Latent membrane protein 1 of Epstein–Barr virus interacts with JAK3 and activates STAT proteins

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Latent membrane protein 1 (LMP1) acts like a permanently activated receptor of the tumor necrosis factor (TNF)-receptor superfamily and is absolutely required for B cell immortalization by Epstein–Barr virus. Molecular and biochemical approaches demonstrated that LMP1 usurps cellular signaling pathways resulting in the induction of NF- κ B and AP-1 via two C-terminal activating regions. We demonstrate here that a third region encompassing a proline rich sequence within the 33 bp repetitive stretch of LMP1's C-terminus is required for the activation of Janus kinase 3 (JAK3). The interaction of LMP1 and JAK3 leads to the enhanced tyrosine auto/transphosphorylation of JAK3 within minutes after crosslinking of a conditional NGF-R:LMP1 chimera and is a prerequisite for the activation of STAT transcription factors. These results reveal a novel activating region in the LMP1 C-terminus and identify the JAK/STAT pathway as a target of this viral integral membrane protein in B cells.

Keywords: B lymphocyte/EBV/JAK3/LMP1/STAT protein

Introduction

Epstein–Barr virus (EBV) is a widespread human γ herpes virus, which is associated with several human malignancies such as infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease (for review see Farrell, 1995; Kieff, 1996). The infection of primary B cells with EBV *in vitro* leads to the immortalization of these cells generating lymphoblastoid cell lines with indefinite life span (for review see Farrell, 1995). Nine viral proteins are expressed in established lymphoblastoid cell lines, five of which appear to be absolutely required for B cell immortalization: EBNA1, 2, 3a, 3c and LMP1 (Hammerschmidt and

Sugden, 1989; Cohen *et al.*, 1991; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993; Kilger *et al.*, 1998). Latent membrane protein 1 (LMP1) is the only EBV protein which has a transforming potential in established rodent cell lines (Wang *et al.*, 1985; Baichwal and Sugden, 1988; Moorthy and Thorley-Lawson, 1993). Interestingly, LMP1 induces the expression of the B cell activation markers CD23 and CD40, cell adhesion molecules ICAM1, LAF1 and LFA3 (Wang *et al.*, 1990), and the epithelial growth factor receptor (Miller *et al.*, 1997). LMP1 is composed of a short cytoplasmic N-terminus of 25 amino acids, a transmembrane domain with six membrane-spanning segments, and a long cytoplasmic C-terminus of 200 amino acids (Liebowitz *et al.*, 1986). Molecular analysis identified two cellular signaling pathways targeted by LMP1 and demonstrated that it shares functional characteristics with members of the tumor necrosis factor (TNF) receptor superfamily, with CD40 in particular (Zimber-Strobl *et al.*, 1996; Kilger *et al.*, 1998). LMP1 patches in the plasma membrane via its transmembrane domain (Gires *et al.*, 1997). Signal transduction is mediated by the C-terminus which interacts with members of the TNF receptor-associated factors (TRAF) family (Mosialos *et al.*, 1995) and with the TNF receptor-associated death domain protein TRADD (Izumi and Kieff, 1997). These are features reminiscent of TNF receptors although differences between LMP1 and TNF receptors in the architecture of signaling complexes and mechanisms emerge (Kieser *et al.*, 1999). The induction of the transcription factors NF- κ B (Hammarskjöld and Simurda, 1992; Laherty *et al.*, 1992; Mitchell and Sugden, 1995) and AP-1 (Kieser *et al.*, 1997; Eliopoulos and Young, 1998) may account for many of the cellular changes observed in response to LMP1.

The patched phenotype, as well as the binding of molecules involved in signal transduction cascades, are characteristics reminiscent of receptor molecules. Unlike other receptors, whose activation depends upon ligand-binding, LMP1 is constitutively active without the need for a ligand (Gires *et al.*, 1997; Eliopoulos and Rickinson, 1998). Studies using cellular systems and biochemical approaches clearly demonstrated that the membrane localization and the aggregation of the cytoplasmic C-terminus of LMP1 are necessary and sufficient for the induction of signaling events in epithelial and lymphoid cells. Additionally, LMP1's N-terminus/transmembrane domain has the intrinsic capacity to form homoaggregates in the plasma membrane which results in a constitutively active pseudo-receptor (Gires *et al.*, 1997; Eliopoulos and Rickinson, 1998).

In the present study we investigated the potential of LMP1 to induce other cellular signaling cascades besides activation of NF- κ B and AP-1. Since LMP1 encompasses a long (200 amino acids) cytoplasmic signaling domain, it was tempting to speculate that additional pathways are

induced by LMP1. We show here that LMP1 triggers the JAK/STAT pathway, which has been shown to be activated by almost all known cytokines (for review see Briscoe *et al.*, 1996), growth factors, and also by CD40 (Hanissian and Geha, 1997; Karras *et al.*, 1997). The JAK/STAT signaling cascade involves members of the Janus kinase (JAK) family of protein tyrosine kinases (PTK), which are constitutively bound to the receptors mentioned above. Ligand-induced aggregation of receptor molecules leads to the autophosphorylation of JAKs and subsequently to the activation of STAT (signal transducers and activators of transcription) proteins. Tyrosine phosphorylated STAT proteins dimerize via their respective SH2 domains and subsequently translocate into the nucleus where they bind to specific DNA motifs to induce gene transcription (for review see Briscoe *et al.*, 1996). We show here that endogenous LMP1 and Janus kinase 3 (JAK3) interact in the EBV-immortalized B cell line 1852.4 (Kilger *et al.*, 1998) as well as in 293 cells following ectopic expression. This interaction leads to an enhanced tyrosine phosphorylation of JAK3 which requires a proline rich motif and surrounding sequences within LMP1's 33 bp repeat region. Tyrosine phosphorylation of JAK3 occurs within minutes after activation of the chimeric NGF-R:LMP1 receptor which is a time course reminiscent of cytokine receptors. Concomitantly, the expression of LMP1 leads to the rapid activation of STAT proteins. Thus, LMP1 is revealed to be a pseudo-receptor which induces multiple and distinct signaling pathways. The simultaneous or sequential activation of pathways involved in cell proliferation or protection from apoptosis is very likely to be the molecular basis for the function of LMP1 during B cell immortalization and tumor formation.

