Epstein–Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor

Ellen Kilger, Arnd Kieser, Matthias Baumann and Wolfgang Hammerschmidt¹

GSF-National Research Center for Environment and Health, Institut für Klinische Molekularbiologie und Tumorgenetik, Marchioninstr. 25, D-81377 Munich, Germany

¹Corresponding author
e-mail: hammerschmidt@gsf.de

The Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1) is essential for the immortalization of human B cells and is linked etiologically to several human tumors. LMP1 is an integral membrane protein which acts like a constitutively active receptor. It binds tumor necrosis factor (TNF)-receptor-associated factors (TRAFs), activates NF- κ B and triggers the transcription factor AP-1 via the c-Jun N-terminal kinase (JNK) cascade, but its specific contribution to B-cell immortalization has not been elucidated fully. To address the function of LMP1, we established B cell lines with a novel mini-EBV plasmid in which the LMP1 gene can be regulated at will without affecting the expression of other latent EBV genes. We demonstrate here that continuous expression of LMP1 is essential for the proliferation of EBV-immortalized B cells *in vitro*. Re-induction of LMP1 expression or activation of the cellular CD40 receptor both induce the JNK signaling cascade, activate the transcription factor NF- κ B and stimulate proliferation of these B cells. Our findings strongly suggest that LMP1 mimics B-cell activation processes which are physiologically triggered by CD40–CD40 ligand signals. Since LMP1 acts in a ligand-independent manner, it replaces the T cell-derived activation signal to sustain indefinite B-cell proliferation.

Keywords: B cells/CD40 receptor/Epstein–Barr virus/LMP1

Introduction

Epstein–Barr virus (EBV) is a ubiquitous human herpes virus associated with a number of malignant diseases including Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma (for a review, see Rickinson and Kieff, 1996). Infection of primary human B lymphocytes *in vitro* with EBV leads to B-cell immortalization and lymphoblastoid cell lines. Four of the 11 viral genes expressed in the immortalized B cells have been found to be essential genetically for the process of immortalization: the EBV nuclear antigens EBNA2, EBNA3a and EBNA3c, and the latent membrane protein 1 (LMP1) (Cohen *et al.*, 1989; Hammerschmidt and Sugden, 1989; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993; Kempkes *et al.*, 1995c). Since EBNA1 maintains the status of the EBV genomes

in the proliferating B cells, it might also be indispensable (for a review, see Yates, 1996).

Among the proteins involved in B-cell immortalization, LMP1 is the only protein that has oncogenic effects in non-lymphoid cells. When expressed in rodent fibroblast cell lines, LMP1 induces growth transformation (Wang *et al.*, 1985; Baichwal and Sugden, 1988; Moorthy and Thorley-Lawson, 1993). Expression of LMP1 in Burkitt's lymphoma cell lines induces many of the phenotypic changes observed in EBV infection such as up-regulation of the B-cell activation markers CD23 and CD40 and the cell adhesion molecules ICAM1, LFA1 and LFA3 (for a review, see Kieff, 1996). Although LMP1 plays an important role in B-cell immortalization and is also implicated in EBV-associated malignancies like Hodgkin's disease, its mechanism of action has not yet been elucidated fully.

LMP1 is an integral membrane protein composed of a short cytoplasmic N-terminus of 24 amino acids, six transmembrane domains of 186 amino acids and a cytoplasmic C-terminus of 200 amino acids (Fennewald *et al.*, 1984; Hennessy *et al.*, 1984; Liebowitz *et al.*, 1986). It has been shown recently that LMP1 acts like a constitutively active receptor (Gires *et al.*, 1997) which shares certain characteristics with members of the tumor necrosis factor receptor (TNFR) family. Similarly to CD30, CD40 and TNFRs, LMP1 binds certain TNFR-associated factors (TRAFs) (Mosialos *et al.*, 1995; Devergne *et al.*, 1996; Sandberg *et al.*, 1997) and activates the transcription factor NF- κ B (Laherty *et al.*, 1992; Huen *et al.*, 1995) by a pathway which might involve TRAF molecules (Hsu *et al.*, 1995; Rothe *et al.*, 1995; Duckett *et al.*, 1997; Tsitsikov *et al.*, 1997). In addition, the stress-activated protein kinase JNK1 is activated by both LMP1 (Kieser *et al.*, 1997) and CD40 (Berberich *et al.*, 1996). In EBV-immortalized B cells lacking a functional EBNA2 protein, CD40 activation and LMP1 expression result in the same phenotype of prolonged cell survival and DNA synthesis (Zimmer-Strobl *et al.*, 1996). Although CD40 and LMP1 share very little protein homology, their analogous functions provide circumstantial evidence that CD40 and LMP1 might engage similar signaling pathways to target common effector genes.

In order to characterize the role of LMP1 in B-cell immortalization, we have now established B-cell lines with a conditional LMP1 gene on the basis of a mini-EBV plasmid. Mini-EBVs are recombinant *Escherichia coli* plasmids which contain all elements of EBV necessary for immortalization of human B lymphocytes. Upon introduction into primary B cells, mini-EBV plasmids induce proliferation as efficiently as wild-type virus (Kempkes *et al.*, 1995a,b). In a newly constructed mini-EBV plasmid, the LMP1 gene is under control of a conditional, artificial promoter which permits the selective repression of the LMP1 gene without affecting the expression of other viral genes in EBV-immortalized B cells.

