NF-κB only partially mediates Epstein–Barr virus latent membrane protein 1 activation of B cells

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The latent membrane protein 1 (LMP1) of Epstein–Barr virus (EBV) is required for EBV-induced immortalization of human B cells and causes tumorigenic transformation of cell lines. LMP1 expression induces phenotypic changes resembling B cell activation, such as cell size increase and up-regulation of cell surface activation markers. LMP1 contains two domains that activate the transcription factor NF-κB, one through interactions with TRAF proteins and the other with the TRADD protein. The purpose of the present study was to investigate the importance of NF-κB induction in the up-regulation of the B cell activation markers ICAM-1 and CD71 by LMP1.

This study shows that expression of LMP1 activates transcription from p50/p65- and c-Rel-responsive promoters, and that this activity can be completely inhibited by expression of a dominant inhibitory IκB mutant. ICAM-1 and CD71 are nevertheless up-regulated by LMP1 in primary B cells and cell lines expressing the dominant IκB. Furthermore, LMP1-induced cell size increase of primary B cells was unaffected by IκB expression. It was concluded that even when LMP1 is unable to activate NF-κB, it is still capable of inducing certain characteristics of activated B cells, strongly suggesting that LMP1 can also activate cells independently of NF-κB.

Introduction

Epstein–Barr virus (EBV) is a herpesvirus carried by the majority of the human population. The virus immortalizes human primary B cells in vitro, and the resulting lymphoblastoid cell lines (LCLs) express a small subset of the EBV genome (reviewed in Sugden, 1989; Kieff & Liebowitz, 1990), including the latent membrane protein 1 (LMP1). Expression of LMP1 is crucial for immortalization to take place (Kaye et al., 1993). LMP1 can, by itself, transform cell lines (Wang et al., 1985; Baichwal & Sugden, 1988) and LMP1 is frequently found in EBV-associated malignancies such as Burkitt’s lymphomas (Epstein & Achong, 1979), nasopharyngeal carcinomas (Fähræus et al., 1988), T cell leukemias (Chen et al., 1993; Anagnostopoulos et al., 1992; Su et al., 1991) and in Reed–Sterberg cells of Hodgkin’s lymphomas (Herbst et al., 1991). LMP1 expression induces many phenotypic changes resembling EBV-immortalization of B lymphocytes in a variety of cell types. The expression of LMP1 in type I Burkitt’s lymphoma cell lines, which have several characteristics of resting B cells (Rowe et al., 1987), leads to cell size increase, up-regulation of cell surface activation markers such as ICAM-1, LFA-1, CD23, CD21 and CD71 (Wang et al., 1988), as well as expression of the bcl-2 oncogene (Henderson et al., 1991; Martin et al., 1993; Rowe et al., 1994). Importantly, transient expression of LMP1 in human primary B cells also leads to induction of activation markers, cell size increase and DNA synthesis (Peng & Lundgren, 1992).

