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CELLULAR SIGNALING PATHWAYS ENGAGED BY THE EPSTEIN-BARR VIRUS TRANSFORMING PROTEIN LMP1

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1. ABSTRACT

The latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) is an essential component of the viral machinery that orchestrates cellular transformation and oncogenesis. The critical role of LMP1 in transformation has been established through recombinant genetic analysis of the EBV genome, ectopic expression in cell lines and transgenic mice and immunohistochemical analysis of EBV- associated tumor specimens. The principal mechanism of LMP1 function is based on mimicry of activated cell surface receptors of the tumor necrosis factor superfamily. LMP1 signaling culminates in the activation of transcription factors NF-κB, AP1 and STAT1/3, which have been tightly linked to prevention of apoptosis and malignant transformation. The molecular mechanisms of LMP1 function will be reviewed in this report.

2. INTRODUCTION

Epstein-Barr virus (EBV) is a highly prevalent human herpesvirus, which infects epithelial cells and B-lymphocytes and it has been associated with a number of human malignancies (1). The infection of epithelial cells is primarily lytic whereas the infection of B-lymphocytes is usually latent thus allowing the virus to establish a persistent infection. In healthy individuals EBV is restricted by the immune system to a very small number of memory B-lymphocytes that does not exceed approximately 50 cells per one million of peripheral blood B-lymphocytes (2, 3). To avoid further the immune surveillance the virus expresses only two out of nine antigens that represent the full spectrum of latent antigen gene expression (4-7). The delicate balance that is established between the virus and the immune system allows the infection to persist for the life of the host. However in immune deficient patients EBV may escape from the strict control of the immune system and express up to 10 latent genes most of which have been implicated in the process of tumorigenesis (8).

The oncogenic factors of EBV have been identified through the recombinant genetic analysis of the viral genome coupled with EBV-mediated growth transformation of primary B-lymphocytes *in vitro* into long-term proliferating lymphoblastoid cell lines (LCL) (8). These studies have demonstrated that the latent EBV antigens LMP1, EBNA2, EBNALP, EBNA3A and EBNA3C are essential for the process of B cell transformation *in vitro*. EBNA1 is required for the maintenance of the viral genome in latently infected cells and its direct involvement in the process of transformation could not be addressed in these studies. The transforming potential of EBV latent antigens has been evaluated further through the phenotypic analysis of cells expressing individual antigens and immunohistochemical characterization of EBV-associated tumor specimens. All the evidence accumulated so far support a role for LMP1 as the principal transforming factor of EBV. LMP1 is essential for EBV-mediated B lymphocyte transformation *in vitro* since viral strains lacking LMP1 expression are unable to transform B-lymphocytes (9). LMP1 is expressed in most human malignancies associated with EBV,

