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EBNA2 Is Required for Protection of Latently Epstein-Barr Virus-Infected B Cells against Specific Apoptotic Stimuli

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In addition to functioning as a transcriptional transactivator, Epstein-Barr virus EBNA2 interacts with Nur77 to protect against Nur77-mediated apoptosis. Estrogen-regulated EBNA2 in EREB2-5 cells was replaced by either EBNA2 or EBNA2 with a deletion of conserved region 4 (EBNA2ΔCR4). Both EBNA2-converted and EBNA2ΔCR4-converted EREB2-5 cells grew in the absence of estrogen and expressed LMP1. Treatment with tumor necrosis factor alpha did not induce apoptosis of EBNA2- or EBNA2ΔCR4-expressing cells, but EBNA2ΔCR4 cells were susceptible to etoposide and 5-fluorouracil, Nur77-mediated inducers of apoptosis. Thus, EBNA2 protects B cells against specific apoptotic agents against which LMP1 is not effective.

Epstein-Barr virus (EBV) infection of B cells in culture leads to the outgrowth of immortalized lymphoblastoid cell lines (LCLs) that express the EBV latency genes encoding EBV nuclear antigen 1 (EBNA1), EBNA2, EBNA3A, EBNA3B, and EBNA3C, EBNA leader protein, latent membrane protein 2A (LMP2A) and LMP2B, the products of the BamHI-A rightward transcripts, and the noncoding polymerase III EBV-encoded RNAs (28, 48). EBNA2 is essential for B-cell immortalization (7, 14). EBNA2 acts as a transcriptional transactivator to regulate EBV latency gene expression in B cells and to modify cellular gene expression with a resultant stimulation of G_0-to-G_1 cell cycle progression (24, 27, 37). In its transcriptional role, EBNA2 mimics the effects of activated NotchIC (11, 19, 21, 39). Notch is an evolutionarily conserved surface receptor that influences cell fate and developmental decisions and is frequently activated in human cancers (33, 36). EBNA2 and NotchIC both target responsive promoters through the cellular DNA binding protein CBF1/CSL/RBP-Jk (16). CBF1 functions as a transcriptional repressor through interactions with an mSin3-histone deacetylase-containing complex (20, 22, 25, 52). EBNA2 and NotchIC activate expression by displacing the corepressor complex (20, 25, 50, 51) and by contacting the basal transcriptional machinery (40, 42) and recruiting coactivators, which in the case of EBNA2 include pCAF, p300/CBP (43), p100 (41), the SWI/SNF complex (45), survival motor neuron protein (1), and EBNA leader protein (15, 34, 35, 47).

Comparison of the EBNA2 amino acid sequence with that encoded by the EBV-related baboon virus herpesvirus papio identified nine regions (32) that are also conserved in the EBNA2 proteins of other old world primate lymphocryptoviruses (4, 35). These conserved regions mediate nuclear localization and transactivation (5, 32), interaction with CBF1 and the repression-to-activation switch protein SKIP (13, 18, 46, 50), and interaction with Nur77 (29). Binding of EBNA2 to Nur77 is a property shared with NotchIC (23) and is mediated by EBNA2 conserved region 4 (CR4). The interaction blocks Sindbis virus-induced apoptosis and Nur77-mediated apoptosis in transfected cells by preventing mitochondrial targeting of Nur77 (29). A tumorigenic phenotype is associated with increased resistance to apoptosis in addition to increased proliferation. To further evaluate the potential contribution of EBNA2's antiapoptotic activity to EBV-associated disease, we used the EREB2-5 transcomplementation assay (11, 12) to examine EBNA2 function in an EBV-infected B-cell background. EREB2-5 is an EBV-immortalized B-cell line in which EBNA2 is expressed as an estrogen receptor binding domain-EBNA2 fusion (EREBNA2) (27). EBNA2 function is dependent on the presence of estrogen in the culture medium, and in the absence of estrogen, EREB2-5 cells undergo growth arrest and apoptosis (26, 27). Lentivirus transduction of wild-type or immortalization-competent mutant EBNA2 can rescue cell growth in the absence of estrogen, whereas transduction of an immortalization-incompetent EBNA2 mutant fail to compensate for estrogen withdrawal (11). Thus, the transcomplementation approach can be used to study the properties of EBNA2 mutant proteins in the context of latent EBV infection.

