Latent membrane protein 1 of Epstein–Barr virus mimics a constitutively active receptor molecule

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Latent membrane protein 1 (LMP1) of Epstein–Barr virus (EBV) is an integral membrane protein which has transforming potential and is necessary but not sufficient for B-cell immortalization by EBV. LMP1 molecules aggregate in the plasma membrane and recruit tumour necrosis factor receptor (TNF-R) -associated factors (TRAFs) which are presumably involved in the signalling cascade leading to NF-κB activation by LMP1. Comparable activities are mediated by CD40 and other members of the TNF-R family, which implies that LMP1 could function as a receptor. LMP1 lacks extended extracellular domains similar to β-adrenergic receptors but, in contrast, it also lacks any motifs involved in ligand binding. By using LMP1 mutants which can be oligomerized at will, we show that the function of LMP1 in 293 cells and B cells is solely dependent on oligomerization of its carboxy-terminus. Biochemically, oligomerization is an intrinsic property of the transmembrane domain of wild-type LMP1 and causes a constitutive phenotype which can be conferred to the signalling domains of CD40 or the TNF-2 receptor. In EBV, immortalized B cells cross-linking in conjunction with membrane targeting of the carboxy-terminal signalling domain of LMP1 is sufficient for its biological activities. Thus, LMP1 acts like a constitutively activated receptor whose biological activities are ligand-independent.
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Introduction

Epstein–Barr virus (EBV) immortalizes human B lymphocytes *in vitro* and latent membrane protein 1 (LMP1) is one of the EBV genes which is genetically essential to this process (Kaye *et al.*, 1993). LMP1 is a membrane protein composed of a short cytoplasmic amino-terminus linked to a transmembrane domain, with six membrane-spanning segments separated by short reverse turns of ~185 amino acid residues, and a cytoplasmic carboxy-terminal domain of 200 residues (Fennewald *et al.*, 1984; Hennessy *et al.*, 1984; Liebowitz *et al.*, 1986).

Several lines of evidence suggest that LMP1 might act like an activated receptor, perhaps similar to CD40 which is a member of the tumour necrosis factor (TNF) receptor family. First, the expression of certain cellular surface activation markers, adhesion molecules, and growth factors are induced in B cells by expression of LMP1 or activation of CD40 by its ligand CD40L (Wang *et al.*, 1990; Banchereau and Rousset, 1991; Hammarskjold and Simurda, 1992; Kieff, 1996; Rickinson and Kieff, 1996; Miller *et al.*, 1997). Second, LMP1 molecules aggregate and form patches in the plasma membrane, attach to the cytoskeleton, and turn over rapidly—properties which are shared with activated growth factor receptors (Hennessy *et al.*, 1984; Mann *et al.*, 1985; Liebowitz *et al.*, 1986; Baichwal and Sugden, 1987; Martin and Sugden, 1991). Third, similar to many members of the TNF-receptor family including CD40, LMP1 activates NF-κB (Laherty *et al.*, 1992; Huen *et al.*, 1995; Mitchell and Sugden, 1995). Fourth, TNF-receptor-associated factors (TRAFs) were found to bind to amino acids (aa) 199–231 of the LMP1 cytoplasmic domain (Mosialos *et al.*, 1995; Devergne *et al.*, 1996; Sandberg *et al.*, 1997) which might partially explain the activation of NF-κB and up-regulation of several cell surface markers by the recruitment of upstream protein kinases (Winston *et al.*, 1995; Hanissian and Geha, 1997; Lee *et al.*, 1997; Malinin *et al.*, 1997). Fifth, activation of CD40 and expression of LMP1 in EBV-immortalized B cells exhibited comparable phenotypes, indicating that both LMP1 and CD40 might engage the same signal transduction pathway to activate B cells (Zimber-Strobl *et al.*, 1996).

These findings supported the idea that LMP1 might mimic the function of a ligand-activated receptor as had been proposed several years ago (Sugden, 1989). In this paper we provide experimental proof for this hypothesis which has been a long-standing open issue.

Results

Conditional 'one-finger' mutants of LMP1 induce NF-κB activity at will

Recent reports indirectly support the idea that LMP1 acts like a TNF-receptor-type molecule which is constitutively signalling due to its inherent tendency to aggregate in patches in plasma membranes. It is known for molecules like the TNF- and CD40-receptors that their activation is mediated by the binding of the ligand to the extracellular portion to induce oligomerization of the cytoplasmic part of the receptor (Banner *et al.*, 1993; Rosette and Karin, 1996). Ligand-mediated oligomerization of these receptors and membrane patching of LMP1 could be functionally equivalent. However, LMP1 does not bear any resemblance to receptor molecules based on the examination of its amino acid sequence. In particular, LMP1 lacks any

extracellular domains and does not contain any motifs which can be implicated in ligand binding; as a consequence, no ligand (or even candidate) has been identified.

LMP1 localizes in the plasma membrane to form spots or patches consistent with spontaneous oligomerization of LMP1 molecules, which seems to be a necessary condition for efficient signalling, i.e. activation of NF-κB. Genetic evidence indicates that the six transmembrane domains of LMP1 could mediate its aggregation (Hammerschmidt *et al.*, 1989; Liebowitz *et al.*, 1992; Mitchell and Sugden, 1995). To test this hypothesis, we generated LMP1 mutants lacking transmembrane segments 1 to 4 (Figure 1A, 'one-finger' mutant). Derivatives of this 'one-finger' mutant carry the haemagglutinin (HA) epitope recognized by the 12CA5 monoclonal antibody between the fifth and sixth transmembrane segments (Figure 1B, 'one-finger'-HA) or a trimer of FKBP12 (Spencer *et al.*, 1995) located at the carboxy-terminus of LMP1 (Figure 1C, 'one-finger'-3×FKBP12). The coding sequences of the LMP1 mutants were fully sequenced and expression was confirmed by Western blot analysis (data not shown). These LMP1 derivatives can be cross-linked *in vivo* using the 12CA5 monoclonal antibody or two pharmaceutical compounds, AP1510 or FK1012, which bind to FK506-binding protein 12 (FKBP12). FKBP12 has been engineered to provide conditional alleles of *src*-like tyrosine kinases (Spencer *et al.*, 1995). All three 'one-finger' mutants of LMP1 were impaired in their capacity to activate NF-κB as compared with wild-type LMP1 when analysed in transient transfection assays in 293 cells (Figure 1). Either extracellular cross-linking of the LMP1 'one-finger' mutant ('one-finger'-HA) with the 12CA5 antibody or intracellular cross-linking ('one-finger'-3×FKBP12) with a dimerizing drug (Spencer *et al.*, 1995) substantially enhanced NF-κB activation up to nearly wild-type levels (Figure 1B and C). The induction of NF-κB reporter activity in response to artificial cross-linking was due to an increase in the activity of the LMP1 molecule rather than to an increase of the steady-state levels of LMP1 expression in this and the following experiments, as determined by Western blotting (data not shown).

