Functional analysis of the mutated Epstein-Barr virus oncoprotein LMP1$_{69del}$: implications for a new role of naturally occurring LMP1 variants

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Background and Objectives. The role of carboxyterminal deletions of the latent membrane protein-1 (LMP1) in Epstein-Barr virus (EBV) infection and oncogenesis is unclear. Here we describe functional properties of a rare 69-bp LMP1 deletion mutant (LMP1$_{69del}$) isolated from a patient with polyclonal B-cell lymphocytosis.

Design and Methods. Colony focus assay was used to evaluate the transforming capacity of LMP1$_{69del}$ in comparison to that of wild-type LMP1 from EBV strain B95/8. Transient transfectants of B -, T -, epithelial and 3T3 cells, and stable transfectants with ecdyson-inducible LMP1 expression were produced. The signaling capacity of both LMP1s on nuclear transcription factors NFκB and AP-1 were studied. Secretion of matrix metalloproteinase MMP-9, apoptosis, and EBV lytic and latent gene expression were also investigated.

Results. LMP1$_{69del}$ showed transforming properties comparable to those of the wild-type oncoprotein. Induction of NFκB but a markedly reduced influence on AP-1 were observed. Both oncoproteins induced secretion of MMP-9, and enhanced pre-apoptotic effects in Jurkat-T cells leading to increased Fas/Apo-1 and doxorubicin-mediated apoptosis. Furthermore, LMP1$_{69del}$ showed a more effective down-regulation of the EBV lytic cycle master gene BZLF1(Zebra) than did wild-type LMP1.

Interpretation and Conclusions. (i) LMP1$_{69del}$ possesses oncogenic properties, (ii) the observed impaired activity on AP-1 does not interfere with MMP-9 induction, (iii) the enhanced inhibition of BZLF1 could compensate for previously described mutations of our isolate leading to a more lytic phenotype and may be responsible for counteracting permanent virus replication in the chronic active EBV syndrome observed in this patient.

Key words: EBV, LMP1, 69 bp deletion mutant, PPBL, lymphocytosis.

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Naturally occurring sequence variations of the Epstein-Barr virus (EBV) latent membrane protein-1 (LMP1) have been described in different EBV-associated malignancies. Special focus has been put on the C-terminal 30-base pair (bp) deletion (LMP1$_{30del}$) which leads to the lack of amino acids 346-355 of the LMP1 oncoprotein. This LMP1$_{30del}$ was initially described in nasopharyngeal carcinoma (NPC) and has been suggested to be more oncogenic *in vitro*[1] and in animal models.[2] However, there is no indication that it is a requirement for NPC isolates.[3] LMP1$_{30del}$ has been detected in human immunodeficient virus (HIV)-negative lymphoproliferations,[4] oral hairy leukoplakia,[5] Burkitt's lymphoma in Turkish patients[6] and other lymphoproliferative disorders.[7] A correlation with HIV-associated and childhood Hodgkin's disease was observed[8,9] but EBV with a LMP1$_{30del}$ is also frequently found in normal tissue, with large regional differences observed worldwide.[8,10] The lack of consistent signaling patterns of the different isolates also makes it difficult to regard the 30bp deletion as a marker of a predisposition to malignant disorders.[11,12]

In contrast to the frequent 30bp-deletions regularly found in EBV isolates, 69bp deleted mutants (LMP1$_{69del}$), leading to deletion of amino acids 333-355 in the same region of LMP1, are rare. 69bp deletion mutants have been described in lymphoproliferative disorders and tumors.[4,6,7,13] The functional properties of LMP1$_{69del}$ remain unclear. The only functional differences reported originate from tumorigenicity studies, in which the 30bp deletion had no influence on lymphocytic cell lines (LCL), which were tumorigenic in severe combined immunodeficiency (SCID) mice only. However, the LCL harboring the 69bp mutant were tumorigenic in both SCID and nude mice.[14] In another study a 63bp-deleted LMP1 mutant (amino acids 334-354) isolated from a patient with Hodgkin's disease was found to be tumorigenic in nude mice, to enhance colony formation and to induce expression of adhesion molecules. The authors interpreted these results as indicating a possible enhanced oncogenic capacity of this particular isolate.[15]

LMP1 is a pleiotropic protein which acts as an oncogene leading to immortalization and transformation of latently infected cells. It resembles a continuously activated CD40 molecule, a membrane receptor belonging to the tumor necrosis factor receptor (TNF-R) family. The signaling activity of LMP1 has been mainly attributed to two important C-terminal activation regions

(CTAR-1: amino acids 187-231; CTAR-2: amino acids 351-386) mediating binding of TNF-R-associated factors and death domain proteins (TRAFs, TRADD); very recently the transmembrane spanning domains have been suggested to contribute to LMP1 signaling. EBV-mediated growth transformation has been closely linked to LMP1-induced activation of nuclear transcription factor κ B (NFκB) and constitutive MAP-kinase phosphorylation by LMP1, as shown using artificial deletion mutants.[16]

LMP1 also activates nuclear transcription factor AP-1, through activation of the c-Jun N-terminal kinase (JNK) cascade; this function has been attributed to the CTAR2 region but not the CTAR1 region.[17] NFκB and AP-1 activation have been shown to be involved in inducing expression of matrix metalloproteinase-9 (MMP-9) in LMP1-transfected cells,[18] suggesting a contribution to metastasis of EBV-associated tumors. This concept is supported by a recent study showing that strong MMP-2 expression correlated with a favorable prognosis, while expression of MMP-9 was associated with a tendency towards an adverse outcome in Hodgkin's disease.[19] Furthermore, salicylates inhibit LMP1-induced tumor invasiveness *in vitro* through suppression of MMP-9 expression and of NFκB and AP-1 activity.[20]

We previously isolated a stable cell line (SM) from a patient with persistent polyclonal B-cell lymphocytosis (PPBL) , an obscure chronic disorder, with a questionable association with EBV, observed in middle-aged female smokers with HLA-DR7 phenotype.[21,22] This cell line showed expression of LMP1 in a patchy peri-nuclear location similar to Golgi staining and a deletion of 69bp leading to a lack of 21 amino acids of the oncoprotein.[23] As artificial deletion mutants of the C-terminus have defects in their cell transforming capacity we decided to investigate the transforming and signaling properties of this naturally occurring deletion mutant. In most cases 69bp deletions have been associated with malignancy; PPBL is normally regarded as a benign condition although rare conversions into malignant lymphoma have been described.[24] Thus, one of the aims of this study was to assess the risk of malignancy in this particular case and in PPBL in general.

Design and Methods

Virus strains, cell culture

The PPBL cell line SM has been described previously.[21] The EBV-positive cell line B95/8 (ATCC #CRL-1612) was used as a reference for wild-type LMP1. Additionally we used different cell lines which are representative of possible target cells of EBV *in vivo*. The EBV-negative B-cell lymphoma lines RAEL and BJAB, the T-cell leukemia line Jurkat (ATCC #TIB-152) and EREB2-1 cells were grown in RPMI 1640 medium. EREB2-1 is a lymphocytic cell line expressing a conditional EBNA2/estrogen-receptor fusion protein.[25,26] In these cells the lytic cycle of EBV can be induced when EBNA2 is shut off.[27] The human laryngeal epithelial cell line HEp2 (ATCC # CCL-23) and mouse fibroblast cell line Balb/3T3 (ATCC #CCL-163) were grown in DMEM. A NIH/3T3 cell line containing the ecdyson receptor was kindly provided by Dr. M. Allday, Imperial College of Science, Technology and Medicine, London, UK.

Expression vectors, reporter genes

The LMP1 gene from laboratory strain B95/8 ($LMP1_{B95/8)}$, which is representative of wild-type LMP1, and the 69bp-deleted gene ($LMP1_{69del}$) from isolate SM,[23] were inserted into pCR®3.1 plasmids under the control of the strong CMV-IE promoter. Additionally, the RSV promoter of pREP7 was used, a plasmid with episomal persistence based on a truncated EBNA1 gene. The resulting plasmids, as well as controls containing irrelevant genes (such as green fluorescence protein; GFP) or the ancestor plasmids, were used in transformation assays and transient transfection assays.