Ectopically expressed EBNA2ΔCR4 supports growth of EREB2-5 cells in estrogen-free medium. Recombinant lentiviruses expressing EBNA2 and EBNA2ΔCR4 (with a deletion of amino acids 123 to 147) were constructed in the vector pLK2. The IRES-eGFP fragment of the lentivirus vector pLVEF.GFP (8) was replaced with the IRES-hrGFP cassette from pIRES-hrGFP1a (Stratagene) to generate pLK2. A BglII fragment en-

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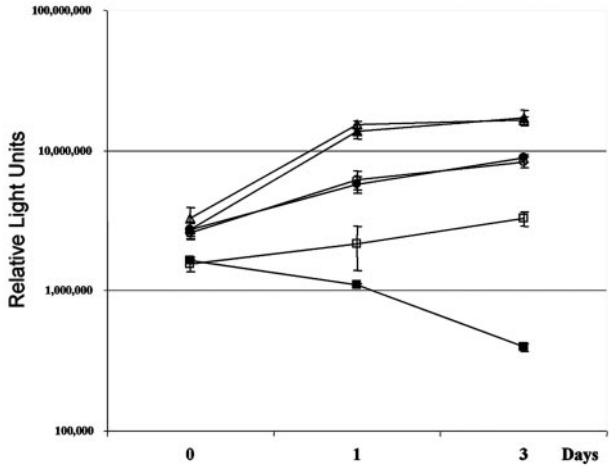

FIG. 1. Effect of estrogen starvation on growth of EBNA2-transduced EREB2-5 cells. Ten thousand cells/well were plated into 96-well plates, and cell proliferation was monitored using the CellTiterGlo assay to measure ATP usage. LK2-EREB2-5 cells with estrogen (□) and without estrogen (■), LK2-EBNA2 cells with estrogen (△) and without estrogen (▲), and LK2-EBNA2ΔCR4 cells with estrogen (○) and without estrogen (●) are shown. Data shown are the means for three assays, with standard deviations provided.