Fig. 1. Induction of NF-κB activity by LMP1 and derivatives of 'one-finger' LMP1 mutants. (**A**) The 'one-finger' mutant of LMP1 was impaired in its ability to activate NF-κB compared with wild-type LMP1. 293 cells were transfected with increasing amounts of either the 'one-finger' mutant or wild-type LMP1 together with 50 ng 3×-κB-Luc reporter DNA (Mitchell and Sugden, 1995). Expression levels of both proteins were comparable and a function of the amount of expression plasmid transfected (data not shown). Shown are the mean values of three independent experiments, each carried out in duplicate. Error bars indicate standard deviation. (**B**) External cross-linking of 'one-finger' LMP1-induced NF-κB. 293 cells were transfected with the indicated amounts of expression vector DNA encoding the 'one-finger' LMP1 mutant with the influenza haemagglutinin (HA) epitope in the extracellular loop between the fifth and sixth transmembrane segments. At 6 h after transfection, cells were incubated with 1 μg of 12CA5 antibody together with 5 μg of secondary cross-linking antibody or left untreated. NF-κB activity and protein amounts of the cell extracts were analysed 24 h after transfection. (**C**) Same experiment as in (B) with a 'one-finger' mutant of LMP1 carrying a trimer of FKBP12 at the C-terminus ('one-finger'-3×FKBP12). Intracellular cross-linking was achieved by the addition of the dimerizing compound AP1510. In (B) and (C), both 'one-finger' mutants appear to be constitutively more active at highest input doses, whereas the parental 'one-finger' LMP1 was not, for unknown reasons.

The amino/transmembrane domain of LMP1 confers an activated phenotype to cytoplasmic domains of CD40 and TNF-R2

Since NF-κB activation was dependent on the aggregation of LMP1, its transmembrane domain was suspected to be responsible for this phenomenon. Therefore, the LMP1 transmembrane domain was fused to the cytoplasmic signalling domains of CD40 receptor and TNF-R2 to generate chimeric receptors termed LMP1:CD40 and LMP1:TNF-R2 (Figure 2). Expression of LMP1:CD40 resulted in NF-κB activities similar to those observed with CD40 plus CD40-L-expressing cells or CD40 plus CD40-L trimer (Figure 2A and data not shown). NF-κB reporter assays with the LMP1:TNF-R2 chimeric receptor also conferred a robust induction of NF-κB in the absence of TNFα (Figure 2B). Since 293 cells express TNF-R1, their stimulation with TNFα was not informative. Thus, the transmembrane domain of LMP1 was capable of substitut-
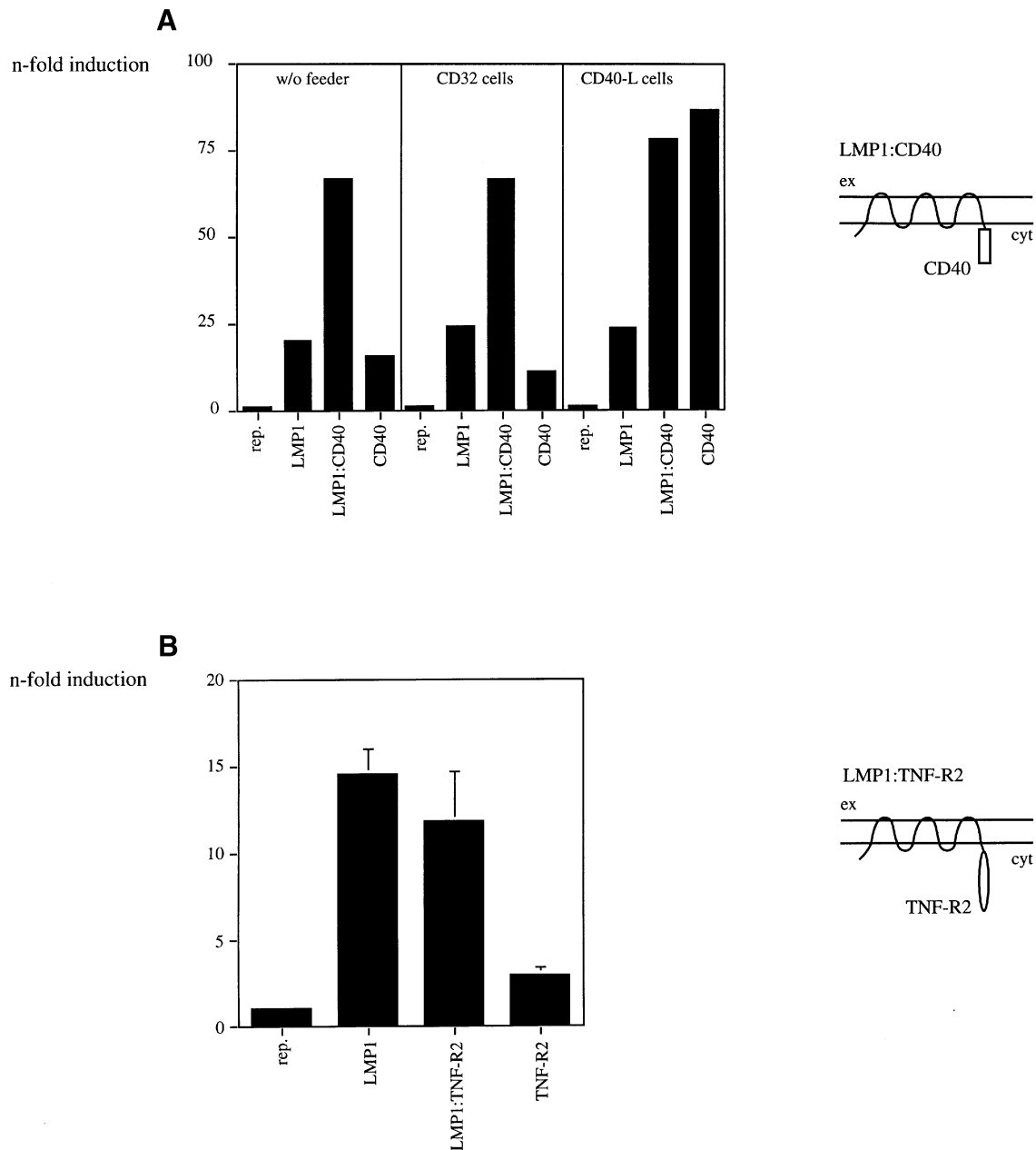
A