Here we show that continuous expression of LMP1 is essential for the proliferation of EBV-immortalized B cells *in vitro*. B cells which express very reduced levels of LMP1 cease to proliferate, but survive in a resting state. Upon re-induction of LMP1, the B cells return to normal proliferation. The same growth-promoting effect was achieved by activation of the endogenous cellular CD40 receptor in the absence of LMP1. Since the *in vivo* signal for B-cell activation involves triggering of CD40 by the CD40 ligand (CD40-L) present on activated CD4⁺ T cells (Banchereau and Rousset, 1991; Noelle *et al.*, 1992; Laman *et al.*, 1996), our finding strongly suggests that LMP1 functionally substitutes an activated CD40 receptor to sustain B-cell proliferation *in vitro*. Since LMP1 signals in a ligand-independent manner (Gires *et al.*, 1997), it replaces a T cell-derived activation signal to induce indefinite proliferation of EBV-infected cells.

Results

Construction of a conditional LMP1 gene on a mini-EBV plasmid

To study the role of LMP1, we established a conditional expression system on the basis of a mini-EBV plasmid. Mini-EBV plasmids carry all viral genes found expressed in *in vitro* immortalized B cells to induce and maintain B-cell proliferation as efficiently as wild-type EBV (Kempkes *et al.*, 1995a,b). Moreover, mini-EBV plasmids can be engineered to carry additional genes, and their overall composition can be modified in *Escherichia coli* with conventional genetic techniques (O'Connor *et al.*, 1989).

The conditional expression system for LMP1 consists of the coding sequence of the wild-type gene under the control of an artificial promoter whose activity can be regulated by a chimeric transcriptional repressor, tetR-KRAB, in a tetracycline-dependent manner (Figure 1) (Deuschle *et al.*, 1995). We used the TP1 promoter of EBV with the two RBP-J κ sites (Zimber-Strobl *et al.*, 1994) replaced by 10 copies of the *tet* operator (*tetO*) analogous to that used in a publication by Gossen and Bujard (1992). This cassette was then moved onto a plasmid that allows homologous recombination in *E.coli* with the mini-EBV plasmid p1478A. Since this mini-EBV plasmid is fully competent to immortalize human primary B cells *in vitro*, its wild-type LMP1 gene was replaced by the conditional LMP1 gene via allelic exchange in *E.coli*. The expression cassette with the chimeric repressor tetR-KRAB (Deuschle *et al.*, 1995) was added onto this mini-EBV plasmid in a second step with the chromosomal building technique (O'Connor *et al.*, 1989) to yield the final construct p1852 (Figure 1A). tetR-KRAB consists of the KRAB domain, a potent repressor of transcription in mammalian cells, fused to the Tn10-derived DNA-binding domain of *tetR* which binds to *tetO* sites (Deuschle *et al.*, 1995). Binding of tetR-KRAB in the absence of tetracycline leads to transcriptional repression of the LMP1 gene. Since tetracycline prevents the binding of tetR-KRAB to *tetO*, LMP1 is expressed from the active TP1 promoter. Figure 1B shows a schematic illustration of the conditional expression system.

Conditional LMP1 expression in EBV-immortalized B cells

The mini-EBV plasmid p1852 carrying the conditional LMP1 allele was packaged into virions as described earlier

(Kempkes *et al.*, 1995b; Zimber-Strobl *et al.*, 1996) (E.Kilger, A.Schwenk, G.Pecher and W.Hammerschmidt, in preparation). Virus stocks were used to infect primary human B lymphocytes, and cell clones were expanded in medium containing tetracycline to allow LMP1 gene expression. Single cell clones were confirmed to contain only mini-EBV plasmid by PCR and Southern blot analyses (data not shown).

Individual cell clones immortalized by p1852 were kept in the presence or absence of tetracycline in the culture medium and LMP1 expression was monitored in total protein extracts by Western blotting. After withdrawal of tetracycline, all clones showed a clear down-regulation of LMP1 expression with slight clonal variations (Figure 2A). LMP1 was not shut off completely but LMP1 levels were considerably lower than in normal, EBV-immortalized B cells (Figure 2A). To confirm that only LMP1 expression is regulated by tetracycline, protein extracts were also analyzed for expression of EBNA1 and EBNA2. As expected, the expression of these proteins was not affected by tetracycline (Figure 2A). In Northern blot analyses, transcription of LMP2 was found to be reduced ~2- to 3-fold, but the steady-state levels of LMP2A were not altered detectably in Western blots (data not shown).

We characterized the expression of LMP1 within the cell population of 1852 cells further by flow cytometry. Cells of clone 1852.4 were kept in the presence of tetracycline, or tetracycline was omitted for 7 days to down-regulate LMP1 expression. Cells were fixed, permeabilized and stained with an LMP1-specific antiserum and a phycoerythrin-conjugated secondary antibody or with the secondary antibody alone. FACS analysis of the cells was performed and the results are shown in Figure 2B. In the presence of tetracycline, LMP1 expression was high in most of the cells, with a mean fluorescence value of 550. After cultivation without tetracycline, the expression level of LMP1 decreased significantly within the cell population. The mean fluorescence value dropped to 102 and the majority of the cells expressed no detectable or low levels of LMP1. However, the distribution curve also shows that a small portion of the cells still expressed LMP1, resulting in only reduced expression levels as detected in Western blot analyses (Figure 2A).

