Role of Epstein–Barr virus-encoded latent membrane protein 2A on virus-induced immortalization and virus activation

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To quantitatively evaluate the role of Epstein-Barr virus (EBV)-encoded latent membrane protein 2A (LMP2A) in immortalization of peripheral B-lymphocytes, we used the Akata cell system to generate an EBV recombinant in which the first exon of the LMP2A gene was disrupted. The results indicated that deletion of the LMP2A gene did not affect the immortalization efficiency of EBV in B-lymphocytes. Deletion of the LMP2A gene made EBV-transformed lymphocytes more permissive for virus replication in response to surface immunoglobulin cross-linking. On the other hand Akata cells, in which LMP2A expression was much lower than in EBV-transformed lymphocytes, were equally permissive for virus replication whether they were infected with wild EBV or LMP2A-knockout EBV. The results raise a question as to the role of LMP2A in inhibition of disruption of virus latency in vivo, where LMP2A expression has been expected to be low as in Akata cells.

Epstein–Barr virus (EBV), a human herpesvirus, is the causative agent of infectious mononucleosis, and is associated with various malignancies such as Burkitt’s lymphoma and nasopharyngeal carcinoma (Rickinson & Kieff, 1996). EBV is ubiquitous in humans, establishes latent infection in peripheral B-lymphocytes following primary infection, and persists for the remainder of the host’s lifetime. In vitro, EBV transforms peripheral B-lymphocytes into indefinitely growing lymphoblastoid cells (LCL). LCL maintain the entire viral genome mostly in a plasmid form and express a limited number of virus gene products, including six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP), three latent membrane proteins (LMP1, LMP2A and LMP2B), two small nonpolyadenylated RNAs (EBER1 and EBER2) and the transcripts from the BamHI-A region (BARF0) (Kieff, 1996).

LMP2A and LMP2B are transcribed from unique and common exons (Laux et al., 1988; Sample et al., 1989). The first exon is unique to LMP2A and encodes a 119 amino acid cytoplasmic amino-terminal domain. The remaining eight exons are common with LMP2B, and encode 12 hydrophobic membrane-spanning domains and a 27 amino acid cytoplasmic carboxy-terminal domain. The amino-terminal part of LMP2A is associated with src family protein tyrosine kinases (Burkhardt et al., 1992; Longnecker et al., 1991). LMP2A is a substrate for these protein kinases, acts as a dominant negative regulator of lyn and fyn protein kinase activities, and interferes with signal transduction after cross-linking of cell surface immunoglobulin (slg) (Miller et al., 1993, 1994a, 1995). Cross-linking of slg efficiently activates a switch to lytic EBV gene expression in latently EBV-infected cells (Takada, 1984; Takada & Ono, 1989). LMP2A prevents lytic reactivation in response to slg cross-linking in EBV-immortalized B-lymphocytes (Miller et al., 1994b), and is thought to maintain a latent state of EBV infection in vivo, because only EBNA1 and LMP2A were reported to be expressed in latently EBV-infected B-cells in vivo (Chen et al., 1995; Miyashita et al., 1997; Qu & Rowe, 1992; Tierney et al., 1994).

