Epstein–Barr virus latent membrane protein 2A mimics B-cell receptor-dependent virus reactivation

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Latent membrane protein 2A (LMP2A) of Epstein–Barr virus (EBV) shares protein motifs with the B-cell receptor that play a role in B-cell receptor signalling and has been shown to mimic an activated B-cell receptor by providing a survival signal for mature B cells in transgenic mice. Conversely, LMP2A has been reported not to support but to inhibit B-cell receptor signalling with respect to virus reactivation and to block lytic virus induction after anti-Ig treatment of EBV-infected B cells. To solve this apparent paradox, the role of LMP2A in lytic-cycle induction was re-examined in B cells conditionally immortalized by EBV. It was shown that, in the absence of other stimuli, LMP2A expression alone could lead to induction of the virus lytic cycle. Similarly to B-cell receptor stimulation by anti-Ig treatment, this LMP2A-mediated reactivation was dependent on the mitogen-activated protein kinase pathway and could be inhibited by the viral LMP1. Our data reinforce the notion that LMP2A is a functional homologue of the B-cell receptor, not only with respect to B-cell survival but also with respect to regulation of the lytic cycle.

INTRODUCTION

Epstein–Barr virus (EBV) is a ubiquitous human lymphotropic herpesvirus. In the vast majority of cases, viral infection is benign, yet EBV is associated with a number of human malignancies, including Burkitt’s lymphoma, nasopharyngeal carcinoma, lymphoproliferative diseases in immunocompromised individuals and Hodgkin’s disease (Rickinson & Kieff, 2001). After primary infection, EBV persists for the life of the host in the peripheral blood in resting memory B cells (Thorley-Lawson, 2001). Virus reactivation from these cells is strictly controlled and mainly observed in immunocompromised individuals (Lam et al., 1991; Decker et al., 1996; Babcock et al., 1999). Latent membrane protein 2A (LMP2A) transcripts have been detected in memory B cells, the site of EBV persistence in the periphery, by RT-PCR (Qu & Rowe, 1992; Tierney et al., 1994; Miyashita et al., 1997; Babcock et al., 1998, 1999), yet analysis of the EBV latent gene-expression pattern at the single-cell level has revealed that none of the viral latent genes is expressed in latently infected memory B cells (Hochberg et al., 2004a, b). In contrast to peripheral EBV-positive B cells, memory B cells in tonsils exhibit consistent expression of LMP2A together with the latent viral nuclear protein EBNA1 and the viral LMP1 (Babcock & Thorley-Lawson, 2000; Babcock et al., 2000). The expression of LMP2A and LMP1 in tonsillar memory B cells is believed to provide a survival signal to peripheral latently EBV-infected cells and to rescue the cells from apoptosis when they migrate through secondary lymphoid organs. LMP1 and LMP2A may thus contribute significantly to long-term survival of EBV-positive memory B cells in vivo.

LMP2A is an integral membrane protein with 12 hydrophobic transmembrane domains and cytoplasmic N and C termini (Laux et al., 1988, 1989; Rowe et al., 1990). Similarly to the intracellular domain of the B-cell receptor, the N terminus of LMP2A contains a number of motifs involved in protein–protein interactions with different phosphotyrosine kinases and motifs involved in immunoreceptor tyrosine-based activation motif formation (Longnecker, 2000) and it resides in lipid rafts (Higuchi et al., 2001). In transgenic mice, LMP2A provides signals for B-cell survival allowing B-lymphocyte development to proceed in the absence of a functional B-cell receptor (Caldwell et al., 1998). Studies with chimeric proteins expressing the LMP2A N terminus have shown that the N terminus can induce cytokine expression and calcium release (Alber et al., 1993; Beaufils et al., 1993). Recently, it has additionally
been shown that expression of LMP2A in epithelial cells leads to activation of the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways (Chen et al., 2002).

Cross-linking of the B-cell receptor leads to activation of a complex signalling cascade resulting in calcium mobilization and activation of the ERK and JNK pathways and of protein kinase C (Campbell, 1999). It has long been known that stimulation of the B-cell receptor induces EBV reactivation in virus-infected B cells (Tovey et al., 1978; Takada, 1984). Even though LMP2A mimics a functional B-cell receptor, it has been shown that in LMP2A-expressing B cells, B-cell receptor-dependent activation of the lytic cycle is inhibited (Miller et al., 1993, 1994, 1995; Dykstra et al., 2001). LMP2A expression interferes with calcium mobilization and EBV reactivation after cross-linking of surface Ig. This is presumably due to a dominant-negative effect on phosphotyrosine kinase activation and interference with the transfer of the B-cell receptor to lipid rafts. The inhibitory effect is dependent on the level of expression of LMP2A (Konishi et al., 2001).

