p107, consistent with earlier reports and indicating that p107 can interact with the E2F-1 transactivation domain, even though p107 has been reported to bind specifically to E2F complexes containing E2F-4. The results indicate that, if the associations of EBNA-LP with pRB and p53 are physiologically relevant, they most likely affect other functions of these proteins or modulate their gene regulatory functions in ways that cannot be detected by transfection into cycling transformed cells.

Introduction

Infection of resting primary B lymphocytes in vitro by Epstein–Barr virus (EBV) drives the infected cells into the cell cycle and frequently results in the production of immortalized lymphoblastoid cell lines (LCLs). The LCL cells resemble activated B blasts and it may be that the EBV immortalization mechanism is cell type-specific. Nevertheless the virus is likely to be overcoming the same basic cell cycle checkpoint mechanisms that have been characterized in other cell types (reviewed in Farrell, 1995). The small DNA tumour viruses, including the adenoviruses, papovaviruses and papillomaviruses, have evolved common mechanisms to deregulate the host cell DNA synthesis machinery to allow efficient replication of their own DNA. The adenovirus E1A, human papillomavirus (HPV) E7 and the simian virus (SV40) large T antigen can all induce cell DNA synthesis and act as potent deregulators of cell cycle control (reviewed in Moran, 1993). Part of the mode of action of these viral oncoproteins is to associate with key cellular regulators such as the pocket proteins pRb, p107 and p130.

pRb and p107 are believed to function, at least in part, by regulating the E2F cellular transcription factor family (reviewed in Lam & La Thangue, 1994). E2F sites have been found in the promoters of several genes that are important for DNA synthesis. E2F-1 binds preferentially to hypophosphorylated pRb, which inhibits the transactivation function of E2F-1. Phosphorylation of pRb is probably controlled by the action of cdk factors (reviewed in Sherr, 1994) which can relieve the pRb-mediated repression of E2F-1 activity (Dynlacht et al., 1994). Transient transfection studies have demonstrated that p107 can also inhibit the E2F transactivation function (Cress et al., 1993; Schwarz et al., 1993; Zamanian & La Thangue, 1993; Zhu et al., 1993). E1A, E7 and SV40 large T antigen can dissociate E2F-pocket protein complexes to liberate free E2F which is the postulated active form of this transcription factor (reviewed in Lam & La Thangue, 1994). It is therefore likely that the ability of small DNA tumour virus oncoproteins to associate with pocket protein family members is important in the virus-mediated induction of cell DNA synthesis.

Of the approximately 84 genes encoded by EBV only 11 have been demonstrated to be expressed in established LCLs, suggesting that the genes responsible for the immortalization process are contained within this subset. Mutational analysis has demonstrated that at least six of these genes are required for efficient immortalization
(reviewed in Farrell, 1995), including the EBNA-LP gene (Hammerschmidt & Sugden, 1989; Mannick et al., 1991). EBNA-LP (also known as EBNA-5) is one of the first genes to be expressed upon viral infection and cooperates with EBNA-2 to drive the resting B lymphocyte into the G1 phase of the cell cycle (Sinclair et al., 1994). Immunofluorescence analysis has shown that EBNA-LP colocalizes accurately with pRb in nuclear dots in LCLs (Jiang et al., 1991) and EBNA-LP has been shown to associate with pRb in vitro using bacterial fusion proteins or in vitro-translated material (Szekely et al., 1993). The characteristics of this association were different from the binding of other pocket-binding proteins in that binding was prevented by an HPV E7 peptide corresponding to the pocket-binding site but was not affected by a pocket mutation of pRb that prevents other pocket-binding proteins associating. The reported association of pRb and EBNA-LP in vitro and the disruption of this interaction with an E7 peptide suggests that EBNA-LP might interfere with pocket protein control of the E2F-1 transcription factor. Most of the studies characterizing this control have been carried out in the pRb-negative SAOS-2 osteosarcoma cell line or in the F9 embryonic carcinoma cell line. However, because of the cell-type specificity of EBV, we wished to assay the effects of EBNA-LP in lymphoid cells. We therefore employed a GAL4-E2F-1 fusion protein transient transfection system which has previously been demonstrated to support a large activation of GAL4 reporter activity, dependent on the GAL4-E2F-1 fusion protein, when assayed in the EBV-negative Burkitt’s lymphoma cell line DG-75 (Flemington et al., 1993).