Concerning the role of LMP2A in B-cell immortalization, there have been reports from two groups. R. Longnecker and colleagues reported that LMP2A was dispensable for B-cell immortalization. Two EBV mutants were generated: one a knockout of the first exon of the LMP2A gene (Longnecker et al., 1992; Speck et al., 1999), and the other of the last seven transmembrane and carboxy-terminal cytoplasmic domains (Longnecker et al., 1993), which are shared with LMP2B, and therefore the latter mutant is deficient in both LMP2A and LMP2B genes. An EBV recombinant was generated in EBV-producing P3HR-1 cells and B95-8 cells as a mixture of wild EBV and mutant EBV, which was then used to immortalize primary B-lymphocytes. LMP2A- or LMP2A/2B-knockout EBV released from immortalized B-cells was demonstrated to have an ability to immortalize primary B-lymphocytes. However, the assays were not quantitative because the virus preparations always contained both wild and mutant EBV. On the other hand, W. Hammerschmidt and colleagues (Brielmeier et al., 1996) reported that LMP2A was important for efficient B-
Fig. 1. (A) Schematic representation of the plasmid construct used to generate LMP2A-knockout EBV. The structures of LMP2A and LMP2B mRNAs are shown above the map of wild EBV. The targeting plasmid is the BglII fragment encompassing the first exon of LMP2A, which is disrupted by insertion of the neomycin resistance gene (neo'). When the plasmid recombines into the targeting site, novel 8–3 kb EcoRI–BamHI and 11–3 kb EcoRI–SacII fragments will appear. Probes used for Southern blot analysis are indicated at the bottom. B, BamHI; E, EcoRI; Bgl, BglII; M, MunI; S, SacII; TR, terminal repeat. (B, C) Southern blot analysis of a targeted Akata cell clone and LMP2A-knockout EBV-infected Akata− clones. Two µg of cellular DNA was doubly digested with EcoRI/BamHI (B) or EcoRI/SacII (C), blotted, and hybridized with the EcoRI/BamHI or neo' probe (B) or SacII or neo' probe (C) by the procedure described previously (Shimizu et al., 1994) (see Fig. 1A). (D) LMP2A expression in LMP2A-knockout EBV-infected Akata cell clones by RT–PCR analysis. Total cellular RNA was isolated by guanidium isothiocyanate–phenol extraction using TRIzol reagent (GibcoBRL) according to the manufacturer's protocol. One µg of total RNA was treated with DNase I (GibcoBRL) and incubated with Moloney murine leukaemia virus reverse transcriptase (GibcoBRL). The cDNA (corresponding to 100 ng of total RNA) was subjected to PCR using primers 5' CCCTAGAAATGGTGCCAATG 3' and 5' CATGTTAGGCAAATTGCAAA 3'. One-tenth of the PCR product was electrophoresed in a 2% agarose gel, blotted, and hybridized with a 32P-labelled oligonucleotide probe (5' ATCCAGTATGCCTGCCTGTA 3'). LCL immortalized by Akata EBV were used as a positive control. The EBV-negative B-lymphoma line BIB was used as a negative control. (E) LMP2A expression in LMP2A-knockout EBV-immortalized LCL by immunoblot analysis. Protein samples (corresponding to 4 × 10^5 cells) were separated in 10% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell). LMP2A was detected with a rabbit polyclonal antibody. LCL immortalized by LMP2A-positive Akata EBV were used as a positive control. BIB cells were used as a negative control.

cell immortalization. Knockout EBV was generated in an E. coli F-factor-based plasmid and packaged in P3HR-1 cells, which were infected with EBNA2-deleted, transformation-incompetent EBV. This assay, however, could not evaluate the function of LMP2A, because the knocked-out LMP2 regions were common to both LMP2A and LMP2B.

Our present study aimed to quantitatively determine the role of LMP2A in B-cell immortalization by using a pure recombinant EBV preparation generated by an Akata cell system (Shimizu et al., 1996). We also studied whether the small amount of LMP2A expressed in Akata cells, which must be similar to that in latently EBV-infected B cells in vivo, had any effect on preventing activation of latent EBV.

The Akata cell line, derived from Burkitt's lymphoma, was originally 100% EBV-positive (Takada et al., 1991). We isolated EBV-positive and -negative subclones from the parental Akata
Table 1. Effects of LMP2A expression on calcium mobilization and EBV lytic antigen expression after surface immunoglobulin cross-linking

Akata cells were treated with 1% (v/v) anti-IgG, BJAB cells with 1% (v/v) anti-IgM, and LCL with 20 ng/ml 12-O-tetradecanoylphorbol 13-acetate and 3 mM sodium butyrate. After 48 h incubation at 37 °C, cells were harvested and examined for the expression of EA and VCA by the immunofluorescence assay.