LMP1 is essential for continuous proliferation of B cells

Next, the phenotype of the 1852-immortalized B cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,2,5-diphenyl tetrazolium bromide (MTT) conversion (Mosmann, 1983) to monitor the viability and proliferation of several 1852 B-cell clones in the presence and absence of tetracycline. The results with three independent clones are shown in Figure 3. They all proliferated in the presence of tetracycline, similarly to a control clone expressing LMP1 from its wild-type promoter in the context of a mini-EBV plasmid (LCL 1736.18). In contrast, withdrawal of tetracycline had a dramatic effect on cell proliferation. In the absence of tetracycline, the viability of all 1852 cell clones stayed at about the same level for 12 days, indicating that cells expressing very low LMP1 levels survive but lack an important proliferation signal. Direct counting of

Fig. 1. Generation of B-cell clones with the mini-EBV plasmid p1852 carrying a conditional LMP1 gene. **(A)** Composition of the p1852 mini-EBV plasmid. Two elements, a promoter with *tetO* sites and the conditional repressor *tetR*-KRAB, necessary for conditional LMP1 expression, are circled with a black line. These elements were added onto the mini-EBV plasmid p1478A using the chromosomal building technique (O'Connor *et al.*, 1989). p1478A carries all elements for *in vitro* B-cell immortalization and is identical to the mini-EBV plasmid p1495.4 (Kempkes *et al.*, 1995a) except that the last chromosomal building step was omitted. The artificial promoter for the LMP1 gene consists of the EBV TP1 promoter containing 10 *tet* operator sites (*tetO*). The plasmid also carries an expression cassette for the chimeric factor *tetR*-KRAB expressed from the CMV promoter (Deuschle *et al.*, 1995). The 11 viral genes (EBNA1, EBNA-LP, EBNA2, EBNA3A, -B, -C, LMP1, LMP2A, -B, EBER1 and EBER2) generally expressed in the latent phase of the EBV life cycle are denoted as gray boxes or are too small to be represented (EBER1 and EBER2). The map also shows the *cis*-acting elements (open boxes) *oriP*, *oriLyt* and TR which constitute the plasmid origin of replication in latently infected cells, the lytic origin of replication and the terminal repeats, involved in packaging of the plasmid into virions, respectively. **(B)** Schematic representation of the conditional expression system for LMP1. LMP1 gene expression is mediated by an artificial promoter containing 10 *tetO* sites (only five are shown here). Transcription can be repressed by a chimeric factor that binds via its *tet* repressor (*tetR*) domain to the *tetO* sites in the promoter in the absence of tetracycline. Repression of transcription is then mediated by the KRAB domain, a potent repressor of transcription in mammalian cells (upper panel). Addition of tetracycline prevents binding of the chimeric repressor to the promoter and allows LMP1 gene expression from the constitutively active promoter (lower panel).

the cells confirmed that LMP1 levels were critical for continuous proliferation (data not shown).

In the absence of tetracycline, viability as measured by MTT conversion might also reflect a mixed population of slowly growing, resting and dying cells. In fact an increasing number of dead cells could be noticed in the absence of tetracycline during the course of the experiments (data not shown). This situation could be due to the heterogeneous LMP1 expression within the cell population observed in FACS analyses (Figure 2B). The expression of the *tetR*-KRAB repressor itself had no influence on cell proliferation in the absence or presence of tetracycline (data not shown).

EBV-immortalized B cells return to normal proliferation after re-induction of LMP1 expression

Next, we investigated whether LMP1 expression could be re-induced by re-addition of tetracycline. Tetracycline was omitted for 5 days in different 1852 B-cell clones, then re-added, and protein extracts were analyzed in parallel for LMP1 expression by Western blot analysis on five consecutive days. As shown in Figure 4A (top panel), LMP1 expression was re-induced and the initial expression level was reached ~4 days after re-addition of tetracycline. The delay in LMP1 re-expression is probably due to a slow response of the conditional expression system which had been observed earlier (Deuschle *et al.*, 1995).

To address whether re-expression of LMP1 would have

an effect on cell proliferation, we determined the cellular viability by MTT conversion as described above. As shown in Figure 4A, lower panel, cells were kept without tetracycline for 5 days and then tetracycline was re-added. After re-induction of LMP1, the B cells started to proliferate and returned to normal growth characteristics after ~3 days. Cells without tetracycline did not proliferate during a period of 14 days. Cells kept in the presence of tetracycline for the whole time were used as a control for the normal proliferation rate under similar conditions. In a parallel experiment, incorporation of bromodeoxyuridine (BrdU) and DNA content were measured by FACS analysis after immunodetection of BrdU and propidium iodide staining (Figure 4B). Withdrawal of tetracycline led to a dramatic decrease of cells in S phase and an increase in G₁ phase of the cell cycle consistent with the effects observed in MTT assays. After re-induction of LMP1, the number of cells in S increased while cells in G₁ decreased as expected, and the cell cycle profile returned to the normal initial distribution.