Szekely et al. (1993) have recently also reported that EBNA-LP can bind to p53 in vitro. Many studies have demonstrated that wild-type p53 is a tumour suppressor protein and that it can act as a negative growth regulator, arresting cells in G1 or causing them to apoptose in response to DNA damage and/or growth factor withdrawal (reviewed in Picken & Larr, 1994). One biochemical mechanism by which p53 functions is by acting as a transcription factor. p53 can transactivate promoters containing its binding sites in transient transfection assays and in response to DNA damage.

Szekely et al. (1993) showed that glutathione S-transferase-EBNA-LP constructs could bind 53 kDa proteins from extracts of Namalwa and IARC139 cells and associate with wild-type p53 produced in bacteria. Transcriptional regulation by p53 is believed to be important in its role as a tumour suppressor gene, and complex formation of MDM-2 (Momand et al., 1992), HPV-16 E6 (Mietz et al., 1992; Crook et al., 1994a), SV40 large T antigen (Bargonetti et al., 1992; Farmer et al., 1992; Segawa et al., 1993) and adenovirus E1B (Yew & Berk, 1992; Yew et al., 1994) with wild-type p53 leads to an inhibition of p53-mediated transactivation. Therefore, transient transfection studies were also performed to test the ability of EBNA-LP to affect p53-mediated transcriptional regulation in lymphoid cells.

**Methods**

**Plasmids.** The plasmids p3.1W-LP and p3.1W-LPrev, which contain the EBV Wp promoter and a seven-repeat EBNA-LP cDNA cloned in the forward and reverse orientations respectively, have been described previously (Sinclair et al., 1994). pCB6+E6 expresses an HPV-16 E6 cDNA from the cytomegalovirus immediate early (CMV IE) promoter (Crook et al., 1994a). SV40 large T antigen vectors expressing wild-type T antigen (SV40T) and the non-pRb-binding K1 point mutant G1107Lys (SV40K) under the control of the SV40 early promoter/ enhancer sequences (Flemington et al., 1993), the GAL4/E2F-1(368–437) expression vector (Kaelin et al., 1992) and p3j, which contains the SV40 early promoter/enhancer (Morgenstern & Land, 1990), have been described. pSNOC-p3wt expresses a wild-type p53 cDNA from the CMV IE promoter and was constructed by subcloning the EcoRI fragment containing p53 from p4-BL2 (Vousden et al., 1993) into the EcoRI site of pSNOC (Allan et al., 1992). In both the pCMVRb human pRb expression plasmid (Qin et al., 1992) and the pCMVp107 plasmid expressing human p107 (Zhu et al., 1993), the CMV IE promoter controls expression of the Rb and p107. pUSCAT contains five copies of the GAL4 DNA-binding site cloned upstream of the herpes simplex virus type 1 thymidine kinase promoter (Watson et al., 1993). pG13CAT was used to assay p53 transactivation (Kern et al., 1992). pCMV/GAL was constructed by cloning the HidIII-BamHI fragment of pCH110 (Pharmacia) which contains the β-galactosidase reporter gene and SV40 polyadenylation signals, into pCMV19 (Gries & O’Harc, 1989).

**Cell culture and electroporation of cell lines.** The EBV-negative Burkitt’s lymphoma cell line DG-75 (Ben-Bassat et al., 1977) and the Akata Group I EBV-positive cell line (Takada & Ono, 1989) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM-glutamine, 200 μg/ml streptomycin and 60 μg/ml penicillin.

Cell lines were transfected as described previously (Buschle et al., 1990). Cells were harvested 18–24 h post-transfection and assayed for β-galactosidase activity (Miller et al., 1972). The extracts were normalized for transfection efficiency using β-galactosidase activity and then assayed for CAT activity (Sleigh, 1986).

**Western blotting.** Cell extracts were separated by electrophoresis on 10% polyacrylamide gels and analysed by Western blotting as described previously (Sinclair et al., 1994). After blocking in milk, filters were probed as indicated with anti-SV40 large T antigen monoclonal antibody (Mab) 419 (Oncogene Science) or anti-EBNA-LP Mab 1F86 (Finke et al., 1987). Bound antibody was detected using rabbit anti-mouse IgG (Z259; Dako) at 1:2500 followed by addition of 50 nCl/ml 125I-labelled Protein A and autoradiography.

**Results**

**E2F-1 transactivation assays in DG-75 cells**

A plasmid expressing the transactivation domain of E2F-1 fused to the GAL4 DNA-binding domain, pGAL4/E2F-1(368–437) (Flemington et al., 1993) was transiently co-transfected into the EBV-negative
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Burkitt's lymphoma cell line DG-75 (which expresses wild-type pRb) with a reporter gene construct containing five GAL4 DNA-binding sites, pUASCAT. Preliminary titration experiments (data not shown) demonstrated that 0.5 µg of pGAL4/E2F-1 gave a consistent 15-30-fold submaximum activation of reporter gene expression relative to control levels (Fig. 1), in agreement with the findings of Flemington et al. (1993).