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ca²⁺ mobilization</th>
<th>Antigen-positive cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of cells</td>
<td>% change in intracellular Ca²⁺</td>
</tr>
<tr>
<td>Akata−/wild EBV</td>
<td>73.4 ± 0.4</td>
<td>181.3 ± 6.8</td>
</tr>
<tr>
<td>Akata−/LMP2A− EBV</td>
<td>67.4 ± 4.9</td>
<td>182.8 ± 28.2</td>
</tr>
<tr>
<td>LCL/wild EBV</td>
<td>32.7 ± 0.8</td>
<td>45.2 ± 9.5</td>
</tr>
<tr>
<td>LCL/LMP2A− EBV</td>
<td>64.8 ± 5.1</td>
<td>300.6 ± 12.4</td>
</tr>
<tr>
<td>Akata+/LMP2A</td>
<td>37.7 ± 13</td>
<td>913 ± 6.8</td>
</tr>
<tr>
<td>Akata+/neo</td>
<td>73.0 ± 0.4</td>
<td>180.5 ± 7.4</td>
</tr>
<tr>
<td>BJAB/LMP2A</td>
<td>60.2 ± 8.8</td>
<td>108.8 ± 21.6</td>
</tr>
<tr>
<td>BJAB/neo</td>
<td>81.2 ± 8.4</td>
<td>382.0 ± 95.2</td>
</tr>
<tr>
<td>Akata−/LMP2A</td>
<td>56.2 ± 1.7</td>
<td>110.0 ± 7.3</td>
</tr>
<tr>
<td>Akata−/neo</td>
<td>68.0 ± 4.7</td>
<td>171.7 ± 21.7</td>
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Table 1 shows the effects of LMP2A expression on calcium mobilization and EBV lytic antigen expression after surface immunoglobulin cross-linking. The data indicate that LMP2A expression has a significant impact on calcium mobilization and antigen expression. The table compares various cell lines, including Akata, BJAB, and LCL, infected with wild-type or recombinant EBV. The results suggest that LMP2A expression increases calcium mobilization and enhances the expression of EBV lytic antigens.

Akata cells express a small amount of LMP2A that is not sufficient to support viral replication alone. However, in combination with EBV, LMP2A expression greatly enhances the efficiency of EBV immortalization. This finding highlights the importance of LMP2A in the process of EBV immortalization. Further studies are needed to understand the mechanisms behind the enhanced EBV immortalization by LMP2A expression.
Western blot analysis of LMP2A expression in LMP2A-transfected Akata<sup>+</sup> (A), BJAB (B) and Akata<sup>−</sup>(C) cell clones. Akata<sup>+</sup>, BJAB and Akata<sup>−</sup> cells were transfected with the LMP2A expression plasmid carrying the neomycin resistance gene (neo<sup>r</sup>). Cell clones that stably expressed LMP2A protein were selected and analysed. Protein samples (corresponding to 4 x 10<sup>5</sup> cells) were separated in 10% polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher and Schuell). LMP2A was detected with a rabbit polyclonal antibody. LCL immortalized by LMP2A-positive Akata EBV were used as a positive control. BJAB cells were used as a negative control.

As compared with LMP2A expression in Akata cells, LCL expresses a higher level of LMP2A, and it has been reported that LMP2A in LCL blocks sIg-mediated signalling (Miller et al., 1993, 1994<sup>b</sup>). The responses to anti-Ig treatment of LCLs immortalized by LMP2A-knockout EBV were compared. (Table 1). LCLs immortalized by LMP2A-knockout EBV displayed increased calcium mobilization in response to anti-Ig compared with LCL immortalized by wild EBV. Both the percentage of cells responding and the percentage change in the intracellular calcium level increased in LCL transformed by LMP2A-knockout EBV compared with wild EBV.