Results

The C-terminus of LMP1 contains JAK interaction motifs

So far, two C-terminal activating regions (CTAR1 and 2) in the cytoplasmic domain of LMP1 have been described. They are located in the proximity of the transmembrane domain and the distal C-terminus, respectively (Figure 1) (Huen *et al.*, 1995). Both CTAR1 and 2 are necessary for full activation of NF- κ B and efficient B cell immortalization (Kaye *et al.*, 1995; Devergne *et al.*, 1996; Izumi *et al.*, 1997); whereas CTAR2 appears to be the sole mediator of JNK-1 and AP-1 activation by LMP1 (Kieser *et al.*, 1997, 1999; Eliopoulos and Young, 1998). In order to identify additional potential signaling mediators engaged by LMP1, we screened its C-terminus for consensus sequences which might be important for the interaction with signal transducers. Besides the already known PXQXT/S motifs for TRAF binding (within CTAR1) and the YYD motif for TRADD binding (within CTAR2), LMP1 has additional consensus sites which are characteristic for the interaction with members of the JAK family (Figure 1) (Murakami *et al.*, 1991; Ihle *et al.*, 1995). These consensus sites are classified as box 1 motif, which is a proline-rich sequence (PXXPP) believed to serve as a docking site for JAKs and as box 2 motif which may play an important role in kinase activation although its exact role is less well documented (Ihle *et al.*, 1995; Ivashkiv, 1995). As shown in Figure 1, the C-terminus of

Fig. 1. Consensus sequence motifs for JAK binding within the C-terminus of latent membrane protein 1 (LMP1). Top: schematic representation of the LMP1 protein of the EBV strain B95.8 with consensus binding sequences. Shown are the two C-terminal activating regions 1 and 2 (CTAR1/2; filled rectangles) and box 1 and box 2 motifs (open rectangles). Bottom: the amino acid sequence of the cytoplasmic C-terminus of LMP1 (aa 185–386) is depicted with the consensus binding sequences for JAK protein tyrosine kinases. Two identical box 1 motifs (PXXPP) are located at amino acid positions 275–280 and 302–307. The single box 2 motif (PPQLTEEEVENK) is located at amino acid position 320–330 and precedes the last 55 amino acids, which are important for TRADD binding and NF- κ B and AP-1 activation. The two box 1 motifs are located within the heterogeneous stretch of 33 bp repeats whereas the box 2 motif localizes to a non-repetitive segment further downstream. LMP1 of the B95.8 strain has four and a half repetitive sequence units as indicated by arrowheads. The repetitive elements consist of three perfect copies of a simple 33 bp motif, one extended version with a single box 1 consensus sequence, and a truncated repeat also encoding a box 1 motif.

LMP1 contains two identical box 1 motifs (PHDPLP, amino acids 275–280 and 302–307) as well as one box 2 motif (PPQLTEEEVENK, amino acids 320–330). Thus, LMP1 might interact with PTKs of the JAK family leading to the activation of STAT proteins. Interestingly, the box 1 consensus motifs are located within a highly repetitive region of LMP1 termed '33 base pair repeats' whose function is unknown so far.

LMP1 expression in B cells activates the STAT proteins

The consensus motif search prompted us to investigate the activation status of STAT proteins in EBV-immortalized B cells in conjunction with LMP1 expression. Primary B lymphocytes which had been immortalized with a mini-EBV plasmid carrying a conditional LMP1 allele were used for this purpose. The LMP1 gene is under control of a tetracycline-dependent promoter and withdrawal of tetracycline leads to the transcriptional downregulation of the LMP1 gene (Kilger *et al.*, 1998). To minimize LMP1 expression initially, the 1852.4 cells were kept in the

absence of tetracycline for 4 days. Subsequently, LMP1 was reinduced by adding tetracycline to the culture medium from day 1 to 4 with the intention to boost and synchronize LMP1 re-expression. The controls were kept under identical conditions except that they did not receive tetracycline. Simultaneous activation of the JAK/STAT pathway was assessed in cell lysates by performing a gel electrophoretic mobility shift assay (GEMSA) using a probe specific for STAT1 and 3. As a control the human embryonic kidney cell line 293 was treated with IFN γ for 15 min (Figure 2A, right panel). In human cells, IFN γ strongly induces STAT1 which leads to the formation of STAT1-homodimers. Reinduction of LMP1 in 1852.4-immortalized B lymphocytes led to the appearance of a complex similar in size to that observed in IFN γ -treated 293 cells (Figure 2A). The DNA-binding activity of STATs peaked at day 1 after tetracycline readdition and declined over the following 3 days, but was still substantial at day 4 after tetracycline readdition (Figure 2A). The addition of α -STAT1 antibody led to the disruption of the observed complex, while α -JAK3 antibody as a control antibody had no effect. The readdition of tetracycline to the culture medium of 1852.4 cells induced the expression of LMP1 (Figure 2B) as expected without affecting the expression levels of STAT1 (Figure 2C). These results show that LMP1 induces the binding activity of at least STAT1 to specific DNA sequences suggesting that LMP1 engages the JAK/STAT pathway in EBV-immortalized B cells.