CD40 activation and LMP1 expression have similar growth-promoting effects

CD40 activation by the CD40 ligand (CD40-L) or through antibody-mediated cross-linking triggers cell proliferation of resting human B cells *in vitro* (Banchereau and Rousset, 1991; Banchereau *et al.*, 1991; Noelle *et al.*, 1992). In addition, LMP1 expression or CD40 activation both led

Fig. 2. Expression of LMP1, EBNA1 and EBNA2 in immortalized 1852 B-cell clones with or without tetracycline. **(A)** Three different cell clones immortalized with the mini-EBV p1852 were kept in the presence or absence of tetracycline in the culture medium for 3 days and protein extracts were analyzed for expression of LMP1, EBNA1 and EBNA2. All three clones showed a clear down-regulation of LMP1 expression in the absence of tetracycline. A minimal expression of LMP1 was still detected in cells cultivated without tetracycline, but the level was dramatically decreased compared with the LMP1 level found in a control cell line immortalized by a mini-EBV plasmid expressing LMP1 from its wild-type promoter (LCL). As expected, expression levels of EBNA1 and EBNA2 were unaffected by tetracycline. An EBV-negative cell line (DG75) served as a negative control. **(B)** Cells of clone 1852.4 were kept in the presence (+tet) or absence of tetracycline (-tet) for 7 days and expression of LMP1 was analyzed by flow cytometry. The dark curves show the fluorescence of cells stained with an LMP1-specific antiserum and phycoerythrin-conjugated secondary antibody, the light curves show the control staining with secondary antibody alone. When cultivated in the presence of tetracycline (+tet), most of the cells express high levels of LMP1 with a mean fluorescence of 550. In comparison, after removal of tetracycline (-tet), cells express less LMP1 with a mean fluorescence of 102.

to a very similar phenotype, i.e. prolonged viability in the absence of functional EBNA2, in *in vitro* immortalized B cells (Zimber-Strobl *et al.*, 1996). Since LMP1 mediates

Fig. 3. LMP1 is essential for B-cell proliferation in EBV-immortalized B cells. The three 1852 cell clones (clone 4, clone 16 and clone 28) shown to express LMP1 conditionally (Figure 2) and a control clone immortalized with a mini-EBV plasmid with wild-type LMP1 (LCL) were plated at a concentration of 6×10^3 cells/well in a 96-well cluster plate and grown in the presence (+tet) or absence (-tet) of tetracycline for 12 days. At the indicated time points, cell viability was determined by MTT conversion. To compare different experiments, the rate of MTT conversion at day 1 was set to 1 and OD values were normalized accordingly. One representative experiment out of three is shown. All three 1852 clones with down-regulated LMP1 in the absence of tetracycline (-tet) did not proliferate, while cells expressing normal levels of LMP1 [1852 clones in the presence of tetracycline (+tet) and the LCL control clone] proliferated normally. Tetracycline had no effect on the growth of the LCL control clone.

an essential B-cell proliferation signal in 1852 B cells, we asked whether CD40 activation could functionally replace LMP1 to sustain cellular proliferation. Cells of the 1852 clones 4 and 16 were kept with or without tetracycline in the presence of soluble trimerized CD40-L (Hollenbaugh *et al.*, 1992). To monitor cell proliferation, MTT conversion assays were conducted over a period of 12 days. Activation of the CD40 receptor restored the ability of the cells to proliferate normally for up to 3 weeks (Figure 5 and data not shown), compensating for the lack of LMP1 signals. Remarkably, CD40 activation of cells in the presence of tetracycline, i.e. at functional levels of LMP1, had no additional or synergistic effects on cellular proliferation rates (Figure 5). Consistent with this observation, B cells expressing constitutive, normal

Fig. 4. LMP1 re-induction in 1852-immortalized B cells. **(A)** The 1852 clone 4 was grown without tetracycline for 5 days (–tet) then tetracycline was re-added. The time course of LMP1 expression after re-induction was analyzed by Western blot analysis. After 4 days, LMP1 expression reached the initial level. A mini-EBV-infected lymphoblastoid cell line (LCL) serves as a control. In a parallel experiment, cells were plated at 5×10^3 cells/well in a 96-well cluster plate and either grown continuously in the presence (+tet) or absence (–tet) of tetracycline or tetracycline was re-added after 5 days to cells kept without tetracycline (+tet at day 5). Cell viability was determined by MTT conversion and is shown below. Parallel to re-induction of LMP1 expression, the cells returned to normal growth compared with cells grown with tetracycline. Cells left without tetracycline for the same period of time did not proliferate. **(B)** Cell cycle analysis of the 1852 clone 4 grown in the absence of tetracycline (–tet) for 3 days and re-addition of tetracycline (+tet) at day 3 for up to 5 days. Shown is the percentage of cells in G₁ and S-phase of the cell cycle determined by FACS analysis as described in Materials and methods. Down-regulated LMP1 leads to an increase in the number of cells in G₁ phase and a decrease in cells in S phase, which is reversed to initial levels after re-induction of LMP1 expression.

LMP1 levels did not respond significantly to activation of their endogenous CD40 receptor (Figure 5).