We have recently shown that expression of LMP1 can inhibit induction of the EBV lytic cycle (Adler et al., 2002). Comparing in a conditional lymphoblastoid cell line (LCL) the inhibitory effect of LMP1 with the long-known effect of LMP2A on virus reactivation by 12-O-tetradecanoylphosphol-13-acetate (TPA) or anti-Ig, we previously showed that, in this cell system, LMP2A also interferes with virus reactivation, although to a lesser extent than LMP1. Here, we have demonstrated that, in EBV-positive cells that are permissive for virus reactivation, expression of LMP2A alone induced entry into the lytic cycle in the absence of other inducers. LMP2A thus has a dual role with respect to lytic cycle induction: it induces entry into the lytic cycle in the absence of B-cell receptor stimulation and it impedes lytic-cycle induction through the B-cell receptor when both signals act simultaneously.

**METHODS**

**Cells and cell lines.** All cell lines were grown in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Oestrogen was added to the medium of EREB2-1 cells to a final concentration of 1 μM. Akata and P3HR1 cells were electroporated at 250 V and 975 μF, and EREB2-1 cells were electroporated at 220 V and 975 μF. The mitogen-activated protein kinase (MAPK) kinase inhibitor PD98059 (50 μM; Calbiochem) was added to the cell-culture medium directly after transfection. Cells co-transfected with pEGFP-C1 were harvested 72 (BZLF1 detection) or 96 [virus capsid antigen (VCA) detection] h after transfection. For additional stimulation with anti-Ig antibodies, EREB2-1 and Akata cells were stimulated 48 h after transfection with plate-bound anti-IgM (Sigma) or plate-bound anti-IgG (ICN Biomedicals), respectively. Anti-IgM was used at 20 μg per well and anti-IgG at 72 μg per well in six-well plates. Cells co-transfected with NGFR were purified by microbead-assisted cell sorting (MACS; Miltenyi Biotec) using an anti-NGFR antibody (HB8737; ATCC) and anti-mouse IgG microbeads. Purification was performed 48 h after transfection and before additional stimulation.

**FACS analysis.** For staining of GFP-positive cells for EBV antigens, cells were fixed in 4% paraformaldehyde, washed in staining buffer (PBS with 1% BSA and 0.03% saponin) and incubated with a mouse mAb to VCA (gp125, BALF4; Chemicon) or a mouse mAb to BZLF1 (B21; a kind gift of M. Rowe, University of Wales, Cardiff). Phycocerythrin-conjugated goat anti-mouse IgD (Dianova) was used as a secondary antibody for staining positive cells. Isotype controls were either mouse IgG1 (Dianova) or mouse IgG2a (Pharmingen). LMP2A-expressing cells were stained with a rat mAb to LMP2A (Fruehling et al., 1996) and an anti-rat IgG Cy3-conjugated secondary antibody (Dianova). Cells were analysed using a Becton Dickinson FACScan with CELLQUEST analysis software.

**Immunoblot analysis.** Cellular extracts were prepared by sonication in sample buffer (0·13 M Tris/HCl pH 6·8, 6% SDS, 10% glycerol), separated on 10% polyacrylamide gels and transferred to nitrocellulose (Hybond ECL; Amersham Pharmacia). Filters were blocked in 5% low-fat milk in PBS and then incubated with mouse mAb to BZLF1 or a human antiserum recognizing VCA. Immunoreactive proteins were detected by peroxidase-conjugated goat anti-mouse IgG antibody or rabbit anti-human IgG (Sigma) and enhanced chemiluminescence (ECL system; Amersham Pharmacia). An anti-mouse mAb (C-2; Santa Cruz Biotechnology) was used to stain the blots for equal loading.