B
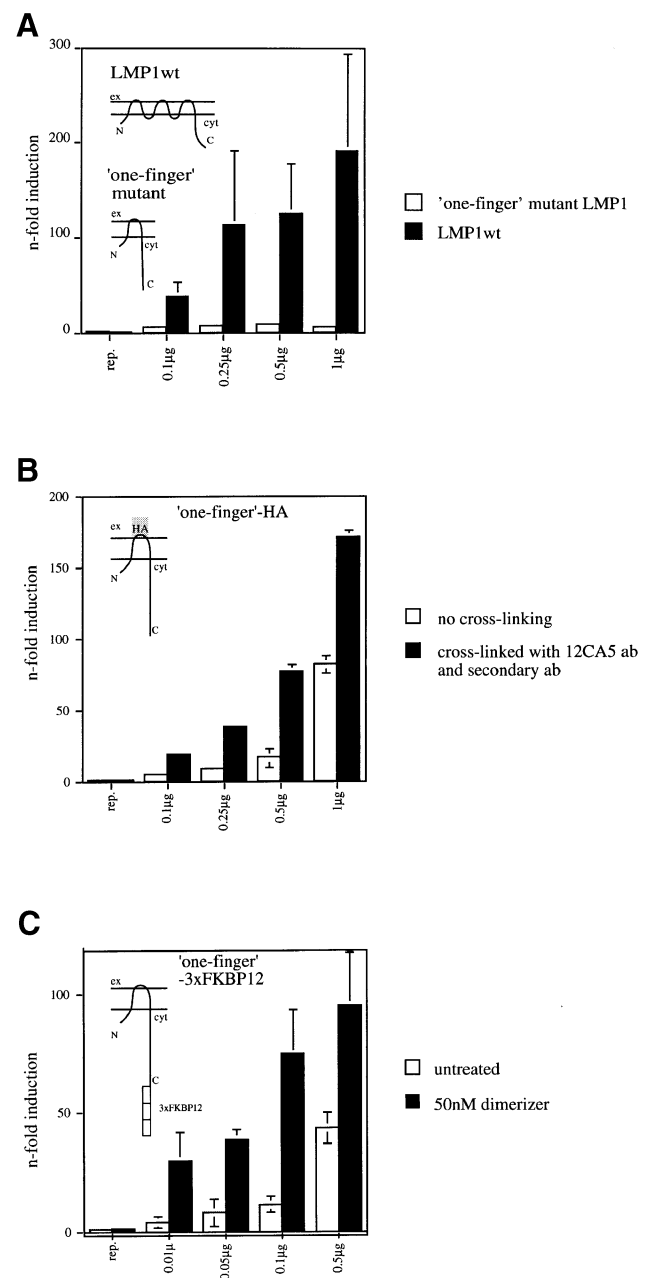
Fig. 2. Induction of NF-κB activity by chimeric LMP1:CD40 and LMP1:TNF-R2 receptors. (**A**) The chimeric receptor LMP1:CD40 and a transfected CD40 receptor stimulated by its ligand (CD40-L) induced a level of NF-κB activity which was in the same range. 293 cells were transfected with 0.5 μg of wild-type LMP1, LMP1:CD40, or CD40 expression vector DNA together with 0.5 μg of 3×-κB-Luc reporter DNA (10-fold more reporter DNA as in Figures 1, 2 and 6). At 16 h after transfection, cells were co-cultivated with CD32 or CD40-L expressing mouse L-cells (Galibert *et al.*, 1996). Induction of NF-κB activity was measured 40 h after transfection. (**B**) The chimeric receptor LMP1:TNF-R2 and LMP1 wild-type induced NF-κB to a similar level, whereas TNF-R2 hardly induced NF-κB in the absence of TNFα. 293 cells were transfected with 0.5 μg of wild-type LMP1, LMP1:TNF-R2 or TNF-R2 expression vector DNAs together with 0.5 μg NF-κB reporter DNA as in (A). The activation is ~10- to 20-fold less than that observed in Figure 1, which is a function of the amount of reporter plasmid used here. The results of one representative experiment out of four are shown.

ing for the ligand-dependent activation of both CD40 and TNF-R2, leading to constitutively activated receptors. This finding suggested that the transmembrane domain of LMP1 can cause self-aggregation, thereby mimicking ligand-induced oligomerization.