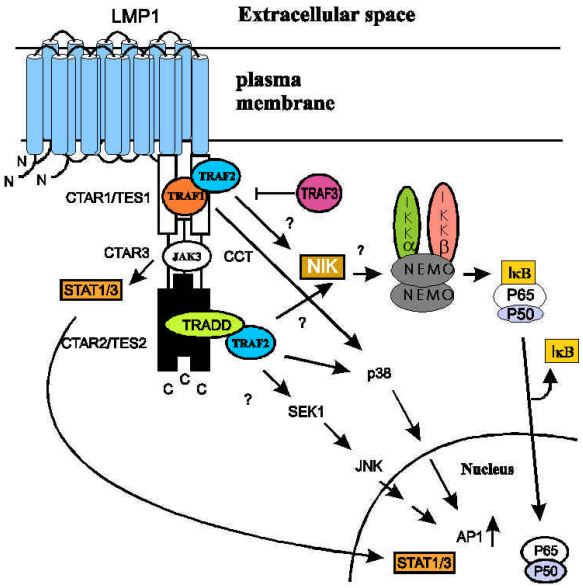

Figure 1. Signal transduction mechanisms of LMP1. A trimer of LMP1 is depicted at the plasma membrane (the stoichiometry of signaling LMP1 complexes at the plasma membrane is not known). The amino (N) and carboxyl (C) termini of LMP1 are located in the cytoplasm. The six transmembrane domains (vertical cylinders inserted in the plasma membrane) enable the protein to oligomerize at the plasma membrane. Three signaling domains (CTAR1/TES1, CTAR2/TES2 and CTAR3) have been characterized in the cytoplasmic carboxyl terminus (CCT) of LMP1. CTAR1 (white rectangular) interacts with TRAF1 and TRAF2 to mediate activation of NF-κB, a process that is inhibited by TRAF3, which interacts with CTAR1 as well. CTAR2 (black rectangular) interacts with TRADD and mediates a more powerful activation of NF-κB via a pathway that apparently involves also TRAF2. The activation of NF-κB by CTAR1 and CTAR2 involves the activation of NIK or a related MAP kinase kinase kinase and the subsequent activation of the IκB kinase complex (IKKα/IKKβ/NEMO). Activation of the IκB kinase complex results in the phosphorylation and degradation of the NF-κB inhibitor IκB and the release of active NF-κB heterodimers (p50/p65), which can translocate into the nucleus and activate transcription. CTAR2 activates also the AP1 transcription factor through the activation of protein kinases p38 and JNK1. P38 and AP1 are also activated by CTAR1. Activation of AP1 occurs through upregulation of the genes that constitute the DNA-binding subunits of AP1 (*cjun*, *cfos*) and phosphorylation of the transactivation domain of cJun.

including anaplastic nasopharyngeal carcinoma (NPC), Hodgkin's disease (HD) and immunoblastic lymphomas developed in immunosuppressed patients (1). LMP1 expression has been associated with the generation of precancerous lesions of NPC and their progression to malignancy, indicating a fundamental role of this protein in deregulation of cellular growth control that leads to tumorigenesis (10). Expression of LMP1 in established rodent fibroblast lines leads to loss of contact inhibition, anchorage independent growth and tumorigenicity in nude mice demonstrating an autonomous transforming activity, which is unique among latent EBV antigens (11, 12). In addition transgenic mice expressing LMP1 under the control of immunoglobulin heavy chain promoter/enhancer develop B cell lymphomas at higher frequency than control animals (13). These findings established the tumorigenic potential of LMP1 *in vivo*. Furthermore expression of LMP1 in epithelial and B-lymphoid cell lines causes extensive phenotypic changes characterized by induction of cell surface activation markers, growth factor receptors and antiapoptotic proteins. In B-lymphocytes LMP1 induces the activation markers CD21, CD23, CD30, CD40, CD44 and Fas the cell adhesion molecules ICAM1, LFA1, LFA3 and the antiapoptotic factors A20, Bcl2, Bfl1 and Mcl1 (14-21). In epithelial cells LMP1 prevents differentiation and induces the expression of epidermal growth factor receptor and A20 (22, 23). The upregulation of cell adhesion molecules in LMP1-expressing B cells leads to increased homotypic adhesion allowing autocrine growth circuits to operate efficiently. The induction of antiapoptotic factors inhibits programmed cell death by LMP1 in response to p53 activation or reduced serum (16, 24). The pleiotropic involvement of LMP1 in EBV-mediated deregulation of growth control has placed this molecule at the core of intensive research efforts aiming at the understanding of the molecular mechanisms that underlie tumorigenesis associated with EBV infection.

3. STRUCTURAL AND FUNCTIONAL FEATURES OF LMP1

LMP1 is an integral membrane protein that consists of a short 24 amino acid cytoplasmic amino terminus, six hydrophobic transmembrane domains and an approximately 200-amino acid cytoplasmic carboxyl terminus (CCT) which may vary in length depending on the viral strain (Figure 1). The cytoplasmic amino terminus does not appear to contain a functional domain and most likely it secures the position of the first transmembrane domain (25, 26). The six transmembrane domains enable the protein to oligomerize constitutively at the plasma membrane and form characteristic patches and caps in the absence of a ligand (27). Constitutive oligomerization of LMP1 at the plasma membrane is essential for its transforming activity in rodent fibroblasts and B-lymphocytes (9, 14, 28). Mutated EBV strains lacking even the first transmembrane domain of LMP1 are unable to transform primary B lymphocytes and display diffuse localization throughout the cytoplasm and plasma membrane (9, 28). Approximately 10% of LMP1 localize in discrete plasma membrane regions known as lipid rafts or detergent resistant membranes (29-32). Plasma membrane lipid rafts are enriched in cholesterol and sphingolipids and have attracted particular attention because a large number of signaling proteins are located in these domains. These include tyrosine kinases, G proteins and G-protein coupled receptors, the T cell receptor and the B cell receptor. The six transmembrane domains mediate the association of LMP1 with lipid rafts. Palmitoylation of LMP1 is not necessary for raft association even though it plays an important role for raft association of other

transmembrane proteins (30). The presence of LMP1 in lipid rafts may facilitate the assembly of specific LMP1-containing signaling complexes. The amino terminal and transmembrane domains of LMP1 also confer a cytostatic activity, which prevents overexpression of the protein and may limit its signaling activity to an optimal range for cellular growth (33, 34). The same regions of LMP1 have been associated with transcription repression of certain EBV promoters (35). The molecular mechanisms of growth arrest and transcription repression by the LMP1 amino terminal and transmembrane domains are not clear at present.