**Luciferase reporter assays.** Hygromycin B-resistant Raji DR-LUC cells (2·5×10⁶) were co-incubated for 72 h with supernatants of P3HR1 cells transfected with SVLMP2A or pHEBop5. Cells were lysed in luciferase lysis buffer (100 mM potassium phosphate pH 7·8, 1 mM DTT, 1% Triton X-100). Luciferase activity was measured in cleared lysates. Luciferase assays were performed in luciferase assay buffer [25 mM glycyglycine pH 7·8, 15 mM MgSO₄, 5 mM ATP and 15 μg d-luciferin (Roche Diagnostics) per sample]. Inductibility of luciferase activity was controlled by treatment of the cells with 20 ng TPA ml⁻¹.

**RESULTS**

**Transient expression of LMP2A can induce expression of viral BZLF1 and VCA**

We have recently shown that LMP1 can inhibit induction of the EBV lytic cycle (Adler et al., 2002). To compare the inhibitory effect of LMP1 with the effect of LMP2A on
anti-IgM-induced EBV reactivation, we transiently transfected SVLMP1 and SVLMP2A into EREB2-1 cells, a conditionally immortalized LCL. The EREB2-1 cell line was established by co-infection of primary B cells with EBNA2-deficient P3HR-1 virus and recombinant EBV expressing an EBNA2-oestrogen receptor fusion protein (Kempkes et al., 1995, 1996a). Upon oestrogen withdrawal, EBNA2 is inactivated and EBNA2-dependent genes are downregulated, including the genes encoding the viral membrane proteins LMP1 and LMP2A. In the absence of EBNA2, these cells are highly permissive for virus reactivation. Transient transfection assays were performed in freshly oestrogen-depleted EREB2-1 cells co-transfected with plasmids expressing either LMP1 or LMP2A under the control of the SV40 promoter and a plasmid expressing GFP. Two days after transfection, cells were stimulated with anti-IgM or left untreated, and 2 days later, GFP-positive cells were analysed by FACS for VCA expression. VCA induction by anti-IgM was in the range of 10–30 % of the whole cell population (data not shown). Both LMP1 and LMP2A inhibited EBV reactivation, although LMP1 was much more efficient than LMP2A (Adler et al., 2002) (Fig. 1). The background of spontaneous VCA production of pHEBop5-transfected cells without anti-IgM stimulation was in the range of 10 % of that of IgM-stimulated cells and this spontaneous VCA production was also inhibited by LMP1 (Fig. 1, column 5) (Adler et al., 2002). In cells not treated with anti-IgM, LMP2A increased rather than decreased the number of VCA-positive cells, in contrast to LMP1 (Fig. 1, column 6). Transfection of LMP2A alone also induced expression of the viral immediate-early protein BZLF1 (Fig. 2). Fig. 2(a) shows a comparison of LMP2A- versus anti-IgM-dependent BZLF1 induction in EREB2-1 and Akata cells. This demonstrated that LMP2A significantly induced the viral lytic cycle as measured by BZLF1 expression, although to a lesser extent than anti-Ig treatment. Expression of LMP1 alone did not result in BZLF1 induction (Fig. 2a). The efficiency of BZLF1 induction increased with increasing amounts of transfected LMP2A expression vector in both EREB2-1 and Akata cells (Fig. 2b). LMP2A expression was controlled in Akata cells by indirect immunofluorescence (data not shown), as well as by FACS analysis (Fig. 3). The number of LMP2A-positive cells corresponded to the number of GFP-positive cells after transfection of a GFP expression plasmid (Fig. 3). Transfection rates were poor in Akata cells (Fig. 3) as well as in EREB2-1 cells (data not shown) and resulted in only about 2–5 % GFP-positive cells. At a transfection efficiency of, for example, 5 %, and with 5 % of the transfected cells becoming BZLF1-positive, this corresponded to 0.25 % BZLF1-positive cells in the total population and to an absolute number of 500 BZLF1-positive cells analysed within 10 000 transfected GFP-positive cells. Due to the strong fluorescence of GFP and the weak fluorescence of LMP2A, double staining of GFP- and LMP2A-positive cells was not possible. To verify BZLF1 and VCA expression by biochemical means, increasing amounts of the LMP2A-expression plasmid were transfected into EREB2-1 cells along with a plasmid expressing a truncated NGFR, and the NGFR-positive cells were sorted using magnetic beads. Western blot analysis of the sorted cells revealed that BZLF1 and VCA were clearly induced by LMP2A and that this induction was dose dependent at least at concentrations of LMP2A at and below 2–5 μg SVLMP2A DNA (Fig. 4).