Next we examined whether high-level expression of LMP2A comparable to the level in LCL could inhibit calcium mobilization induced by anti-Ig treatment. The LMP2A expression plasmid was generated from cDNA of Akata EBV-immortalized LCL by a PCR-based strategy. Total cellular RNA was reverse-transcribed using the 3' LMP2A primer (GCACATTGGGTTTATTGTAGTGTTTGTAAATAC). The cDNA was then subjected to 35 cycles of PCR using primers 3' LMP2A and 5' LMP2A (TTAGCGCTGCTGAGCTATGGGGTCCCTAG). The amplified DNA was sequenced to confirm the absence of any mutations introduced by PCR. Compared with the nucleotide sequence of the B95-8 strain (Baer et al., 1984), there were three base changes, which resulted in three amino acid changes (tyrosine-23 to aspartate, and serine-348 and -444 to isoleucine). Tyrosine-23 has been reported to be dispensable for blocking B-cell signal transduction (Fruehling et al., 1996). The cDNA of LMP2A was further cloned into the pSG5 vector containing neo<sup>r</sup> and transfected into BJAB, Akata<sup>+</sup> and Akata<sup>−</sup> cells. Cell clones that had LMP2A levels similar to those of LCL were isolated (Fig. 2A, B, C) and examined for their calcium mobilization response to anti-Ig treatment. In all three cell lines, LMP2A-transfected cell clones showed reduced numbers of cells responding, as well as a reduction in the degree to which responding cells mobilized calcium, compared with those of vector-transfected clones (Table 1).

It is well known that Ig receptor signalling induces activation of latently infected EBV (Takada, 1984). Therefore, we compared the levels of lytic antigen expression after anti-Ig treatment between wild EBV-infected and LMP2A-knockout EBV-infected Akata cells. The results are shown in Table 1. There was no difference in the frequency of lytic antigen-positive cells. These results suggest that a small amount of LMP2A in type I latency does not block sIg-mediated signalling.

We then compared the level of lytic antigen expression after anti-Ig treatment between wild EBV-infected and LMP2A-knockout EBV-infected LCL. The results indicated that wild EBV-infected LCLs showed very little or no induction of EBV lytic antigens, while LMP2A-knockout EBV-infected LCLs produced EBV early antigens (EA) and viral capsid antigens (VCA) in 5% and 1% of cells, respectively, after anti-Ig treatment.

Next Akata<sup>+</sup> cells, which expressed a small amount of LMP2A, were transfected with the LMP2A plasmid, and cell clones that constitutively expressed LMP2A at a level similar to LCL were isolated. These cell clones became less permissive for activation of latent EBV genes. The results suggest that LMP2A expression can block the induction of lytic EBV antigens in response to anti-Ig treatment.

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for virus replication as compared with Akata\textsuperscript{+} cell clones transfected with the neo\textsuperscript{+} plasmid as a control.

Our results showed clearly that deletion of the LMP2A gene did not affect the immortalization efficiency of EBV in peripheral lymphocytes. Brielmeier et al. (1996) reported that the deletion of both LMP2A and LMP2B genes from EBV lowered the lymphocyte immortalization efficiency remarkably. It remains to be studied whether LMP2B plays some role in lymphocyte immortalization.

LCL express a high level of LMP2A protein, which can be detected by Western blot. The high-level expression of LMP2A in LCL is caused by transactivation of the EBNA2 protein (Zimber et al., 1993). On the other hand, Akata cells are negative for EBNA2 expression and express little LMP2A. The analysis of peripheral blood lymphocytes by PCR showed that only EBNA1 and LMP2A were expressed in EBV latency in vivo (Chen et al., 1995; Miyashita et al., 1997; Qu & Rowe, 1992; Tierney et al., 1994). Although the level of LMP2A expression in peripheral lymphocytes has not been measured quantitatively, the absence of EBNA2 expression suggests a low level of LMP2A expression in these cells. Our results showed that the low level of LMP2A expressed in Akata cells could not block the EBV activation by cross-linking of slg, suggesting that a low level of LMP2A expression in vivo latency could not block EBV activation.

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References


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