Epstein–Barr virus (EBV) is a human B-lymphotropic \(\gamma\)-herpesvirus that, following primary infection, persists latently in the host's memory B-cell pool for life and may switch periodically to lytic infection (Rickinson & Kieff, 2001). EBV is linked with malignancies including Burkitt's lymphoma (BL), Hodgkin's lymphoma, nasopharyngeal carcinoma and post-transplant lymphoproliferative diseases (Murray & Young, 2002), where it expresses different patterns of latency genes (Fruehling & Longnecker, 1997; Longnecker et al., 1991; Miller et al., 1994b; Swart et al., 2000). Cross-linking of BCR on EBV-infected cells induces lytic EBV replication through transcriptional activation of the EBV immediate-early lytic gene \(BZLF1\). In lymphoblastoid cell lines, LMP2A interferes with this process by blocking the activation of protein tyrosine kinases (Miller et al., 1994a, 1995). This mechanism is thought to prevent lytic EBV replication in latently infected B cells circulating in the body upon encounter of antigens or other ligands that may engage BCR (Portis et al., 2002). Indeed, LMP2A expression is found in EBV-infected tonsillar memory B cells \(ex vivo\) (Babcock & Thorley-Lawson, 2000; Babcock et al., 2000). Furthermore, B cells in \(LMP2A\)-transgenic mice show increased survival (Caldwell et al., 2000; Merchant et al., 2000). These observations suggest a central role for LMP2A to ensure persistence of EBV latency within the infected cell.

LMP2B, as for LMP2A, has been shown to promote spread and motility of epithelial cells (Allen et al., 2005), but little is known about the function of LMP2B in B cells. LMP2B co-localizes with LMP2A in the membrane, where the C terminus of both splice variants can interact and regulate the activity of each other (Lynch et al., 2002; Matskova et al., 2001). Recently, LMP2B was shown to regulate LMP2A activity negatively when these genes were expressed in the EBV-negative BL cell line BIAB (Rovedo & Longnecker, 2007). Nevertheless, the role of LMP2B in the presence of EBV remains unknown.

Silencing of latent membrane protein 2B reduces susceptibility to activation of lytic Epstein–Barr virus in Burkitt’s lymphoma Akata cells

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Epstein–Barr virus (EBV) latent membrane protein 2A (LMP2A) blocks B-cell receptor (BCR) signalling after BCR cross-linking to inhibit activation of lytic EBV, and ectopically expressed LMP2B negatively regulates LMP2A. Here, it is demonstrated that silencing of \(LMP2B\) in EBV-harbouring Burkitt’s lymphoma Akata cells results in reduced expression of EBV immediate-early lytic \(BZLF1\) gene mRNA and late lytic gp350/220 protein upon BCR cross-linking. Similarly, reduction of lytic EBV activation was observed in Akata cells overexpressing LMP2A. In contrast, silencing of \(LMP2A\) expression resulted in higher lytic EBV mRNA and protein expression in BCR cross-linked Akata cells. These observations indicate a role for LMP2B distinct from that of LMP2A in regulation of lytic EBV activation in the host cell and support the hypothesis that LMP2B exhibits a negative-regulatory effect on the ability of LMP2A to maintain EBV latency by preventing the switch to lytic replication.
We hypothesized that LMP2B exhibits a role distinct from that of LMP2A in activation of EBV lytic replication. To test our hypothesis, we compared the impact of LMP2B silencing with the impacts of LMP2A overexpression and LMP2A silencing on activation of EBV lytic replication in the EBV-harbouring BL cell line Akata upon BCR cross-linking (Takada, 1984). Akata cells retain an EBV gene expression pattern in vitro that closely resembles that found in BL biopsies, and are an established model for the study of activation of EBV lytic replication, which can be triggered effectively by BCR stimulation using cross-linking anti-IgG (Daibata et al., 1990).