LMP1 genes and controls were also cloned into an inducible expression system consisting of an ecdyson promoter containing expression vector (pIND) and a plasmid mediating expression of the ecdyson receptor (pVgRXR; all vectors obtained from Invitrogen). These constructs were used to generate stable transfected clones of BJAB, Jurkat and 3T3 cells in which induction of LMP1 expression can be achieved by addition of insect hormones. The expression plasmids used for EREB2-1 cells are based on pHEBo[28] and express $LMP1_{B95/8}$ (pSV-LMP1-B95/8),[29] an artificially constructed carboxy- terminal deletion mutant of LMP1 (pSV-LMP1-[194-386]),[30] or the naturally occurring LMP_{169del} mutant (pSV-LMP1-69del) under control of the SV40 promoter enhancer.

NFκB activation was detected using the κB-CONA-luc vector which carries a firefly luciferase gene under the control of three synthetic copies of the κB consensus sequence of the immunoglobulin γ-chain promoter upstream of the conalbumin transcription site.[31] As a negative control we used the CONA-luc vector lacking the κB sequence (both kindly provided by Dr. F. Arenzana-Seisdedos, Institut Pasteur, Paris, France).

Cellular transcription factor AP-1 activation was detected using a luciferase coupled AP-1 promoter construct as well as a control construct with a deleted AP-1 (dAP-1) binding site, as previously described.[32]

The phRL-TK vector (Promega, Madison, WI, USA) expressing pTK-renilla luciferase, was used as the internal control for standardization of transfection efficiency.

Transfection of cells, induction of stable transfectants, detection of protein expression

Using predetermined optimal conditions, 2×10^6 HEp2, Balb/3T3 and NIH/3T3 cells and 1×10^7 RAEL, BJAB or Jurkat cells were transfected with 10 µg of the different LMP1-expression vectors alone or together with the ecdyson-inducible expression system for transient and stable transformation.

3T3 and HEp2 cells were electroporated with an Electrocell Manipulator 600/BTX (San Diego, CA, USA) at 100 Volt, 1275 µFd and 48 ohm. RAEL, BJAB and Jurkat cells were electroporated with a BTX T820 ElectroSquarePorator (ITC, Biotech, Heidelberg, Germany) at 450 V/cm and 5 pulses of 99 ms. EREB2-1 cells were electroporated with 5 µg of LMP1 constructs, 4 µg of nerve growth factor receptor (NGFR) and 5 µg of GFP containing plasmid using a Biorad Gene Pulser at 220V and 950 µF.

Transiently transfected cells were kept for 48 h in cell culture until analyzed whereas stable transfectants of BJAB or Jurkat cells were obtained by selection of transfectants in Geneticin (G418) and Zeocin (Invitrogen). Cells stably transfected with the ecdyson-inducible $LMP1_{B95/8}$, $LMP1_{69del}$, and GFP genes were incubated for 48h with 1 µM of insect hormones muristerone A or ponasterone A (from Invitrogen) in order to induce LMP1 expression. LMP1 protein was detected by Western blot analysis with anti-LMP1 monoclonal antibody (clone CS1-4, DAKO, Denmark). A monoclonal antibody against tubulin (Eubio, Vienna, Austria) was used as a control. In addition, expression of LMP1 was detected by immunofluorescence with anti-LMP1 antibody followed by fluorescein-labeled anti-mouse rabbit serum (DAKO). Additionally induction of expression of the anti-apoptotic protein bcl-2 was investigated in stable LMP1 transfectants by Western blot analysis using a monoclonal antibody (clone Bcl.2/100, Pharmingen).

Anchorage independent growth assay

Transformation assays with rodent fibroblasts were performed according to the method described for Balb/3T3 cells.[33] Balb/3T3 cells were transfected with pCR3.1 expression vectors containing the different LMP1 genes or the GFP control plasmid and then seeded into soft agar (0.3% agar/DMEM with supplements). After two weeks of culture the numbers and sizes of the colonies were determined.

Promoter reporter gene analysis

Reporter gene expression representative of NFκB and AP-1 activity was measured in RAEL, Jurkat, and HEp2 cells by transient co-transfection assays using 10 µg of the expression vectors $pREP\text{-}LMP1_{B95/8}$, $pREP\text{-}LMP1_{69del}$ or control construct and 10 µg of the relevant promoter constructs in the above described electroporation conditions. The stable LMP1-transfected BJAB cells were electroporated with the reporter plasmids only. The stable LMP1-transfected NIH/3T3 cells were chemically transfected with Metafectene™ according to the manufacturer's instructions (Biontex Lab., Munich, Germany).

For normalization, 2 µg of the renilla luciferase vector were co-transfected. Stably transfected cells containing the ecdyson-inducible expression system were additionally induced by addition of 1 µM ponasterone A or muristerone A for 48 h prior to transfection. Transfected cells were then cultured for another 48 h before lysis using the Dual-Luciferase® Reporter Assay System (Promega). Specific promoter activity was expressed as the ratio of firefly luciferase activity divided by renilla luciferase activity, with the figures representing the means of multiple data points of at least 3 independent transfections.

Expression of matrix metalloproteinase 9

Secretion of MMP-9 was studied in supernatants of LMP1-transfected BJAB and NIH/3T3 cells. Cells were stimulated with 1 µM of muristerone A for 24 h, washed and kept in serum-free medium for 8 hours (Panserin 501, Pan Biotech, Germany). MMP-9 was detected by gelatinolytic activity using enzyme zymography as described previously.[18]

The matrix metalloproteinases were identified according to their relative molecular mass and their ability to cleave the 0.1% gelatine contained in the SDS polyacrylamide gels, resulting in clearly visible defects in staining by Coomassie blue at the positions of enzymatic activity.

Quantification of apoptosis

Apoptosis assays using different induces of programmed cell death were performed as described elsewhere.[34] Stably transfected BJAB- and Jurkat cells expressing LMP1 were incubated with 50 ng/mL of Fas/Apo-1 agonistic antibody CH-11 (Upstate Biotechnology Inc.), 0.5 µg/mL doxorubicin (Eli Lilly, Indianapolis, IN, USA), 10^{-7} M dexamethasone or 5 mM butyrate (Sigma-Aldrich, Vienna, Austria) or 100 ng/mL TNFα (Alexis, Vienna, Austria) for 24 h to 48 h. For detection and quantification of apoptosis, propidium iodide fluorescence staining together with forward/sideward light scattering was used as previously described.[34] The cells (5×10^5) were then permeabilized, stained with propidium iodide and subjected to analysis in an argon laser-equipped FACScan III (Becton Dickinson). Cell debris and small particles were excluded from analysis. Based on propidium iodide staining, cells in the sub-G1 marker window were considered to be apoptotic. Using for-