Titration analysis demonstrated that p107 repressed this E2F-1-mediated transactivation progressively, with 2 µg of p107 expression plasmid reducing CAT activity to basal levels (Fig. 1a). pCMVRb was found to be a less efficient repressor of GAL4/E2F-1-mediated transcriptional activity in DG-75 cells, with 20 µg of pRb expression vector reducing CAT activity by 70% (Fig. 1b).

SV40 large T antigen and a point mutant (SV40K) which does not bind pRb were used to verify further that the p107- and pRb-mediated repression of GAL4/E2F-1 was occurring specifically, in which case the repression should be reversed by wild-type large T antigen but not
the mutant. However, both wild-type and mutant SV40 large T antigen were found to have an additional activity, upregulating CAT activity 2–3-fold from the pUASCAT reporter gene in the presence of the E2F-1 expression vector (data not shown). Similar results were also obtained by Flemington et al. (1993) when these viral proteins were expressed in pRb-negative SAOS-2 cells. The specific effect of SV40 large T antigen mediated through its binding to the pocket proteins was thus observed more readily by allowing for this effect (see legend to Fig. 2). Titration analysis demonstrated that SV40 large T antigen could overcome p107-mediated repression of E2F-1 transactivation in a dose-dependent fashion (Fig. 2a). Similar analysis demonstrated that the pRb-binding defective SV40 large T antigen mutant could not derepress p107-mediated repression of E2F-1 transcriptional activity (Fig. 2a).

Analogous experiments were also performed substituting the pRb for the p107 expression plasmid. Co-expression of SV40 large T antigen (Fig. 2b), but not the mutant T antigen defective in pRb binding (Fig. 2b), was able to overcome the weak pRb-mediated repression of E2F-1 transactivation. The inability of the SV40 T antigen mutant to overcome pRb- and p107-mediated repression of E2F-1 activity was not due to lack of expression of this mutant as the SV40T and SV40K expression plasmids were found to express similar levels of T antigen, as measured by Western blot analysis (Fig. 3).

The potential ability of EBNA-LP to derepress pRb- and p107-mediated repression of E2F activity was then investigated using the same system as described above. EBNA-LP was found to have no significant effect on E2F-1 transactivation (Fig. 4). EBNA-LP was also found to have no effect on the p107- (Fig. 4a) or the pRb-mediated (Fig. 4b) repression of E2F-1 transactivation. Western blot analysis using the EBNA-LP MAb JF186 demonstrated that EBNA-LP was expressed in these experiments (Fig. 5) and that expression of EBNA-LP from p3.1W7rLP was not affected significantly by the presence of the GAL4/E2F-1 fusion, pRb and p107 expression plasmids.
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Fig. 5. EBNA-LP protein expression detected by Western blotting in the transfection experiment shown in Fig. 4. Lanes 1 contain molecular mass markers. (a) Left-hand and middle EBNA-LP titration series in Fig. 4 (b) (lanes 1–8, pCMV vector control; lanes 9–15, pGAL4/E2F-1). (b) EBNA-LP titration series in the presence of pCMVRb (lanes 2–8) or pCMVp107 (lanes 9–15). Lanes 2 and 9 have no p3.1W7rLP, lanes 3 and 10 have 0.1 lag, lanes 4 and 11 have 0.5 lag, lanes 5 and 12 have 1 lag, lanes 6 and 13 have 2 lag, lanes 7 and 14 have 5 lag and lanes 8 and 15 have 10 lag of p3.1W7rLP.

**p53 transactivation assays in Akata cells**

Sequencing and Southern blotting analysis in our laboratory has demonstrated previously that the Akata Group I Burkitt's lymphoma cell line contains a frameshift mutation on one allele of the p53 gene (at amino acid position 190 of p53, resulting in premature termination of the protein after a further 57 amino acids) and has the other allele deleted (Farrell et al., 1991). Western blotting analysis has shown that this cell line expresses no detectable p53 protein; the level of p53 mRNA is very low and presumably the truncation also may render the expressed protein unstable (Farrell et al., 1991). This cell line does not express EBNA-LP and was therefore chosen to study potential effects of EBNA-LP on the transcriptional properties of transfected wild-type p53.