Activation of the JAK/STAT pathway is a rapid process involving JAK3

The activation of STAT proteins in the 1852.4 cell line could result either from autocrine loops in which LMP1 induced the release of cytokines such as IL-6 (Tosato *et al.*, 1990; Eliopoulos *et al.*, 1997) or from a direct activation following LMP1 re-expression. To distinguish between these possibilities a conditional LMP1 molecule was used which can be activated at will within minutes (Gires *et al.*, 1997). This LMP1 molecule is a chimera between the low affinity nerve growth receptor (NGF-R) and the C-terminal signaling domain of LMP1. The chimeric receptor localizes to the plasma membrane where it can be aggregated by an antibody directed against the extracellular part of the NGF-R:LMP1 molecule, thereby mimicking receptor activation (Gires *et al.*, 1997). 293 cells were transiently transfected with expression plasmids encoding wild-type LMP or NGF-R:LMP1. One day after transfection, NGF-R:LMP1 molecules were cross-linked for 15 and 45 min using a murine α -NGF-R antibody in combination with an α -mouse immunoglobulin secondary antibody. Cell lysates were then subjected to GEMSA using a probe specific for STAT1/3 as described above. Surprisingly, no specific band shift could be observed in either of the single transfectants, as shown in Figure 3A. We reasoned that one or more components involved in the JAK/STAT signaling pathway might be missing in 293 cells. Since JAK3 is lymphocyte specific (Tortolani *et al.*, 1995) it was the prime candidate for complementation in 293 cells. Additionally, JAK3 has been reported to be constitutively activated in EBV-immortalized B cells as well as in HTLV-immortalized T cells (Xu *et al.*, 1995; Weber-Nordt *et al.*, 1996; Murata and Puri, 1997). Therefore, JAK3 was co-transfected

Fig. 2. Activation of STAT proteins following the expression of LMP1 in the 1852.4-immortalized B cells. (A) 1852.4 cells are immortalized human B cells carrying a conditional, tetracycline-regulated LMP1 gene in the context of a mini-EBV plasmid (Kilger *et al.*, 1998). 1852.4 cells were kept in the absence of tetracycline for four days (-Tet) which leads to transcriptional downregulation of the LMP1 gene. The expression of LMP1 was reinduced upon readdition of tetracycline for four days (Tet 1d, 2d, 3d or 4d) and cell extracts prepared as described in Materials and methods. As a control, 293 cells were treated for 15 min with recombinant IFN γ and the cells lysed under the same conditions. GEMSAs were performed using a probe specific for STAT1 and STAT3 proteins. Where indicated antibodies specific for JAK3 (α -JAK3) or STAT1 (α -STAT1) were incubated with the extracts. The STAT-specific complex is indicated by an arrow. One representative experiment out of three is shown. (B) Expression levels of LMP1 as a function of the presence or absence of tetracycline were assessed. Proteins were separated by 10% SDS-PAGE and LMP1 was detected using the CS1-4 antibody mix in combination with a HRP-conjugated secondary antibody. The LMP1 control lane contains the crude extract of 293 cells transiently transfected with an expression plasmid for wild-type LMP1. (C) Expression levels of immunoprecipitated STAT1 was assessed as a function of LMP1 re-expression. Proteins were immunoprecipitated from 1852.4 B cell lysates using an α -STAT1 antibody and separated by 10% SDS-PAGE. STAT1 levels were detected with specific antibodies and show a double band of 91 and 84 kDa as expected.

together with expression plasmids encoding LMP1 or NGF-R:LMP1 in the next set of experiments (Figure 3B). Twenty-four hours after transfection, NGF-R:LMP1 molecules were cross-linked and cell lysates were analyzed as described above. The induction of a band shift comparable to that observed in the 1852.4 B cell line was clearly dependent upon cross-linking of NGF-

Fig. 3. Rapid activation of STAT proteins by LMP1 in the human embryonic kidney cell line 293 is dependent on JAK3. Short-term cross-linking of the conditional NGF-R:LMP1 chimera activates STAT proteins in 293 cells and is dependent on the cotransfection of JAK3. (A) 293 cells were transiently transfected with expression plasmids for LMP1 (1 μ g) or NGF-R:LMP1 (0.5 μ g) as indicated. One day after transfection cells were treated with cross-linking antibodies (murine α -NGF-R in combination with α -mouse immunoglobulin antibody as indicated by '+') or with secondary antibody alone (α -mouse Ig as indicated by '-') for the time period indicated. Subsequently, cells were lysed and GEMSA was performed as described in Materials and methods using a probe specific for STAT1 and STAT3 proteins. (B) As in (A), with additional cotransfection of an expression plasmid for JAK3 (0.5 μ g). The shifted complexes specific for STAT1/3 are indicated with an arrow. One representative experiment out of three is shown. (C) As in (B), with untreated or cross-linked NGF-R:LMP1 molecules. The expected size of the STAT complex is indicated with an arrow. The question mark indicates an unidentified shift complex which seems to be induced by LMP1 in conjunction with JAK3 in 293 cells only, as described in the Results.

R:LMP1 and co-expression of JAK3. Activation of STAT proteins in these experiments was a rapid process occurring within 15 min after LMP1 cross-linking, while the shifted complexes were still present with lower intensity after 45 min. In the absence of primary α -NGF-R antibody no significant induction of STAT DNA-binding activity was observed (Figure 3C). Transfection of LMP1 together with JAK3 induced a weak band shift only, which most likely reflects the steady-state activation level of the JAK/STAT pathway by constitutively active LMP1. The expression levels of JAK3 were critical in these experiments since overexpression led to a constitutive activation of STAT proteins (data not shown) which is expected due to autophosphorylation of JAK3 at high expression levels (Silvennoinen *et al.*, 1993; see also Figure 4). Furthermore, in the presence of LMP1 we consistently observed the induction of a lower migrating band whose nature is unknown (Figure 3).