The LMP1 CCT plays an essential role in LMP1 function. EBV strains expressing LMP1, which lacks the CCT, fail to transform primary B-lymphocytes in vitro (36). Furthermore the LMP1 CCT is essential for the engagement of cellular signaling pathways leading to activation of transcription factors NF-κB, AP1 and STAT1/3 (see below). Activation of NF-κB or AP1 by LMP1 has been linked to upregulation of cellular proteins and inhibition of apoptosis. Two functional domains associated with the transforming potential of LMP1 have been mapped in the CCT. Transformation effector site 1 (TES1, Figure 1) consists of the membrane proximal 45 amino acids of CCT (amino acids 187-231) whereas transformation effector site 2 (TES2, Figure 1) spans the last 35 amino acids of LMP1 (amino acids 352-386) (37, 38). Deletion of TES1 abolishes B-lymphocyte transformation by EBV in vitro (38). TES2 plays a critical role in the long-term growth potential of EBV-transformed B-lymphocytes (36, 39). The LCLs that are derived by EBV clones lacking TES2 have a significantly shorter life span compared to those derived from WT EBV and require fibroblast feeders or high cell density for survival unless high viral titer is used in the infection. These findings suggest that TES2 compensates for paracrine growth factor activity that is essential for long term growth of EBV-transformed LCLs. TES1 and TES2 overlap with two domains (CTAR1 and CTAR2, respectively) associated with activation of transcription factors NF-κB and AP1 (Figure 1) (40-44). These findings implicate the activation of NF-κB and AP1 in the process of B-lymphocyte transformation by EBV. However the exact role of these transcription factors in EBV-mediated lymphocyte transformation remains the subject of intense investigation. More recently a third domain of the LMP1 CCT was linked to activation of transcription factor STAT1. The STAT-activating domain (also known as CTAR3) is located between CTAR1 and CTAR2 and is associated with two motifs at amino acid positions 275-280 and 302-307 (Figure 1) (45). These sequences constitute binding sites for the tyrosine kinase JAK3 that mediates phosphorylation and activation of STATs. The functional significance of CTAR3-mediated signal transduction is unclear since this domain appears to be dispensable for *in vitro* B-lymphocyte transformation by EBV (46). Furthermore recent evidence suggests that CTAR3 is not absolutely essential for STAT activation whereas intact CTAR1 and CTAR2 are required for STAT activation (see below).

Variations in LMP1 sequence in different EBV isolates from tumors have attracted particular attention recently in an effort to correlate particular signaling properties of LMP1 with oncogenicity (47-54). Most functional studies of LMP1 have used the gene encoded by the B95.8 strain of EBV. A recent systematic analysis of LMP1 variants from european and chinese isolates has classified the various LMP1 molecules in 5 groups (A, B, C, D and Chinese) based on consistent amino acid differences from the B95.8 prototype (55). Chinese and group D variants activate NF-κB more potently than B95.8 LMP1 whereas Chinese, group B and group D variants were stronger activators of AP1 compared to B95.8 LMP1. No significant differences were noted in the ability of different LMP1 molecules to activate STATs. Differences in NF-κB and AP1 activation could not be attributed to alterations in core CTAR1 and CTAR2 elements, which were identical or to specific amino acid differences. Apparently multiple amino acid differences in the amino terminal cytoplasmic domain, the transmembrane regions and the CCT contribute to differences in NF-κB and AP1 activation. This study revealed also that most likely unidentified signaling pathways are engaged by LMP1 and contribute to phenotypic alterations occurring in LMP1-expressing cells.

