Physical interaction of Epstein–Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) with human oestrogen-related receptor 1 (hERR1): hERR1 interacts with a conserved domain of EBNA-LP that is critical for EBV-induced B-cell immortalization

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Epstein–Barr virus (EBV) nuclear antigen leader protein (EBNA-LP) consists of W1W2 repeats and a unique C-terminal Y1Y2 domain and plays a critical role in EBV-induced transformation. To identify the cellular proteins associating with EBNA-LP, we performed a yeast two-hybrid screen using EBNA-LP cDNA containing a single W1W2 domain as bait and an EBV-transformed human peripheral blood lymphocyte cDNA library as the source of cellular genes. Our results were as follows. (i) A cDNA in the positive yeast colony was found to encode a cellular protein, human oestrogen-related receptor 1 (hERR1), which is a constitutive transcriptional activator of the various types of oestrogen response elements. (ii) A purified chimeric protein consisting of glutathione S-transferase (GST) fused to hERR1 specifically formed complexes with EBNA-LPs containing one (EBNA-LPR1), two (EBNA-LPR2) or four W1W2 repeats (EBNA-LPR4) transiently expressed in COS-7 cells. Reciprocally, GST fused to EBNA-LPR1 or EBNA-LPR2 pulled down hERR1 transiently expressed in COS-7 cells. (iii) Mutational analyses of EBNA-LP revealed that the Y2 domain of EBNA-LP is responsible for the interaction with hERR1 and two leucines in the Y2 domain (Leu-78 and -82), which are conserved among a subset of primate gammaherpesviruses, are interactive sites for hERR1. So far, it has been reported that the only domain of EBNA-LP critical for EBV-induced transformation is the Y1Y2 domain. Potential roles of hERR1 in EBV-induced transformation are discussed.

INTRODUCTION

Epstein–Barr virus (EBV) is a human gammaherpesvirus that is closely associated with infectious mononucleosis and a wide variety of human malignancies such as endemic Burkitt’s lymphoma, nasopharyngeal carcinoma, Hodgkin’s disease, gastric carcinoma and various lymphomas (reviewed in Rickinson & Kieff, 2001). In vitro, EBV can readily infect resting human B-cells and efficiently immortalize them. The resultant lymphoblastoid cell lines (LCLs) express only a subset of viral genes including six viral nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and LP), three integral latent membrane proteins (LMP-1, -2A and -2B) and two small RNAs (EBERs), possibly for effective stimulation of cell growth and maintenance of the viral episome in proliferating cells (reviewed in Rickinson & Kieff, 2001; Kieff & Rickinson, 2001). Among these latency-associated EBV proteins, EBNA-1, EBNA-2, EBNA-3A, EBNA-3C, EBNA-LP and LMP-1 are critical for EBV-induced B-cell immortalization, whereas EBNA-3B, LMP2A, LMP2B and EBERs are not (reviewed by Kieff & Rickinson, 2001).

The first gene products expressed immediately after the infection by EBV of B-cells are EBNA-LP and EBNA-2 (Alfieri et al., 1991). These genes are transcribed from the BamHI W promoter (Wp), and from the BamHI C promoter (Cp) followed by the gradual waning of Wp activity (Fig. 1A) (Woisetschlaeger et al., 1990, 1991). EBNA-2, a multiple transactivator, stimulates transcription from all
promoters of the latency-associated viral genes in LCLs as well as cellular gene expression including c-myc, c-fgr, CD21 and CD23 (reviewed in Kieff, 1996). The other protein, EBNA-LP, a subject of this report, contains multiple copies of a 66-amino-acid W1W2 domain and a unique 45-amino-acid C-terminal Y1Y2 domain (Fig. 1) (Sample et al., 1986). Reverse genetic studies using recombinant EBVs unable to express the C-terminal Y1Y2 domain revealed that the mutants showed severely impaired transforming activity, indicating that EBNA-LP plays an important role in the EBV-induced immortalization process (Allan et al., 1992; Hammerschmidt & Sugden, 1989; Mannick et al., 1991). Although the actual roles of EBNA-LP in EBV-induced B-cell immortalization remain to be elucidated, the relevant background information listed below suggests a biological function for EBNA-LP.