The amino/transmembrane domain of LMP1 oligomerizes in the plasma membrane

Activation of receptors like CD40 or the TNF-2 receptor occurs naturally through binding of the cognate ligand and subsequent oligomerization. Since the amino/transmembrane domain of LMP1 can confer an activated phenotype as shown above, we reasoned that this domain of LMP1 might be responsible for homotypic oligomerization. Therefore, we performed co-immunoprecipitation assays with wild-type LMP1 (LMP1wt) or the one-finger LMP1 mutant together with a FLAG-tagged amino/trans-membrane domain of LMP1 which lacks the carboxy-terminus (FLAG:LMP1tm) (Figure 3A). 293 cells were transiently transfected with expression plasmids in the
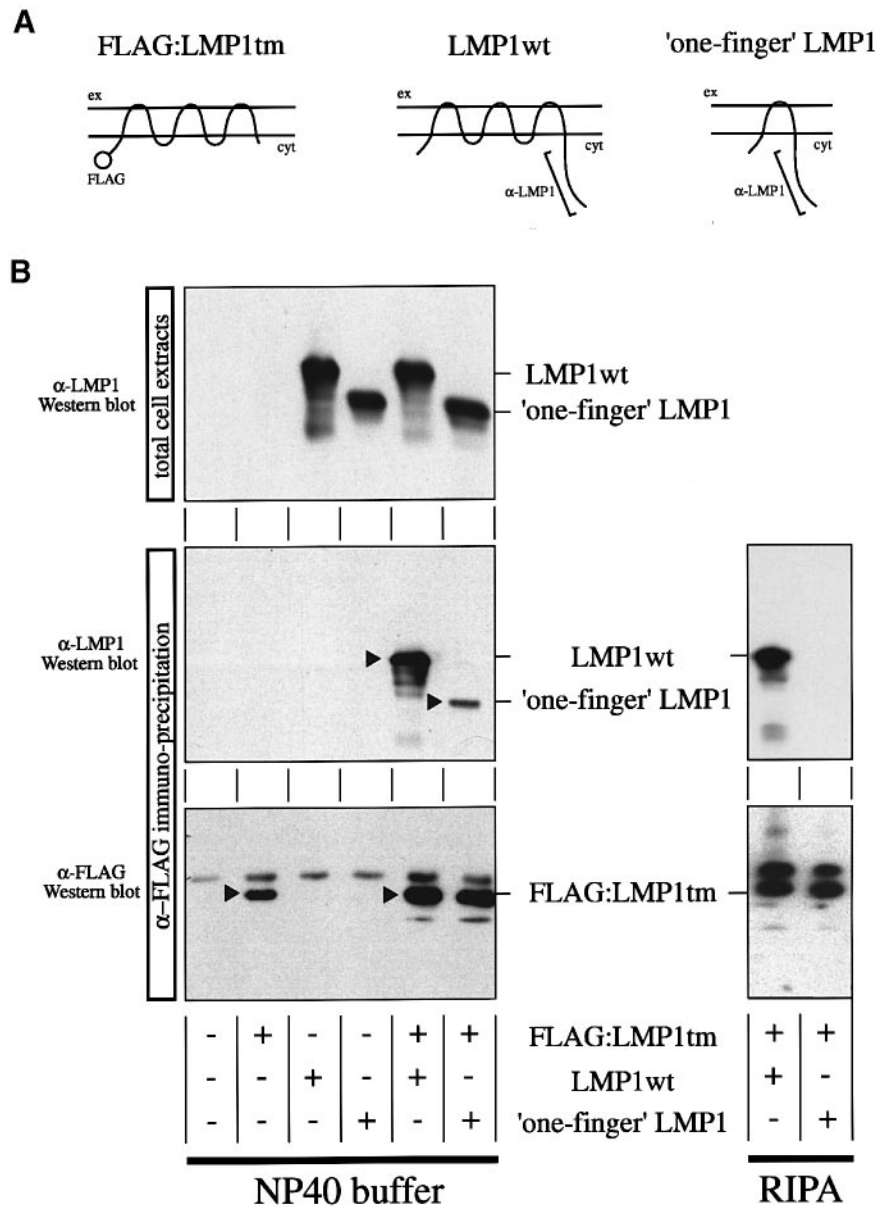
Fig. 3. Co-immunoprecipitation of wild-type and 'one-finger' LMP1 with a FLAG-tagged transmembrane domain of LMP1. Wild-type LMP1 preferentially precipitated with the FLAG-tagged transmembrane domain of LMP1 whereas the 'one-finger' mutant of LMP1 associated less avidly (in the presence of the non-ionic detergent NP40, middle panel, left) or not at all (in the presence of ionic detergents in RIPA buffer, middle panel, right) with the FLAG-tagged transmembrane domain of LMP1. As controls, immunoprecipitated FLAG:LMP1tm was also visualized with an anti-FLAG monoclonal antibody in a Western blot (lower panel) which showed equal amounts of immunoprecipitated FLAG:LMP1tm. As controls for the expression of wild-type LMP1 and 'one-finger' LMP1, crude lysates of transiently transfected 293 cells were analysed by Western blotting before immunoprecipitation with an antibody directed against the cytoplasmic, carboxy-terminal domain of LMP1 (upper panel).

combination shown in Figure 3 and its legend. After 20 h, transfected cells were lysed either in RIPA buffer or a buffer containing only non-ionic detergents, as described in Materials and methods. The amounts of protein expression were comparable, as seen by Western blot analysis (Figure 3B, top panel). Cell lysates were then immunoprecipitated with the FLAG M2 affinity matrix to bring down the FLAG-tagged amino/transmembrane domain of LMP1. Precipitates were separated on SDS–PAGE and the co-precipitates detected with a monoclonal antibody directed against the carboxy-terminus of LMP1 after Western blotting.

Wild-type LMP1 preferentially co-precipitated with FLAG:LMP1tm compared with the 'one-finger' mutant of LMP1 in the presence of non-ionic detergents. No co-precipitation could be observed between the 'one-finger' LMP1 mutant and FLAG:LMP1tm in the presence of ionic detergents, indicating only a weak interaction (Figure 3B). It was clear from these experiments that LMP1 transmembrane domains aggregate *in vivo* to form oligomers and that the intact transmembrane domain of LMP1 is necessary for efficient and stable oligomerization.

Oligomerization of the cytoplasmic domain of LMP1 is sufficient for NF-κB activation