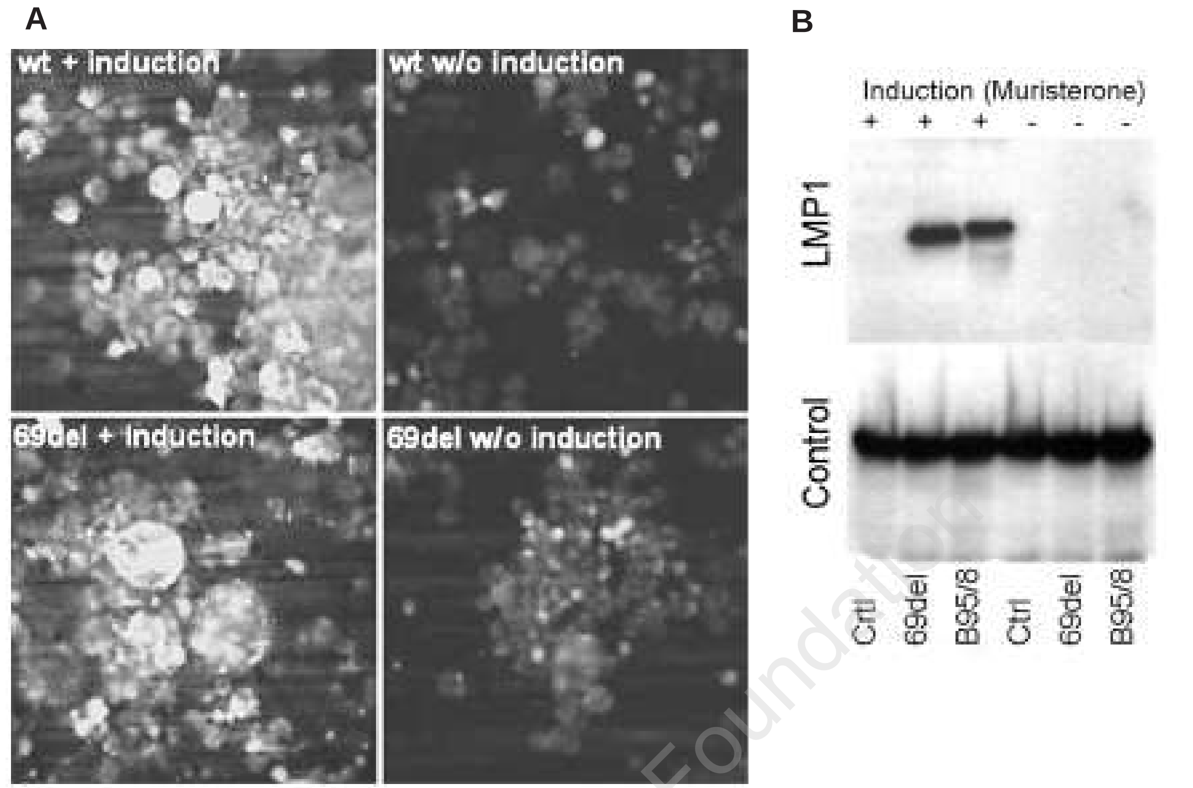


Figure 1. Transfected cells express LMP-1 protein. A: Indirect immunofluorescence showing expression of LMP1 in BJAB cells. Cells were transfected with the inducible-ecdyson system together with wild-type LMP1 from EBV strain B95/8 (wt), 69-del LMP1 from SM isolate (69del) or GFP as control (not shown). Stable transformed cells were induced with muristerone A to produce LMP1 (+induction). Controls without induction are shown for comparison (w/o induction). B: Western blot analysis of NIH/3T3 cells showing expression of LMP1 and tubulin as control. Cells were transfected with the inducible-ecdyson system together with wild-type LMP1 from EBV strain B95/8 (B95/8), 69-del mutated LMP1 from the SM isolate (69del) or GFP as control (ctrl). Stable transformed cells were induced with muristerone A to produce LMP1 (+ induction). Controls without induction are shown for comparison (– induction). Note the differences in size of LMP1 from strain B95/8 and the 69del mutant visible in the Western blot.

ward/sideward light scattering as parameters, apoptotic cells appeared smaller (lower forward scatter values) and more granulated (higher sideward scatter values) than living cells.

Stimulation for EBV reactivation, measurement of BZLF1 expression

Reactivation of the EBV lytic cycle, stimulated by cross-linking of B-cell surface IgM, was measured in EREB2-1 cells in which EBNA2- and EBNA2-dependent genes such as LMP1 were shut off after withdrawal of estrogen. These cells were transfected with pHEBo plasmids expressing LMP1 or LMP1 mutants, or pHEBo as a control. The transfected cells were co-transfected with GFP or with a nerve growth factor receptor (NGFR) construct enabling selection. Two days after transfection half of the cells were subjected to microbead-assisted cell sorting (MACS, Miltenyi Biotech GmbH Germany) using an anti-NGFR antibody. Both the wash fraction (NGFR negative) as well as the eluted fraction (NGFR positive) were analyzed for expression of LMP1, GFP, and actin by Western blot analysis. The other half of the cells were stimulated with plate-bound anti-IgM (20 μg per well on 6-well plates) for 24 hours. EBV reactivation was followed by expression of the EBV immediate early protein BZLF1 (Zebra) which functions as the major viral control protein of the EBV lytic cycle. BZLF1-expressing cells were detected by intracellular staining for BZLF1 of the GFP positive cell population and quantified by FACS analysis as described previously.[27] The number of BZLF1-expressing cells in the transfection experiment using the control plasmid (pSV) was set at 100%.

Results

Inducible expression of LMP-1 in transfected cells

Stably transfected BJAB, Jurkat and NIH/3T3 cells showed expression of LMP1 upon induction with muristerone A in indirect immunofluorescence analysis as well as in Western blots. In the selected high producing cell clones almost 100% of the cells stained positive with anti-LMP1 monoclonal antibodies. Interestingly, induction and overexpression of $LMP1_{69del}$ in the B-cell line BJAB led to a change in phenotype and an increase in cell volume in certain cells, which was more pronounced than with the wild-type protein $LMP1_{B95/8}$ (Figure 1A). Basal expression of LMP1 in the transfected cells, as detected by Western blot analysis, was very low. The transfected NIH/3T3 cells, in particular, turned out to have an exceptionally tight expression system. Zero background expression was observed in the uninduced state (Figure 1B).

No apparent differences in the quantity of the expression of the different sized full-length and truncated LMP1 proteins were observed in the tested cell lines. Slightly more protein degradation was observed with the wild-type protein than with the mutant one, which suggests that the protein stability of the 69bp mutant is comparable to that of the wild-type protein.

Transforming capacity of LMP1 constructs

The LMP1 isolates were assayed for their ability to induce anchorage-independent growth in the rodent fibroblast cell line Balb/3T3. After two weeks we observed outgrowth of colonies of Balb/3T3 cells with both wild-type and mutated LMP1 plasmids, whereas no colonies were observed with the control vector. This indicates that the $LMP1_{69del}$ possesses significant transforming capacities. Approximately 55-65 cell clones with a diameter of 3-5 mm were visible in the wells of Balb/3T3 cells transfected with both types of LMP1. Compared to the colonies with the wild-type protein, those transformed with $LMP1_{69del}$ were marginally smaller and slower in growth (Figure 2). This could be explained by reports on artificial mutants lacking amino acids 334-364 which seem to generate a more toxic LMP-1. The slower growth might also be related to the observation that LCL containing a 69-del variant are more heavily dependent on serum than LCL with the wild type protein.[14]

Induction of transcription factor NFκB in transiently transfected cells and stable transfectants by both LMP1 proteins

The C-terminal CTAR2 region has been shown to be involved in NFκB activity: we, therefore, studied this aspect of the deletion mutant. In reporter gene assays using RSV promoter constructs, both LMP1 proteins induced activation of NFκB in Jurkat, RAEL and HEp2 cells. RAEL and HEp2 cells showed higher overall expression levels of NFκB reporter activity and an induction of 6- to 12 -fold by $LMP1_{B95/8}$ and 4- to 7-fold by $LMP1_{69del}$, whereas Jurkat cells exhibited a 2- to 3-fold induction by $LMP1_{B95/8}$ and $LMP1_{69del}$ (Figure 3A). No big differences between $LMP1_{B95/8}$ and $LMP1_{69del}$ could be observed in transiently transfected 3T3 cells although NFκB induction levels appeared to be slightly lower using the mutant protein (*not shown*). A clear induction of NFκB reporter activity was also observed upon induction with muristerone using the stably trans-