It is now accepted that a major biological activity of EBNA-LP is to stimulate EBNA-2-mediated transcriptional activation of viral and cellular gene expression, including LMP1 and cyclin D2. Initial domain mapping of EBNA-LP identified the W1W2 repeat domain as a functional domain required for transcriptional cooperation with EBNA-2 (Harada & Kieff, 1997; Nitsche et al., 1997). Subsequent and more detailed analyses revealed that, in the W1W2 repeat domain, regions named CR1 to CR3 and a serine residue at position 35 which are conserved among a subset of primate gammaherpesviruses are critical to the co-activator function of EBNA-LP (McCann et al., 2001; Peng et al., 2000b; Yokoyama et al., 2001a, b). CR2 is a multifunctional domain that also mediates nuclear localization, nuclear matrix association and self-association of EBNA-LP (McCann et al., 2001; Peng et al., 2000b; Tanaka et al., 2001; Yokoyama et al., 2001a, b). Serine-35 is the site for phosphorylation by cellular kinase(s) (Yokoyama et al., 2001b). These observations suggest that the co-activator activity of EBNA-LP is regulated by cellular localization, phosphorylation and protein complex formation mediated by EBNA-LP.

Although the biochemical function of EBNA-LP by itself is being gradually unveiled, the mechanisms by which EBNA-LP acts in EBV-induced B-cell immortalization remain largely unknown at present. Because recombinant EBVs having mutations in each W1W2 repeat domain of EBNA-LP are quite difficult to generate, there is no direct evidence showing a correlation between the co-activator function of EBNA-LP and the EBV-induced B-cell immortalization process. So far, the only available information in this regard is that the Y1Y2 domain of EBNA-LP is critical for the immortalization process (Mannick et al., 1991). It is conceivable that EBNA-LP functions in the immortalization process through the interaction of its Y1Y2 domain with host cellular protein(s). However, the only cellular protein that interacts with the Y1Y2 domain of EBNA-LP is the 70 kDa family of heat shock proteins (Hsp70s) and the biological significance of the interaction is unclear (Kitay & Rowe, 1996). These observations prompted us to hypothesize that additional cellular targets that interact with the Y1Y2 domain of EBNA-LP remain and that further understanding of the role of EBNA-LP in the immortalization process requires their identification. We report here the identification of a novel EBNA-LP binding partner, human oestrogen-related receptor-1 (hERR1), that is associated with the Y1Y2 domain of EBNA-LP.