As shown above, the amino/transmembrane domain of LMP1 is capable of forming oligomers in the plasma membrane, and this appeared to be a prerequisite for
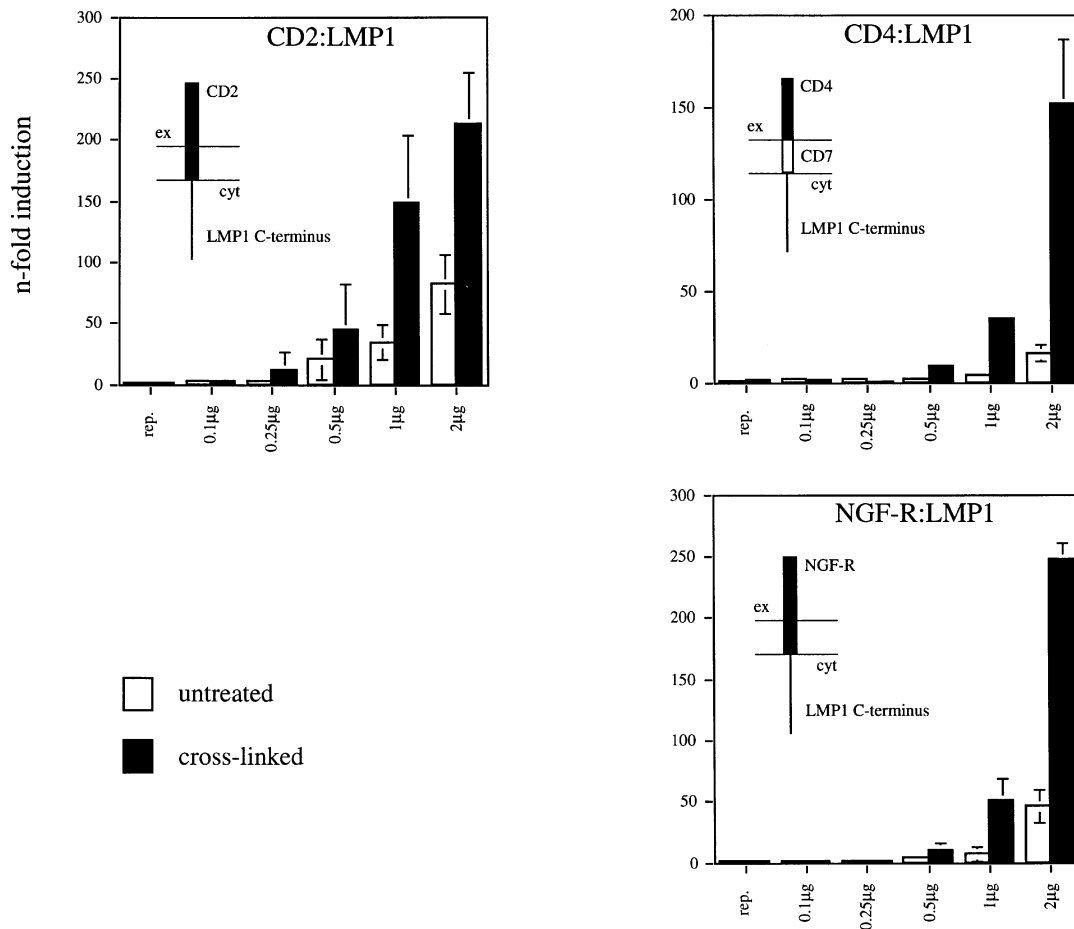
Fig. 4. Induction of NF-κB activity by CD2:LMP1, CD4:LMP1 and NGF-R:LMP1 constructs in 293 cells. External cross-linking of chimeric receptor molecules consisting of extracellular portions of CD2, CD4 or NGF-R fused to the cytoplasmic, carboxy-terminal domain of LMP1 induced NF-κB activity, indicating that this part of LMP1 acts as a signalling domain. 293 cells were transfected with indicated amounts of CD2:LMP1, CD4:LMP1 or NGF-R:LMP1 expression vector DNA together with 50 ng NF-κB reporter as described in Figure 1. At 16 h after transfection, cells were incubated with 1 μg monoclonal antibody directed against the extracellular domains of the various receptor molecules and 5 μg of cross-linking secondary antibody. NF-κB activity was determined as described in Figure 1. Shown are mean values and standard deviations of three independent experiments in which each set of transfections was carried out in duplicate.

activation of NF-κB. These data also suggest that the signalling function of LMP1 is solely confined within its carboxy-terminal, cytoplasmic domain. To investigate this hypothesis we constructed chimeric LMP1 receptors consisting of the extracellular and transmembrane domains of the CD2, CD4 and low-affinity p75 NGF receptors and the cytoplasmic domain of LMP1, termed CD2:LMP1, CD4:LMP1 and NGF-R:LMP1, respectively (Figure 4). Expression of the chimeric receptors was assessed by Western blot analysis and was unaffected after cross-linking (data not shown). Increasing amounts of the chimeric LMP1 constructs were co-transfected together with the NF-κB-luciferase reporter plasmid. Transfected cells were left untreated or incubated with cross-linking monoclonal antibodies directed against the extracellular portions of the LMP1 receptor fusion proteins. Cross-linking of the extracellular domains led to a significant increase in NF-κB activity compared with untreated cells (3- to 10-fold, depending on the construct). These experiments demonstrated that NF-κB induction was strictly dependent on the oligomerization of the cytoplasmic, carboxy-terminal domain of LMP1.

Membrane localization of the carboxy-terminal domain of LMP1 is a prerequisite for its signalling capacity

An important question arose whether membrane localization of the cytoplasmic carboxy-terminal domain of LMP1 is mandatory for signalling as would be expected from a molecule which mimics an activated receptor. Two mutants of LMP1, consisting only of the cytoplasmic domain of LMP1, were constructed which carry a trimer of FKBP12 at the amino-terminus (3×FKBP12:LMP1) or carboxy-terminus (LMP1:3×FKBP12) that can be cross-linked by a dimerizing drug (Spencer *et al.*, 1995). Transfection of 3×FKBP12:LMP1 or LMP1:3×FKBP12 into 293 cells, together with the NF-κB reporter plasmid followed by cross-linking, did not lead to any increase in NF-κB activity (Figure 5). In order to target and aggregate the cytoplasmic tail of LMP1 to the membrane, 3×FKBP12:LMP1 and LMP1:3×FKBP12 were co-transfected with a membrane-anchoring construct expressing an amino-terminally myristoylated FKBP12 trimer (Spencer *et al.*, 1995). Aggregation and membrane recruitment of 3×FKBP12:LMP1 induced NF-κB activity ~70-fold
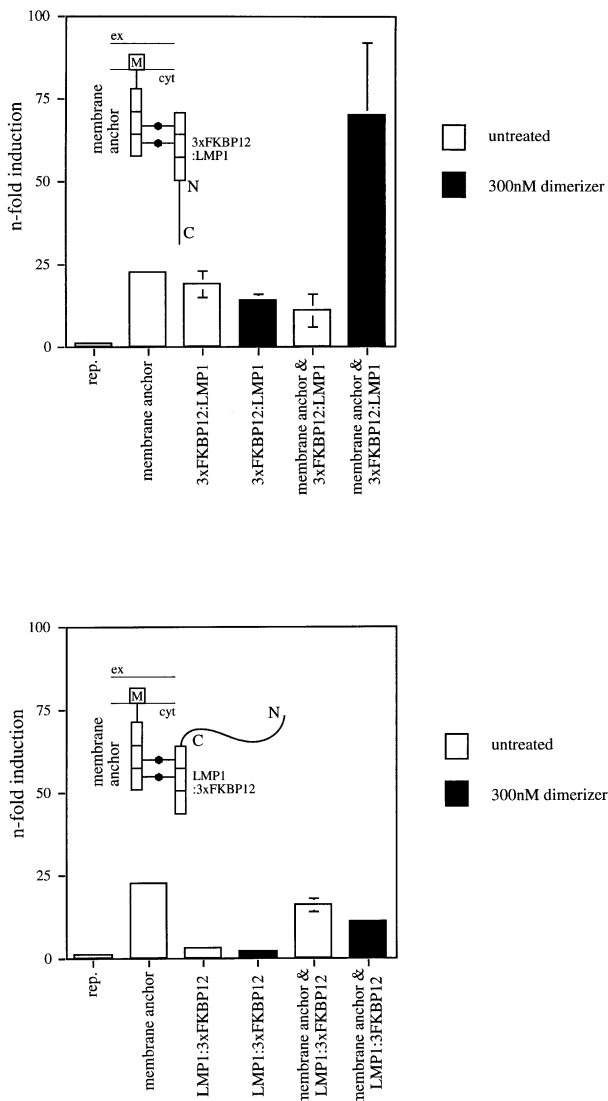
Fig. 5. Membrane localization of LMP1 is essential for its NF-κB activation capacity. Oriented targeting of the cytoplasmic, carboxy-terminal domain of LMP1 to the plasma membrane is a prerequisite for LMP's induction of NF-κB in 293 cells. Only 3×FKBP12:LMP1 was capable of inducing NF-κB when targeted to the plasma membrane whereas LMP1:3×FKBP12 was not, indicating that the relative orientation of LMP's signalling domain is critical. Cells were transfected with combinations of membrane anchor (consisting of a myristoylated trimer of FKBP12) and two versions of the carboxy-terminal part of LMP1 fused to a FKBP12 trimer (LMP1:3×FKBP12 and 3×FKBP12:LMP1). Fifty ng of the NF-κB reporter plasmid was co-transfected with 1 μg of the expression constructs encoding the different proteins. Cross-linking of LMP1:3×FKBP12 or 3×FKBP12:LMP1 in the cytoplasm was initiated 6 h after transfection by the addition of 300 nM AP1510 dimerizer (Spencer *et al.*, 1995). Membrane targeting of LMP1:3×FKBP12 or 3×FKBP12:LMP1 was achieved by co-transfection of 0.5 μg plasmid DNA expressing the myristoylated membrane anchor in combination with 300 nM AP1510 dimerizer. All values shown are mean values and standard deviations of three independent experiments in which each transfection was carried out in duplicate.