Akata cells were co-transfected with 5 µg of pG13CAT, which contains 13 copies of the ribosomal gene cluster p53 consensus p53-binding site (Kern et al., 1992) and increasing amounts of the wild-type p53 expression plasmid pSNOC-p53wt. pSNOC-p53wt was found to increase CAT activity up to about 100-fold relative to control levels (Fig. 6). We found that 0.5 µg of pSNOC-p53wt activated reporter gene expression approximately 40-fold and this was selected as an amount to transfet in subsequent experiments as it fell within the linear range of the p53 response.

Increasing amounts of the EBNA-LP expression vector p3.1W7rLP and correspondingly decreasing amounts of the control vector p3.1WRev7rLP were co-transfected with 0.5 µg of the pSNOC-p53wt wild-type p53 expression plasmid or the control vector pSNOC and the pG13CAT reporter gene. EBNA-LP did not prevent p53-mediated transactivation of reporter gene expression (Fig. 7a) and in fact caused a slight increase. Western blotting analysis with the EBNA-LP-specific MAb JF 186 demonstrated that EBNA-LP was expressed in these samples (Fig. 8). Similar experiments were also performed using the pCB6 + E6 plasmid which expresses wild-type HPV-16 E6 protein. E6 was found to prevent wild-type p53 transactivation efficiently in this system (Fig. 7b).

**Discussion**

Determining the mechanism by which EBV immortalizes B lymphocytes and how the latent genes of EBV contribute to this process are major objectives in EBV
Fig. 7. HPV E6 protein prevents p53 activation of pG13CAT but EBNA-LP does not. Akata cells were transfected with 5 μg of pG13CAT, 1 μg pCMVβGal, either 0.5 μg of pSNOC-p53wt or the control vector pSNOC as indicated and (a) 1, 5 or 10 μg of p3.1W7rLP as shown or (b) 1, 5 or 10 μg of pCB6+E6 as shown. The variable amounts of p3.1W7rLP and pCB6+E6 were balanced using p3.1W7rLP Prev or the pCB6+ vector, respectively, to give a constant amount of DNA in each transfection. After normalization for transfection efficiency using β-galactosidase activity, CAT activity was assayed and expressed as fold activation in response to pSNOC-p53wt, the mean of two experiments being shown.

Research. EBNA-LP plays an important role in the immortalization process (Hammerschmidt & Sugden, 1989; Mannick et al., 1991; Allan et al., 1992) and our earlier analysis demonstrated that the presence of EBNA-LP may enable saturation-arrested EBV-immortalized cell lines to enter the S phase of the cell cycle more rapidly and synchronously following release from saturation than control cultures (Allan et al., 1992). Entry into the S phase of the cell cycle is believed to be controlled by the E2F family of transcription factors (reviewed in La Thangue, 1994) and this family has been demonstrated to be an important target of pRb family member proteins (reviewed in Goodrich & Lee, 1993). Associations of pRb and its family member p107 with various forms of E2F have been shown to inhibit E2F-mediated transactivation. Therefore, because association of E1A, E7 and SV40 large T antigen with pocket proteins leads to a derepression of E2F activity (reviewed in Moran, 1993), we sought to assay the effects of EBNA-LP on E2F-1-mediated transactivation in lymphoid cells.

The pRb protein was found to repress the transactivation function of a GAL4 DNA-binding-E2F-1 transactivation domain fusion protein, in agreement with previous findings (Helin et al., 1993; Flemington et al., 1993). This repression was, however, weaker than described in assays performed with similar plasmids in the pRb-negative SAOS-2 cell line (Helin et al., 1993; Flemington et al., 1993). The p107 plasmid repressed GAL4-E2F-1 transactivation much more effectively than the pRb plasmid in this cell line. Four members of the E2F transcription factor family, E2F-1, E2F-2, E2F-3 and E2F-4 have been cloned (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1994; Ivey-Hoyle et al., 1993; Lees et al., 1993; Beijersbergen et al., 1994; Ginsberg et al., 1994). Immunoprecipitation analysis has demonstrated that pRb interacts with E2F-1, E2F-2, E2F-3 and weakly with E2F-4 and that p107 interacts only with E2F-4 (Dyson et al., 1993; Lees et al., 1993; Beijersbergen et al., 1994; Ginsberg et al., 1994). Our results show that p107 can efficiently inhibit transcripational activity by a GAL4 DNA-binding-E2F-1 transactivation domain fusion protein when its corresponding expression plasmids are co-transfected into DG-75 cells, consistent with earlier work in other cell types (Zamanian & La Thangue, 1993; Cress et al.,...
pRb can bind to the c-Abl tyrosine kinase (Welch & EBNA-LP binding to pRb may disrupt these complexes and ATF-2 transcription factor and stimulating its transactivation properties (Kim). Transcription from SP-1 sites by binding to SP-1I, an activator, and pRb can positively stimulate TGF-β1 gene expression (Kim et al.).