**METHODS**

**Cells.** HeLa and COS-7, the monkey kidney epithelial cell line, were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS).
Plasmids. The construction of pGBT9-EBNA-LPR1, pGBT9-EBNA-LPR2, pGBT9-EBNA-LPR4 and pZipEBNA-LPR1 was as described previously (Kawaguchi et al., 2000). To generate plasmids encoding the GAL4 DNA binding domain fused to various EBNA-LP (pGBT9-EBNA-LPR1, -LPR2, -LPR1Y1Y2, -LPR1Y1Y2-LPY1Y2, -LPY2), fragments amplified by PCR with appropriate primer pairs were cloned into pGBT9 (Clontech). Amino acid substitution mutants (pGBT9-EBNA-LPR1W81A, pGBT9-EBNA-LPR2L78A, pGBT9-EBNA-LPR2L78/82, pGBT9-EBNA-LPR2L82A, pGBT9-EBNA-LPR4L78A, pGBT9-EBNA-LPR4L78/82, pGBT9-EBNA-LPR4L82A, pGBT9-EBNA-LPR4L82/82A, pGBT9-EBNA-LPR4L82/82A) were generated using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. pGEX-EBNA-LPR1 and pGEX-EBNA-LP-LPR4 are cloned into pGBT9 (Clontech). Amino acid substitution mutants (pGBT9-EBNA-LPR1W81A, pGBT9-EBNA-LPR1L78A, pGBT9-EBNA-LPR1L82A, pGBT9-EBNA-LPR1W81A, pGBT9-EBNA-LPR1L78A, pGBT9-EBNA-LPR1L82A, pGBT9-EBNA-LPR2L78A, pGBT9-EBNA-LPR2L78/82A, pGBT9-EBNA-LPR2L82A, pGBT9-EBNA-LPR4L78A, pGBT9-EBNA-LPR4L78/82A and pGBT9-EBNA-LPR4L82A) were used for GST pull-down experiments and purified on glutathione-Sepharose beads (Amersham Pharmacia) and quantified by the b-mercaptoethanol method as described previously (Kawaguchi et al., 1995). To isolate cDNA whose encoded proteins are able to interact with EBNA-LP, the bait plasmid pGBT9-EBNA-LPR1 and an EBV-transformed human peripheral blood lymphocyte cDNA library (Clontech), fused to the GAL4 transcriptional activation domain in pACT, were sequentially co-transformed into yeast strain HF7C (Clontech).

Yeast two-hybrid library screen. The yeast two-hybrid system was employed as described previously (Kawaguchi et al., 1997a). To isolate cDNA whose encoded proteins are able to interact with EBNA-LP, the bait plasmid pGBT9-EBNA-LPR1 and an EBV-transformed human peripheral blood lymphocyte cDNA library (Clontech), fused to the GAL4 transcriptional activation domain in pACT, were sequentially co-transformed into yeast strain HF7C (Clontech).

GST pull-down experiments. GST fusion proteins were expressed in E. coli DH5α transformed with either pGEX-hERR1, pGEX-EBNA-LPR1, pGEX-EBNA-LPR2 or pGEX-EBNA-LPR4, purified on glutathione–Sepharose beads (Amersham Pharmacia) and quantified as described previously (Kawaguchi et al., 1997a). In the experiments in Figs 2 and 3, COS-7 cells were transfected with either pME-hERR1(F), pME-EBNA-LPR1, pME-EBNA-LPR2 or pME-EBNA-LPR4 by DEAE-dextran methods. After 72 h transfection, the cells were harvested and lysed in HEPES buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 10 mM MgCl₂ and 1 mM PMSF) containing 1 % Triton X-100. In the experiments in Fig. 7, HeLa cells were transfected with either pME-EBNA-LPR2, pME-EBNA-LPR2L78/82A, pME-EBNA-LPR4 or pME-EBNA-LPR4L78/82A by the calcium phosphate method as described previously (Kawaguchi et al., 1995). At 72 h post-transfection, the cells were lysed in TNE buffer (10 mM Tris/HCl, pH 7.8, 150 mM NaCl, 1 mM EDTA and 1 % Nonidet P-40). The cell lysates were incubated with GST alone or an appropriate combination of GST fusion proteins. The proteins bound to GST fusion proteins were separated in a denaturing gel and reacted with a mouse monoclonal antibody to EBNA-LP (JF186). Lysates of COS-7 cells transfected with pME-EBNA-LPR1 were reacted with GST or GST–hERR1 chimeric protein immobilized on glutathione–Sepharose beads. The beads were pelleted, rinsed extensively, subjected to electrophoresis on a denaturing gel and reacted with the mouse monoclonal antibody to EBNA-LP. Lane 1, whole cell extract (WCE) from COS-7 cells transfected with pME-EBNA-LPR1 used in the binding reaction; lanes 2 and 3, WCE from COS-7 cells transfected with pME-EBNA-LPR1 bound to GST and GST–hERR1, respectively. (B, C) hERR1 binds to EBNA-LP with multiple W1W2 repeat domains. Experiments were done exactly as described in (A) except that COS-7 cells were transfected with either pME-EBNA-LPR2 (B) or pME-EBNA-LPR4 (C). Molecular masses (kDa) are shown on the left.
Monoclonal antibody to EBNA-LP (JF186) was kindly provided by G. Klein. A mouse monoclonal antibody to the Flag epitope (M2) was commercially provided by Sigma. Immunoblotting was done as described previously (Kawaguchi et al., 1997b).