To silence expression of LMP2B specifically, we constructed a short hairpin (sh) RNA targeting exon 1 of LMP2B. The shRNA was designed by using the software RNAi Central (http://katahdin.cshl.org) and is available upon request. We cloned the shRNA into the lentiviral vector pSICOR (Ventura et al., 2004), verified it by sequencing and named the construct pSICOR-LMP2B-68 (Table 1). After LMP2B-68 shRNA lentiviral production in HEK 293T cells (Graham et al., 1977), we incubated wild-type Akata (Akatawt) cells with concentrated virus-containing supernatant and amplified them for 1 month in culture using medium supplemented with G418 (0.4 mg ml\(^{-1}\)). To monitor uptake of pSICOR-LMP2B-68, we tested transduced Akata cells for expression of enhanced green fluorescence protein (EGFP) by flow cytometry. To obtain a homogeneous EGFP-expressing cell population (99% purity), we enriched EGFP-expressing cells by fluorescence-activated cell sorting (FACS) twice and selected them for 1 month in culture using medium supplemented with G418 (0.4 mg ml\(^{-1}\)). To determine the consistency of the degree of silencing justified the use of Akata-LMP2BshRNA pools 1, 2 and 3 as biological replicates in all subsequent experiments.

EBV lytic replication is initiated by upregulation of BZLF1. Thus, based on own previous observations (Bernasconi et al., 2006), we determined BZLF1 mRNA expression levels before and 6, 24, 48 and 72 h after BCR cross-linking by using qRT-PCR. \(\Delta C_t\) values from unstimulated cells were subtracted from \(\Delta C_t\) values from stimulated cells, resulting in \(\Delta \Delta C_t\) values. mRNA expression of the vector control was set to 100% and the percentages of reduction of mRNA expression in LMP2B-silenced cells were evaluated. BZLF1 mRNA levels in LMP2B-silenced Akata-LMP2BshRNA cells were 25–35% of those in control Akata-scrshRNA cells from 6 to 72 h after BCR cross-linking (Fig. 1b). Using flow cytometry, we found similar frequencies of surface IgG-positive cells in Akata-LMP2BshRNA and Akata-scrshRNA

To assess the efficiency and specificity of LMP2B silencing, we isolated total RNA from Akata-LMP2BshRNA and control Akata-scrshRNA cells. After cDNA synthesis, we used quantitative real-time (qRT)-PCR (Taqman) targeting an LMP2B-specific sequence (available upon request) to determine LMP2B mRNA expression levels. Results were normalized to housekeeping gene hydroxymethylbilane synthase (HMBS) mRNA expression, resulting in \(\Delta C_t\) (cycle threshold) values. Akata cells are considered to be in latency I (EBNA1 expression only). However, even though no LMP2 protein can be detected, we observed low but significant levels of LMP2A and LMP2B mRNA expression (\(C_t\) range, 32–34; data not shown). Silencing of LMP2B in the three Akata-LMP2BshRNA cell pools showed LMP2B mRNA expression levels to be 25.3 \(\pm\) 2.3 % of those of Akata-scrshRNA cells (Fig. 1a), but there was no change in LMP2A mRNA expression (not shown). The lack of available anti-LMP2B antibody precluded substantiation of the above qRT-PCR data by immunoblotting. Nevertheless, we considered the degree of silencing sufficient for our purposes, and the consistency of the degree of silencing justified the use of Akata-LMP2BshRNA pools 1, 2 and 3 as biological replicates in all subsequent experiments.

**Table 1. Characteristics of Akata cells used for silencing and overexpression experiments**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Cell line</th>
<th>Vector construct</th>
<th>EGFP+ (%)*</th>
<th>sIgG+ (%)*</th>
<th>gp350/220 staining (% of cells)*</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
<td>0 h</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stimulated</td>
</tr>
<tr>
<td>Silencing</td>
<td>Akatawt</td>
<td>None</td>
<td>NM</td>
<td>96</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Vector control</td>
<td>Akata-scrshRNA</td>
<td></td>
<td></td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>LMP2B pool 1–3</td>
<td>pSICOR-scrRNA</td>
<td>99</td>
<td>91</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>LMP2A pool 1–3</td>
<td>pSICOR-LMP2B-68</td>
<td>99 ± 1</td>
<td>90 ± 4</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Overexpression</td>
<td></td>
<td></td>
<td></td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td>Vector control</td>
<td>Akata-neoect</td>
<td>NM</td>
<td>91</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LMP2A</td>
<td>pEneo</td>
<td>NM</td>
<td>92</td>
<td>–</td>
</tr>
</tbody>
</table>

*Values are presented as means \(\pm\) SD.
This excluded the possibility that the differences in BZLF1 mRNA expression levels upon BCR cross-linking were due to differences in surface IgG expression. Thus, the reduced BZLF1 mRNA expression in Akata-LMP2BshRNA cells was probably a direct consequence of LMP2B silencing.