4. THE NF-kB PATHWAY

The critical role of the LMP1 CCT in B-lymphocyte transformation and signal transduction prompted further investigation into the molecular events that mediate these functions. The principal goal of these studies was the identification of cellular signaling proteins that interact with the LMP1 CCT. These experiments led to the identification of members of the TRAF (tumor necrosis factor receptor associated factors) family of proteins and TRADD (tumor necrosis factor receptor 1 associated death domain protein) as mediators of LMP1 signaling that leads to NF-κB activation (Figure 1) (38, 56). TRAFs interact with CTAR1, which mediates approximately 20-30% of NF-κB activation by LMP1 whereas TRADD binds to CTAR2, which transduces 70-80% of the LMP1-mediated NF-κB activation (38, 40, 41, 57-59). The engagement of TRAFs and TRADD by LMP1 immediately prompted to an association between the signaling mechanisms of LMP1 and members of the tumor necrosis factor receptor (TNFR) superfamily (56).

The mammalian TRAF family consists of six members that have been implicated in NF-κB regulation by various TNFR family members including TNFRI, TNFRII, CD40, CD30, lymphotoxin β receptor (LTβR), CD27, 4-1BB and OX40 (reviewed in (60)). TRAF6 has been implicated in NF-κB activation by IL1/Toll receptors. TRAF2, TRAF5 and TRAF6 can activate the NF-κB pathway upon overexpression and appear to be the principal mediators of NF-κB activation by TNFR family members. TRAF proteins consist of a highly conserved carboxyl terminal TRAF domain (TD), a coiled-coil domain located in the central part of the protein and a cysteine-histidine-rich amino terminal region containing multiple putative zinc-finger motifs and in some cases a RING finger motif. The TD is required for the interaction

of TRAFs with the cytoplasmic tails of the receptors. The RING finger and the amino terminal two zinc fingers are essential for the activation of the NF-κB pathway. The crystal structure of the TD of TRAF2 indicates a mushroom-like trimeric structure with three receptor-binding sites (one per monomer) (61). The affinity of each TRAF receptor-binding site for the receptor tail is very weak (the dissociation constant is in the millimolar range) essentially preventing association of non-activated monomeric receptors with TRAFs (62). However ligand-dependent receptor trimerization should permit binding of TRAFs to the receptor tail and initiation of signal transduction. Experimental data obtained with agonist-mediated activation of TNFRI, CD40 and LTβR support the model of ligand-dependent recruitment of TRAFs to the receptor as the initial step in the process of signal transduction (63-66). In the case of LMP1 TRAFs are constitutively associated with the CCT presumably because of the constitutive oligomerization of LMP1 by the six transmembrane domains (57).

LMP1 interacts with TRAF1, TRAF2, TRAF3 and TRAF5 through CTAR1 (20, 57-59). The core of the TRAF-binding domain of LMP1 spans amino acids 201 to 210 and encompasses the motif P204QQATD209, which falls within the TRAF2-binding consensus sequence PXQXXD (where X is any amino acid) (57, 67). Alteration of P204 and Q206 to alanine residues abolishes association of LMP1 with TRAFs and severely impairs LMP1-mediated NF-κB activation by CTAR1 and reduces NF-κB activation by full length LMP1 (20, 57). Furthermore overexpression of a dominant negativeTRAF2 mutant inhibits NF-κB activation by CTAR1 and CTAR2 thus implicating TRAF2 in the NF-κB-activating signal that emanate from these LMP1 domains (68, 69). In addition NF-κB activation by CTAR1 is enhanced by TRAF1 overexpression and inhibited by TRAF3 overexpression (23, 57). These findings indicate that TRAF1 and TRAF2 mediate NF-κB activation by CTAR1 whereas TRAF3 may have an inhibitory role in this process. TRAF2 or a related TRAF is also implicated in NF-κB activation by CTAR2 (68). Clearly the definitive role of each TRAF molecule in NF-κB activation by LMP1 will be established only upon investigation of NF-κB activation by LMP1 in cells lacking the corresponding *traf* gene or multiple *traf* genes. For example recent evidence indicates that there is redundancy in the role of TRAF2 and TRAF5 in NF-κB activation by TNF (70).