Expression of LMP2A in a permissive cellular background leads to production of infectious virus

We next addressed the question of whether LMP2A-mediated lytic-cycle induction would remain abortive or would progress to production of infectious virus progeny. To test whether LMP2A expression resulted in the production of infectious virus, P3HR1 cells were transfected with 5 μg of an LMP2A expression vector or an empty vector as a control. Supernatants of transfected cells were harvested 4 days after transfection and the amount of virus in the supernatant was quantified by infection of Raji DR-LUC cells. Quantification of virus in the supernatant was based on the finding that virus released from P3HR1 cells can induce EBV early-antigen expression in Raji cells (Rabson et al., 1983). Raji DR-LUC cells are stably transfected with a vector expressing a luciferase reporter gene under the control of the EBV DR promoter (Laux et al.,...
Virus in cell-culture supernatants can thus be quantified by measuring the luciferase activity in Raji DR-LUC cells infected with cell-culture supernatants of P3HR1 cells. In four independent experiments, we consistently observed an increase in luciferase activity in the expected range (Table 1). P3HR1 cells showed spontaneous reactivation in 1\%\(\pm\)0\% of the cells as revealed by VCA staining. Since the transfection efficiency of P3HR1 cells was 5\%\(\pm\)1\% and about 5\% of SVLMP2A-transfected P3HR1 cells became BZLF1-positive (Fig. 2b), we expected to induce the lytic cycle in 0\% to 1\% of the cells. This would correspond to an increase in VCA-positive cells from 1\% to 1\% (increase by a factor of 1\%). Our experiments showed a mean increase of 1\%\(\pm\)0\% (P = 0\%, unpaired Student’s t-test) (Table 1), which was thus in the expected range. These data therefore support the notion that lytic-cycle induction by LMP2A progresses to the production of infectious virus.

Fig. 2. Transient expression of LMP2A induces BZLF1 expression. (a) Akata cells or freshly oestrogen-depleted EREB2-1 cells were transiently transfected with 1 or 5 \(\mu\)g, respectively, of the indicated expression vectors and 5 \(\mu\)g pEGFP-C1. At 48 h post-transfection a pHEBop5 control was either stimulated with plate-bound anti-IgM (EREB2-1) or anti-IgG (Akata). Twenty-four hours later, cells were harvested, intracellularly stained for BZLF1 expression and GFP-positive cells were analysed by FACS analysis. Data shown are means \(\pm\) SD of three or four independent experiments, except for the anti-IgG stimulation of Akata cells, which is the mean of two independent experiments. BZLF1 expression significantly different from pHEBop5 controls is indicated (*, P < 0.05, unpaired Student’s t-test). (b) EREB2-1, Akata and P3HR1 cells were transfected with 5 \(\mu\)g pEGFP-C1 and increasing amounts of SVLMP2A, and stimulated and analysed as described in (a). One representative experiment per cell line is shown.
LMP2A-dependent induction of BZLF1 can be inhibited by the MAPK kinase inhibitor PD98059

It has been shown that phorbol ester-dependent induction of BZLF1 expression can be inhibited by inhibition of the ERK1/ERK2 pathway (Fenton & Sinclair, 1999). Virus reactivation after phorbol ester treatment of LCLs could be inhibited by the chemical inhibitor PD98059, which specifically blocks the activity of the MAPK kinase MEK1, which activates ERK1 and -2. As B-cell receptor signalling also leads to induction of the ERK pathway, we asked whether anti-IgM- and LMP2A-mediated BZLF1 induction also depended on MAPK activation. As shown in Fig. 5, treatment of cells with PD98059 inhibited BZLF1 induction in EREB2-1 cells, not only after anti-IgM treatment but also after transfection of SVLMP2A. The basal level of BZLF1 expression in oestrogen-depleted EREB2-1 cells was not affected by PD98059 at the concentration used in our experiments. This indicated that both inducers of BZLF1 expression, anti-IgM and LMP2A, acted through the ERK signalling pathway.