**Reporter gene assays.** COS-7 cells were seeded 1 day before transfection and transfected using DMRIE-C (Gibco BRL) according to the manufacturer's instructions. Briefly, COS-7 cells in six-well plates were transfected with 200 ng of pG5 E1b Luc and 500 ng of pM or pM-hERR1, together with 1.3 μg of pME18S or pME-EBNA-LPR2. 50 ng of pRL-tk (Promega) was also co-transfected as an internal control for transfection efficiency. For reporter assays, transfected COS-7 cells were harvested at 48 h post-transfection and the luciferase activity in total cell extracts was analysed using the Dual-luciferase reporter assay system (Promega). Fold activation activity was relative to that of control expression vectors pME18S and pM.

### RESULTS

**EBNA-LP interacts with hERR1 in the yeast two-hybrid system**

Previously, we had performed exactly the same yeast two-hybrid screening except that we used EBNA-LP containing more W1W2 repeats (four copies) as bait, and found that EBNA-LP interacts with HAX-1 (Kawaguchi et al., 2000). To find different cellular proteins, we used EBNA-LP containing a single W1W2 domain as bait (Fig. 1B) in the present study. The evidence that EBNA-LP interacted with hERR1 in the yeast two-hybrid system was as follows. Of 2.4 x 10⁶ colonies, two were histidine-positive and of these one colony expressed strong β-galactosidase activity. The cDNA isolated from the positive colony was positive for interaction with EBNA-LP and negative for interaction with human lamin, murine p53 or the GAL4 DNA binding domain alone (Fig. 1C). Partial sequence analysis of the plasmid from the positive yeast revealed that the plasmid contains a partial cDNA encoding hERR1, an orphan nuclear receptor (Giguere et al., 1998; Yang et al., 1996).

**EBNA-LP specifically forms complexes with hERR1 in reciprocal GST pull-down experiments**

To verify the observed interaction between EBNA-LP and hERR1 in yeast, we expressed GST fusion proteins in E. coli and performed two series of GST pull-down experiments. Because it has been shown that EBNA-LP with a single W1W2 repeat exhibits biological activities different from those of EBNA-LPs with multiple W1W2 repeats (Kawaguchi et al., 2000; Nitsche et al., 1997; Peng et al., 2000b; Yokoyama et al., 2001a, b), we also tested whether or not EBNA-LPs containing more than two copies of W1W2 repeats interact with hERR1.

In the first series of experiments, we examined whether GST–hERR1 pulls down EBNA-LP expressed in mammalian cells. The GST–hERR1 or GST bound to glutathione–Sepharose beads was reacted with extract of COS-7 cells transfected with pME-hERR1(F) used in the binding reaction; lanes 2 and 3, WCE from COS-7 cells transfected with pME-hERR1(F) used in the binding reaction; lanes 2 and 3, WCE from COS-7 cells transfected with pME-hERR1(F) used in the binding reaction; lanes 2 and 3, WCE from COS-7 cells transfected with pME-EBNA-LPR1 bound to GST and GST–EBNA-LP, respectively. Experiments were done exactly as described in (A) except that lysates of the transfected cells were reacted with GST or GST–EBNA-LP2. Molecular masses (kDa) are shown on the left.
The conserved leucines in the Y2 domain of EBNA-LP are the sites of interaction with hERR1