Co-transfection of SV40 large T antigen was found to alleviate pRb-mediated suppression of GAL4-E2F-1 activity, in agreement with previous findings (Flemington et al., 1993). The mutant SV40 large T antigen (SV40K), which does not bind pRb, was unable to perform this function. Wild-type but not mutant SV40 large T antigen derepressed p107-mediated repression of E2F-1 transactivation, indicating that SV40 large T antigen is able to interact functionally with p107 in vitro. Although SV40 large T has previously been shown to bind to p107 (Ewen et al., 1991) and to disrupt E2F complexes in non-lymphoid cells, our results are the first report that SV40 large T antigen is able to alleviate p107-mediated repression of E2F activity. Co-transfection of EBNA-LP was found to have no effect on GAL4-E2F-1-mediated transactivation or its repression by pRb or p107 and this was not due to a lack of expression of EBNA-LP.

Data presented here suggest that EBNA-LP is unable to dissociate E2F-1 complexes from their negatively regulating proteins pRb and p107. The results indicate that the effect of EBNA-LP on induction of DNA synthesis (Allan et al., 1992) and its role in the efficient immortalization of primary B cells in culture (Hammerschmidt & Sugden, 1989; Mannick et al., 1991) are unlikely to be due to direct deregulation of E2F-1. Although our results argue against any physiological significance of the EBNA-LP-pRb interaction so far as E2F-1 regulation is concerned, our data do not preclude the direct interaction of pRb and EBNA-LP described in vitro (Szekely et al., 1993) from occurring in vivo, as it is likely that pRb plays roles other than mediating cell cycle regulation of E2F. For example, the PU-1 (Hagemeir et al., 1993) and elf-1 (Wang et al., 1993) transcription factors have recently been demonstrated to interact with pRb. Also, pRb can positively stimulate transcription from SP-1 sites by binding to SP-1, an inhibitor of SP-1-mediated transactivation (Chen et al., 1994). Similarly, pRb positively regulates expression of TGF-β1 gene expression (Kim et al., 1991) and TGF-β2 expression, in the latter case by associating with the ATF-2 transcription factor and stimulating its transactivation properties (Kim et al., 1992). It is possible that EBNA-LP binding to pRb may disrupt these complexes and consequently interfere with these mechanisms of pRb action. Recent experiments have also shown that pRb can bind to the c-Abl tyrosine kinase (Welch & Wang, 1993). c-Abl-pRb association is mediated by the C terminus of pRb and is pocket-independent. EBNA-LP binding to pRb in vitro was not disrupted by a pocket point mutation that inhibits the LXCXE-mediated pRb binding function of E1A, SV40 large T antigen and E7 (Szekely et al., 1993). EBNA-LP might thus contribute to B cell immortalization by disrupting pRb control of c-Abl tyrosine kinase or by changing protein complex formation with pRb.

Previous studies have demonstrated that HPV-16 E6 can prevent both transcriptional transactivating and transrepressing activities of wild-type p53 in a variety of human and rodent cell lines (Lechner et al., 1992; Mietz et al., 1992; Crook et al., 1994a). Data presented here extend these studies to demonstrate that HPV-16 E6 can function efficiently to prevent the wild-type p53 transactivating function in the human BL cell line Akata.

EBNA-LP expression was found to be unable to prevent p53-mediated transactivation in our assays. If EBNA-LP does interact with p53 in vivo, this interaction does not appear to prevent p53-mediated transactivation but might affect some p53 function other than transcription factor activity. In our assays there was a slight enhancement of p53 transactivation by EBNA-LP (Fig. 7a) but the small magnitude of this effect makes it of doubtful significance. Our recent report that full latent EBV gene expression is unable to prevent p53-mediated apoptosis in response to DNA damage (Allday et al., 1995) indicates that EBNA-LP does not interfere with apoptosis mediated by p53. The ability of p53 to suppress transformation of rodent cells by small DNA tumour virus oncoproteins in conjunction with the ras oncogene does not, however, appear to be dependent on the transcriptional properties of p53 (Crook et al., 1994b) and we have not yet excluded the possibility that EBNA-LP might interfere with this effect of p53.

The only functional response to EBNA-LP reported in a transient transfection system is the requirement for EBNA-LP, in cooperation with EBNA-2, for induction of cyclin D2 and entry into the cell cycle in primary B cells (Sinclair et al., 1994). It is possible that this assay will eventually reveal the biochemical mechanism of EBNA-LP action.

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References


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