Expression of the Epidermodysplasia Verruciformis-Associated Genes EVER1 and EVER2 Is Activated by Exogenous DNA and Inhibited by LMP1 Oncoprotein from Epstein-Barr Virus

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EVER1 and EVER2 are mutated in epidermodysplasia verruciformis patients, who are susceptible to human betapapillomavirus (HPV) infection. It is unknown whether their products control the infection of other viruses. Here, we show that the expression of both genes in B cells is activated immediately after Epstein-Barr virus (EBV) infection, whereas at later stages, it is strongly repressed via activation of the NF-κB signaling pathway by latent membrane protein 1 (LMP1). Ectopic expression of EVER1 impairs the ability of EBV to infect B cells.

Epidermodysplasia verruciformis (EV) is a rare primary immune-deficiency, and EV patients have increased susceptibility to infection with human betapapillomaviruses (beta HPVs) (1, 2). The familial occurrence of the disease led to the search for an EV gene involved in the control of HPV infection. In 2002, Ramoz et al. described that mutations in two adjacent novel genes called EVER1 (TMC6) and EVER2 (TMC8) are considered the two genetic etiologies of EV (3–5). Until now, the role of the two proteins EVER1 and -2 has been exclusively circumscribed to keratinocytes, in which they control the intracellular levels of zinc (2).

However, in a recent report a link between EVER proteins and intracellular zinc homeostasis was documented in T cells from an EVER-deficient patient (6). In addition, peripheral blood T cells of EV patients showed a biased memory/naive T cell ratio and increased skin homing surface markers, although no alteration in cell proliferation was observed (7). These new data justify further investigations to evaluate whether EVER protein functions are relevant in other cells and/or are linked with infection with pathogens other than beta HPVs. Interestingly, human lymphocyte populations (6) and, in particular, human B cells, are among the cells carrying the highest levels of EVER1 and EVER2 genes (8).

We first evaluated whether these genes play a role at an early stage of HPV infection and transiently transfected spontaneously immortalized keratinocytes (NIKS) with a linearized 8-kb fragment of the beta HPV38 genome. EVER1 and EVER2 mRNA levels were increased 24 h posttransfection (Fig. 1A). Upregulation of both genes was observed also in NIKS transfected with a plasmid containing the green fluorescent protein (GFP) gene (data not shown), suggesting that EVER1 and EVER2 expression could be activated when cells are exposed to exogenous DNA. To corroborate our data, we repeated the experiments in B lymphocytes, which are known to express very high levels of EVER1 and EVER2 (6, 8, 9). Myeloma-derived RPMI-8226 cells were transfected with the bacterial plasmid pBluescript SK (pBSK), which due to the absence of eukaryotic promoters in its sequence, does not produce any transcript in mammalian cells. pBSK transfection into RPMI-8226 cells resulted in a rapid increase in EVER1 and EVER2 mRNA levels that persisted up to 48 h posttransfection (Fig. 1B). In addition, exposure of the same cells to the Toll-like receptor 3 (TLR3) ligand polyinosinic poly(C) [poly(I·C)], which mimics the double-stranded RNA structure, did not stimulate transcription of EVER1 or EVER2 (Fig. 1E). Thus, our data indicate that the activation of EVER1 and EVER2 expression is mainly associated with the sensing of foreign DNA in the cells.

To validate our initial results in a more relevant experimental model, we infected primary B cells with recombinant EBV expressing GFP (EBV-GFP) (10). The presence of the GFP gene in the EBV genome enabled the monitoring of infection efficiency by determining the percentage of green cells. In a first set of experiments, we observed that infection with EBV resulted in increased EVER1 and EVER2 mRNA levels at early time points postinfection, whereas expression of the two genes was strongly inhibited after 24 h (Fig. 1C and D). To gain more insights into the phenomenon, we inactivated EBV particles with heat or UV treatment. Heat-inactivated EBV particles lose the ability to enter into the cell due to denaturation/unfolding of the glycoproteins of the envelope and other viral proteins. In contrast, the UV-exposed EBV retains the ability to infect the cells but loses expression of the viral genes due to substantial fragmentation of viral DNA and RNA. Upregulation of EVER1 and EVER2 expression is dependent on viral entry, since heat-inactivated EBV-GFP did not induce the event. UV-inactivated EBV-GFP retained a similar efficiency to the untreated virus in promoting upregulation of EVER1 and