Autophosphorylation of JAK3 on tyrosine residues is rapidly induced by ectopic expression of LMP1 in 293 cells

Ligand binding by cytokine receptors leads to an increased phosphorylation of JAK proteins on tyrosine residues which results in an enhanced kinase activity. Since JAK3 expression was mandatory for the activation of STAT proteins by LMP1 as shown in transient transfection experiments in 293 cells we investigated the activation status of JAK3 as a function of LMP1 expression. LMP1 and murine JAK3 expression plasmids were transfected in 293 cells in the combinations as indicated in Figure 4A. Twenty hours post transfection, cells were lysed in the presence of phosphatase and protease inhibitors and

tyrosine phosphorylated proteins were detected using α -phosphotyrosine antibodies. Transfection of JAK3 alone resulted in the appearance of a tyrosine phosphorylated protein of ~125 kDa which is the expected molecular weight of JAK3 (Figure 4A, upper panel). Cotransfection of LMP1 with JAK3 strongly increased the phosphorylation of this protein. Immunoprecipitation using antibodies directed against JAK3 and subsequent detection of phosphorylated tyrosine residues clearly identified the 125 kDa protein as JAK3 (Figure 5B, right panel and data not shown). The expression of LMP1 was analyzed following immunoprecipitation and was found to be similar in the LMP1 transfectants (Figure 4A, lower panel). Next, we cotransfected a kinase-defective dominant-negative mutant of JAK3 (Kawahara *et al.*, 1995) (JAK3 Δ KD) together with wild-type LMP1. As shown in Figure 4B, tyrosine phosphorylation was completely blocked by JAK3 Δ KD, demonstrating that JAK3 phosphorylation was the result of auto/transphosphorylation. *In vitro* translation of JAK3 Δ KD, resulted in a protein of 87 kDa as predicted (data not shown).

In the following experiment we analyzed the kinetic JAK3 phosphorylation after activation of LMP1 signaling. LMP1, NGF-R:LMP1 and JAK3 expression plasmids were cotransfected in 293 cells in combinations as indicated in Figure 4C. Twenty-four hours post transfection NGF-R:LMP1 molecules were either cross-linked for 15 min with the antibody directed against the external NGF-R part in conjunction with the secondary antibody or treated with secondary antibody only. Cells were lysed and tyrosine phosphorylation was assessed by Western blotting and immunodetection with α -phosphotyrosine antibodies. As shown in Figure 4C, strong tyrosine phosphorylation of

Fig. 4. Fast tyrosine autophosphorylation of JAK3 in the presence of LMP1. Tyrosine autophosphorylation of JAK3 is strongly enhanced in the presence of LMP1 and occurs within minutes after LMP1 aggregation. (A) 293 cells were transiently transfected with LMP1 (1.0 μ g) and JAK3 (0.5 μ g) expression plasmids as indicated. One day after transfection, cells were lysed in RIPA buffer containing protease/phosphatase inhibitors. An aliquot of each crude extract (10%) was separated by 10% SDS-PAGE and tyrosine phosphorylated proteins were detected using the PY20/4G10 antibody mix (upper panel). Remaining extracts were immunoprecipitated using LMP1-specific antibodies and LMP1 expression was assessed (lower panel). (B) 293 cells were transiently transfected with expression plasmids for LMP1 (1 μ g), wild-type JAK3 (0.5 μ g), or dominant-negative JAK3 Δ KD (0.5 μ g) in the combinations indicated. Tyrosine phosphorylated proteins were detected as described in (A). (C) 293 cells were transiently transfected with expression plasmid encoding LMP1 (1.0 μ g), NGF-R:LMP1 (0.5 μ g) and JAK3 (0.5 μ g) in the combinations indicated. One day after transfection cells were left untreated (un), incubated with cross-linking antibodies (+), or with secondary antibody alone (–) for 15 min as described (Gires *et al.*, 1997). Tyrosine phosphorylated proteins were detected as in (A). Each panel shows one representative experiment out of three.

JAK3 was a rapid process occurring within 15 min after NGF-R:LMP1 cross-linking. Continuous expression of wild-type LMP1 resulted in a much weaker autophosphorylation of JAK3, which most likely reflects a combinatorial effect of phosphorylation and subsequent activation of phosphatases as is the case with numerous cytokine receptors (Darnell, 1997). This finding further supported the idea that LMP1 directly activates the JAK/STAT pathway as short term cross-linking of NGF-R:LMP1 displayed a very strong tyrosine phosphorylation of JAK3

Fig. 5. Tyrosine phosphorylation of endogenous JAK3 and interaction with LMP1. (A) 1852.4 cells are immortalized human B cells carrying a conditional tetracycline regulated LMP1 gene in the context of a mini-EBV plasmid. Cells were grown in the absence of tetracycline for four days (–Tet) which leads to transcriptional downregulation of the *LMP1* gene. Subsequently, LMP1 expression was reinduced upon readdition of tetracycline to the culture medium for up to four days (+Tet). Alternatively, tetracycline deprived cells were kept in the presence of CD40-ligand expressing mouse fibroblasts (+CD40-L). Cell extracts from 1×10^7 cells were analyzed by immunoblotting using antibodies against tyrosine phosphorylated proteins. The expected size of JAK3 is indicated with an arrow. (B) Endogenous JAK3 was immunoprecipitated from proliferating 1852.4 cells (1×10^8 cells per sample) and analyzed by immunoblotting using antibodies against JAK3 (left) or tyrosine-phosphorylated proteins (right). Crude extracts from 293 cells transfected with expression plasmids for LMP1 and murine JAK3 were analyzed in parallel. Direct visualization of ectopically expressed murine JAK3 in 293 cells with the available antibodies was not feasible which is probably due to the insufficient affinity of the JAK3 antibody for the murine protein under Western blot conditions. (C) 1852.4 cells (1×10^8 cells per sample) were grown in the presence of tetracycline and lysed in RIPA buffer containing protease/phosphatase inhibitors. Precleared cell lysates were immunoprecipitated using α -LMP1 (CS1-4; DAKO) or α -JAK3 (C21; Santa Cruz) antibodies. Immunoprecipitates were separated by 10% SDS-PAGE and analyzed by immunoblotting using α -JAK3 (Santa Cruz; upper panel) or α -LMP1 (S12-biotinylated; middle panel) or α -phosphotyrosine (PY20/4G10; lower panel) antibodies. Each panel shows one representative experiment of three.

in the absence of secondary downregulating events (e.g. phosphatase activity).