The results show that LMP1 and CD40 signals are interchangeable for triggering B-cell proliferation. Our findings also suggest that proliferative signals by either one (pseudo-) receptor, CD40 or LMP1, is sufficient to promote B-cell proliferation since no additive effect by the stimulation of both molecules could be detected. Together, these data provide functional evidence that LMP1 and CD40 might function via similar mechanisms in B cells.

LMP1 expression and CD40 activation both induce the JNK1 pathway and activate NF- κ B

Although it is known that LMP1 and CD40 activate the transcription factor NF- κ B complex and interact with TRAF family members, the signal transduction pathways which are triggered by CD40 and LMP1 have not yet been elucidated fully. It has been shown that LMP1 induces the transcription factor AP-1 via the c-Jun N-terminal kinase (JNK) cascade (Kieser *et al.*, 1997) and that CD40 can activate JNK1 in certain B-cell lines (Berberich *et al.*, 1996). In addition to the activation of

the JNK1 signaling pathway, the induction of NF- κ B might be influential in triggering B-cell proliferation by both LMP1 and CD40. We therefore tested if JNK1 and NF- κ B are activated in our cell system after re-induction of LMP1 expression or stimulation of CD40 by CD40-L.

1852.4 cells were kept without tetracycline for 4 days to down-regulate LMP1 expression. Subsequently, LMP1 expression was re-induced by addition of tetracycline or cells were plated onto a feeder cell layer expressing CD40-L (Banchereau and Rousset, 1991; Galibert *et al.*, 1996; Zimmer-Strobl *et al.*, 1996) in the absence of tetracycline. At different time points, cells were lysed and endogenous JNK1 was immunoprecipitated. The activity of JNK1 was measured in *in vitro* kinase assays using GST-c-Jun as a substrate (Figure 6A). JNK1 activity was barely detectable at reduced LMP1 levels in the absence of tetracycline. Activation of CD40 as well as induction of LMP1 expression both led to a 4- to 6-fold activation of JNK1 (Figure 6A). After CD40 stimulation, JNK1 activation is already detectable at day 1, while the kinetics of JNK1 induction follow the relatively slow LMP1 re-expression after addition of tetracycline. This result could also be interpreted as an indirect effect of LMP1. However,

Fig. 5. CD40 activation can functionally replace LMP1 in mediating B-cell proliferation. The 1852 clones 4 and 16, and a mini-EBV-immortalized control clone (LCL) were plated at a concentration of 1×10^4 cells/well in a 96-well cluster plate and grown in the presence (+tet) or absence of tetracycline (-tet) for 12 days. In an identical setting, soluble CD40 ligand (+CD40-L) was added. MTT conversion was measured at the indicated time points and normalized to the OD value at day 1. A representative of three independent experiments is shown. Activation of the CD40 receptor could compensate for the effect of low level LMP1 expression as shown by adding soluble CD40 ligand to cells which had been cultivated without tetracycline. CD40 activation of 1852 cells expressing LMP1 at the wild-type level (+tet) had no significant or reproducible additive effect on cell proliferation. The control LCL clone (LCL) showed no significant differences in proliferation when grown in the presence or absence of tetracycline and/or CD40 ligand.

we were able to show recently that LMP1 signaling to JNK1 follows a direct pathway involving the kinase SEK1 (Kieser *et al.*, 1997).

To test if LMP1 re-induction or CD40 activation also lead to activation of NF- κ B, we prepared nuclear extracts and analyzed the NF- κ B DNA-binding activity in electrophoretic mobility shift assays (EMSAs; Figure 6B). In the absence of tetracycline, cells with greatly reduced LMP1 expression levels showed little DNA-binding activity of NF- κ B, but both re-induction of LMP1 and activation of CD40 led to induction of nuclear NF- κ B which was detectable after 1 day of stimulation. After 3 days of CD40 activation, a maximum number of nuclear NF- κ B complexes was detected, which decreased afterwards, presumably due to an increase in I- κ B expression. At least two different complexes bound to the NF- κ B-binding motif, as indicated by two bands of different size detected on the gel. Complexes of the upper band could be supershifted with an anti-p65 antibody showing that LMP1 and CD40 induced DNA binding of p65-containing NF- κ B complexes. Supershifts with an anti-p50 antibody showed that the majority of the induced NF- κ B complexes

Fig. 6. (A) CD40 and LMP1 both induce endogenous JNK1 activity in 1852.4 B cells. 1852.4 cells were cultured in the absence of tetracycline for 4 days to down-regulate LMP1 expression. Subsequently, cells were either plated onto a feeder layer of CD40 ligand-expressing fibroblastic L cells (Banchereau and Rousset, 1991; Galibert *et al.*, 1996; Zimmer-Strobl *et al.*, 1996) in the absence of tetracycline (shown on the left) or stimulated with 1 μ g/ml tetracycline to re-induce LMP1 expression (shown on the right). At the indicated time points, cells were lysed and endogenous JNK1 protein was immunoprecipitated using the anti-JNK1 C-17 antibody. Kinase assays were performed with the immunoprecipitated JNK1 using purified GST-c-Jun as a substrate. Top panels: autoradiographs of JNK1 kinase assays. Middle panels: x-fold induction of JNK1 kinase activities as quantitated by phosphorimager analysis. Bottom panels: immunoblot analysis of LMP1 expression using the anti-LMP1 antiserum CS1-4. (B) CD40 and LMP1 both activate NF- κ B in 1852.4 B cells. 1852.4 cells were treated as in (A). At the indicated time points, nuclear extracts were prepared and electrophoretic mobility shift assays were performed with a probe containing an NF- κ B-binding site. Supershift analyses were performed with antibodies against p65 (+p65 Ab) and p50 (+p50 Ab) where indicated. Induced DNA-bound NF- κ B complexes are marked with black triangles. Supershifted complexes are marked with open triangles.

contain p50; presumably complexes of the lower band consist mainly of p50-p50 homodimers.