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EVER2 expression. However, UV-treated EBV-GFP was unable to downregulate EVER1 and EVER2 mRNA levels (Fig. 1C and D), indicating that the phenomenon is dependent on viral genome replication and/or viral transcription.

Time course experiments indicated that downregulation of EVER1 and EVER2 mRNA levels correlates with the expression of the latent membrane protein 1 (LMP1) viral oncogene (Fig. 2A and B). To further evaluate the role of LMP1, we compared EVER1 and EVER2 mRNA levels in RPMI-8226 cells infected with EBV-GFP or an EBV-GFP mutant lacking the LMP1 gene (EBVΔLMP1). As expected, infection with both viruses led to a strong increase in transcript levels of the two cellular genes at early time points postinfection (Fig. 2B, left and middle graphs). However, only wild-type EBV-GFP was able to efficiently downregulate EVER1 and EVER2 expression at late stages postinfection, whereas in EBVΔLMP1-infected cells, the mRNA levels of the two cellular genes remained high (Fig. 2B, left and middle panels). This scenario resembled the one observed in cells infected with
EBV-mediated downregulation of EVER1 and EVER2 via the oncoprotein LMP1. (A and B) RPMI-8226 cells were infected with the wild type or EBV-GFP mutant lacking the LMP1 gene, ΔLMP1 (DLMP-1) EBV (MOI, 1). At the indicated times, cells were processed for the determination of EVER1, EVER2, and LMP1 mRNA levels by RT-qPCR. Data are presented as means ± SD from three independent experiments. In panel A, the decrease in EVER1 or EVER2 mRNA levels was statistically significant: time zero versus 24, 48, or 72 h, P < 0.02. (C) ΔLMP1 EBV-infected cells were transduced with empty retrovirus (pLXSN) or a retrovirus expressing LMP1. After establishment of stable cell lines, cells were processed for the determination of EVER1, EVER2, and LMP1 mRNA levels by RT-qPCR. Data are presented as means ± SD from three independent experiments. The decrease in EVER1 or EVER2 mRNA levels was statistically significant: pLXSN versus LMP1, P = 0.0001 and P = 0.005, respectively. (D) Primary fibroblasts were infected with HSV-1 (MOI, 10). At the indicated times, cells were processed for the determination of EVER1 mRNA levels by RT-qPCR. Data are the means from three independent experiments performed in duplicate. Data are presented as means ± SD from 3 independent experiments. The increase in the EVER1 mRNA level was statistically significant: time zero versus 1.5, 7, 18, or 24 h, P < 0.02.
FIG 3 EBV LMP1 represses EVER1 and EVER2 expression via activation of the canonical NF-κB signaling pathway. (A) RPMI-8226 cells stably infected with EBV were cultured in appropriate medium containing the indicated chemical inhibitors. After 6 h, cells were processed for the determination of the EVER1 mRNA level by RT-qPCR. Data are presented as means ± SD from two independent experiments. The increase in EVER1 mRNA levels was statistically significant: dimethyl sulfoxide (DMSO)-treated EBV-infected cells versus Bay11-7082-treated EBV-infected cells, \( P < 0.0001 \). (B) RPMI-8226 cells were transduced with empty retrovirus (pLXSN) or a retrovirus expressing wild-type LMP1 or CTAR-1, CTAR-2, or CTAR-1/2 LMP1 mutants. After establishment of stable cell lines, cells were processed for the determination of EVER1, EVER2, and LMP1 mRNA levels by RT-qPCR. Data are presented as means ± SD from two independent experiments. (C) Western blot analyses were performed on 70 μg of cellular protein extracts from RPMI-8226 cells stably transduced with empty retrovirus (pLXSN) or a retrovirus expressing wild-type LMP1 or CTAR-1, CTAR-2, or CTAR-1/2 LMP1 mutants. Membranes were probed with anti-LMP1 S12 (1:2) or anti-β-actin (1:5,000) antibody. Membranes were then washed and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000) (Promega). Membranes were then developed using the Clarity Western ECL kit (Bio-Rad). (D) RPMI-8226 cells (Mock), RPMI-8226 cells stably infected with EBV, LMP1-expressing RPMI-8226 cells, and LCL cells were transfected with scramble or NF-κB siRNA. At 48 h posttransfection, cells were processed for the determination of EVER1 mRNA level by RT-qPCR. Data are presented as means ± SD from two independent experiments. The increase in EVER1 mRNA levels was statistically significant in the EBV-infected RPMI-8226 cells, RPMI-8226 cells stably transduced with virus expressing LMP1, and LCL cells: scramble siRNA versus siNF-κB, \( P < 0.02 \). (E) Western blot analyses were performed on 50 μg of cellular extracts from RPMI-8226 cells, RPMI-8226 cells stably infected with EBV, RPMI-8226 cells stably transduced with a retrovirus expressing wild-type LMP1, or LCL cells transfected with scramble or NF-κB siRNA for 48 h. Membranes were probed with anti-p65, anti-p50 (1:2,000) (Cell Signaling) or anti-β-actin (1:5,000) antibody. Membranes were then washed and incubated with anti-mouse or rabbit horseradish peroxidase-conjugated secondary antibodies (1:5,000) (Promega). Membranes were then developed using the Clarity Western ECL kit (Bio-Rad).
EBV LMP1 Downregulates EVER1 and EVER2 Expression