CTAR2 is the second NF-κB-activating domain of the LMP1 CCT and spans amino acids 352 to 386. The core NF-κB-activating element of CTAR1 consists of amino acids P379, V380, Q381, S383 and Y384 (37, 71, 72). CTAR2 does not appear to interact directly with TRAF proteins. Instead CTAR2 interacts with TRADD, another mediator of NF-κB activation that is also utilized by TNFRI (37). TRADD is a 312 amino acid protein that contains a carboxyl terminal 111-amino acid "death domain" which is responsible for interaction with TNFRI, oligomerization, induction of apoptosis and NF-κB activation (73). The amino terminal 169 amino acids of TRADD can bind to TRAF2 (74). NF-κB activation by TRADD is mediated by TRAF2 since a dominant negative mutant of TRAF2 inhibits NF-κB activation by TRADD (74). TRADD has been implicated in CTAR2-mediated NF-κB activation because it interacts directly with CTAR2 and synergizes with CTAR2 towards NF-κB activation (37). Furthermore truncated TRADD proteins consisting of amino acids 122-293 or 1-194 inhibit LMP1-mediated NF-κB activation presumably acting as dominant negative mutants (75, 76). The immediate downstream effector of NF-κB activation by CTAR2 and TRADD appears to be TRAF2 since a dominant negative TRAF2 mutant can inhibit NF-κB activation by CTAR2 (68). Surprisingly TRADD does not induce apoptosis upon binding to LMP1 as it does when it is recruited to TNFRI highlighting a fundamental difference between the two cell surface receptors (75). It is conceivable that the conformation or posttranslational modification of TRADD in the LMP1-TRADD complex does not allow efficient recruitment of downstream effectors of apoptosis such as FADD and caspases. Alternatively a cofactor that is critical for TRADD-mediated apoptosis may not be present in the LMP1-TRADD complex. It is also possible that LMP1 recruits an inhibitor of the apoptotic pathway or induces a powerful antiapoptotic process. The latter may be dependent on strong activation of NF-κB. CTAR2 interacts also with RIP, a "death domain"-containing protein kinase, which is also recruited to TNFRI and is essential for NF-κB activation by TNFRI and apoptosis mediated by TNFRI and TNFRII (75, 77-79). However the role of RIP in LMP1 signaling remains unclear since NF-κB activation by LMP1 does not require the presence of RIP as it has been determined in a mutated Jurkat cell line which lacks RIP (75).

Induction of NF-κB activity by LMP1 depends on the activation of the IκB kinase (IKK) complex which phosphorylates the inhibitor of NF-κB, IκB, leading to its ubiquitination and degradation (reviewed in (80)). Upon degradation of IκB, heterodimers of NF-κB subunits are released from IκB and translocate from the cytoplasm into the nucleus where they bind and regulate responsive promoters. The IKK complex consists of two catalytic subunits (IKKα and IKKβ) and a non-catalytic subunit (NEMO/IKK?), which is essential for the assembly of a functional IKK complex. Kinase inactive, dominant negative mutants of IKKα or IKKβ inhibit NF-κB activation by LMP1 suggesting an involvement of the IKK complex in NF-κB activation by LMP1 (81). Dominant negative mutants of IKKα or IKKβ inhibit NF-κB activation by both CTAR1 and CTAR2 indicating that the two signaling domains of LMP1 transmit signals that converge on the IKK complex (81). Since TRAF2 appears to be a common mediator of NF-κB activation by CTAR1 and CTAR2 it is conceivable that signal transduction by these domains is identical downstream of TRAF2. The biochemical steps that mediate IKK activation following TRAF and TRADD recruitment to LMP1 remain unclear. A MAP kinase kinase kinase (MAP3K) known as NIK (NF-κB inducing kinase) appears to be involved in NF-κB activation downstream of TRAF2 by certain TNFRs such

as LTβR (82). NIK can phosphorylate IKKα on serine 176 located on its activation loop and may also phosphorylate IKKβ (83-85). A catalytically inactive dominant negative mutant of NIK (NIK K429A/K430A) inhibits NF-κB activation by both CTAR1 and CTAR2 (81). NF-κB activation by LMP1 is also inhibited by a dominant negative mutant of NIK consisting of its carboxyl terminal 322 amino acids (NIK624-947) but not a mutated form of NIK624-947 (*aly* NIK624-947), which contains a mutation (*aly*) that inactivates NIK (86). The *aly* mutation abolishes the binding of NIK to IKKα whereas it has only a marginal effect on the association of NIK with IKKβ. These findings implicate NIK or a related MAP3K in NF-κB activation by LMP1 and highlight an important role for IKKα in LMP1-mediated NF-κB activation.