LMP1 is a multiple transmembrane-domain protein oriented in the plasma membrane in such a way that both the C and N termini are cytoplasmic (Liebowitz et al., 1986; Fennewald et al., 1984; Hennessy et al., 1984). LMP1 is distributed as discrete patches in the plasma membrane, and associates with the cytoskeleton in lymphocytes and transfected fibroblasts (Liebowitz et al., 1987; Martin & Sugden, 1991). The short N-terminal part is responsible for patching of the LMP1 molecules, but has not been shown to be important for the function of LMP1 in transformation assays (Izumi et al., 1994) or B cell activation (Peng-Pilon et al., 1995). The 200 amino acid cytoplasmic C-terminal domain appears to contain most of the elements necessary for LMP1 function, and deletion of this domain abolishes the ability of LMP1 to transform Rat-1 fibroblasts (Moorthy & Thorley-Lawson, 1995).
LMP1 triggers the activation of the transcription factor NF-κB (Hammarskjöld & Simurda, 1992; Laherty et al., 1992), and the NF-κB proteins p50, p65 and c-Rel have been shown to mediate activation of the surface marker ICAM-1 (Jahnke & Johnson, 1994). The C-terminal domain of LMP1 contains two NF-κB-activating sub-domains, CTAR1 and CTAR2 (C-terminal activating regions 1 and 2). CTAR1 is membrane-proximal, located between amino acids 194 and 232, and CTAR2 is at the C terminus between amino acids 351 and 386 (Huen et al., 1995; Mitchell & Sugden, 1995). CTAR1 interacts directly with TNF receptor-associated factor (TRAF) proteins of the TNF receptor signalling pathway and activates NF-κB to a low degree (Mosialos et al., 1995; Devergne et al., 1996). CTAR2, which does not interact directly with TRAFs, seems to be the major site for NF-κB activation (Huen et al., 1995; Mitchell & Sugden, 1995). This domain has recently been shown to activate NF-κB through binding of the TNF receptor-associated death domain (TRADD) protein (Izumi & Kieff, 1997).

The present study was designed to investigate if, or to what degree, up-regulation of B cell activation markers upon transient expression of LMP1 is dependent on NF-κB activation. We show that expression of LMP1 activates NF-κB, and that expression of a dominant mutant of λBz, an inhibitor of NF-κB, totally abolishes LMP1-induced NF-κB activity in DG75 Burkitt’s lymphoma and K562 pre-erythroblastoid cell lines. Flow cytometric analysis of B cell activation markers shows that the markers CD71 and ICAM-1 are to a large extent up-regulated by LMP1 even when the NF-κB activity is decreased below basal levels. Furthermore, the activation of primary B cells by LMP1 as measured by cell size increase could not be inhibited by expression of the dominant λBz. The results strongly suggest that B cell activation by LMP1 is dependent on an additional signal(s) separate from NF-κB.

Methods

**Cells.** DG75 is an EBV-negative Burkitt’s lymphoma cell line (Rowe et al., 1986) and K562 is a pre-erythroblastoid cell line (Gauwerky & Golde, 1980). CBM1 Ral-STO is an LCL (Ennberg et al., 1989). Primary B cells were isolated from donated blood as previously described (Pilon et al., 1991). Yields were 25–45 x 10⁶ cells per 500 ml blood sample. At 18 h prior to transfection, the primary B cells were treated with 5 ng/ml PDB (phorbol 12,13-dibutyrate, Sigma).

All cells were maintained at 1 x 10⁶/ml in a growth medium that consisted of RPMI 1640 supplemented with 5% FCS (10% for primary B cells) and antibiotics at 37 °C in a humidified atmosphere containing 6% carbon dioxide.

**Plasmids.** The LMP1 expression plasmid pEFLMP1 was obtained by ligating the LMP1 gene from pCMVLMP1 (Peng & Lundgren, 1992) as a blunt-ended XhoI–SmaI fragment into XhoI-digested and blunt-ended pEFC-X, carrying the strong polypeptide chain elongation factor 1α promoter. This plasmid is a derivative of pEF-BOS (Mizushima & Nagata, 1990), where a Clal/SalI/BamHI/XbaI polylinker is introduced between the two XbaI sites.

The p50 expression plasmid pCMV50 consists of the cDNA encoding amino acids 1–400 of p105 (Kieran et al., 1990) cloned into pRc/CMV (Invitrogen). The p65 expression plasmid pCMV65 has been described previously (Mercuro et al., 1992). The c-Rel cDNA was amplified by PCR from an LPS- and PDB-stimulated murine B cell line (a kind gift from P. Siders, Division of Tumour Biology), with primers designed to add HindIII and NotI sites flanking the cDNA. The PCR product was cloned between the HindIII and NotI sites of pRc/CMV. The isolated cDNA was identical to the sequence described by Bull et al. (1990).