LMP1 and JAK3 interact in 293 and B cells

The most obvious scenario which would explain tyrosine phosphorylation of JAK3 by LMP1 is the direct binding of JAK3 to the C-terminal domain of LMP1. The constitutively aggregated LMP1 molecules would tether JAK3 molecules to the plasma membrane inducing them in a constitutive manner. As anticipated, immunoprecipitation of LMP1 revealed a weak interaction with an ~125 kDa phosphorylated protein in 293 cells only when they had been transfected with a JAK3 expression vector. In contrast, no such interaction was observed between LMP1 and JAK1 or JAK2 (data not shown). These results strongly suggested that JAK3 might be bound to LMP1 but these co-immunoprecipitation assays were based on overexpression of both LMP1 and JAKs in 293 cells. To make use of a less artificial experimental setting, the phosphorylation status of endogenous JAK3 and its interaction with LMP1 were investigated in the EBV-immortalized B cell line 1852.4. Cells were deprived of tetracycline in order to downregulate LMP1 expression (see Figure 2B). LMP1 re-expression was achieved by addition of tetracycline to the culture medium. Alternatively, tetracycline-deprived cells were kept on a CD40-L expressing mouse fibroblasts feeder layer (Kilger *et al.*, 1998). Cell lysates were then tested for tyrosine-phosphorylated proteins by immunoblotting. As shown in Figure 5A, reinduction of LMP1 as well as treatment with CD40-L induced the tyrosine phosphorylation of several cellular proteins, one migrating with an apparent molecular weight of 125 kDa. Subsequent immunoprecipitation with a JAK3-specific antibody confirmed that the 125 kDa protein was JAK3 which becomes phosphorylated at tyrosine residues only after LMP1 re-expression or CD40 activation. Reinduction of LMP1 expression did not alter the steady-state levels of JAK3 (data not shown).

The activation of JAK3 following CD40 ligation has been reported recently (Hanissian and Geha, 1997), supporting the hypothesis of the functional similarity between LMP1 and CD40. Further experiments with immunoprecipitated JAK3 from cycling 1852.4 cells showed that a substantial amount of JAK3 is tyrosine phosphorylated in EBV-immortalized B cells (Figure 5B). Endogenous human JAK3 from B cells and murine JAK3 expressed in 293 cells migrated with the same molecular weight as seen with an anti-phosphotyrosine-specific antibody (Figure 5B). As is apparent in the control lanes in Figure 5B, detection of ectopically expressed murine JAK3 in 293 cells with the available antibodies was not feasible. This is probably due to the insufficient affinity of the JAK3 antibody for the murine protein under Western blot conditions.

To examine the interaction of LMP1 and JAK3 in more detail, 1852.4 cells were grown in the presence of tetracycline to permit LMP1 expression and cell proliferation (Kilger *et al.*, 1998). A total of 1×10^8 cells were lysed and LMP1 was precipitated using a mix of monoclonal antibodies specific for LMP1 (CS1-4). Precipitated proteins were separated by SDS-PAGE, and co-precipitated JAK3 was detected using an α -JAK3 antibody after Western blotting. As shown in Figure 5C, JAK3 co-precipitated with LMP1, demonstrating that LMP1 and endogenous JAK3

physically interact in EBV immortalized B cells. Incubation of cell lysates with beads alone (or with an α -NGF-R antibody; data not shown) precipitated neither JAK3 nor LMP1. Reciprocally, LMP1 was also detectable in α -JAK3 immunoprecipitates as shown in Figure 5C. We could further show that LMP1 associated JAK3 is tyrosine phosphorylated (Figure 5C, lower panel) as anticipated from the cotransfection experiments in 293 cells (Figure 4). These results clearly demonstrate that endogenous LMP1 and JAK3 interact *in vivo* in EBV-immortalized B cells, which is the most likely molecular basis for JAK3 phosphorylation and activation by LMP1.

The JAK3 activation domain maps to the box 1 motifs within the repetitive region of LMP1

The C-terminus of LMP1 was sufficient for the rapid activation of JAK3 and STAT proteins (Figures 3 and 4). To confirm our initial hypothesis of JAK activation via the box 1 motifs of LMP1, we generated a LMP1 mutant that lacked the entire JAK-binding sequences, as depicted in Figure 1. This mutant, termed LMP1 Δ 33bp, was transiently transfected in 293 cells together with the JAK3 expression vector and tyrosine autophosphorylation of JAK3 was analyzed. Both wild-type LMP1 and LMP1 Δ 33bp were expressed as FLAG epitope-tagged variants to permit the detection of both proteins with equal sensitivity. As shown in Figure 6A, FLAG-tagged LMP1 Δ 33bp (F-LMP1 Δ 33bp) was unable to induce the phosphorylation of JAK3 at tyrosine residues, in contrast to FLAG-tagged wild-type LMP1 (F-LMP1), although both molecules were expressed at comparable levels (Figure 6A). The modifications in F-LMP1 Δ 33bp had no consequences in terms of activation of the c-jun N-terminal kinase 1 pathway (Figure 6B) or induction of NF- κ B as anticipated (Figure 6C). This was expected since neither CTAR1 nor CTAR2 are affected by the deletion in F-LMP1 Δ 33bp. Thus, the LMP1 Δ 33bp mutation displays a very discrete phenotype that selectively affects the induction of the JAK/STAT pathway.