LMP1 can inhibit LMP2A-dependent induction of BZLF1 expression

We have recently shown that LMP1 interferes with phorbol ester- and anti-Ig-mediated induction of BZLF1 expression in permissive cell lines such as EREB2 cells and Burkitt’s lymphoma cell lines (Adler et al., 2002). It is not known at

Table 1. LMP2A can induce virus production in P3HR1 cells as indicated by luciferase activity (relative light units)
P3HR1 cells were transfected with 5 μg SVLMP2A or pHEBop5 as a control. Four days after transfection, 0·3 ml cell-culture supernatant was co-cultured with 2·5 × 10⁵ Raji DR-LUC cells for 72 h. Cells were lysed and luciferase activity was determined as described in Methods.

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<th>Supernatant from P3HR1 cells transfected with:</th>
<th>Experiment 1</th>
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Fig. 4. LMP2A can induce BZLF1 and VCA expression. Freshly oestrogen-depleted EREB2-1 cells were transfected with pHEBop5 (control) and increasing amounts of SVLMP2A and 5 μg NGFR-expressing plasmid. For all transfections, the total amount of plasmid was adjusted to 10 μg with pHEBop5. At 48 h post-transfection, NGFR-expressing cells were purified by MACS. One of the control transfections was stimulated with plate-bound anti-IgM. At 24 or 48 h after sorting, cells were harvested for BZLF1 or VCA Western blot analysis, respectively. The positions of the molecular mass standards are indicated on the left. The BZLF1 blot was stripped and stained for actin expression to give a loading control.

Fig. 3. Expression of GFP and LMP2A in Akata cells after transient transfection. Akata cells were transiently transfected with 5 μg pHEBop5 as a control (left panels), or with 5 μg pEGFP-C1 or 5 μg SVLMP2A (right panels). At 48 h post-transfection, cells were either intracellularly stained for LMP2A expression (FL-3) or screened for GFP expression (FL-1). GFP-expressing cells had to be fixed with 4% paraformaldehyde in order not to lose the GFP expression. Cells stained for LMP2A were not fixed, as the LMP2A staining appeared to be sensitive to paraformaldehyde fixation. The different fixation procedures resulted in different cellular scatters.
which level LMP1 interferes with induction of BZLF1. We show here that also the LMP2A-mediated induction of BZLF1 expression can be inhibited by LMP1. Different amounts of LMP1- and LMP2A-expression vector were co-transfected into EREB2-1 cells and the percentage of BZLF1-positive cells was monitored by indirect immunofluorescent staining. Co-transfection of SVLMP1 compared with co-transfection of the empty vector led to a significant reduction of the LMP2A-mediated induction of BZLF1 expression (Fig. 6). To exclude the possibility that the inhibition observed simply reflected cytostatic activity of the N terminus and the membrane-spanning domain of LMP1 (Kaykas & Sugden, 2000), 5 μg of a C-terminal deletion mutant of LMP1 lacking aa 194–386 (Kieser et al., 1999) was co-transfected as a control. This deletion mutant did not inhibit BZLF1 induction by either anti-IgM or LMP2A (Fig. 6). This indicated that a signal coming from the C terminus of LMP1 interferes with LMP2A- and anti-IgM-dependent EBV reactivation and that both inducers of reactivation use similar signalling pathways.

**DISCUSSION**

After primary infection, EBV persists lifelong in its host. In the peripheral blood, a stable number of latently infected B cells in the range of 0.5–50 per 10⁶ B cells is detectable. These cells are resting memory B cells in which it is probable that no latent viral genes are expressed (Hochberg et al., 2004a, b). According to the scenario proposed by Thorley-Lawson (2001), persistence of EBV in this pool of long-lived B cells is accomplished by a complex viral gene-expression programme, which is activated when infected...
B cells enter secondary lymphoid organs and which is shut off in the peripheral blood. In this model, LMP1 and LMP2A are assumed to play a crucial role in driving EBV-infected B cells into the memory compartment by providing signals that are normally provided by antigen and T-cell help. LMP2A is thus believed to mimic an activated B-cell receptor and LMP1 the CD40 receptor that senses the help of activated T cells. It has been shown that chimeric receptors expressing the N-terminal cytoplasmic domain of LMP2A can trigger calcium responses and cytokine production similarly to the B-cell receptor (Alber et al., 1993; Beaufils et al., 1993). In transgenic mice expressing LMP2A in B cells, LMP2A signalling leads to a bypass of normal B-lymphocyte development in the absence of the B-cell receptor and allows Ig-negative cells to colonize peripheral lymphoid organs (Caldwell et al., 1998). Also similarly to the B-cell receptor, LMP2A localizes to lipid rafts (Higuchi et al., 2001). In LMP2A-expressing cells, the signalling molecules Syk, phosphatidylinositol 3-kinase, phospholipase C-γ2 and Vav, which are also activated through the B-cell receptor and antigen, are constitutively phosphorylated (Miller et al., 1993, 1994, 1995).