To substantiate the above qRT-PCR results, we stained Akata-LMP2BshRNA and Akata-scrshRNA cells before and 24 h after BCR cross-linking with a fluorescein isothiocyanate-labelled antibody against EBV late lytic gp350/220 protein in three independent experiments performed in triplicate (Table 1). Both unstimulated Akata-LMP2BshRNA and
Akata-scr-srRNA cells showed a similar low basal percentage of cells staining for gp350/220 (mean ± SD, 0.3 ± 0.1%). Consistent with the qRT-PCR data, 24 h after BCR cross-linking, Akata-LMP2B-srRNA cells showed a 4- to 5-fold lower number of cells positive for gp350/220 than Akata-scr-srRNA cells (0.3 ± 0.1% versus 1.7 ± 0.5%). Thus, upon BCR cross-linking, induction of EBV lytic replication in LMP2B-silenced Akata-LMP2B-srRNA cells is lower than in Akata-scr-srRNA cells, not only at the mRNA expression level of the immediate-early lytic gene BZLF1, but also at the level of the late lytic protein gp350/220, indicating a reduced susceptibility of LMP2B-silenced EBV-harbouring cells to activation of EBV lytic replication.

Next, we reasoned that if LMP2B regulates LMP2A negatively, which prevents activation of lytic EBV, over-expression of LMP2A should yield outcomes similar to those of LMP2B silencing. Therefore, we amplified the sequence encoding LMP2A from the vector pSG5-LMP2 (Sample et al., 1989) by PCR and cloned the PCR product via Pmel/Xho into the Moloney murine leukemia virus-derived pEneo bicistronic expression vector containing an internal ribosome entry site with a neomycin selection marker (Schaefer et al., 2001). Positive clones were verified by sequencing. The parental pEneo plasmid was used as control vector. Akata-vt cells were transfected by electroporation with the pEneo-LMP2A expression vector or the parental vector pEneo. After 2 days recovery, the transfected cells were subjected to G418 selection. Stably transfected cells emerged after 2–3 weeks. Expression of LMP2A mRNA was confirmed by qRT-PCR (not shown) and immunoblotting showing expression of the correct-sized protein at 43 kDa (Fig. 1c). These cells were named Akata-LMP2A-ex, and the control cells Akata-neo-ex (Table 1). Immunofluorescence staining showed expression of LMP2A in all Akata-LMP2A-ex cells, with heterogeneous high and low LMP2A expression levels reflecting a polyclonal population (Fig. 1d), but no expression in the control Akata-neo-ex cells (not shown). These populations showed no up- or down-regulation of LMP2B mRNA expression by qRT-PCR (not shown) and similar levels of surface IgG (Table 1), confirming that LMP2A overexpression had not affected LMP2B mRNA or surface IgG expression.

To compare susceptibility to activation of lytic EBV in LMP2A-overexpressing Akata cells with the above-assessed activation in LMP2B-silenced Akata cells, we stimulated Akata-LMP2A-ex and control Akata-neo-ex cells by BCR cross-linking. We chose 24 h after BCR cross-linking for the comparison because LMP2B-silenced Akata and control cells had shown a suitable difference in BZLF1 mRNA expression at 24 h (Fig. 1b, c). Stimulated and unstimulated Akata cells were collected and examined by qRT-PCR for BZLF1 mRNA expression. The data were normalized to HMBS mRNA and further normalized to mRNA levels in unstimulated cells as mentioned above. The results are presented as ratios of stimulated over unstimulated Akata cells. As expected from published data (Fukuda & Longnecker, 2005; Konishi et al., 2001), BZLF1 mRNA expression in LMP2A-overexpressing cells was reduced to 3.3 ± 5.7% of that in control cells in three independent experiments (Fig. 1f). This superior reduction, compared with the reduction to 33.3 ± 6.7% seen in LMP2B-silenced cell pools (Fig. 1c), was probably intrinsic to the nature of the experiments, with overexpression of LMP2A being more effective than LMP2B silencing. Nevertheless, the reduced BZLF1 mRNA expression upon BCR cross-linking in EBV-harbouring LMP2B-silenced and LMP2A-overexpressing cells indicated that LMP2B modulates LMP2A negatively in preventing activation of EBV lytic replication.