The λBz cDNA was amplified from a Bc (EBV-immortalized human lymphoblastoid) cell line (a kind gift from P. Siders), with primers designed to add HindIII and NotI sites flanking the cDNA. The PCR product was identical to the MAD-3 sequence described by Haskell et al. (1991), except for a C instead of a T at nucleotide position 400, which does not change the amino acid sequence. The PCR product was cloned between the HindIII and NotI sites of pRc/CMV, and it was moved as a HindIII (blunted)–XhoI fragment to SalI (blunted) and Xbal-digested pEFC-X to generate pEFlxBz. pEFlxBzDM was derived from pEFlxBz by mutating serines 32 and 36 to alanines by PCR-directed mutagenesis.

pGL2(λgB) carries a luciferase gene downstream of two Xb elements derived from the immunoglobulin kappa light chain enhancer (Pierce et al., 1987). The BgII–Spel fragment carrying a hexameric µE5–µE2 site (Cornelussen et al., 1994) of a derivative of pGL2-basic was replaced by the sequence 5’GGGGCTTTCCAGAGGCGGGCTTCCTCGAGAATGCGCGG3’ (underlined). The sequence 3’ of the two Xb sites is identical. The sequence 5’GGGGCTTTCCAGAGGCGGGCTTCCTCGAGAATGCGCGG3’ (underlined). The sequence 3’ of the two Xb sites is identical.

**Transfection by electroporation.** Electroporations were performed under optimized conditions using 10⁷ cells in 500 µl culture medium in 1 ml cuvettes with a 0.4 cm space between the electrodes (BioRad). After the addition of DNA, the samples were gently mixed and kept at room temperature for 5 min. Electroporation was performed at 950 µF using 300 V for DG75, 330 V for K562 and 340 V for primary B cells. After electroporation, the samples were kept at room temperature for 5 min before being diluted with 10 ml culture medium. The amount of DNA per transfection was always adjusted to 25–30 µg using pUC19 as a carrier when necessary. The number of viable cells was determined 24 h after electroporation using the trypan blue exclusion method and was 67% for DG75, 70% for K562 and 13% for human primary B cells. Viability did not differ significantly between transfections with the various constructs. Transfection efficiency was determined by flow cytometric analysis, where cells bearing the co-transfection marker CD2 were analyzed in parallel with cells expressing the XhoI–E2 control plasmid.
antigen were scored as positive, and was 48% for DG75, 58% for K562 and 3% for primary B cells. Transfection efficiency did not vary significantly between transfections with the various constructs.

- **Western blotting.** Proteins from total cell extracts of $2 \times 10^6$ transfected DG75 cells, prepared 24 h after transfection with the indicated plasmids, were separated by SDS–PAGE according to the method of Laemmli (1970) and blotted onto nitrocellulose filters (Amersham), basically according to the method of Towbin et al. (1979). Excess protein-binding sites were blocked by incubation of the filters for 1 h in blocking reagent (Boehringer Mannheim). The filters were incubated overnight with an appropriate dilution of the anti-LMP1 monoclonal antibody reagent (Boehringer Mannheim) according to the manufacturer’s instructions.

- **Assay for NF-κB activity.** NF-κB activity was determined by quantifying luciferase expressed from the transfected NF-κB reporter plasmids. At 24 h post-transfection, cells were washed in PBS and lysed in 100 µl cell culture lysis buffer (Promega). Luciferase activity was measured from 10 µl cell lysate in 100 µl luciferase assay reagent (Promega) in a BioOrbit 1250 luminometer.