A. EVER1

B. %GFP positive cells

C. EBER

D. Percentage of GFP-positive cells

E. EBER

mock

Ectopic EV1(cl2)

Ectopic EV1(cl5)
UV-inactivated EBV-GFP (Fig. 1C and D). Together, these findings show that LMP1 plays a key role in the repression of these two cellular genes. Accordingly, transduction of EBV/LMP1-infected cells with a recombinant retrovirus expressing LMP1 restored the EBV wild-type efficiency in inhibition to decrease EVER1 and EVER2 mRNA levels (Fig. 2C). We next examined whether another member of the Herpesviridae family (i.e., herpes simplex virus 1 [HSV-1]) is able to interfere with EVER1 expression. Primary human fibroblasts were infected with HSV-1, and EVER1 expression levels were determined by real-time reverse transcription-quantitative PCR (RT-qPCR). Like EBV, HSV-1 infection induced an increase in expression of both genes. However, we did not observe any downregulation of their expression at least up until 24 h postinfection (Fig. 2D). Together, these data demonstrate that EBV or HSV-1 infection induces an upregulation of EVER1 and EVER2 expression. However, only infection with wild-type EBV via the expression of viral LMP1 strongly counteracts this event.

It has been shown that LMP1 has the ability to activate a variety of signaling pathways, including NF-κB, mitogen-activated protein kinase (MAPK) p38, Jun N-terminal protein kinase (JNK), and MAPK/extracellular signal-regulated kinase (ERK). This activation is mediated by its C-terminal cytosolic domains (11–14). To determine which signaling pathway is affecting the regulation of EVER1 and EVER2 expression, we first treated EBV-infected RPMI-8226 cells with chemical inhibitors that specifically inhibit the activity of the NF-κB, MAPK p38, or JNK pathways (Bay11-7082 or H9260). It has been shown that two of the cytoplasmic domains of LMP1, named C-terminal activation region 1 (CTAR-1) (residues 194 to 232) and CTAR-2 (residues 351 to 386) (15), have the ability to activate the noncanonical and canonical NF-κB pathways, respectively (16, 17). Using recombinant retroviruses, we transduced RPMI-8226 cells with mutant LMP1 mutant AxAxAX (CTAR-1), 378 STOP (CTAR-2), or AxAxAX/378 STOP (CTAR-1/2). Levels of expression of these different constructs were analyzed by immunoblot analyses, and the constructs showed a similar pattern of expression (Fig. 3C). Both CTAR-2 and CTAR-1/2 LMP1 mutants failed to inhibit EVER1 and EVER2 expression, whereas the CTAR-1 mutant was still able to elicit this inhibition (Fig. 3B). Therefore, we conclude that LMP1 downregulates EVER1 and EVER2 expression through its CTAR-2 domain, which activates the canonical NF-κB pathway. To confirm this result, we silenced the expression of the NF-κB subunits by using a pan-NF-κB small interfering RNA (siRNA) oligonucleotide (18) in a lymphoblastoid cell line (LCL) as well as in RPMI-8226 cells which either expressed only LMP1 or were infected with the entire EBV. Following siRNA treatment, levels of expression of p65/RelA and p50 were monitored by immunoblot analyses and showed a decrease in expression (Fig. 3E). We observed a strong upregulation of EVER1 mRNA levels in all cells treated with NF-κB siRNA, whereas control scramble siRNA did not show any effect (Fig. 3D), confirming that the downregulation of EVER1 and EVER2 expression by EBV LMP1 is mediated by the activation of the canonical NF-κB signaling pathway.