The results demonstrate that both CD40 activation and LMP1 expression lead to JNK1 and NF- κ B activation, concomitant with the induction of B-cell proliferation. These results also suggest that both signals might be involved in mediating B-cell proliferation in a complementary fashion.

Discussion

LMP1 has been shown to mimic a ligand-activated receptor (Gires *et al.*, 1997) and shares several characteristics with members of the TNFR superfamily, CD40 in particular. Both, LMP1 and CD40 interact with TRAFs (Mosialos *et al.*, 1995; Devergne *et al.*, 1996; Sandberg *et al.*, 1997), activate the transcription factors NF- κ B (Hammariskjold and Simurda, 1992; Laherty *et al.*, 1992) and AP-1 (Kieser *et al.*, 1997), and up-regulate similar cell surface markers on B cells (for reviews, see Kieff, 1996; Laman *et al.*, 1996). However, nearly all of these observations have been made by transient or stable transfection of the LMP1 gene into 293 human embryonic kidney cells or Burkitt's lymphoma cell lines. Recently, we found that LMP1 and activated CD40 cause a comparable phenotype in EBV-immortalized B cells lacking functional EBNA2, leading to prolonged survival and DNA synthesis (Zimber-Strobl *et al.*, 1996). This was the first observation made in the natural context of an EBV-immortalized B cell, suggesting that LMP1 expression and CD40 receptor activation could be functionally similar.

To address further the function of LMP1 in B-cell immortalization and its similarity to CD40, we established immortalized B cell lines with a conditional LMP1 gene. For the first time, it was possible to study the effect of LMP1 selectively in B cells expressing all other latent EBV genes. We could show that B cells with a greatly reduced level of LMP1 cease to proliferate, indicating that LMP1 constitutes an essential proliferative signal. The B cells did survive under these conditions and most of them remained in a resting state which was reflected by an increased number of cells in G₁. The cell cycle profile did not show a complete G₁ arrest because a small number of cells still continued to progress through S phase, probably due to a heterogeneity of LMP1 expression in the cell population, such that some cells still had elevated LMP1 levels. Nevertheless, the results clearly demonstrate that cells with reduced LMP1 expression were greatly impaired in proliferation. The state of these cells resembles primary resting B cells which enter the cell cycle when activated through their CD40 receptor (Banchereau *et al.*, 1991). B-cell activation *in vivo* is mediated by contact with CD40-L-bearing activated CD4⁺ T cells (Armitage *et al.*, 1992) and plays an important role in the immune response. Signals mediated through CD40–CD40-L induce the expression of activation markers, immunoglobulin production and isotype switching, and are also required for formation of B memory cells in germinal centers presumably because CD40 activation prevents apoptosis of germinal center B cells (for a recent review, see Laman *et al.*, 1996). Here we show that LMP1 expression leads to cellular proliferation to the same extent as does activation of CD40. This result strongly indicates that LMP1 simulates B-cell activation processes which are physiologically triggered by CD40–CD40-L signals, thereby rendering B-cell proliferation independent of extracellular stimuli.

There are several hints that LMP1 and CD40 may address the same signaling pathway(s), but the signal cascades linked to the phenotypic effects of both molecules are not yet fully understood. TRAFs have been reported to interact with the C-terminus of CD40 and be involved

in NF- κ B activation (Cheng *et al.*, 1995; Rothe *et al.*, 1995; Kuhné *et al.*, 1997). The C-terminus of LMP1 also binds TRAF molecules and promotes NF- κ B activation by TRAF-dependent and -independent mechanisms (Huen *et al.*, 1995; Mitchell and Sugden, 1995; Devergne *et al.*, 1996; Brodeur *et al.*, 1997; Sandberg *et al.*, 1997). While activation of NF- κ B by LMP1 leads to induction of the anti-apoptotic A20 gene (Laherty *et al.*, 1992), NF- κ B activation does not seem to account for the transforming potential of LMP1 since it transforms BALB/3T3 cells without inducing NF- κ B (Mitchell and Sugden, 1995). While searching for new nuclear targets for LMP1 signaling, we found that LMP1 activates the transcription factor AP-1, a dimer composed of Jun or Fos family proteins, via the JNK cascade (Kieser *et al.*, 1997). We show here that in EBV-immortalized B cells, activation of CD40 as well as induction of LMP1 expression induces JNK1, the kinase phosphorylating and activating c-Jun. JNK1 activation might be involved in triggering the proliferation signals mediated by LMP1 and CD40. Consistent with previous reports on CD40 (Berberich *et al.*, 1994; Rothe *et al.*, 1995) and LMP1 (Huen *et al.*, 1995), we found that in our mini-EBV-immortalized B cells, either activation of CD40 or re-expression of LMP1 lead to the activation of NF- κ B transcription factor complexes. The activation of NF- κ B probably contributes to enhanced cell survival where induction of anti-apoptotic genes (Laherty *et al.*, 1992; Sarma *et al.*, 1995), cell adhesion molecules (Barrett *et al.*, 1991; Kansas and Tedder, 1991) and cytokines (Clark and Shu, 1990; Kieff, 1996; Eliopoulos *et al.*, 1997) might be involved. However, more work needs to be done to elucidate the distinct functions of the JNK1 pathway and the activation of NF- κ B in CD40- and LMP1-mediated proliferation.