compared with the reporter levels (Figure 5), which represents ~50% of LMP1 wild-type level (see Figure 1A). No induction was observed when either the dimerizer, 3×FKBP12:LMP1 or the myristoylated membrane anchor were omitted, indicating that membrane localization of the cytoplasmic domain of LMP1 is a prerequisite for its

signalling capacity. Under all circumstances, LMP1:3× FKBP12 was unable to induce NF-κB activity, suggesting that the signalling complex might require a spatially ordered assembly similar to the orientation in wild-type LMP1. Alternatively, the FKBP12 trimer might have different steric effects on the adjacent LMP1 domains such that the NF-κB domain (Huen *et al.*, 1995; Sandberg *et al.*, 1997) located at the carboxy-terminus of LMP1 is non-functional in this particular fusion.

Conditional B-cell activation by the NGF-R:LMP1 chimera

The experiments described so far were obtained by using mainly one parameter, NF-κB activation in the cell line 293. Therefore, it was mandatory to investigate conditional LMP1 chimeras in an appropriate B-cell system. EREB2-5 cells (Kempkes *et al.*, 1995) are *in vitro*-immortalized B cells which carry an oestrogen-dependent EBNA2 gene. Withdrawal of oestrogen inactivates EBNA2 and down-regulates LMP1 at the transcriptional level. Experiments with EREB2-5 cells stably transfected with LMP1 demonstrated that it contributes to B-cell survival, but is not sufficient for proliferation in the absence of EBNA2 (Zimber-Strobl *et al.*, 1996). This phenotype in EREB2-5 cells is consistent with an increase in cell vitality similar to that seen after activation of CD40 (Banchereau and Rousset, 1991), suggesting that both molecules have similar functions in B cells (Zimber-Strobl *et al.*, 1996). In order to assess LMP1's function in this system, EREB2-5 cells were stably transfected with plasmids encoding the 'one-finger' mutant of LMP1 or the chimeric receptor NGF-R:LMP1. The cell surface expression of NGF-R:LMP1 was assessed by flow cytometry analysis using antibodies directed against the extracellular part of NGF-R:LMP1 (Figure 6A). The expression of the LMP1 'one-finger' mutant was confirmed by Western blot analysis (data not shown). Clone D6 expressed high amounts of NGF-R:LMP1 at the surface (~50% of the cells were positive). In contrast, other clones such as clone 9.1 showed only low surface expression (Figure 6A). The viability of these EREB transfectants was tested in MTT assays in the absence of oestrogen as described by Zimber-Strobl *et al.* (1996). Cross-linking of the extracellular NGF-R domain of clone D6, but not clone 9.1, led to a significant increase in vitality although it was less pronounced (~70%) when compared with controls expressing LMP1 wild-type (Figure 6A). Despite the fact that the 'one-finger' mutant of LMP1 was expressed at elevated levels in EREB2-5 cells (data not shown), their phenotype was unchanged compared with the parental EREB2-5 cells (Figure 6B). Although the initial density of chimeric LMP1 molecules at the cell surface seemed to be critical for functionality, these experiments clearly showed that signalling through the cytoplasmic domain of LMP1 depended only on artificial cross-linking leading to homotypic oligomerization of LMP1's signalling domain.

Discussion

Since 1989, when it was hypothesized that LMP1 affected cells as if it were a constitutively active receptor (Sugden, 1989), no direct evidence for that hypothesis has been presented. Elliott Kieff's group has demonstrated recently
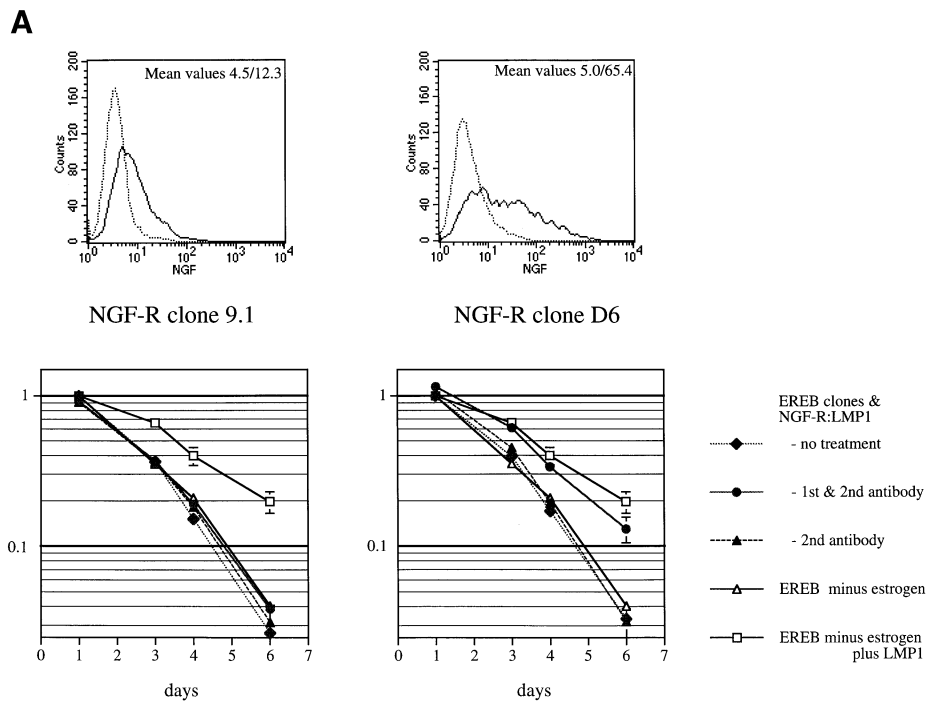
A