To verify our results by using an opposite approach, we constructed a silencing pSICOR vector specific for LMP2A, targeting exon 1 of LMP2A (Stewart et al., 2004), and transduced it virally into Akata-vt cells as described above for LMP2B silencing to obtain pools 1, 2 and 3 of Akata-LMP2A-srRNA (Table 1). Akata-LMP2A-srRNA cell pools showed LMP2A mRNA expression levels of 15.9 ± 11.1% of those in control cells (Fig. 1g), but no effect on LMP2B mRNA expression levels (not shown), confirming the specificity of silencing. To assess the effect of LMP2A silencing on induction of EBV lytic replication, we measured BZLF1 mRNA expression levels 24 h following BCR cross-linking in the three biological replicates and compared the levels with those in the corresponding control cells. Activation of EBV lytic replication in LMP2A-silenced cells was 2- to 6-fold higher than in control cells, as illustrated by the increase of BZLF1 mRNA to 552.3 ± 89.9% compared with vector-control Akata-scr-srRNA cells (Fig. 1h). Increased activation of EBV lytic replication in LMP2A-silenced cells was confirmed at the protein level by the measurement of the expression of gp350/220 in Akata-LMP2A-srRNA cells (Table 1). Akata-LMP2A-srRNA cells expressed higher basal levels of gp350/220 before stimulation (1.2 ± 0.6%) and at 24 h unstimulated (1.1 ± 0.5%) than did Akata-scr-srRNA cells (0.3 ± 0.1 and 0.4 ± 0.2%, respectively). Twenty-four hours after stimulation, the detected envelope protein reached levels of up to 2.2 ± 0.3% in Akata-LMP2A-srRNA-pool 1 cells, compared with 1.7 ± 0.5% of Akata-scr-srRNA cells. Thus, stimulated Akata-LMP2A-srRNA cells show a 1.3-fold increase of gp350/220 expression compared with Akata-scr-srRNA cells, similar to the data obtained from qRT-PCR. This indicates that silencing of LMP2A increases the susceptibility to activation of EBV lytic replication. Therefore, silencing of LMP2B and LMP2A exhibits opposite effects on susceptibility to activation of EBV lytic replication.

LMP2A plays a relevant role in controlling the switch from EBV latency to lytic replication in B cells through modulation of the activity of cellular kinases, including the Src family of phosphotyrosine kinases (Mancao et al., 2005; Portis et al., 2004), and calcium mobilization following BCR cross-linking. This results in a decreased activation of lytic EBV. LMP2B lacks an N-terminal signalling domain and cannot therefore influence any signalling cascade directly. Rovedo & Longnecker (2007) showed that LMP2B and LMP2A can interact with each other in the EBV-negative BL cell line...
BJAB, resulting in decreased phosphorylation of the LMP2A cytoplasmic N-terminal domain and the block of cytoplasmic calcium release upon BCR cross-linking.

Here, we show that silencing of LMP2B reduces activation of EBV lytic replication in the BL cell line Akata, as does overexpression of LMP2A. This indicates that LMP2B decreases the block of LMP2A on BCR activation. In contrast, specific silencing of LMP2A in Akata cells had exactly the opposite effect of increasing induction of lytic EBV replication upon stimulation, as documented by BZLF1 mRNA expression and gp350/220 protein expression. Hence, given that LMP2A prevents lytic EBV activation (Miller et al., 1994b, 1995), our results support the notion that LMP2B negatively affects LMP2A regulation of activation of lytic EBV upon BCR cross-linking.

Akata cells are known to maintain latency I in culture (Takada, 1984) and express low levels of LMP2A (Konishi et al., 2001), and are negative for LMP2A protein expression when probed by immunoblotting. More sensitive tools like Southern–PCR (Tao et al., 1998) or qRT–PCR, as shown here, are able to detect low mRNA expression levels of both LMP2A and LMP2B in exponentially growing Akata cells. We were able to detect LMP2A after immunoprecipitation of resting Akata cells, but could not observe any quantitative changes upon activation of lytic EBV (M. Bernasconi & J. A. Sigrist, unpublished observation). Low levels of LMP2A do not seem sufficient to block activation upon BCR cross-linking in Akata cells (Konishi et al., 2001). However, our results indicate that, for as low as the protein expression level is, both LMP2A and LMP2B influence the susceptibility to activation of lytic EBV infection.

In conclusion, our results suggest that LMP2B adds a novel layer of complexity to the regulation of lytic EBV infection in B cells. Together with the observations made in the EBV-negative cell line BJAB (Rovedo & Longnecker, 2007), we suggest that LMP2B under some circumstances impacts on the activity of LMP2A, resulting in increased susceptibility to activation of lytic EBV replication through modulation of BCR and downstream signalling.

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