An important issue that needs to be elucidated is the biochemical role of TRAFs in the signaling process that leads to NF-κB activation by LMP1. TRAFs could play the role of a scaffold or adaptor molecule that allows the assembly of a multisubunit-signaling complex ("signalsome") that includes the IKKs. Indeed TRAF2 is one of the components of the "signalsome" that is recruited to TNFRI following ligand binding and includes IKKα, IKKβ, NEMO/IKK? and RIP (87-89). TRAF2 interacts with IKKα and IKKβ? through its RING-finger domain. The complex of TRAF2 with IKKα, IKKβ and NEMO/IKK? is stabilized by RIP. It is not known whether the IKK complex is recruited to LMP1. In addition to their potential role as building blocks of the signalsome, TRAF2 and TRAF6 also possess an enzymatic activity that has been implicated in NF-κB activation. TRAF2 and TRAF6 are components of ubiquitin-ligase complexes (E3s) which act in concert with Ubc13 and Uev1A (E2) to catalyze protein polyubiquitination (90). Addition of ubiquitin to proteins occurs through the coordinated activity of three enzymes, a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligating enzyme which attaches ubiquitin moieties to lysine residues on the substrate. The ubiquitinating activity of TRAF2 and TRAF6 depends on the integrity of the RING finger domain and a dominant negative mutant of Ubc13 blocks NF-κB activation by TRAF2 and TRAF6. TRAF2 and TRAF6-mediated polyubiquitination differs from the typical polyubiquitination reaction that leads to protein degradation by the fact that ubiquitin moieties are connected through lysine 63 in polyubiquitin chains formed by TRAFs whereas in polyubiquitin associated with degradation ubiquitin branching typically occurs at lysine 48. Furthermore TRAF degradation does not appear to be essential for IKK activation by TRAFs. TRAF2 and TRAF6 are likely to be the actual targets of their ubiquitinating activity. Existing evidence suggests that autoubiquitination of TRAF2 or TRAF6 activates downstream kinases such as TAK1, which subsequently activate IKK directly or indirectly (91). TRAF autoubiquitination may create novel protein binding sites that recruit downstream kinases or mediate activation of an associated kinase through conformational changes. Currently it is not known whether TRAF2-associated ubiquitin ligase activity is essential for LMP1-mediated NF-κB activation and it is unclear how the ubiquitinating activity of TRAF2 is regulated. Nevertheless it has been shown that oligomerization of TRAF6 triggers its autoubiquitination (91). Therefore it is conceivable that constitutive oligomerization of LMP1 results in constitutive oligomerization and activation of the TRAF2-associated ubiquitin ligase and propagation of an IKK and NF-κB activating signal.

Activation of NF-κB by LMP1 has pleiotropic effects on the EBV-infected cells. NF-κB mediates directly or indirectly the upregulation of a number of LMP1-induced genes including those encoding for ICAM-1, LFA-3, A20, Bfl1, Bcl2, CD40, IL-6, Fas, TRAF1, EBI3 and cyclooxygenase-2 (20, 69, 92-94). These proteins are involved in cell adhesion (ICAM-1, LFA-3), prevention of apoptosis (A20, CD40, TRAF1), epithelial cell growth (IL-6) possible restriction of NK and CTL responses to EBV infection (EBI3), self-limitation of infected-B-lymphocyte expansion to avoid immune surveillance (Fas) and angiogenesis (cyclooxygenase-2). Most importantly, NF-κB plays an essential role in the prevention of apoptosis of EBV-transformed B-lymphocytes and in fibroblast transformation. Inhibition of NF-κB through overexpression of IκB leads to programmed cell death of EBV-transformed LCLs and suppresses transformation and tumorigenicity of LMP1-expressing Rat1 fibroblasts (92, 93, 95).