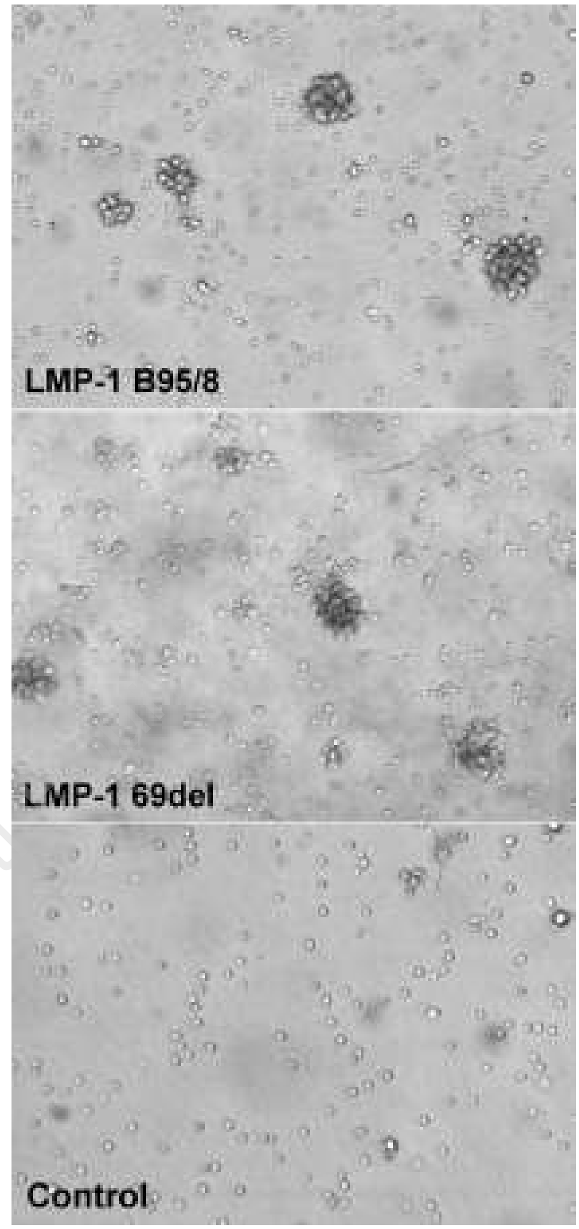


Figure 2. LMP1 containing the naturally occurring 69bp deletion transforms rodent fibroblasts. High-power photographs of wild-type LMP1 B95/8 and LMP1 69del transformed foci of Balb/3T3 cells are shown in soft agar. A plasmid containing GFP was used as a control. Colonies of attachment-independent growth versus areas of non-transformed cells are visible in a monolayer.

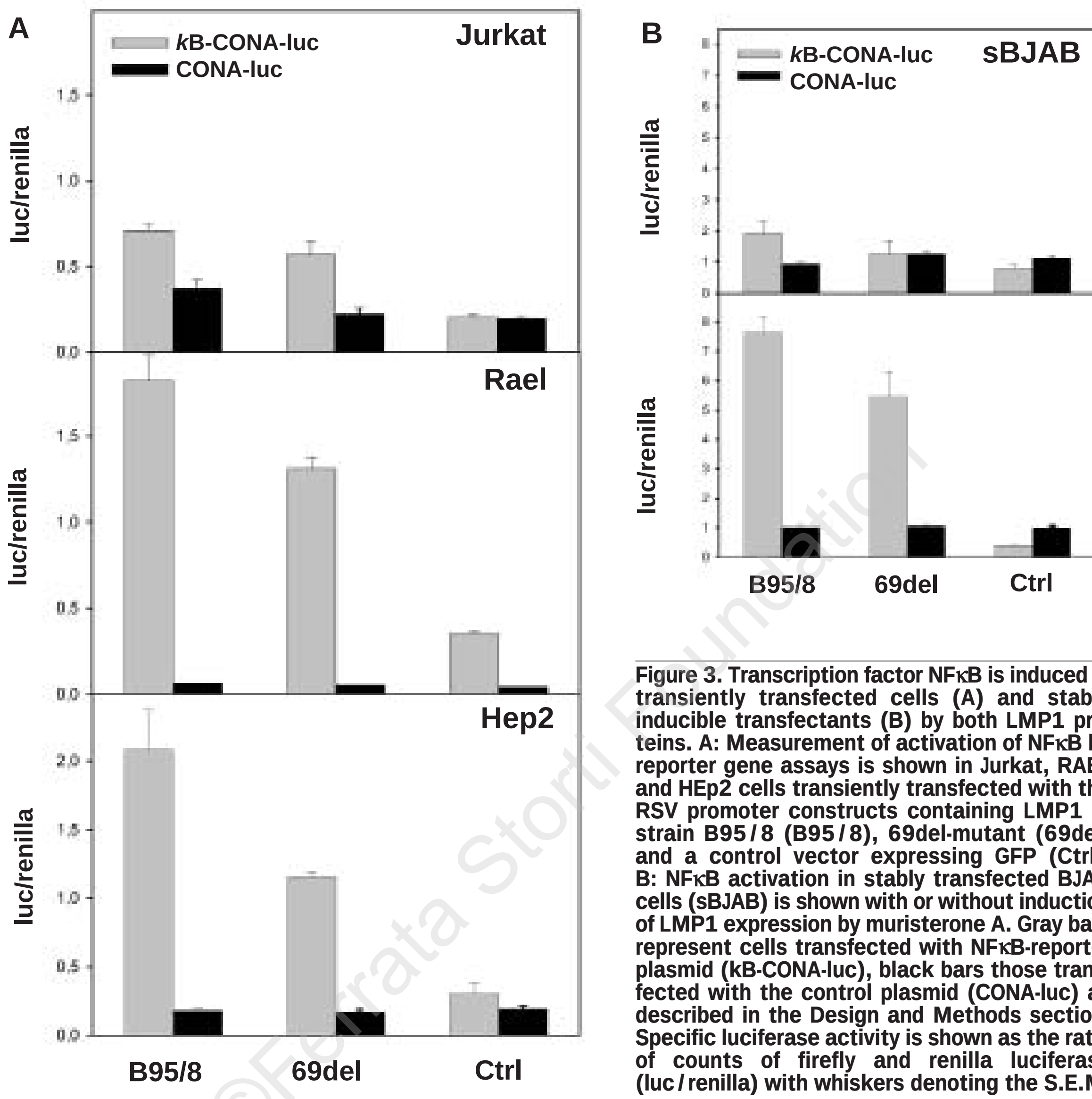


Figure 3. Transcription factor NFκB is induced in transiently transfected cells (A) and stable inducible transfectants (B) by both LMP1 proteins. A: Measurement of activation of NFκB by reporter gene assays is shown in Jurkat, RAEL and HEp2 cells transiently transfected with the RSV promoter constructs containing LMP1 of strain B95/8 (B95/8), 69del-mutant (69del) and a control vector expressing GFP (Ctrl). B: NFκB activation in stably transfected BJAB cells (sBJAB) is shown with or without induction of LMP1 expression by muristerone A. Gray bars represent cells transfected with NFκB-reporter plasmid (kB-CONA-luc), black bars those transfected with the control plasmid (CONA-luc) as described in the Design and Methods section. Specific luciferase activity is shown as the ratio of counts of firefly and renilla luciferase (luc/renilla) with whiskers denoting the S.E.M.

fected BJAB cell line, the induction levels increasing between 6- and 8-fold with both LMP1 constructs as compared to with the control cells (Figure 3B).

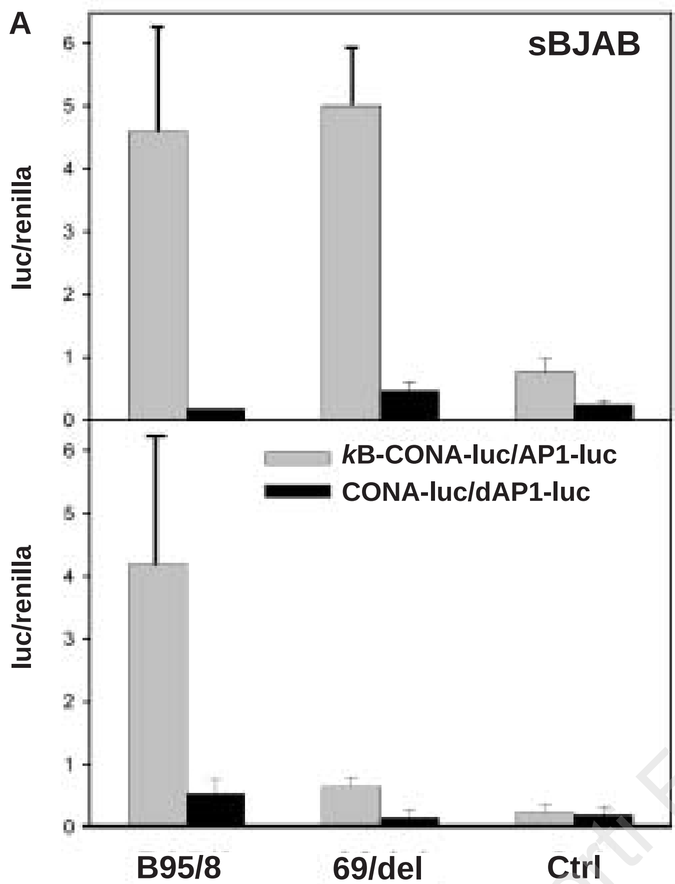
Different induction of transcription factor AP-1 by $LMP1_{B95/8}$ and $LMP1_{69del}$

Since the C-terminal CTAR-2 region has also been shown to be crucial for AP-1 activation, we investigated the behavior of the deletion variant on AP-1 induction. The $LMP1_{69del}$ isolate which was found to induce activation of NFκB to levels comparable with those of the wild-type protein, showed a 4 to 12-fold decreased induction of cellular transcription factor AP-1 in stable transfected BJAB cells (Figure 4A). Similar results were observed in stable transfected NIH/3T3, in which mutated LMPI1 caused a significantly less activation of AP-1 than did the wild-type LMP1 (Figure 4B).