In EBV-positive Hodgkin's disease cases, the Reed–Sternberg cells which constitute the malignant cell population all express LMP1 at high levels together with a very limited number of other EBV genes (Rickinson and Kieff, 1996). The growth-promoting effect of LMP1 described here is likely to play an important role in the malignant phenotype in Hodgkin's disease as well as in other EBV-associated malignancies where LMP1 is expressed, such as nasopharyngeal carcinoma and certain T-cell lymphomas.

Materials and methods

Plasmids

Construction of the mini-EBV plasmid p1852 (Figure 1A) is based on the plasmid p1478A, the precursor of p1495.4 which is capable of inducing and maintaining B-cell proliferation *in vitro* (Kempkes *et al.*, 1995a). p1478A is very similar to p1495.4 except it lacks the last building step of p1495.4 which includes the addition of the gene for hygromycin resistance (Kempkes *et al.*, 1995a) dispensable for B-cell immortalization. The artificial promoter for LMP1 on p1852 consists of the EBV TP1 promoter with its two RBP-J κ sites (Zimber-Strobl *et al.*, 1994) replaced by 10 successive copies of the *tet* operator (Gossen and Bujard, 1992). The wild-type LMP1 gene present on p1478A was replaced by the LMP1 gene with the artificial promoter by allelic exchange in *E.coli* using the chromosomal building technique (O'Connor *et al.*, 1989). The LMP1 gene under control of the artificial promoter was cloned onto the shuttle vector pMBO96 (O'Connor *et al.*, 1989) together with flanking EBV sequences to promote homologous recombination events with homologous sequences on p1478A, as described previously (Zimber-Strobl *et al.*, 1996), and the correct recombinant mini-EBV plasmid was isolated. The expression cassette for the chimeric factor tetR-KRAB expressed from the cytomegalovirus (CMV) promoter

was derived from the plasmid CMV-tetR-KRAB (Deuschle *et al.*, 1995) and cloned onto the tet shuttle vector p1242.1 (Kempkes *et al.*, 1995b) to yield the plasmid p1846.5. The expression cassette was added onto the mini-EBV plasmid by homologous recombination in *E.coli* to yield the mini-EBV plasmid p1852 (Figure 1A).

Cell lines

All cell lines were grown in RPMI 1640 culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. HH514 is a single cell clone of the Burkitt's lymphoma cell line P3HR1 (Rabson *et al.*, 1982). WI38, a human fibroblast cell line, was obtained from the American Type Culture Collection, ATCC. CD40-L-expressing L-cells (Banchereau and Rousset, 1991; Galibert *et al.*, 1996) were a kind gift from Dr J.Banchereau. The cell line 1736.18 are *in vitro* immortalized, lymphoblastoid cells generated by infection of primary human B cells with the mini-EBV plasmid p1736 (E.Kilger, A.Schwenk, G.Pecher and W.Hammerschmidt, in preparation) and was used as LCL control where indicated. Human primary B lymphocytes were prepared and purified from routine tonsillectomies by generating single cell suspensions and depletion of T lymphocytes by rosetting with sheep red blood cells as described (Zeidler *et al.*, 1997).

Transformation of primary B cells

HH514 cells (1×10^7) were co-transfected with 20 µg of CsCl-purified p1852 plasmid DNA and 10 µg of pCMV-BZLF1 (Hammerschmidt and Sugden, 1988), an expression vector for the viral BZLF1 gene to induce the lytic phase of EBV's life cycle. Electroporation was performed with a Bio-Rad gene pulser at 960 µF and 250 V in a total volume of 250 µl RPMI of 1640. Virus released into the supernatant was harvested 5 days later. Primary human B lymphocytes were infected with filtered virus stocks from the transiently transfected HH514 cells and plated at a dilution of 3.5×10^5 cells per well in 96-well cluster plates in RPMI 1640 medium supplemented with 1 µg/ml tetracycline on a lethally irradiated (50 Gy) human fibroblast feeder cell layer (WI38) as described previously (Kempkes *et al.*, 1995a,b). The absence of P3HR1 helper virus in immortalized B-cell clones was verified by PCR with appropriate primer pairs and Southern blot analysis as described (E.Kilger, A.Schwenk, G.Pecher and W.Hammerschmidt, in preparation).