To gain insights into the biological role of EVER1 in the context of EBV infection, RPMI-8226 cells were first transduced with recombinant lentiviruses expressing two different EVER1 small hairpin RNAs (shRNAs) (EV1 sh1 and sh2). Efficiency of EVER1 gene silencing was demonstrated by reverse transcription-quantitative PCR (RT-qPCR) (Fig. 4A). Then, after EBV infection, we monitored the susceptibility of the transduced RPMI-8226 cells to EBV-GFP infection in EVER1-silenced versus scramble siRNA-transduced cells. We observed that shRNA-mediated knockdown of EVER1 expression increased the number of GFP-positive cells and the expression levels of the small noncoding viral RNA EBER (Fig. 4B and C). Finally, RPMI-8226 cells were transduced with recombinant EVER1-expressing lentivirus and then infected with EBV-GFP recombinant virus. We observed that RPMI-8226 EVER1-expressing cells decreased EBV infection efficiency and EBER expression compared with mock-transduced cells (Fig. 4D and E). In conclusion, these data indicate that EVER1 expression is able to decrease the efficiency of EBV infection.

Mutations in EVER1 and EVER2 have been clearly associated with an increased susceptibility to cutaneous beta HPV infections in EV patients (4). In addition, it has been suggested that both encoded proteins are involved in the innate immune response (19). Consistent with this hypothesis, EVER1 and EVER2 have been found to be highly expressed in T and B lymphocytes, natural killer cells, and dendritic cells (9). Although EV patients also appear to be permissive hosts for other non-beta HPV types, such as HPV3 and -10, there is no clear evidence that the infection prevalence of other viruses is increased in these patients compared with that in healthy individuals (4). One possible explanation is that many viruses, in contrast to beta HPV types, have the ability to counteract EVER1 and EVER2 function, and therefore mutations of their genes will not be essential for the establishment of an infection and/or completion of the viral life cycle. Here, we show for the first time that EBV, via the oncoprotein LMP1, strongly represses the transcription of EVER1 and EVER2. This transcriptional inhibition is mediated by the activation of the canonical NF-κB signaling pathway. Although our initial data suggest that these two cell-
lular proteins are part of the innate immune response activated by exogenous DNA, the precise mechanisms and the cellular pathways involved remain to be elucidated.

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