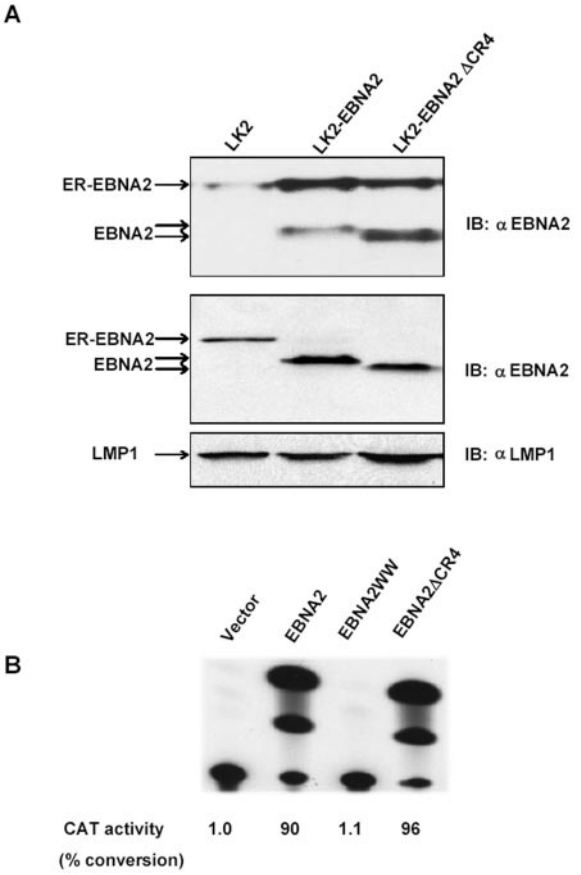

FIG. 2. EBNA2ΔCR4 is transcriptionally competent in supporting LMP1 expression and Cp promoter activity. (A) Upper panel, Western blot probed with anti-EBNA2 PE2 monoclonal antibody (DAKO). ER-EBNA2 is expressed in EREB2-5 cells transduced with LK2. ER-EBNA2 plus EBNA2 are expressed in LK2-EBNA2- and LK2-EBNA2ΔCR4-transduced cells. Middle panel, Western blot showing loss of ER-EBNA2 expression in LK2-EBNA2 and LK2-EBNA2ΔCR4 cells grown continuously in the absence of estrogen. LK2-EREB2-5 cells maintained in estrogen-containing medium retain ER-EBNA2 expression. Lower panel, the membrane shown in the middle panel was stripped and reprobed with anti-LMP1 S12 monoclonal antibody (32a). LK2-EBNA2ΔCR4 cells showed no deficit in the ability to mediate LMP1 expression. (B) Transient expression assay in which HeLa cells were cotransfected with the EBV Cp promoter reporter 4× Cp-CAT and either control vector or an expression plasmid for EBNA2, the EBNA2 mutant (EBNA2WW), or EBNA2ΔCR4. EBNA2ΔCR4 was as effective as EBNA2 in activating expression of 4× Cp-CAT.

coding EBNA2 was introduced into the BamHI site of pLK2 to form wild-type EBNA2-expressing virus (LK2-EBNA2), and an EcoRI/BglII fragment encoding EBNA2ΔCR4 was ligated as a blunt-end fragment into EcoRV-cleaved pLK2 to form LK2-EBNA2ΔCR4. LK2, LK2-EBNA2, and LK2-EBNA2ΔCR4 viruses were produced by transient transfection of 293T cells as previously described (8). EREB2-5 cells were transduced with the recombinant lentiviruses, and cells expressing green fluorescent protein were enriched using fluorescence-activated cell sorting. After expansion for 3 weeks in the presence of estrogen, fluorescence-activated cell sorting analysis indicated that the resulting cell pools had similar mean fluorescence intensities and were more than 95% green fluorescent protein positive (data not shown). The transduced cultures were then tested for their ability to survive in estrogen-free conditions, using the CellTiter-Glo luminescent cell assay (Promega) to measure viable cell number. After estrogen withdrawal, LK2-transduced cells stopped growing and died, whereas the growth of LK2-EBNA2- and LK2-EBNA2ΔCR4-transduced cells was unaffected by the absence of estrogen, although viable LK2-EBNA2ΔCR4 cells did accumulate at a slower rate than LK2-EBNA2 cells (Fig. 1). The ability of EBNA2ΔCR4 to support LCL growth and LMP1 expression is consistent with observations previously made by using a recombinant EBV carrying a larger EBNA2 deletion encompassing the CR4 region (6).

Selection of EREB2-5-transduced cells lacking ER-EBNA2 expression. We wished to examine the importance of the Nur77-EBNA2 interaction for protection against apoptosis, using the EBNA2ΔCR4-transduced EREB2-5 cells. However, the continued expression of the ER-EBNA2 fusion protein in these cells complicated their utility. The EBNA2- and EBNA2ΔCR4-transduced EREB2-5 cells were cultured in estrogen-free medium for 2 months, after which clones were identified that no longer expressed ER-EBNA2 proteins (Fig. 2A, upper and middle panels). The level of LMP1 expression in the selected EBNA2-transduced cells was similar to that in the EBNA2ΔCR4-transduced cells (Fig. 2A, lower panel), indicating that there was no defect in regulation of LMP1 by EBNA2ΔCR4. The ability of EBNA2ΔCR4 to activate the EBV latency Cp promoter was also checked in HeLa cells cotransfected with a 4× Cp-CAT reporter and expression plasmids for EBNA2, EBNA2ΔCR4, or the non-CBF1-binding EBNA2(WW) mutant as previously described (3). EBNA2ΔCR4 was shown to be as effective as EBNA2 in transactivation of 4× Cp-CAT expression (Fig. 2B). These results reinforce the point that the EBNA2ΔCR4 protein does not suffer from any defects in transactivation function.