Influence of $LMP1_{69del}$ on the expression of matrix metalloproteinase 9 activity

AP-1 has been suggested to mediate the activity

A



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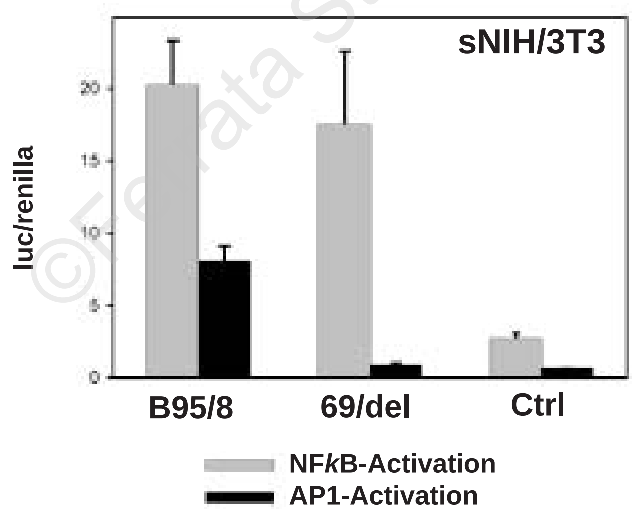


Figure 4. Transcription factor AP-1 is only induced efficiently by LMP1$_{B95/8}$ but not by LMP1$_{69del}$. A: Measurement of activation of NFκB and AP-1 by reporter gene assays using the stable transfected BJAB (sBJAB) cell line expressing LMP1 of strain B95/8, the 69del-mutant, and GFP as the control (Ctrl). In the upper part, gray bars show BJAB cells transfected with NFκB-reporter plasmid (kB-CONA-luc), black bars represent cells which were transfected with the control plasmid (CONA-luc). In the lower part, gray bars represent cells transfected with AP-1 reporter plasmid (AP1-luc), black bars cells which were transfected with the control plasmid (dAP1-luc), both after induction of LMP1 expression with muristerone A. B: Measurement of NFκB and AP-1 activation by reporter gene assays using stable NIH/3T3 transfectants (sNIH/3T3), induced by muristerone for expression of LMP1 of strain B95/8, 69del-mutant, and GFP as control (Ctrl). Gray bars represent NIH/3T3 cells transfected with the NFκB-reporter plasmid, black bars indicate transfection with the AP-1 reporter plasmid.

of MMP-9. We investigated whether LMP1$_{69del}$ had retained the MMP-9-inducing function of the wild-type LMP1. As shown in Figure 5, supernatants of stable transfected 3T3 cells expressing LMP1$_{B95/8}$ as well as LMP1$_{69del}$ showed a dramatic increase in enzymatic activity of MMP-9 upon induction with muristerone, whereas control constructs and non-induced constructs did not show gelatinolytic activity. In contrast, minor basal enzymatic activity of MMP-2 was detected in all cells, and no enhancement by LMP1 induction was observed. Stable BJAB cells also showed similar expression of

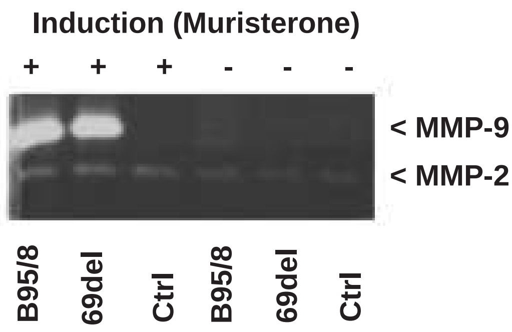


Figure 5. Induction of matrix metalloproteinase 9 (MMP-9) by both LMP1 constructs. Zymography showing the induction of gelatinolytic activity of LMP1 of B95/8 strain, the 69del mutant and a GFP construct (ctrl) in stable NIH/3T3 cells. The gelatinase B enzymatic activity of MMP-9 in cell culture supernatants is visualized as an intense band of 93 kD against a dark background of stained gelatin only in induced cells. No MMP-9 bands are visible in non-induced cells. In contrast, basal expression of MMP-2 (gelatinase A) is seen in both cells induced with muristerone A (+) as well as in non-induced controls (–).

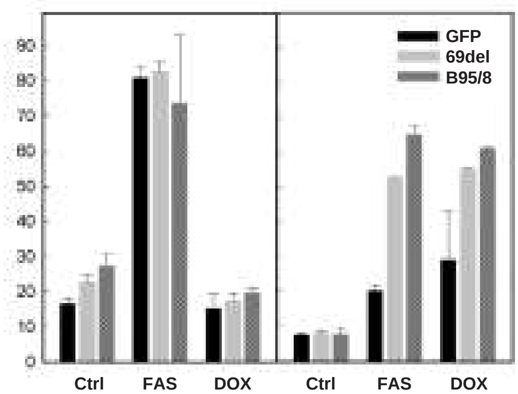


Figure 6. Influence of LMP1$_{B95/8}$ and LMP1$_{69del}$ on apoptosis of BJAB and Jurkat cells. Enhancement of Fas- and doxorubicin-mediated apoptotic effects in Jurkat cells. The percentage of apoptosis in stably transfected BJAB (left side) and Jurkat cells (right side) which have been treated with different apoptosis-inducing agents is shown. Cell lines expressing both LMP1 and GFP as control (Ctrl) were induced to produce LMP1 by addition of muristerone A and then apoptotic cells determined by FACScan analysis. In the triple columns the first bar denotes GFP transfected cells, the second bar cells transfected with the 69del mutant strain, and the third bar B95/8 strain LMP1-transfected cells. The inducers of apoptosis were Fas/Apo-1 agonistic antibody (FAS) and doxorubicin (DOX), the control (Ctrl) was merely cell culture medium.

MMP-9 enzymatic activity following LMP1 induction (*not shown*).

Influence of LMP1$_{B95/8}$ and LMP1$_{69del}$ on apoptosis of Jurkat cells

The apoptotic effects of Fas and doxorubicin were enhanced in Jurkat T-cells but not in stable transfected BJAB B-cells (Figure 6). No enhancement of apoptosis was observed using dexamethasone, butyrate and TNF-α in either Jurkat or BJAB cells (*not shown*). The slight increase of spontaneous apoptosis in the control group of BJAB cells (i.e. without an apoptosis-inducing agent) indicates possible toxicity upon induction of LMP1 expression, with higher protein levels obtained in the B-cell line than in the Jurkat T-cells.

No significant differences in expression of anti-apoptotic Bcl-2 protein were observed between LMP1$_{B95/8}$, LMP1$_{69del}$ and control cells, in both stable transfected BJAB and Jurkat cells (*not shown*).

Influence of LMP1$_{69del}$ on the EBV lytic cycle; enhanced down-regulation of BZLF1

The EBV lytic cycle in EREB2-1 cells can be induced when EBNA2 is shut off. Expression of LMP1$_{B95/8}$ and LMP1$_{69del}$ protein was detected in transfected EREB2-1 cells as was GFP and actin expression in the transfected cell preparations enriched by magnetic cell sorting via co-transfected NGFR expression (Figure 7A).