Protein immunoblots

For Western blot analysis, cellular extracts were prepared by sonification in H8 lysis buffer containing 20 mM Tris pH 7.0; 2 mM EGTA, 2 mM EDTA, 6 mM β-mercaptoethanol, 50 mM NaF, 100 mM NaCl and 1% SDS. The protein concentration was determined, and equal amounts of protein were separated on SDS–10% polyacrylamide Laemmli gels. Proteins were transferred onto nitrocellulose membrane (Amersham Hybond C) and protein expression was analyzed with antiserum directed against LMP1 (polyclonal rabbit antiserum kindly provided by Bill Sugden), or monoclonal antibodies against EBNA1 (anti-EBNA1-H1) or EBNA2 (anti-EBNA2-R3). Horseradish peroxidase-conjugated secondary antibodies were used (1:2000 dilution) for detection with the enhanced chemoluminescence system (ECL system; Amersham).

MTT assays

Between 5×10^3 and 1×10^4 cells were seeded in triplicate in a total volume of 100 µl of cell culture medium in 96-well cluster plates in the absence or presence of tetracycline for different time periods. Soluble trimerized CD40-L (Immunex) was added at a final concentration of 1 µg/ml where indicated. After incubation with MTT (0.5 mg/ml) for 4 h, MTT conversion, which correlates with the number of living cells in the assay, was determined in an enzyme-linked immunosorbent assay (ELISA) reader as described (Mosmann, 1983). Each experiment was repeated at least twice. In order to compare different experiments, the OD of the first day was set to 1, and normalized OD values are given for the following days.

Cell cycle analysis

For analysis of the cell cycle profile, cells were kept in the absence of tetracycline for 3 days. After 3 days, cells were divided and tetracycline was added to one part while the other stayed without tetracycline. To allow continuous growth conditions, cells were diluted with fresh medium to a concentration of 5×10^5 cells/ml every day. At the indicated time points, cells in the absence or presence of tetracycline were incubated in the presence of BrdU for 4 h, fixed with ethanol and stained with anti-BrdU antibodies conjugated with fluorescein isothiocyanate and

propidium iodide as described (Lowe *et al.*, 1993). The stained cells were analyzed on a Becton Dickinson FACS analyzer.

Analysis of LMP1 expression by flow cytometry

1852.4 cells were treated as described in the legend of Figure 2B. For analysis of LMP1 expression, cells were fixed in 4% paraformaldehyde for 10 min on ice, washed with phosphate-buffered saline (PBS) and blocked with blocking solution (0.1% saponine, 10% human AB-serum, 50 µg/ml goat IgG, 0.01% HEPES) for 10 min at room temperature. Cells were washed with saponine buffer (0.1% saponine, 0.1 M HEPES) and incubated with antiserum directed against LMP1 (polyclonal rabbit antiserum kindly provided by Bill Sugden) for 45 min at room temperature. After washing with saponine buffer, cells were incubated with a phycoerythrin-conjugated secondary antibody [R-PE-conjugated F(ab)₂ fragment donkey anti-rabbit IgG (Dianova)] for 30 min at room temperature. Parallel control samples were incubated with the R-PE-conjugated secondary antibody only. All antibodies were diluted in saponine buffer. The stained cells were washed, resuspended in 1 ml of saponine buffer and analyzed on a Becton Dickinson FACS analyzer.

JNK1 kinase assays

Cells were treated as described in the legend of Figure 6. Immunoprecipitations were performed as described (Kieser *et al.*, 1996) with the rabbit anti-JNK1 antibody C-17 (Santa Cruz Biotech.) immobilized to protein G-Sepharose beads (Pharmacia) to immunoprecipitate endogenous JNK1. *In vitro* immunocomplex kinase assays with the immunoprecipitated JNK1 were performed under conditions as described (Kieser *et al.*, 1996) using GST-tagged c-Jun (purified from *E.coli*) as a substrate. As indicated, kinase reactions or total cell lysates were separated by SDS-PAGE and blotted onto Hybond-C membranes (Amersham). Kinase reactions were analyzed by autoradiography and phosphorimager scanning. LMP1 expression was detected on immunoblots using the anti-LMP1 antibodies CS1-4 (Dako) and the ECL system (Amersham).

Electrophoretic mobility shift assays

Cells were treated as described in the legend of Figure 6. Nuclear extracts were prepared as described previously (Avvedimento *et al.*, 1991), normalized for protein concentration, and 4 µg of protein were incubated with 1 µg of poly(dGdC), 1.2 µg of calf thymus DNA, 3.5 µg of bovine serum albumin, 75 mM KCl and a ³²P-labeled probe in a volume of 20 µl, adjusted with 10–15 µl of buffer [20 mM HEPES pH 7.9, 20% glycerol, 20 mM KCl, 1 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] for 20 min at room temperature. In supershift reactions, 1 µl of anti-p65 antibody (sc-109, Santa Cruz) or 5 µl of anti-p50 antibody (5E6 rat monoclonal antibody, kindly provided by E.Kremmer) were added after 10 min of incubation for another 10 min prior to loading. The probe containing an NF-κB-binding site (5'-TCGACAGAGGGGATTTCGAAGAGCC-3'/5'-TCGAGGCCTCTTGGAAATCCCCCTCTG-3') was labeled with [α-³²P]dCTP (Amersham) by standard procedures using Klenow enzyme (Boehringer Mannheim). DNA-protein complexes were resolved on a 4.5% non-denaturing polyacrylamide gel containing 0.2× TBE (1× TBE: 89 mM Tris, 89 mM borate and 2 mM EDTA). Dried gels were exposed to X-ray film for autoradiography.

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