5. THE AP1 PATHWAY

AP1 consists of homodimers or heterodimers of transcription factors that belong to the Jun, Fos, Jun dimerization partners, ATF and Maf families (reviewed in (96)). It plays a critical role in promoting cell growth since multiple growth factors and oncoproteins induce its activity. Furthermore it was recognized early on that certain AP1 subunits such as Jun and Fos members represent cellular protooncogene products. The pleiotropic association of AP1 activity with cell growth and tumorigenesis and its induction by TNF prompted an investigation on the relationship of LMP1 signaling with AP1 (42, 43, 97). LMP1 expression induces AP1 activity at two levels. It increases the DNA binding activity of AP1 and enhances its transactivation potential. CJun and ATF2 have been identified as components of the AP1 DNA-binding activity that is induced by LMP1 in epithelial cells (44). Furthermore LMP1 induces the transactivation potential of cJun presumably through phosphorylation of its amino terminal-transactivating domain. The activation of AP1 is likely to be a direct effect of the LMP1 signaling process. This has been demonstrated by crosslinking of a chimeric molecule (CD2LMP1) consisting of the extracellular and transmembrane domains of CD2 fused to the LMP1 CCT. Crosslinking of the CD2LMP1 molecule by an antibody that recognizes the extracellular domain of CD2 activates the LMP1 signaling pathway and induces JNK1 activation within 30 min (72).

AP1 activity is induced by CTAR1 and CTAR2 (42-44). CTAR2 induces AP1 through the sequential activation of protein kinases SEK1 and JNK1 (42, 43). The

core sequence that mediates JNK1 activation by CTAR2 overlaps with the NF-κB-inducing element and consists of P379, Q381, S383 and Y384 (72, 76). JNK1 is capable of phosphorylating and activating the transactivation domains of cJun and ATF2 (96). CJun-ATF2 heterodimers may also induce the expression of *cjun* through AP1-responsive elements in its promoter (96). JNK1 activation by CTAR2 appears to be mediated at least in part by TRADD and TRAF2 (72). Low levels of TRADD expression augment JNK1 activation by LMP1 and a dominant negative TRAF2 mutant partially inhibits JNK1 activation by LMP1. CTAR1 does not activate JNK1. However both CTAR1 and CTAR2 activate kinase p38, which is another mediator of AP1 activity (44). P38 can activate AP1 through transactivation of the *cfos* and *cjun* promoters and increase in the levels of the corresponding proteins (96). Activation of p38 appears to be mediated by TRAF2 since a dominant negative mutant of TRAF2 inhibits LMP1-induced activation of p38 (44). Despite the involvement of common LMP1 domains and effectors in AP1 and NF-κB activation the two pathways are distinct and most likely diverge downstream of TRAF2. This is supported by the fact that a dominant negative mutant of NIK inhibits NF-κB activation but does not affect JNK1 activation by LMP1 (72). Furthermore addition of SB203580, a specific inhibitor of p38 does not affect NF-κB activation by LMP1 (44).

AP1 activation mediates upregulation of IL10, IL6 and IL8 by LMP1 (44, 98). IL10 suppresses macrophage and T cell effector functions by inhibiting cytokine synthesis and may promote B cell growth. IL6 and IL8 are involved in inflammatory responses which may facilitate the growth of EBV infected cells. IL8 is also involved in the promotion of angiogenesis, which could play a favorable role in the growth of EBV associated tumors.

6. THE JAK/STAT PATHWAY

A third signaling pathway that is activated by LMP1 leads to activation of STAT (signal transducers and activators of transcription) transcription factors. STATs mediate pleiotropic effects by cytokines and growth factors including prevention or promotion of apoptosis, induction of DNA synthesis and upregulation of protooncogenes (reviewed in (99, 100)). Activation of STATs occurs by phosphorylation on tyrosine residues mediated by members of the JAK (Janus kinase) tyrosine kinase family, growth factor receptor tyrosine kinases or non-receptor tyrosine kinases. JAKs are associated with cytokine receptor cytoplasmic tails and upon receptor oligomerization they are activated by autophosphorylation. Subsequently JAKs phsphorylate the STATs on tyrosine residues. Tyrosine phosphorylation of STATs leads to their homo or heterodimerization and translocation from the cytoplasm to the nucleus where they activate transcription. Constitutive STAT activation has been associated with tumorigenesis. The identification of two putative JAK binding sites in the LMP1 CCT and the association of a JAK/STAT pathway with CD40, a TNFR family member, prompted an investigation into a potential relationship between LMP1 and JAK/STAT-mediated signal transduction (45). Indeed expression of LMP1 in B-lymphocytes leads to activation of JAK3 followed by STAT1 and STAT3 activation. JAK3 interacts with CTAR3 (amino acids 275-307), which is located between CTAR1 and CTAR2. Deletion of CTAR3 prevents activation of JAK3 by LMP1 demonstrating an essential role of this domain in LMP1-mediated JAK/STAT activation. However in addition to CTAR3 an essential role of CTAR1 and CTAR2 in JAK/STAT activation by LMP1 was revealed by the inability of LMP1 with mutated CTAR1 and CTAR2 to activate the JAK/STAT pathway (101). The role of the JAK/STAT pathway in the process of EBV-mediated transformation remains questionable since deletion of CTAR3 does not affect B lymphocyte transformation by LMP1 in vitro (46). Furthermore EBV can transform in vitro B-lymphocytes from patients with defective JAK3 (102, 103). Nevertheless an important role of the JAK/STAT pathway in the oncogenic activity of EBV in vivo cannot be excluded.