Both LMP1 constructs were found to be capable of interfering with the EBV lytic cycle, significantly reducing the expression of EBV immediate early transactivator protein, BZLF1. Interestingly, the mutant LMP1$_{69del}$ was even more effective in reducing the IgM-mediated activation of BZLF1 than was the B95/8 protein, leading to reductions of 19.3% and 32.6%, respectively, of the original activity observed with the control plasmid (pSV). Thus it appears that the ability of the LMP1$_{69del}$ to suppress BZLF1 production is greater than that of the LMP1$_{B95/8}$ isolate, despite the fact that, in EREB2-1 cells, protein expression levels of the mutant LMP1$_{69del}$ appeared to be lower than those of the wild-type protein. The artificially constructed carboxy-terminal deletion mutant of LMP1 lacking amino acids 194-386, which was used for comparison, did not show any inhibitory effect (Figure 7B).

Discussion

The functional and oncogenic properties of the naturally occurring LMP1 deletion variants remain unclear. Results from a study in laboratory animals

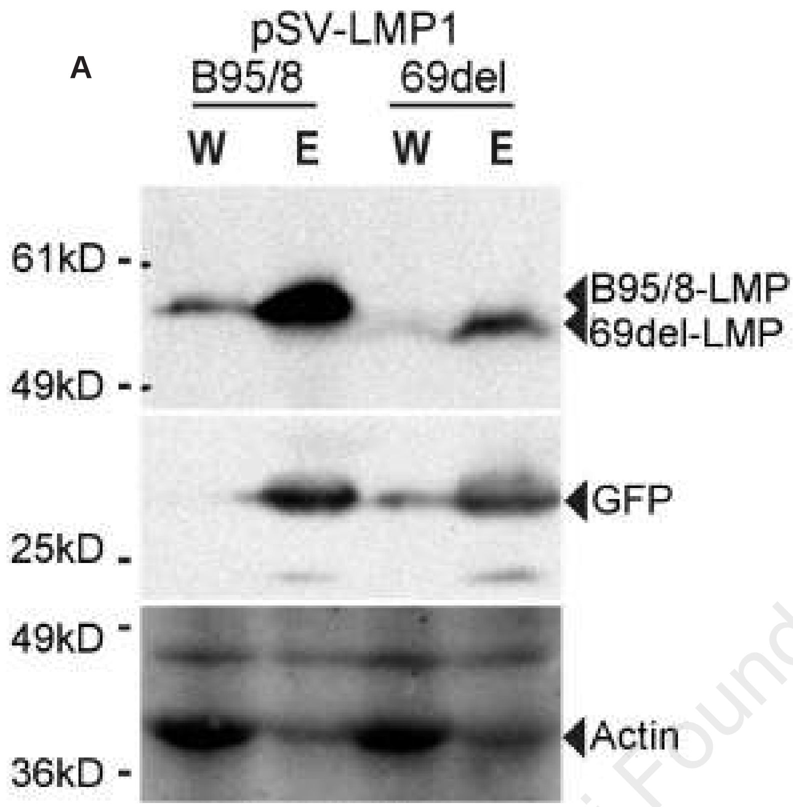
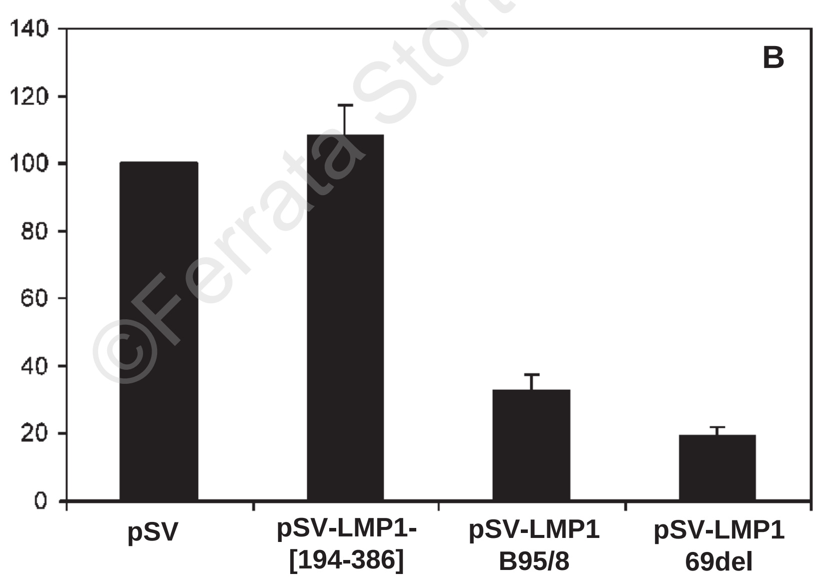


Figure 7. Inhibition of the EBV lytic cycle protein, BZLF1, by LMP1$_{69del}$. Estrogen depleted EREB2-1 cells were transfected with constructs containing LMP1 genes together with GFP and NGFR. Taking half of the cells, the NGFR-expressing cells were separated by MACS. The wash fraction (W = NGFR negative) as well as the elute fraction (E = NGFR positive) were analyzed for expression of LMP1$_{B95/8}$ and LMP1$_{69del}$, GFP, and actin by Western blotting (A). The other half of the cells was stimulated with anti-IgM, and BZLF1-expressing cells were detected by intracellular staining for BZLF1 and FAC-Scan analysis of the GFP-positive cell population. The number of BZLF1-expressing cells in the cell population transfected with the control plasmid was set at 100% (B).



have suggested that the 69del LMPl1 mutant has a higher oncogenic potential.[14] However, we had found that the capacity of a mutated EBV strain to infect cord blood lymphocytes was impaired.[35] This mutant originated from cell line SM and was established from a patient presenting with symptoms of chronic active EBV replication and PPBL,[21,22] which is a presumed benign disorder.[24] The LMP1$_{69del}$ of this patient was studied. The results indicate that LMP1$_{69del}$ has considerable immortalizing and oncogenic potential. This also applies to the expression of matrix metalloproteinases since induction of

NFκB alone seems to be sufficient for MMP-9 secretion. These results contrast with those of previous papers suggesting the necessity of both NFκB and AP-1-dependent pathways for MMP-9 induction.[36] The observed lack of AP-1 activation by $LMP1_{69del}$ is consistent with work using artificially deleted LMP1-constructs, which attributed AP-1 function to the CTAR2 (amino acids 351-386) region of LMP1,[17] which matches relatively closely to the naturally occurring 69bp deletion (amino acids 333-355). The reduced activation of AP-1 was observed in several cell types including NIH/3T3 and BJAB cells, with the latter cells showing a higher basal secretion of MMP-2 (gelatinase A) as compared to the NIH/3T3 cells (*not shown*). Interestingly, published data also suggest a possible anti-apoptotic response by activation of extracellular MMP-2 and subsequent initiation of intracellular survival signals.[37] Whether this is also the case for MMP-9 (gelatinase B) remains to be clarified. There are, however, hints that MMP-9 is also involved in angiogenetic and cell differentiation processes.[38,39] Imbalance of MMP-9 regulation and a possible IL-10-mediated autocrine regulation mechanism of metalloproteinase inhibitor synthesis may play roles in EBV-immortalized B lymphocytes, controlling B-cell growth and apoptosis[40] and regulation of MMP-9 by anti-apoptotic protein Bcl-2, which has been reported in gliomas.[41]

Expression of LMP1 has been shown to have anti-apoptotic effects in Jurkat T-cells, rendering clones that express the protein more resistant to apoptosis induced by serum deprivation.[42] Our observation that LMP1 showed enhanced pre-apoptotic effects in non-B-cells seems contradictory but is not too surprising, and under certain circumstances could be beneficial for replication and spread of the virus, since T cells and epithelial cells usually do not harbor EBV in its latent state. Our observation that LMP1 enhanced Fas- and doxorubicin-mediated apoptosis in Jurkat cells is supported by recent data from the epithelial HeLa cell line.[43] In this system, expression of LMP-1 potentiated apoptosis which was triggered by ligation of the death receptor (Fas). Interestingly, no effect was observed in this study on TNF-induced apoptosis, despite Bcl-2 expression decreasing upon induction of LMP1 in HeLa cells.[43] We, however, found no differences in Bcl-2 expression in our stable transfectants, which is consistent with earlier data showing that LMP1 only induced a transient increase of Bcl-2 proteins in these cell types.[44] These results indicate that LMP1, resembling the mammalian homolog CD40, can exert different effects on cell survival depending on the nature of the apoptosis inducer, in particular in non-B-cells.