- **Flow cytometric analyses.** Live-cell fluorescence was carried out using saturating amounts of FITC-conjugated anti-ICAM-1, anti-CD71 and an isotype-matched control antibody (all from Immunotech) and with saturating amounts of biotinylated anti-cCD2 antibody followed by streptavidin–phycoerythrin to specifically label transfected cells. Prior to analysis, propidium iodine was added to a final concentration of 10 mg/ml. Binding was quantified using a flow cytometer (FACSCalibur, Becton Dickinson) gated on side and forward light scatter as well as for propidium iodine-emitted fluorescence to monitor only live cells, and gated on phycoerythrin-emitted fluorescence to monitor only cCD2-positive cells (i.e. successfully transfected cells). For each sample, at least 3000 live cCD2-positive cells were counted.

**Results**

LMP1 activates transcription from p50/p65- and c-Rel-specific κB sites

NF-κB belongs to a family of transcription factors with five known mammalian members, including the founding member c-Rel and the classic NF-κB heterodimer of p50 and p65. In order to quantify the effects of LMP1 expression on NF-κB activity, we designed luciferase reporter plasmids with promoters preferred by certain NF-κB family members. The p50/p65 reporter pGL2(κB)2 contains two copies of the κB site from the immunoglobulin kappa light chain enhancer (Pierce et al., 1987), and pGL2(κB1)2 contains two copies of a κB site that has been shown to be c-Rel-specific in a transient transfection assay (Ohmori et al., 1994). The effect of LMP1 expression on activation of transcription from these reporters was investigated. DG75 cells were used because Burkitt’s lymphoma type I cells share many characteristics with resting B cells (Rowe et al., 1987), and K562 was used since this cell-line shows clear effects upon LMP1 expression (Peng & Lundgren, 1993), while having a lower basal NF-κB activity than B cells.

**Fig. 1.** LMP1 activation of NF-κB in K562 and DG75 cells. NF-κB activity was measured using luciferase reporter plasmids (2 µg) with p50/p65-responsive IgκB or c-Rel-responsive κB1 sites (see Methods) 24 h after co-transfection with plasmids expressing LMP1 (pEFLMP1, 0.25 µg) or the parental vector, pEF-CαX (VC, vector control, 0.25 µg). The results from the luciferase measurements are represented in luciferase units. The mean ± SD of three transfections is shown.

LMP1-induced NF-κB activity can be completely inhibited by overexpression of IκBz

One objective of this study was to find a way to selectively inhibit NF-κB induction in LMP1-expressing cells. NF-κB forms a complex with an inhibitory κB protein, IκB, which inactively sequesters NF-κB in the cytoplasm. Activation of NF-κB requires the phosphorylation and subsequent degradation of IκB, releasing NF-κB to translocate to the nucleus and activate its target genes. We therefore co-transfected pEFLMP1 together with vectors expressing the NF-κB inhibitor IκBz. We used vectors expressing both wild-type IκBz (pEFκBz) and a dominant IκBz mutant, IκBzDM, with the inducible serine phosphorylation sites at positions 32 and 36 mutated (pEFκBzDM). This mutant cannot be phosphorylated and is consequently not proteolyzed upon NF-κB stimulation (Brown et al., 1995). This mutant efficiently retains NF-κB in the cytoplasm, unable to bind to the κB sites in the nucleus and induce transcription.
Expression of IκBζDM inhibits the LMP1-induced NF-κB activity from IκBζ sites (Fig. 2a) and κB1 sites (Fig. 2b). The dominant IκBζDM resulted in NF-κB activities below vector control levels when co-expressed with LMP1 in K562 and DG75 (Fig. 2). NF-κB activation by LMP1 was completely inhibited with 0.25 μg pEFLMP1 in K562, whereas 2 μg was needed for total inhibition in DG75. This difference probably reflects the higher basal level of NF-κB in DG75 (Fig. 1). Low transfection efficiency and viability (see Methods) render the corresponding analysis very difficult in primary B cells. However, co-transfections of 4 μg pEFLxBζDM together with pEFLMP1 (0.25 μg) resulted in total inhibition of the LMP1-induced NF-κB activity (data not shown). These inhibitions in primary B cells resulted in NF-κB activity at or below vector control levels.