Next, we selectively reconstituted the deleted repeats within the LMP1 Δ 33bp mutant using oligonucleotides encoding either the simple 33 bp repeat lacking the box 1 motif or the PXXXP box 1 motif in the context of the more complex repeat unit (see Figure 1 for the differences). The purpose of this reconstitution experiment was to perform the fine mapping of the JAK3 activation domain within LMP1. Reconstituted mutants containing one or two copies of the different repetitive sequences were expressed in 293 cells together with JAK3 followed by immunoblotting to detect tyrosine phosphorylated proteins. Enhanced tyrosine phosphorylation of JAK3 was observed in the presence of wild-type LMP1 (Figure 7), which was strongly impaired in the case of the LMP1 Δ 33bp mutant as seen before (see Figure 6A). As expected, reintroduction of box 1 motif sequences had a phenotype almost comparable with wild-type LMP1 in terms of JAK3 phosphorylation. Reintroduction of the simple 33 bp repeat lacking the box 1 consensus motif led to a mediocre but clearly detectable increase of JAK3 phosphorylation (Figure 7). This effect could be due to the presence of box 1-like sequences (PXXP) within the simple 33 bp repeats (Figure 1) or surrounding sequences. Thus, the JAK3 activation domain maps primarily to the box 1 motifs located within the repetitive array of LMP1 although surrounding sequences seem to contribute to

Fig. 6. Tyrosine phosphorylation of JAK3 in the presence of FLAG-LMP1Δ33bp or FLAG-LMP1. The LMP1Δ33bp mutant of LMP1 fails to induce tyrosine phosphorylation of JAK3 although it is as effective as wild-type LMP1 with respect to NF-κB and JNK1 activation. **(A)** Tyrosine phosphorylation of JAK3: 293 cells were transiently transfected with expression plasmids for the FLAG-epitope tagged variant of wild-type LMP1 (F-LMP1) or LMP1Δ33bp (F-LMP1Δ33bp) (0.1 μg) together with murine JAK3 (0.3 μg). Tyrosine phosphorylated proteins were detected as described (left panel). Equal expression of FLAG-LMP1 and FLAG-LMP1Δ33bp was assessed by immunoblotting with M2-FLAG antibody in combination with HRP-conjugated secondary antibody (right panel). One representative experiment out of three is shown. **(B)** Activation of JNK1. 293 cells were transiently transfected with expression plasmids for FLAG-epitope tagged variants of LMP1 (F-LMP1) or LMP1Δ33bp (F-LMP1Δ33bp) (0.125 μg) together with HA-tagged JNK1 (1 μg). Kinase activity of immunoprecipitated JNK1 (upper panel) was assessed as described (Kieser *et al.*, 1997). Expression of HA-JNK1 was equal as assessed by immunoblotting with α-JNK1 antibody (lower panel). **(C)** Activation of NF-κB. 293 cells were transiently transfected with expression plasmids for FLAG epitope-tagged LMP1 or LMP1Δ33bp (0.125 μg) together with an NF-κB reporter plasmid (5 ng). Luciferase activity was measured one day after transfection and normalized for β-galactosidase activity. One experiment out of two is shown in (B) and (C).

maximal JAK3 autophosphorylation. Unexpectedly, the repeat copy number had no discernible effect.

Discussion

In this study, we investigated the potential of LMP1 to induce cellular signaling cascades besides those leading to NF-κB and AP-1 activation. It has been shown recently that LMP1 is a constitutively active pseudo-receptor (Gires *et al.*, 1997; Hatzivassiliou *et al.*, 1998) that acts as a key player during B cell immortalization (Zimber-Strobl *et al.*,

Fig. 7. JAK3-activation domain primarily maps to the box 1 motif. To evaluate the function of the different repetitive sequences for JAK3 activation, FLAG-LMP1Δ33bp (F-LMP1Δ33bp) lacking the entire 33 bp repeat region was reconstituted with synthetic oligonucleotides. They encode one or two copies of the simple 33 bp repeat (F-LMP1/1×33bp and F-LMP1/2×33bp) or the longer repeat encompassing the box 1 motif (F-LMP1/1×box1 and F-LMP1/2×box1) as described in Material and methods. Expression plasmids for each LMP1 variant were transfected together with JAK3 in 293 cells. One day after transfection cells were lysed and tyrosine phosphorylation was assessed by immunoblotting (upper panel). The expression levels of each LMP1 variant were comparable as revealed by immunoblotting with FLAG epitope-specific antibodies (not shown). One representative experiment out of three is shown.

1996; Izumi and Kieff, 1997; Kilger *et al.*, 1998). So far, two signaling domains have been identified, termed C-terminal activating region 1 and 2 (CTAR1 and CTAR2) (Huen *et al.*, 1995), which interact with TRAFs (Sandberg *et al.*, 1997) and TRADD (Izumi and Kieff, 1997; Kieser *et al.*, 1999), respectively, and account for the induction of AP-1 and NF-κB (Mitchell and Sugden, 1995; Kieser *et al.*, 1997). No function could be assigned to the fairly large remaining parts of the C-terminus of LMP1. Here, we identified a third C-terminal activating region located between CTAR1 and CTAR2 (Figure 1). We observed that LMP1 and JAK3 interact following the ectopic expression of both molecules in 293 cells while no interaction was detected between LMP1 and JAK1 or JAK2 (data not shown). More importantly, LMP1 and JAK3 also strongly interact in EBV-immortalized B cells (Figure 5). This protein-protein interaction leads to the enhanced tyrosine phosphorylation of JAK3, which is a hallmark for its activation (Figures 4 and 5), and activation of STAT1 (Figure 2) and STAT3 (data not shown) as indicated by their induced phosphorylation and DNA-binding capacity. In line with our findings, JAK3 and Tyk2 were shown to be constitutively phosphorylated in EBV-immortalized B cells (Murata and Puri, 1997), as was JAK3 in T cells immortalized by HTLV1 (Xu *et al.*, 1995). As a consequence, the downstream targets of JAK3, STAT1 and STAT3 also acquire a permanent activation state in lymphoblastoid cell lines and in Burkitt's lymphoma (Weber-Nordt *et al.*, 1996). Mutational analysis allowed the mapping of LMP1's activation domain to the box 1 consensus motifs (PXXPPX) in the context of the 33 bp repeats of LMP1 (Figure 6) with surrounding sequences contributing to the activation domain (Figure 7). In accordance with LMP1's nomenclature we would suggest naming this newly identified functional domain CTAR3.