To further map the site(s) in EBNA-LP that interact with hERR1, we performed three series of experiments. In the first, we constructed a series of 3' deletion mutants of EBNA-LPR1, as shown in Fig. 6(A), and tested them for interaction with hERR1 in yeast. These results indicated that the domain of EBNA-LP responsible for the interaction with hERR1 is the Y2 domain.
interaction with hERR1 in the yeast two-hybrid system. As indicated in Fig. 6(A), the site in EBNA-LP that interacted with hERR1 mapped to codons between Leu-82 and Trp-81. In the second series of experiments, we mutagenized the cDNA encoding EBNA-LPR1 by replacing with alanine the single codon Leu-82, Trp-81 or Leu-78, or the codons Leu-82 and -78, as described in Methods. In this experiment, mutants with the amino acid substitution EBNA-LPR1L82A, EBNA-LPR1L82A or EBNA-LPL78/82A failed to interact with hERR1 whereas EBNA-LPR1W83A still sustained the ability to bind to hERR1 in the two-hybrid system (Fig. 6A), indicating that either or both of Leu-78 and -82 is critical for interaction with hERR1 in yeast. Interestingly, Leu-78 and -82 are well-conserved among a subset of primate gamma-herpesviruses (Fig. 6B) (Peng et al., 2000a; McCann et al., 2001). In the third series of experiments, we examined whether the amino acid substitution mutants of EBNA-LP with two or four W1W2 repeats also lack the ability to associate with hERR1 in GST pull-down experiments, in order to verify the results obtained in the yeast two-hybrid system. As shown in Fig. 7, GST–hERR1 was able to pull-down wild-type EBNA-LPR2 or EBNA-LPR4, whereas GST alone was not. In contrast, neither GST–hERR1 nor GST pulled-down EBNA-LPR2L78/82A or EBNA-LPR4L78/82. To some extent, GST–hERR1 was able to pull-down EBNA-LPR2L78A and EBNA-LPR4L78A (data not shown). Thus we conclude that the conserved Leu-78 and -82 are the sites in EBNA-LP of interaction with hERR1.

**DISCUSSION**

Like other viral regulatory proteins, EBNA-LP interacts with multiple cellular proteins and structures. It has been reported that EBNA-LP binds to p53 and pRb in _in vitro_ binding assays, and associates with Hsp70s, HSI-associated protein X-1, DNA-dependent protein kinase catalytic subunit, HA95, α- and β-tubulin, and Hsp72 at the cellular level (Han et al., 2001; Kawaguchi et al., 2000; Kitay & Rowe, 1996; Mannick et al., 1995; Szekely et al., 1993). In EBV-infected B cells, EBNA-LP is localized in both the nucleus and the cytoplasm (Kawaguchi et al., 2000) and a portion of the EBNA-LP in the nucleus is localized to a nuclear structure called ND10 which also contain hsp70s, an antigenically distinct form of pRb and CBP/p300 (Bandobashi et al., 2001; Jiang et al., 1991; Szekely et al., 1995, 1996). Thus EBNA-LP appears to be a multifunctional protein which interacts with and modulates various components of the cellular machinery.

Further identification of the cellular proteins that interact with the EBV regulatory protein EBNA-LP would provide a better understanding of its role in the virus life-cycle and perhaps help to reveal the mechanisms by which EBNA-LP acts in the EBV-induced B-cell immortalization process. We report here the identification of a cellular transcription
factor, hERR1, as a new EBNA-LP-binding protein in the yeast two-hybrid system and in in vitro biochemical assays. We have also shown that the interaction is mediated through the region CR4 in the Y2 domain of EBNA-LP, which is well-conserved among a subset of gammaherpesviruses (McCann et al., 2001; Peng et al., 2000a). The salient features of our results are summarized as follows.