Wild-type IκBζ also inhibited the LMP1-induced NF-κB activity. In Fig. 2(a), 3 μg pEFLxBζ was used, resulting in partial inhibition. Addition of higher amounts of this plasmid also leads to total inhibition (data not shown). Transfection of wild-type or mutant IκBζ also totally inhibited the NF-κB activity obtained by transfection of plasmids expressing p50/p65 or c-Rel proteins (data not shown).

IκB expression does not influence LMP1 protein levels

A potential problem with overexpression of IκBζ could be alterations in transcription from the expression vectors due to possible κB sites in promoters or enhancers. We have encountered such problems with the human cytomegalovirus (CMV) promoter (unpublished observations), but as seen in Fig. 3, LMP1 expression from the polypeptide chain elongation factor 1α promoter of pEFLMP1 was unaffected by the simultaneous IκBζDM expression from pEFLxBζDM. The same was seen for the co-transfection marker rCD2, expressed from pEFCD2 (Fig. 4).

LMP1 up-regulates cell surface activation markers

Expression of LMP1 has earlier been shown to lead to up-regulation of cell surface activation markers in cell lines and human primary B cells (Wang et al., 1988; Peng & Lundgren, 1992, 1993). Here we have analysed the expression of ICAM-1 and CD71 (transferrin receptor) in DG75, K562 and human primary B cells after transient expression of LMP1 from pEFLMP1. The rCD2 expression plasmid, pEFC2, was co-transfected to allow specific dual-colour flow cytometric analysis of successfully transfected rCD2-positive cells, as previously described (Pilon et al., 1991; Peng & Lundgren, 1992, 1993).

Representative flow cytometric data are presented in Fig. 4 as dot-plot profiles with logarithmic scales, and expression of the cell surface markers in K562 and DG75 cells is summarized in Table 1. Table 2 shows the corresponding data for human primary B cells, and also the cell size as measured by FSC (light forward scattering count). Values are shown as the percentage of cells scoring positive above an arbitrarily set intensity threshold at which approximately 10% of the vector control

Fig. 2. Inhibition of LMP1-induced NF-κB activity from p50/p65-responsive IκBζ sites (a) and c-Rel-responsive κB1 sites (b) by overexpression of IκBζ. K562 and DG75 cells were transfected with 0.25 μg pEFLMP1 together with the IκBζ or κB1 luciferase reporter (2 μg) and, where indicated, the IκBζ-expressing plasmids pEFLxBζ (3 μg) or sufficient amounts of pEFLxBζDM to decrease the NF-κB activity below vector control levels (0.5 μg in K562 and 2 μg in DG75). The amounts of transfected promoter-carrying plasmids were equalized using pEFC-X. Fold induction after 24 h is compared to the pEFC-X vector control (VC) normalized to 1. The mean ± SD of three transfections is shown.

Expression of IκBζDM was used as a positive LMP1 control.
NF-κB only partially mediates LMP1 effects

Fig. 4. Cell surface activation marker expression. (a) K562 cells co-transfected with 5 µg pEFC-D2 plus 2 µg pEFLMP1 and/or 4 µg pEFlkBαDM. (b) DG75 cells co-transfected with 5 µg pEFC-D2 plus 2 µg pEFLMP1 and/or 12 µg pEFlkBαDM. Expression was analysed 48 h after electroporation of the plasmids indicated in column 1. Columns 2 and 3 show dual-colour flow cytometric data on viable rCD2-positive cells (i.e. successfully transfected cells) double-stained with anti-rCD2 (vertical axis), and anti-ICAM-1 antibody (column 2) or anti-CD71 (column 3) (horizontal axis). The scales are logarithmic, and the vertical lines indicate the threshold level above which lies approximately 10% of the cells that were transfected with the vector control pEFC-X. (c) Cell surface activation marker expression and cell size increase of human primary B cells co-transfected with 20 µg pEFC-D2 plus 4 µg pEFLMP1 and/or 10 µg pEFlkBαDM. Column 3 shows FSC (light forward scattering count) on the horizontal axis (linear scale) plotted against CD71 staining (logarithmic scale). The amounts of transfected promoter-carrying plasmids were equalized using pEFC-X in all transfections.