NGF-R clone 9.1   NGF-R clone D6

B

'one-finger' LMP1

Fig. 6. Efficient cross-linking of LMP1 is essential to prolong the survival of EREB2-5 cells in the absence of functional EBNA2. (**A**) Surface expression of the low-affinity NGF-receptor was analysed in the EREB2-5 cell clones 9.1 and D6 stably transfected with the chimeric NGF-R:LMP1 receptor and compared with the parental EREB2-5 cell line which is negative for this receptor. Clone D6 expressed considerable levels of the NGF-R epitope on its cell surface, whereas only a fraction of cells of clone 9.1 expressed the NGF-R epitope at a low level. In the absence of functional EBNA2 cross-linking of the external NGF-R, part of the chimeric receptor of clone D6 prolonged cell survival similar to EREB2-5 cells expressing wild-type LMP1 constitutively (Zimber-Strobl *et al.*, 1996). No phenotype could be observed for clone 9.1. (**B**) The 'one-finger' mutant of LMP1 is unable to sustain prolonged cell survival of EREB2-5 cells although it is expressed at high levels (data not shown), indicating that it is impaired in homotypic oligomerization and signalling. The results with one clone is shown but all others gave nearly identical results. The parental cell line EREB2-5 and the transfectants were maintained in the absence of oestrogen for up to 6 days and cell viability was determined by MTT conversion on the days indicated. Depicted are MTT values representing viable cells. Results shown are from one representative experiment in which cells were seeded in triplicate. To compare different experiments, the rate of MTT conversion at day 1 was set to 1 and OD values were normalized accordingly.
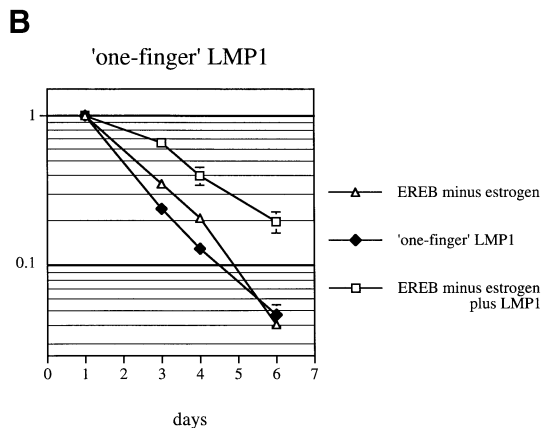
(Mosialos *et al.*, 1995) that LMP1 associates with members of the TRAF family of proteins which have been found to interact primarily with a number of bona fide cell surface receptors (Cheng *et al.*, 1995; Rothe *et al.*, 1995; Cao *et al.*, 1996; Hsu *et al.*, 1996, 1997; Duckett *et al.*, 1997; Tsitsikov *et al.*, 1997). From our own work we found that both LMP1 and CD40 cause the same phenotype in Epstein–Barr virus-immortalized B cells (Zimber-Strobl *et al.*, 1996). These findings, together with descriptive data, supported the assumption that LMP1 might mimic the function of a ligand-activated receptor.

It must be stated, however, that all this previous evidence is circumstantial. Our data provide definitive experimental proof for a hypothesis which has been a long-standing open issue. We can formally show that signalling as

monitored by NF-κB activation in the cell line 293 requires oligomerization of the cytoplasmic carboxy-terminal domain of LMP1. Oligomerization of LMP1 is a process which activates signalling cascades within minutes and does not involve autocrine loops (A.Kieser *et al.*, 1997). Oligomerization is solely encoded in the amino-terminal/transmembrane domain of LMP1, indicating that homotypic aggregation is an intrinsic biochemical characteristic of this domain. Its modular structure can even confer constitutive activation to unrelated receptors which signal upon aggregation of their cytoplasmic domains. LMP1 mutants which can be oligomerized at will further support the view that LMP1 consists of two functionally distinct domains dedicated to either oligomerization or signalling. More importantly, we demonstrate in B cells, i.e. the

target cells of EBV transformation, that conditional cross-linking of LMP1 mutants reproduces the biological effects of wild-type LMP1. This experiment provides genetic proof for the hypothesis that LMP1 mimics the functionality of an activated receptor.

So far, only one Herpes viral protein has been shown to possess the properties of an activated receptor, a gene product encoded by Kaposi's sarcoma-associated Herpes virus (and Herpes virus Saimiri) as has been published recently (Arvanitakis *et al.*, 1997). This viral protein represents a viral homologue of the cellular IL8 receptor. It is constitutively active but it still binds IL8 and related chemokines. Unfortunately, the constitutive nature of the viral IL8 receptor homologue remains undefined.

In contrast, LMP1 does not resemble receptor molecules based on its amino acid sequence. In particular, LMP1 lacks any extracellular domain and does not contain any motifs which can be implicated in ligand binding; as a consequence, no ligand (or even candidate) has been identified. In other words, LMP1 is neither a receptor nor a receptor homologue, although our data support the idea that it functionally substitutes for both a receptor and its ligand.

Most likely, LMP1 abrogates the need for a B cell to become activated through contacting a CD40L-bearing T cell, which enables EBV to confer activation from within a latently infected B cell. LMP1's structure is peculiar to bring about a constitutive, active 'receptor-like' function which feeds into specific signalling cascades (A.Kieser *et al.*, 1997; also data not shown). These findings address a long-standing question in the field, namely, whether the transforming principle of the viral oncogene LMP1 is due to its ability of activating signalling pathways normally used by B-cell survival/growth factors. The significance of these findings is important as LMP1 is likely to be involved in EBV-associated malignancies such as Hodgkin's lymphoma, certain non-Hodgkin's lymphoma, and nasopharyngeal carcinoma.

Materials and methods

Cell lines
EREB2-5 is a lymphoblastoid cell line generated by infection of cord blood human B cells with an Epstein–Barr virus mutant conditional for EBNA2. These cells proliferate in the presence of 1 µM oestrogen, as described by Kempkes *et al.* (1995). 293 is a human embryonal kidney cell line. CD40L- and CD32-expressing L-cells are described by Galibert *et al.* (1996).