Induction levels of NFκB appeared slightly higher with the wild-type protein (30-40% in some experiments), which is in agreement with reports of artificial constructs lacking sequences downstream of amino acid 332 having reduced activity. In AP-1 induction, however, very clear differences were observed, with the levels of wild-type induction being 400 to 1200% of the induction level values of those of the LMP1 mutant. We, therefore, think, that the smaller differences in NFκB values should not be overinterpreted, especially considering the various NFκB results reported by others. Indeed, using artificially constructed LMP1 genes or patient-derived genes, reduced, unchanged or increased values have been found, with the last situation being particularly observed in some of the NPC-derived isolates.[3,12,45] The quantity of LMP1 protein expression has been clearly shown to influence NFκB activity[11] and variations may also be in part due to difficulties in reproducibly adjusting expression levels in transient transfection studies. Furthermore, large differences in NFκB activation among LMP1 constructs have been observed as a result of cell type specific properties[46] which convinced us to include several cell lines of different types in our study.

Down-regulation of the BZLF1 Zp promoter by LMP1 represents a novel function of LMP1 in the virus life cycle.[27] The SM strain of EBV expressing the $LMP1_{69del}$ used in this study shows enhanced activity of the EBV lytic cycle regulator, BZLF1, as we previously demonstrated.[35] Although the $LMP1_{69del}$ exerts an increased inhibitory effect on wildtype BZLF1, the chronic EBV reactivation observed in our PPBL patient suggests that the mutated strain might somehow escape the LMP1 and probably CD40 ligand-dependent control.[27] One possible mechanism could involve the mutations in the BZLF1 promoter region that we had previously found in the SM strain.[35] Chronic reactivation might, therefore, reflect the inability of $LMP1_{69del}$ to efficiently control this particular BZLF1 protein. This may be because LMP1 and activated CD40 control EBV reactivation *in vivo* by blocking the activity of the BZLF1 protein. Thus LMP1 variants may arise as nature´s attempt to counter-regulate an overwhelming replicative activity by selecting viral mutations favoring the development of latency. A LMP1 variant more prone to development of latency would, therefore, make perfect sense in a chronic EBV syndrome. This may also explain why a stable cell line could be outgrown from the peripheral leukocytes of this patient,[21] whereas this is difficult with isolates from patients with chronic active EBV infections.

In summary, the carboxy-terminal region of LMP1 appears to represent a mutational hot spot, with the mutations being stable and independent of the immune response.[7] The higher incidence of LMP1 mutations in aggressive lymphomas suggests that deleted variants might be either preferentially selected in the process of development into lymphomas[47] or could be beneficial for the virus by allowing selection of isolates more prone to latency development and thereby preventing *burn-out* of the virus and destruction of host cells. This complex balance of EBV gene regulation is additionally complicated by the genetic background of the host, as well as a variety of other molecular mechanisms[24] possibly contributing to lymphoproliferative syndromes such as PPBL.

References

1. Hu LF, Zabarovsky ER, Chen F, Cao SL, Ernberg I, Klein G, et al. Isolation and sequencing of the Epstein-Barr virus BNLF-1 gene (LMP1) from a Chinese nasopharyngeal carcinoma. J Gen Virol 1991;72:2399-409.
2. Chen ML, Tsai CN, Liang CL, Shu CH, Huang CR, Sulitzeanu D, et al. Cloning and characterization of the latent membrane protein (LMP) of a specific Epstein-Barr virus variant derived from the nasopharyngeal carcinoma in the Taiwanese population. Oncogene 1992;7:2131-40.
3. Hahn P, Novikova E, Scherback L, Janik C, Pavlish O, Arkhipov V, et al. The LMP1 gene isolated from Russian nasopharyngeal carcinoma has no 30- bp deletion. Int J Cancer 2001; 91:815-21.
4. Barozzi P, Luppi M, Cagossi K, Maiorana A, Marasca R, Artusi T, et al. The oncogenic 30 and 69 bp deletion variants of the EBV LMP-1 gene are common in HIV-negative lymphoproliferations, both malignant and benign. Ann Oncol 1999; 10: 467-9.
5. Palefsky JM, Berline J, Penaranda ME, Lennette ET, Greenspan D, Greenspan JS. Sequence variation of latent membrane protein-1 of Epstein-Barr virus strains associated with hairy leukoplakia. J Infect Dis 1996;173:710-4.
6. Tacyildiz N, Cavdar AO, Ertem U, Oksal A, Kutluay L, Oluoglu O, et al. Unusually high frequency of a 69-bp deletion within the carboxy terminus of the LMP-1 oncogene of Epstein-Barr virus in Burkitt´s lymphoma of Turkish children. Leukemia 1998;12:1796-805.
7. Knecht H, Bachmann E, Brousset P, Rothenberger S, Einsele H, Lestou VS, et al. Mutational hot spots within the carboxy terminal region of the LMP1 oncogene of Epstein-Barr virus are frequent in lymphoproliferative disorders. Oncogene 1995;10:523-8.
8. Dolcetti R, Zancai P, De-Re V, Gloghini A, Bigoni B, Pivetta B, et al. Epstein-Barr virus strains with latent membrane protein-1 deletions: prevalence in the Italian population and high association with human immunodeficiency virus-related Hodgkin's disease. Blood 1997;89:1723-31.
9. Santon A, Martin C, Manzanal AI, Preciado MV, Bellas C. Paediatric Hodgkin's disease in Spain: association with Epstein-Barr virus strains carrying latent membrane protein-1 oncogene deletions and high frequencies of dual infections. Br J Haematol 1998;103:129-36.
10. Dirnhofer S, Angeles-Angeles A, Ortiz-Hidalgo C, Reyes E, Gredler E, Krugmann J, et al. High prevalence of a 30-base pair deletion in the Epstein-Barr virus (EBV) latent membrane protein 1 gene and of strain type B EBV in Mexican classical Hodgkin's disease and reactive lymphoid tissue. Hum Pathol 1999;30:781-7.
11. Fischer N, Kopper B, Graf N, Schlehofer JR, Grasser FA, Mueller-Lantzsch N. Functional analysis of different LMP1 proteins isolated from Epstein- Barr virus-positive carriers. Virus Res1999;60:41-54.
12. Fielding CA, Sandvej K, Mehl A, Brennan P, Jones M, Rowe M. Epstein-Barr virus LMP-1 natural sequence variants differ in their potential to activate cellular signaling pathways. J Virol 2001;75:9129-41.
13. Klein C, Rothenberger S, Niemeyer C, Bachmann E, Odermatt B, Böhm N, et al. EBV-associated lymphoproliferative syndrome with a distinct 69 base-pair deletion in the LMP-1 oncogene. Br J Haematol 1995;91:938-40.
14. Sandvej K, Munch M, Hamilton-Dutoit S. Mutations in the Epstein-Barr virus latent membrane protein-1 (BNLF-1) gene in spontaneous lymphoblastoid cell lines: effect on in vitro transformation associated parameters and tumorigenicity in SCID and nude mice. J Clin Pathol Mol Pathol 1996; 49:290-7.
15. Dolcetti R, Quaia M, Gloghini A, De Re V, Zancai P, Cariati R, et al. Biologically relevant phenotypic changes and enhanced growth properties induced in B lymphocytes by an EBV strain derived from a histologically aggressive Hodgkin's disease. Int J Cancer 1999;80:240-9.
16. Lam N, Sugden B. CD40 and its viral mimic, LMP1: similar means to different ends. Cell Signal 2002;15:9-16.
17. Kieser A, Kilger E, Gires O, Ueffing M, Kolch W, Hammerschmidt W. Epstein-Barr virus latent membrane protein-1 triggers AP-1 activity via the c-Jun N-terminal kinase cascade. EMBO J 1997;16:6478-85.
18. Yoshizaki T, Sato H, Furukawa M, Pagano JS. The expression of matrix metalloproteinase 9 is enhanced by Epstein- Barr virus latent membrane protein 1. Proc Natl Acad Sci USA 1998;95:3621-6.
19. Kuittinen O, Soini Y, Turpeenniemi-Hujanen T. Diverse role of MMP-2 and MMP-9 in the clinicopathological behavior of Hodgkin's lymphoma. Eur J Haematol 2002;69:205-12.
20. Murono S, Yoshizaki T, Sato H, Takeshita H, Furukawa M, Pagano JS. Aspirin inhibits tumor cell invasiveness induced by Epstein-Barr virus latent membrane protein 1 through suppression of matrix metalloproteinase-9 expression. Cancer Res 2000;60:2555-61.
21. Larcher C, Fend F, Mitterer M, Prang N, Schwarzmann F, Huemer HP. Role of Epstein-Barr virus and soluble CD21 in persistent polyclonal B- cell lymphocytosis. Br J Haematol 1995;90:532-40.
22. Mitterer M, Pescosta N, Fend F, Larcher C, Prang N, Schwarzmann F, et al. Chronic active Epstein-Barr virus disease in a case of persistent polyclonal B-cell lymphocytosis. Br J Haematol 1995;90:526-31.
23. Larcher C, McQuain C, Berger C, Mitterer M, Quesenberry PJ, Huemer HP, et al. Epstein-Barr virus-associated persistent polyclonal B-cell lymphocytosis with a distinct 69-base pair deletion in the LMP1 oncogene. Ann Hematol 1997;74:23-8.
24. Reimer P, Weissinger F, Tony HP, Koniczek KH, Wilhelm M. Persistent polyclonal B-cell lymphocytosis: an important differential diagnosis of B-cell chronic lymphocytic leukemia. Ann Hematol 2000;79:327-31.
25. Kempkes B, Spitkovsky D, Jansen-Dürr P, Ellwart JW, Kremmer E, Delecluse HJ, et al. B-cell proliferation and induction of early G1-regulating proteins by Epstein-Barr virus mutants conditional for EBNA2. EMBO J 1995;14:88-96.
26. Kempkes B, Zimber-Strobl U, Eissner G, Pawlita M, Falk M, Hammerschmidt W, et al. Epstein-Barr virus nuclear antigen 2 (EBNA2)-oestrogen receptor fusion proteins complement the EBNA2-deficient Epstein-Barr virus strain P3HR1 in transformation of primary B cells but suppress growth of human B cell lymphoma lines. J Gen Virol 1996;77:227-37.
27. Adler B, Schaadt E, Kempkes B, Zimber-Strobl U, Baier B, Bornkamm GW. Control of Epstein-Barr virus reactivation by activated CD40 and viral latent membrane protein 1. Proc Natl Acad Sci USA 2002;99:437-42.
28. Sugden B, Marsh K, Yates J. A vector that replicates as a plasmid and can be efficiently selected in B-lymphoblasts transformed by Epstein-Barr virus. Mol Cell Biol 1985;5:410-3.
29. Zimber-Strobl U, Kempkes B, Marshall G, Zeidler R, Van Kooten C, Banchereau J, et al. Epstein-Barr virus latent membrane protein (LMP1) is not sufficient to maintain proliferation of B cells but both it and activated CD40 can prolong their survival. EMBO J 1996;15:7070-8.
30. Kieser A, Kaiser C, Hammerschmidt W. LMP1 signal transduction differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2. EMBO J 1999;18:2511-21.
31. Arenzana-Seisdedos F, Fernandez B, Dominguez I, Jacque JM, Thomas D, Diaz-Meco MT, et al. Phosphatidylcholine