EBNA2 CR4 is necessary for inhibition of Nur77-induced

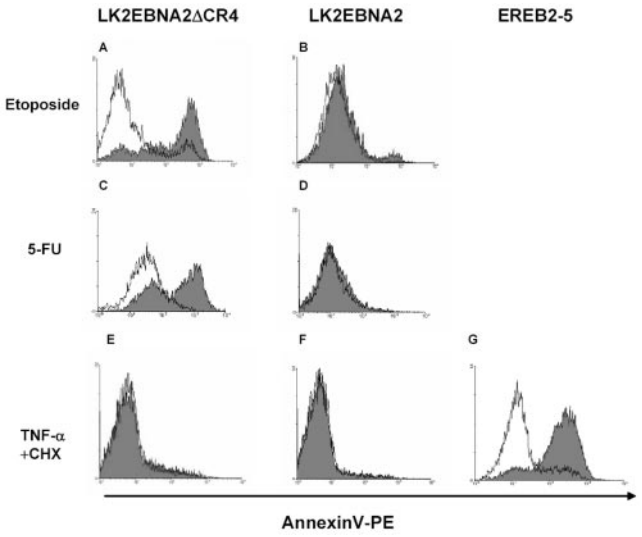

FIG. 3. EBNA2 CR4 is necessary for resistance to etoposide- and 5-FU-induced cell death but not TNF-α-induced cell death. Estrogen-independent LK2-EBNA2ΔCR4 and LK2-EBNA2 cells were treated with (A, B) etoposide (10 μg/ml), (C, D) 5-FU (25 ng/ml), or (E, F) TNF-α (5 ng/ml) plus cycloheximide (10 μg/ml; CalBiochem) for 20 h. (G) EREB2-5 cells were grown in the absence of estrogen for 5 days before treatment with TNF-α plus cycloheximide. Apoptosis was measured by annexin V-phycoerythrin binding. The data shown are representative of three independent experiments. Drug-treated cells, shaded profile; untreated cells, open profile.

cell death but not for TNF-α-induced cell death. LMP1 confers a survival advantage on EBV-infected B cells by activation of NF-κB-upregulated antiapoptotic genes, such as those encoding A20, Bfl-1, and Bcl-2 (2, 9, 10, 17, 38). However, the apoptosis-modulating activity of LMP1 is stimulus dependent. LMP1 expression in HeLa cells protects against apoptosis induced by tumor necrosis factor alpha (TNF-α) but provides no protection against apoptosis induced by Fas or etoposide (49). We previously showed that EBNA2 CR4 binds Nur77 and could protect cells from stimuli, such as treatment with phorbol esters or Sindbis virus infection, that induce apoptosis through activation of Nur77. Nur77 has no role in TNF-α-induced cell death but does mediate apoptosis induced by etoposide and 5-fluorouracil (5-FU) (30, 44). To examine the contribution to B-cell survival of the EBNA2-Nur77 interaction, LK2-EBNA2 and LK2-EBNA2ΔCR4 cells lacking ER-EBNA2 expression were treated with etoposide (10 μg/ml; Sigma), 5-FU (25 μg/ml; Sigma), or TNF-α (5 ng/ml; Sigma) plus cycloheximide (10 ng/ml; Calbiochem). Reverse transcription-PCR analysis using primers 5′-CACCCACTTCTCCACACCTT and 3′-ACAACT TCCTTCACCATGCC showed induction of Nur77 transcripts 2 h after treatment with etoposide or 5-FU (data not shown). Apoptosis was measured by binding of annexin V-PE (BD Pharmingen) (Fig. 3). LK2-EBNA2 cells were resistant to treatment with etoposide and 5-FU (Fig. 3B and D), while LK2EBNA2ΔCR4 cells showed significant etoposide- and 5-FU-induced apoptosis (Fig. 3A and C). In contrast, both LK2-EBNA2 and LK2-EBNA2ΔCR4 cells were resistant to treatment with TNF-α (Fig. 3E and F). Parental EREB2-5 cells grown for 5 days in the absence of estrogen to eliminate EREB2 nuclear activity and LMP1 expression were sensitive to TNF-α-induced apoptosis (Fig. 3G).

In summary, these results show that EBNA2 and LMP1 make separate and complementing contributions to cell survival in EBV-infected LCLs. EBNA2 interaction with Nur77 plays no part in resistance to TNF-α, and LMP1 is sufficient to protect B cells from TNF-α-induced cell death. On the other hand, LMP1 cannot protect against etoposide or 5-FU, which are Nur77-mediated apoptotic stimuli, and EBNA2 is essential for protection against these agents. Interestingly, LMP1 may in fact sensitize cells to Nur77-mediated apoptotic stimuli. Nur77 has recently been shown to bind to Bcl-2 and to convert Bcl-2 from an antiapoptotic protector to a proapoptotic cell death inducer (31). LMP1 is known to upregulate expression of Bcl-2 (17), and hence, additional mechanisms, such as the EBNA2-mediated nuclear retention of Nur77, may be particularly important for the survival of LMP1-expressing latently EBV-infected B cells.

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