Table 1. Summary of flow cytometric data on the effects of LMP1 and IκBαDM expression constructs on cell surface marker expression in K562 and DG75 cells after transfection with the plasmids indicated in column 1

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>K562</th>
<th>DG75</th>
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<tbody>
<tr>
<td></td>
<td>ICAM-1</td>
<td>CD71</td>
</tr>
<tr>
<td>pEFC-X</td>
<td>10·5 (0·2)</td>
<td>10·8 (0·4)</td>
</tr>
<tr>
<td>pEFLMP1</td>
<td>89·3 (4·7)</td>
<td>80·0 (1·6)</td>
</tr>
<tr>
<td>pEFLMP1 + pEFlkBαDM</td>
<td>38·7 (10·0)</td>
<td>85·5 (1·5)</td>
</tr>
<tr>
<td>pEFlkBαDM</td>
<td>6·9 (1·1)</td>
<td>11·0 (1·5)</td>
</tr>
</tbody>
</table>

(pEFC-X) transfectants scored positive. These analyses show that LMP1 induces up-regulation of ICAM-1 cell surface expression in K562 (Fig. 4a; Table 1), and that both CD71 and ICAM-1 are up-regulated in DG75 (Fig. 4b; Table 1) and primary B cells (Fig. 4c; Table 2) (compare pEFLMP1 in row 2 with pEFC-X in row 1). Expression of LMP1 in primary B cells also resulted in cellular enlargement (Fig. 4c; Table 2). The surface markers are up-regulated in a dose-dependent manner, as judged from the correlation between vertical axis (rCD2) and horizontal axis intensity. It should be noted that the scale is logarithmic, so for example, the strongest ICAM-1-staining cells among the pEFLMP1 transfectants show almost 10-fold more staining than vector control transfectants. LMP1 expression did not affect the binding of a control antibody isotype matched with the antibodies toward the cell surface markers (data not shown).

We found that 8-fold more pEFLMP1 (2 µg) was needed to obtain full cell surface marker induction compared to the
amount needed for maximal NF-κB induction (0.25 μg) as measured using the luciferase reporter plasmids. Fig. 5 shows a comparison of the dose-response of NF-κB and ICAM-1 activation by pEFMLP1 in K562 cells. The optimal amounts of pEFMLP1 for these activations are different, with full ICAM-1 up-regulation occurring only at LMP1 levels where the NF-κB activity is already decreased to less than half its maximal level.

Up-regulation of cell surface activation markers and cellular enlargement by LMP1 is only partially dependent on NF-κB activation

In order to investigate if the LMP1-induced up-regulation of the cell surface markers described above is dependent on NF-κB activation, we inhibited the LMP1-induced NF-κB activation by co-expressing IκBαDM from pEFIκBαDM as described above. As can be seen in row 4 of Fig. 4, expression of IκBαDM alone does not affect the expression of the co-transfection marker rCD2, but slightly decreases the expression levels of the analysed LMP1-inducible surface markers in K562, DG75 and primary B cells. This is probably due to the decrease in basal NF-κB activity seen in Fig. 2.

Co-expression of LMP1 with IκBαDM leads to reduction of the LMP1-induced up-regulation of both the analysed cell surface markers (Fig. 4; Tables 1 and 2). The amounts of pEFIκBαDM used (4 μg for K562, 12 μg for DG75 and 10 μg for primary B cells) were sufficient to decrease NF-κB activity below basal levels as measured by both IgκB and κB1 reporters (data not shown). Nevertheless, a substantial part of the LMP1-induced cell surface marker induction is retained in both the cell lines and in primary B cells. In DG75 cells, ICAM-1 is still up-regulated to 40–70% compared to vector control levels, and not even extreme overexpression using up to 20 μg pEFIκBαDM could abolish the LMP1-induced up-regulation of ICAM-1 (data not shown). Furthermore, the LMP1-induced cellular enlargement of primary B cells is not significantly affected by inhibition of NF-κB activation (Fig. 4c; Table 2).