hydrolysis activates NF-κ B and increases human immunodeficiency virus replication in human monocytes and T lymphocytes. J Virol 1993;67:6596-604.
32. Kampfer S, Windegger M, Hochholdinger F, Schwaiger W, Pestell RG, Baier G, et al. Protein kinase C isoforms involved in the transcriptional activation of cyclin D1 by transforming Ha-Ras. J Biol Chem 2001;276:42834-42.
33. Baichwal VR, Sugden B. Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. Oncogene 1988; 2: 461-7.
34. Bernhard D, Ausserlechner MJ, Tonko M, Löffler M, Hartmann BL, Csordas A, et al. Apoptosis induced by the histone deacetylase inhibitor sodium butyrate in human leukemic lymphoblasts. Faseb J 1999;13:1991-2001.
35. Jäger M, Prang N, Mitterer M, Larcher C, Huemer HP, Reischl U, et al. Pathogenesis of chronic Epstein-Barr virus infection: detection of a virus strain with a high rate of lytic replication. Br J Haematol1996; 95:626-36.
36. Takeshita H, Yoshizaki T, Miller WE, Sato T, Furukawa M, Pagano JS, et al. Matrix metalloproteinase 9 expression is induced by Epstein-Barr virus latent membrane protein 1 C-terminal activation regions 1 and 2. J Virol 1999;73:5548-55.
37. Ailenberg M, Silverman M. Cytochalasin D disruption of actin filaments in 3T3 cells produces an anti-apoptotic response by activating gelatinase A extracellularly and initiating intracellular survival signals. Biochim Biophys Acta 2003; 1593: 249-58.
38. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, et al. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. Cell 1998;93:411-22.
39. Kobayashi T, Kishimoto J, Ge Y, Jin W, Hudson DL, Ouahes N, et al. A novel mechanism of matrix metalloproteinase-9 gene expression implies a role for keratinization. EMBO Rep 2001;2:604-8.
40. Gaudin P, Trocme C, Berthier S, Kieffer S, Boutonnat J, Lamy C, et al. TIMP-1/MMP-9 imbalance in an EBV-immortalized B lymphocyte cellular model: evidence for TIMP-1 multifunctional properties. Biochim Biophys Acta 2000;1499:19-33.
41. Oliver L, Tremblais K, Guriec N, Martin S, Meflah K, Menanteau J, et al. Influence of bcl-2-related proteins on matrix metalloproteinase expression in a rat glioma cell line. Biochem Biophys Res Commun 2000;273:411-6.
42. Kawanishi M. Expression of Epstein-Barr virus latent membrane protein 1 protects Jurkat T cells from apoptosis induced by serum deprivation. Virology 1997;228:244-50.
43. Zhang X, Hu L, Fadeel B, Ernberg IT. Apoptosis modulation of Epstein-Barr virus-encoded latent membrane protein 1 in the epithelial cell line HeLa is stimulus-dependent. Virology 2002;304:330-41.
44. Martin JM, Veis D, Korsmeyer SJ, Sugden B. Latent membrane protein of Epstein-Barr virus induces cellular phenotypes independently of expression of Bcl-2. J Virol 1993; 67: 5269-78.
45. Rothenberger S, Bachmann E, Berger C, McQuain C, Odermatt BF, Knecht H. Natural 30 base pair and 69 base pair deletion variants of the LMP1 oncogene do stimulate NF-κB-mediated transcription. Oncogene 1997; 14:2123-6.
46. Huen DS, Henderson SA, Croom-Carter D, Rowe M. The Epstein-Barr virus latent membrane protein-1 (LMP1) mediates activation of NF-κ B and cell surface phenotype via two effector regions in its carboxy-terminal cytoplasmic domain. Oncogene 1995;10:549-60.
47. Kingma DW, Weiss WB, Jaffe ES, Kumar S, Frekko K, Raffeld M. Epstein-Barr virus latent membrane protein-1 oncogene deletions: correlations with malignancy in Epstein-Barr virus–associated lymphoproliferative disorders and malignant lymphomas. Blood 1996;88:242-51.

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Contributions

CL and HPH: conception and design, analysis and interpretation of data, drafting of article and final approval of version to be published; DB, MJA, ES, BA: analysis and interpretation of data, revising the article, and final approval of version to be published; MM: conception and design, revising the article and final approval of version to be published.

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