Plasmids
p432 expresses wild-type LMP1 under the control of the HCMV immediate early promoter. p531 encodes the 'one-finger' mutant of LMP1 (aa 1–24 and 133–386) in pHEBo (Sugden *et al.*, 1985) driven by the HCMV promoter/enhancer. p581 encodes wild-type LMP1 in pHEBo driven by the SV40 promoter/enhancer. Human CD40 cDNA is cloned in pEFBOS and driven by the HCMV promoter/enhancer. The LMP1:CD40 chimeric receptor is driven by the SV40 promoter/enhancer in p581 encoding aa 1–190 of LMP1 (transmembrane domain) fused to aa 223–280 of CD40 (cytoplasmic domain). The full-length TNF-R2 was expressed from the HCMV promoter. LMP1:TNF-R2 is a chimeric receptor with aa 1–190 of LMP1 (transmembrane domain) fused to the carboxy-terminal part of TNF-R2 in p581 driven by the SV40 promoter/enhancer. Human low-affinity NGF-R:LMP1 chimeric receptor consists of aa 1–279 of NGF-R (extracellular and transmembrane domain) fused to aa 191–386 of LMP1 (cytoplasmic domain) expressed by the SV40 promoter/enhancer in pHEBo. Rat CD2:LMP1 chimeric receptor with aa 1–223 of rat CD2 (extracellular and transmembrane domain) fused

to aa 188–386 of LMP1 (cytoplasmic domain) is driven by the SV40 promoter/enhancer. CD4:CD7:LMP1 chimeric receptor consists of aa 1–394 of human CD4 (extracellular domain) and aa 146–202 of human CD7 (transmembrane domain) fused to aa 186–386 of LMP1 (cytoplasmic domain) driven by SV40 promoter/enhancer in p581. FLAG:LMP1tm is the FLAG-epitope fused to aa 1–186 of LMP1 in pCMV2 Flag (Kodak). 'One-finger'-HA is the LMP1 'one-finger' mutant with the 12CA5 epitope between the 5th and 6th transmembrane-spanning domains expressed from the HCMV promoter in pHEBo. 'One-finger'-3×FKBP12 encodes the LMP1 'one-finger' mutant with a trimer of FKBP12 at the C-terminus of LMP1. The membrane anchor was constructed on the basis of pCMF2E (ARIAD Pharmaceuticals) which was linearized with *Spe*I and a third FKBP12 unit was added to obtain a trimer of FKBP12. 3×FKBP12:LMP1 consists of a FKBP12 trimer at the amino-terminus fused to the cytoplasmic carboxy-terminal domain of LMP1 (aa 186–386). LMP1:3×FKBP12 consists of the cytoplasmic domain of LMP1 at the amino-terminal end fused to the FKBP12 trimer at the carboxy-terminus of the fusion protein.

Transfection of 293 cells and luciferase reporter gene assays
293 cells were transfected with the various expression plasmids and a NF-κB reporter plasmid (Mitchell and Sugden, 1995) using Lipofectamin (Gibco) under the conditions recommended by the manufacturer, and luciferase activity was measured. Transfected DNA amounts were equalized by the addition of carrier DNA (pcDNA3). Cell extracts were tested for luciferase activity and protein amounts 24 h after transfection. Relative light units were corrected for protein content and NF-κB activation was calculated as fold induction compared with reporter level which was set to 1.

Cross-linking of chimeric LMP1 receptors
293 and EREB2-5 cells transfected with chimeric receptors were incubated with 1 µg/ml specific monoclonal antibodies [anti-human CD2 (ATCC); anti-human NGF-R (ATCC); anti-human CD4 (Baxter); 12CA5 (Boehringer)], and 5 µg/ml secondary goat anti-mouse F(ab′)$_2$ fragments (Dianova). Cross-linking was carried out 6–16 h after transfection for transient assays and at 3-day intervals for MTT assays with stably transfected EREB2-5 cells. Dimerization of FKBP12-carrying LMP1 chimeras was performed with 50–300 nM AP1510 diluted in standard RPMI medium (ARIAD Pharmaceuticals) 6 h after transfection.

Stable transfection of EREB2-5 cells, flow cytometry, protein blots and MTT assays
EREB2-5 were stably transfected and analysed by flow cytometry as described (Zimber-Strobl *et al.*, 1996) with protein G-purified anti-NGF-R antibody, washed and incubated 30 min in a 1:50 dilution of goat anti-mouse IgG FITC-labelled antibody (Dianova). Analysis was carried out with a FACScan flow cytometer (Becton-Dickinson). Protein expression in Western blots was analysed with polyclonal rabbit serum directed against the carboxy-terminus of LMP1 or S12 mouse monoclonal antibody (Dako). Horseradish peroxidase (HRP) -conjugated secondary antibodies were used (1:2000 dilution) for detection with the enhanced chemiluminescence system (ECL, Amersham). When biotinylated S12 antibody was used, detection was performed with streptavidin–HRP conjugate (1:500). MTT assays were performed as described (Zimber-Strobl *et al.*, 1996; Mosmann, 1983).

Immunoprecipitations
293 cells (1×10^6 cells) were transiently transfected with 5 µg each of the different LMP1 expression constructs together with 5 µg FLAG:LMP1 as described in detail in Figure 3. At 24 h after transfection, cells were harvested, rinsed with cold phosphate-buffered saline (PBS), and kept for 30 min in hypotonic buffer (10 mM HEPES, pH 7.9, 0.5 mM KCl, 0.5 mM MgCl$_2$, 0.1 mM EGTA, 0.5 mM DTT). Cells were Dounce-homogenized using a 27-gauge needle, the lysates were pelleted (3000 *g*, 15 min), and the particulate fraction resuspended in 4 vols cold TBS/0.5% NP40 and recentrifuged. Alternatively, cells were lysed in RIPA buffer containing Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, and protease inhibitors. For immuno-precipitation of FLAG:LMP1, 10 µl α-FLAG affinity gel M2 (Kodak) was incubated with 200 µl cell lysate overnight at 4°C. Following incubation the beads were washed three times in RIPA buffer and once in TBS with 25 mM NaCl. Precipitates were analysed after separation on SDS–PAGE using biotinylated S12 antibody directed against LMP1 (Dako) in combination with streptavidin–HRP (1:500) or M2 antibody in combination with a secondary anti-mouse HRP-conjugated antibody and ECL-mediated detection (Amersham).

Acknowledgements

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