In B cells immortalized with a mini-EBV carrying a conditional LMP1 allele we could demonstrate that the expression of LMP1 induced STAT1 DNA-binding activity (Figure 2). In 293 cells, cross-linking of the NGF-R:LMP1 chimera in the presence of JAK3 led to the appearance of a

similar band shift complex within minutes (Figure 3). In addition, our initial data indicated that LMP1 induced tyrosine phosphorylation of STAT3 in 293 cells (data not shown). Thus, LMP1 expression in the 1852.4 B cell line and in 293 cells clearly induced STAT DNA binding as a function of JAK3 activation.

The kinetics of the JAK/STAT pathway activated by LMP1 were reminiscent of cytokine receptors and occurred rapidly which might represent a very early event in LMP1 signaling, predisposing the cell for additional proliferation signals. In the EBV-immortalized B cell line 1852.4 induction of STAT DNA-binding activity peaked at day 1 but was still substantial at day 4 of LMP1 re-expression. The rapid downregulation of the JAK/STAT pathway after its induction is a common feature of cytokine signaling. The actual induction level of the JAK/STAT signaling cascade is accepted to be the result of activation of PTK in conjunction with counter-regulation by phosphatases and/or pre-disposition of tyrosine phosphorylated STATs for the ubiquitin degradation pathway (Darnell, 1997). Previous work from our group demonstrated that the activation of NF- κ B and JNK1 occurs predominantly between days 2 and 3 in 1852 B cells (Kieser *et al.*, 1997; Kilger *et al.*, 1998), indicating that JAK/STAT activation is a fast event following LMP1 expression.

The JAK/STAT pathway has been shown to be important in several cellular decisions such as proliferation and protection from apoptosis in Baf3 lymphoid cells (Rodriguez Tarduchy *et al.*, 1990; Leonard and O'Shea, 1998). The relevance of JAK3 in cell fate decisions is especially highlighted by the finding of JAK3 deficiency in SCID patients where it plays a key role in human lymphoid development (Macchi *et al.*, 1995; Russell *et al.*, 1995), its ability to induce DNA synthesis and proto-oncogenes such as *c-myc* and *c-fos* (Kawahara *et al.*, 1995), and the contribution of STATs to prevent apoptosis in multiple myelomas (Catlett-Falcone *et al.*, 1999). The activation of the JAK/STAT pathway may therefore contribute to LMP1's ability to transform cells and/or protect B cells from apoptosis in the context of an EBV infection. One could envision that the simultaneous or sequential activation of NF- κ B, AP-1, JAK/STAT (and possibly others) is the molecular basis for the potential of LMP1 to induce growth transformation, expression of cellular surface markers, and B cell immortalization. The recruitment of signal transducing molecules and the balance between these signals will influence cell fate into one or the other direction, promoting cell death or proliferation. Furthermore, the interaction between the CD40 receptor and JAK3 was demonstrated to be important for the induction of cellular surface markers such as CD23, ICAM-1 and LT- α (Hanissian and Geha, 1997). Although LMP1 and CD40 do not display a convincing overall homology at the primary amino acid sequence level, they have analogous functions in B cells (Zimber-Strobl *et al.*, 1996; Kilger *et al.*, 1998). Therefore, it seems conceivable that binding and constitutive activation of JAK3 by LMP1 upregulates the same cellular genes as CD40 when activated by its specific ligand. The identification of downstream target genes of STATs will be essential to reveal the mode of action of LMP1 as a complex, multipotent pseudo-receptor. With the development of recent techniques (Delecluse *et al.*, 1998, 1999),

genetic experiments are now feasible to study the contribution of the different domains within the C-terminus of LMP1 with respect to efficient B-cell immortalization by EBV.