Table 2. Summary of flow cytometric data on the effects of LMP1 and IκBαDM expression constructs on cell surface marker expression and cell size in human primary B cells after transfection with the plasmids indicated in column 1

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>ICAM-1</th>
<th>CD71</th>
<th>FSC</th>
</tr>
</thead>
<tbody>
<tr>
<td>pEFC-X</td>
<td>9.6 (0.3)</td>
<td>10.3 (0.3)</td>
<td>10.3 (0.5)</td>
</tr>
<tr>
<td>pEFMLP1</td>
<td>38.8 (5.4)</td>
<td>32.4 (0.8)</td>
<td>28.2 (4.7)</td>
</tr>
<tr>
<td>pEFMLP1 + IκBαDM</td>
<td>38.8 (11.9)</td>
<td>25.6 (5.5)</td>
<td>23.8 (3.7)</td>
</tr>
<tr>
<td>IκBαDM</td>
<td>4.8 (3.8)</td>
<td>6.9 (2.2)</td>
<td>7.9 (2.2)</td>
</tr>
</tbody>
</table>

Each value is the average of three independent transfections performed as in Fig. 4, and indicates the percentage of the rCD2-positive cells that stained stronger than the threshold level described in Fig. 4. FSC (light forward scattering count) is a measure of cell size. The SD values are given in parentheses.

Discussion

The EBV latent membrane protein LMP1 has profound influences on growth control and other phenotypes of human B cells and cell lines. Apart from acting as an oncogene in mouse fibroblasts (Wang et al., 1985; Baichwal & Sugden, 1988), it activates the expression of numerous cellular proteins, several of them markers found on activated B cells (Wang et al., 1988; Peng & Lundgren, 1992) or involved in protection against apoptosis (Henderson et al., 1991; Martin et al., 1993; Rowe et al., 1994). The present study was designed to investigate the importance of NF-κB induction in the up-regulation of the B cell activation markers ICAM-1 and CD71 by LMP1. We found that LMP1 activates transcription under the control of κB-sites preferentially binding p50/p65 heterodimers as well as sites binding c-Rel in both K562 and DG75 cells (Fig. 1). The p50/p65 preferred site gave the highest transcription in both cell types, but LMP1 gave the highest activation from the c-Rel preferred site. This indicates that LMP1 activation relative to the basal level is higher for c-Rel.

NF-κB induction by LMP1 could be reduced by expressing the NF-κB inhibitor IκBα, and completely inhibited by a dominant mutant, IκBαDM, defective in inducible degradation (Fig. 2). Expression of IκBαDM in cells expressing LMP1 decreased the NF-κB activity to below vector control levels. The ability of this mutant to totally abolish LMP1-induced NF-κB activation gave us a tool to investigate to what degree the LMP1-induced up-regulation of cell surface activation markers and cell size increase are dependent on NF-κB activation. LMP1-induced up-regulation of the surface markers CD71 and ICAM-1 were found to be reduced when IκBαDM was co-
expressed (Fig. 4; Tables 1 and 2). Nevertheless, the surface markers were still up-regulated in all cell types tested. In primary B cells, ICAM-1 was up-regulated to approximately 60% of the level obtained when LMP1 was expressed alone. The up-regulation of CD71 was substantially inhibited by lxBαDM in DG75 cells, but only to a low degree in primary B cells. We also found that lxBαDM was unable to significantly inhibit the LMP1-induced cellular enlargement in primary B cells (Table 2).