(i) A cDNA library from EBV-transformed human peripheral blood lymphocytes was screened in the yeast two-hybrid system, leading to the identification of one interactor for EBNA-LP, hERR1. The interaction of EBNA-LP with hERR1 demonstrated in GST pull-down experiments not only reinforced the evidence of physical interaction between the two proteins but also indicated that the interaction does not depend on the number of W1W2 repeats. Specifically, GST–hERR1 pulled-down EBNA-LP containing one, two or four copies of W1W2 repeats transiently expressed in COS-7 cells and reciprocally, GST–EBNA-LP with one or two W1W2 repeats pulled-down Flag-epitope-tagged hERR1 transiently expressed in COS-7 cells. Furthermore, we demonstrated in the reporter gene assays that transactivation of the reporter gene construct by both EBNA-LP and hERR1 was augmented, compared to that by either EBNA-LP or hERR1 alone. These results are compelling evidence of a physical and functional interaction between EBNA-LP and hERR1.

(ii) Peng et al. (2000a) previously identified five conserved regions (CR1–CR5) among EBNA-LP and its homologues in primate gammaherpesviruses. Genetic analyses of EBNA-LP as a co-activator of EBNA-2 showed that these conserved domains and conserved residues are critical to its function (McCann et al., 2001; Peng et al., 2000a; Yokoyama et al., 2001a, b). Similarly conserved domains of EBNA-2 homologues among primate gammaherpesviruses also link to functional domains of the protein such as those for interaction with cellular proteins (Peng et al., 2000a). Here we demonstrated that the binding site on EBNA-LP for the interaction with hERR1 has conserved residues in the Y2 domain that is critical for EBV-induced B cell immortalization. Thus deletion analyses of EBNA-LP indicated that hERR1 interacts with EBNA-LP through one of the conserved domains, CR4, of EBNA-LP and further fine mapping revealed that two conserved leucines in CR4 are essential for the interaction of EBNA-LP with hERR1. These results further confirm the importance of the interaction between hERR1 and EBNA-LP, possibly in the EBV-induced immortalization process.

(iii) hERR1 is an orphan nuclear receptor ubiquitously expressed in many tissues, relatively abundantly in the nervous system (Bonneye et al., 1997b; Giguere et al., 1998). hERR1 was initially identified by low-stringency screening of cDNA libraries with a probe encompassing the DNA-binding domain of oestrogen receptor, which plays an important role in the differentiation and development of various organs and in the maintenance of proper cellular functions in a wide variety of tissues (Giguere et al., 1998). hERR1 has been reported to regulate the expression of various cellular and viral genes including lactoferrin (Zhang & Teng, 2000), medium-chain acyl coenzyme A dehydrogenase (Sladek et al., 1997), thyroid hormone receptor (Vanacker et al., 1998a), osteopontin (Bonneye et al., 1997a; Vanacker et al., 1998b) and simian virus 40 (Wiley et al., 1993). In the present study we showed that EBNA-LP physically interacts with hERR1 through the conserved residues in EBNA-LP. Although the biological significance of the interaction between EBNA-LP and hERR1 remains uncertain at present, it is conceivable that EBNA-LP affects the regulation of target genes of hERR1 in infected cells. Two targets of hERR1 are of particular interest. One is lactoferrin, which has been reported to suppress both tumour growth and herpesvirus infection (Andersen et al., 2001; Hutchens & Lonnerdal, 1997; Yoo et al., 1998). The other is aromatase,
which is suggested to regulate cancer growth (Chen et al., 1999). It has been reported that the expression of aromatase is unusually up-regulated by hERR1 in cancer cells and there is a significant correlation between aromatase activity and the presence of tumours (Yang et al., 1998). It should also be noted that the aromatase expression is increased by EBV infection (Vottero et al., 1998). This together with the present study suggests that EBNA-LP interacts with hERR1 to affect the expression of hERR1-inducible cellular and viral genes, and the interaction is involved in EBV-induced transformation. Further experiments to unveil the biological significance of the interaction between EBNA-LP and hERR1 by using viruses in which the conserved residues (Leu-78 and -82) of EBNA-LP are mutated are under way in our laboratories.

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