Stimulation of EBV-infected B cells through the B-cell receptor is one of the classical modes of virus reactivation in permissive B cells (Tovey et al., 1978; Takada, 1984). Extrapolating from the extensive similarities between LMP2A and the B-cell receptor, one might postulate that LMP2A should also induce the lytic cycle in permissive B cells in a similar fashion to B-cell receptor stimulation. However, the opposite finding has been reported. LMP2A interferes with B-cell receptor signalling and the LMP2A signalling inhibits rather than induces EBV reactivation (Miller et al., 1993, 1994, 1995). We have now resolved this apparent paradox by providing evidence that LMP2A, when acting alone, can indeed induce viral reactivation in permissive B cells. However, when an activated B-cell receptor and LMP2A act simultaneously, LMP2A does not augment but rather attenuates the signal from the B-cell receptor although it does not block it completely. We additionally showed that LMP2A and the B-cell receptor act through the MEK/MAPK pathway, since both B-cell receptor- and LMP2A-mediated virus reactivation can be inhibited by the MEK1 inhibitor PD98059.

We have described previously that ligands activating the same receptor and the same pathway can interfere with each other if one ligand is providing a much weaker signal than the other (Adler et al., 2001). If, for instance, the effect of the strong inducer on a given phenotype is maximal, any competition with a weak inducer will result in an attenuation of the phenotype imposed by the strong inducer. LMP2A-mediated EBV induction is indeed much weaker than anti-lg-dependent virus reactivation. Inhibition of anti-lg-mediated virus reactivation by LMP2A may thus be the result of sequestration of signalling molecules from the B-cell receptor. The finding that EBV co-opts lipid rafts through LMP2A and thus excludes the B-cell receptor from the intracellular signalling compartment is consistent with this notion (Dykstra et al., 2001). It remains an open question whether the interference of LMP2A with B-cell receptor signalling plays a role in EBV infection. In cells in which LMP2A and LMP1 are expressed simultaneously, e.g. in EBV-infected memory B cells in lymphoid organs, LMP1 should suppress LMP2A-dependent virus reactivation. In resting memory B cells in the peripheral blood, LMP2A has been detected by RT-PCR (Qu & Rowe, 1992; Tierney et al., 1994; Miyashita et al., 1997; Babcock et al., 1998, 1999), yet newer findings indicate that there is no latent gene expression at all and that previous reports are based on an experimental artefact (Hochberg et al., 2004a, b). In immunocompetent individuals, LMP2A-expressing cells are supposed to be eliminated by LMP2A-specific cytotoxic T cells unless specific mechanisms would spare these B cells. In immunocompromised patients, however, LMP2A expression could contribute to spontaneous EBV reactivation. LMP2A thus might play a dual role in EBV infection: it may on the one hand provide a signal for entry of the virus into the memory compartment necessary for long-term survival and persistence of EBV in infected memory B cells, while on the other hand providing a signal that favours sporadic virus reactivation. This spontaneous reactivation should be under the control of antigen-specific cytotoxic T cells in immunocompetent individuals, but might contribute significantly to the strong increase in virus load in immunocompromised patients (Lam et al., 1991; Babcock et al., 1999). It will be of interest to find out when and where in the course of an EBV infection LMP2A expression might contribute to EBV reactivation in immunocompromised patients in vivo. A detailed knowledge of the signalling pathways involved in spontaneous virus reactivation is a prerequisite for the identification of drugs that interfere with this process and that may be used as antiviral agents in immunocompromised individuals.

ACKNOWLEDGEMENTS

This work was supported by Die Deutsche Forschungsgemeinschaft (DFG, SFB455 ‘Virale Funktionen und Immunmodulation’) and Fonds der Chemischen Industrie. B.A. was supported by a grant (Infektionsstipendium) from the German Bundesministerium für Bildung und Forschung. We thank H. Adler for critically reading the manuscript and M. Brielmeier for help with NGFR-based cell sorting.

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