Since we know from the luciferase reporter experiments that the dominant lxBα mutant inhibits detectable NF-κB induction upon LMP1 transfection, and also significantly reduces basal levels of NF-κB, the results strongly indicate that the induction of at least some of the cell surface activation markers by LMP1 is dependent on an additional signal(s) separate from NF-κB. The relatively strong lxBαDM inhibition of CD71 in DG75 cells may agree with a simple model where the LMP1-induced NF-κB activity directly influences the expression of the cell surface molecule at the transcriptional level or by controlling its transport to the cell surface. However, to explain the inability of dominant lxBα to inhibit a substantial part of the LMP1-induced up-regulation of CD71 and cell size increase in primary B cells, and expression of ICAM-1 in K562 and DG75, we propose a scheme whereby LMP1 induces several signals, at least one of which is independent of NF-κB (Fig. 6). This interpretation is strengthened by the observation that several-fold more LMP1 expression plasmid is needed to obtain full up-regulation of the cell surface markers than is needed to fully activate NF-κB. As can be seen in Fig. 5, the dose-response curves for ICAM-1 up-regulation and NF-κB activation by transfection of pEF-LMP1 are strikingly different. NF-κB is activated to the highest levels with 0.1–0.5 μg pEF-LMP1, and higher amounts of plasmid result in substantially lower activity. In contrast, maximal cell surface expression of ICAM-1 is achieved with approximately 10-fold higher amounts of pEF-LMP1, a level of LMP1 expression resulting in less than half maximal NF-κB activation. The failure of LMP1 to up-regulate CD71 in K562 cells indicates that the LMP1-induced up-regulation of CD71 in DG75 and primary B cells involves a signal transduction pathway(s) where a component is lacking or is not active in the non-B-cell line K562.

Our results are in line with growing evidence that LMP1 signalling is rather complex, with two domains, CTAR1 and CTAR2, that signal through different members of the TNF receptor signalling pathway. CTAR1 interacts directly with TRAF proteins (Mosialos et al., 1995), while CTAR2 signals via interaction with the TRADD protein (Izumi & Kieff, 1997). Both domains mediate activation of NF-κB (Huen et al., 1995). It has been shown that mutants lacking CTAR1 or CTAR2 activate ICAM-1 to the same degree in B cell lines (approximately 50% of wild-type LMP1), whereas their ability to activate NF-κB differ and is cell-line-dependent (Huen et al., 1995). Furthermore, it has been demonstrated that LMP1 can induce the epidermal growth factor (EGF) receptor and the A20 zinc finger protein via two different pathways. EGF receptor up-regulation is TRAF-mediated but NF-κB-independent, whereas A20 induction seems to be mediated directly through NF-κB (Miller et al., 1996).

From the results reported here we conclude that even when LMP1 is unable to activate NF-κB, due to an excess of a dominant lxBα mutant, it is still capable of up-regulating cell surface activation markers, strongly suggesting that LMP1 can activate cells independently of NF-κB (Fig. 6). The ICAM-1 and CD71 promoters contain many transcription factor binding sites, including both NF-κB and AP-1 sites (Ouyang et al., 1993; Jahnke & Johnson, 1994; Muñoz et al., 1996). In the case of ICAM-1, activation of the promoter by the antioxidant pyrrolidinedithiocarbamate has been shown to involve the AP-1 site (Muñoz et al., 1996). This makes the recently described LMP1-mediated activation of AP-1 via the JNK kinase (Kieser et al., 1997; Kilger et al., 1998) a strong candidate for an additional signalling pathway involved in the up-regulation of the cell surface activation markers. However, the high number of transcription factor sites in addition to NF-κB and AP-1 in the ICAM-1 and CD71 promoter regions makes it possible that additional signal transduction pathways are involved.

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References


The Epstein–Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-κB and cell surface phenotype via two effector regions in its carboxy terminal cytoplasmic domain. 


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