Materials and methods

Plasmid DNAs

Wild-type LMP1 and NGF-R:LMP1 were expressed from the SV40 promoter/enhancer in pHEBo (Yates *et al.*, 1985). Full-length wild-type LMP1 was expressed as a fusion with the FLAG epitope expressed from the CMV promoter in the FLAG-CMV2 expression plasmid (IBI-Kodak). To create the mutant plasmid FLAG-LMP1 Δ 33bp, the repetitive sequences in FLAG-LMP1 were deleted. A double-stranded synthetic oligonucleotide (upstream oligonucleotide: 5'-GGGGCCGG-GCGACGACCCCCACTCTGCTCTCAAACCTAGGCGCACCTG-GAGGTGGTCCTAGATCTAGATCTCAT-3'; downstream oligonucleotide: 5'-CCCCAATTGTGGAGGGCCTCCATCATTTCAGCAGAGT-CGCTAGGGCTATGAGATCTAGGACCACCTCCAGGTGCGCC-3') was inserted into FLAG-LMP1 which had been cleaved with a combination of *Ngo*M1 and *Mun*I. FLAG-LMP1/1 $\times$ 33bp and FLAG-LMP1/2 $\times$ 33bp are based on FLAG-LMP1 Δ 33bp and contain one or two pairs of oligonucleotides inserted into the *Xba*I-cleaved plasmid (upstream oligonucleotide: 5'-CTAGCGACAATGGCCACAGGACCCGTGACA-ACACTGATT-3'; downstream oligonucleotide: 5'-CTAGAATCAGTGTGTCAGGGTCCTGTGGGCCATTGTGCG-3'). FLAG-LMP1/1 $\times$ box1 and FLAG-LMP1/2 $\times$ box1 were generated similarly (upstream oligonucleotide: 5'-CTAGCGACAATGGCCACATGACCCGCTGCCTCAGGACCTGACAACACTGATT-3'; downstream oligonucleotide: 5'-CTAGAATCAGTGTGTCAGGGTCCTGAGGCAGCGGGTCATGTGGGCCATTGTGCG-3'). Murine JAK3 was cloned in pRK5 and expressed from the CMV promoter (Witthuhn *et al.*, 1994). JAK3 Δ KD was generated by insertion of an *Xba*I nonsense primer (#NEB1062) at the single *Xho*I site 5' of kinase domain (Kawahara *et al.*, 1995).

Cell lines

The cell line 1852.4 was derived from primary human B cells which have been immortalized with a mini-EBV plasmid carrying a conditional LMP1 allele (Kilger *et al.*, 1998). 1852.4 cells were cultured in standard RPMI medium containing 10% fetal calf serum and 1 μ M tetracycline as described (Kilger *et al.*, 1998). The embryonic kidney cell line 293 (Graham *et al.*, 1977) was cultured in standard RPMI containing 10% calf serum. CD40-L-expressing mouse fibroblasts are described by Galibert *et al.* (1996).

Transfection of 293 cells, immunoblots and immunoprecipitation

Transfection of 293 cells was carried out in six well plates at 70% confluency. Plasmid DNAs were transfected using Lipofectamin (Life Technologies) under the conditions recommended by the manufacturer. Protein expression was assessed by immunoblot analysis after separation by 10% SDS-PAGE and electroblotting on a PVDF membrane (Millipore). For the detection of tyrosine phosphorylated proteins the PVDF membrane was first incubated for 1 h at room temperature (RT) in Roti-block buffer (Roth) and then in a 1:1 mixture of PY20 and 4G10 antibodies (1 h RT). Membranes were then washed extensively and incubated with HRP-conjugated α -mouse immunoglobulin secondary antibody for 30 min at RT and detected with the enhanced chemoluminescence system (ECL, Amersham). Expression of LMP1 was detected using biotinylated S12 antibody (1:1000 in TBS-T) in combination with streptavidin-HRP (1:500) and ECL or using the mix of monoclonal antibodies CS1-4 in combination with HRP-conjugated α -mouse Ig antibody. Alternatively, FLAG epitope-tagged variants were detected with M2 α -FLAG antibody (IBI-Kodak; 1:300 in TBS-T; 1 h at RT) in combination with HRP-conjugated α -mouse immunoglobulin. For immunoprecipitation of LMP1, JAK3 and STAT1 transiently transfected 293 cells or 1852.4 B cells were lysed in RIPA buffer containing 0.1 mM vanadate and 0.5 mM phenylmethylsulfonylfluoride (PMSF), and the protein lysates were precleared by centrifugation at 15 300 r.p.m. Precleared lysates were then incubated with protein G-Sepharose beads (Pharmacia) coupled to CS1-4 (Dako), α -JAK3 (Santa Cruz) or STAT1 (Santa Cruz) antibodies (15 μ l beads + 10 μ l antibody for 200 μ l lysate from 0.5–1 $\times$ 10⁶ cells). Immunoprecipitation was carried out overnight at 4°C in an over-head tumbler. Beads were then washed 3 times in

TBS-T, boiled, centrifuged at 15 300 r.p.m. and protein expression was monitored by Western blot immunodetection as described above.

Gel electromobility shift assays

Cells were lysed in ice-cold lysis buffer [0.5% Nonidet P-40 (NP-40), 10% glycerol, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 M EDTA pH 8, 1 mM sodium orthovanadate, 0.5 mM PMSF, 1 mM dithiothreitol] (Schindler *et al.*, 1992). Nuclei were removed by centrifugation. The sequence of the oligonucleotide probe used corresponds to the high-affinity *sis*-inducible element (hSIE) of the *c-fos* gene (SIEM): 5'-GTGACATTTCCCGTAAATC-3' (Sadowski *et al.*, 1993). The probe was end labeled with [γ - 32 P]ATP and aliquots equivalent to 30 000 c.p.m. were used per reaction. Binding reactions were performed in a total volume of 20 μ l containing 10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.1 mM EGTA, 5% glycerol, 1 mg of bovine serum albumin per ml, 0.25 mg of tRNA per ml, and 2% Ficoll. Extracts were preincubated for 5 min at RT with 150 μ g of poly(dI-dC)-(dI-dC) per ml prior to incubation with probe for an additional 20 min at RT. Complexes were separated on 6% non-denaturing acrylamide gels in 0.5 $\times$ Tris-borate-EDTA (TBE) and detected by autoradiography.

Acknowledgements

We thank the members of the Hammerschmidt laboratory for many stimulating discussions and critical reading of the manuscript. We thank Bill Sugden for plasmids encoding various LMP1 and JAK constructs, and Elisabeth Kremer for the supply with biotinylated monoclonal antibodies against LMP1. Our research was supported by Grant CA70723 from the National Institutes of Health, by Grants WI 319/11-3 and Ha 1354/-2 from the Deutsche Forschungsgemeinschaft, by Grant 1-1016-Ze I from the Deutsche Krebshilfe, and by institutional grants.

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Received November 9, 1998; revised and accepted April 13, 1999