7. LMP1 IS A VIRAL PSEUDORECEPTOR OF THE TNFR SUPERFAMILY

The identification of TRAFs and TRADD as mediators of LMP1 signaling provided a clear link between LMP1 and TNFR signal transduction mechanisms and established the basis for the model of a constitutively active pseudoreceptor for LMP1. The amino terminus and the transmembrane domains of LMP1 provide a ligand-independent oligomerization activity. The LMP1 CCT is functionally similar to the cytoplasmic tail of certain TNFRs. This model is supported by a number of experimental approaches. Replacement of the LMP1 CCT with the cytoplasmic tail of CD40 or TNFRII results in chimeric molecules that behave as constitutively active CD40 or TNFRII receptors (97, 104). In addition, fusion of the LMP1 CCT to the extracellular and transmembrane domains of CD2, CD4 or the p75 nerve growth factor receptor results in molecules that mediate LMP1 signaling upon agonist-dependent crosslinking (71, 104).

LMP1 resembles particularly an activated CD40 receptor molecule. CD40 is a TNFR family member that plays a critical role in B cell growth and differentiation (105). LMP1 expression and activation of CD40 cause a number of common phenotypic alterations in B lymphocytes including the induction of the same activation markers (ICAM1, LFA1, CD23 and Fas), secretion of immunoglobulin and IL6, introduction of resting B cells into the cell cycle and prevention of apoptosis (15, 16, 20, 105-110). Furthermore CD40 activation can partially compensate for the lack of LMP1 expression in the maintenance of proliferation of EBV-transformed LCLs (111, 112). At the molecular level LMP1 and CD40 activate common signaling pathways leading to activation of NF-κB, AP1 and JAK/STAT. CD40 is upregulated in a number of human malignancies yet it has not been proven that constitutive CD40 signaling is oncogenic. It should be noted also that despite a number of functional similarities between LMP1 and CD40 the two proteins have significant differences. For example LMP1 expression in B-lymphocytes of transgenic mice suppresses the formation

of germinal centers in lymph nodes whereas CD40 is required for the formation of these structures (110). Furthermore differences in NF-κB activation and induction of ICAM1 in response to LMP1 expression or CD40 activation were noted in certain Burkitt lymphoma cell lines (113). At the molecular level, activation of CD40 leads to degradation of TRAF2 and TRAF3 following their recruitment to CD40 whereas recruitment of TRAFs to LMP1 does not result in their degradation (32). The reason for this difference is not clear but it may explain a more potent signaling process elicited by LMP1 in comparison to CD40 (31). Despite these differences the usurpation of CD40-associated growth and antiapoptotic functional properties by LMP1 offers an attractive framework to built on and improve further our understanding of the oncogenic activity of LMP1.

8. PERSPECTIVES

Significant progress in the understanding of LMP1 function has been made over the past six years. The association of LMP1 with CD40 and other TNFRs at the cellular and molecular levels has provided satisfactory explanations for many functional properties of LMP1. However we are clearly far from a comprehensive understanding of the interaction of LMP1 with cellular survival and growth control mechanisms. Future work must elucidate further the biochemical events that constitute the signaling pathways of LMP1. In particular it will be necessary to identify the steps that mediate activation of IKKs, JNK and p38 following TRAF and TRADD recruitment to LMP1. Gene targeting experiments in the mouse should clarify the role of all the signaling molecules identified so far as players in LMP1 signal transduction. Certain experiments have provided evidence for unknown signaling pathways that must be engaged by LMP1. These pathways must be identified and their involvement in LMP1-mediated transformation should be determined. Finally a systematic and comprehensive analysis of LMP1-induced changes in gene expression must be undertaken using microarray-based methods. These approaches coupled with gene-knock out experiments will identify genes that are targeted by LMP1 and contribute to the establishment of the transformed phenotype.

9. ACKNOWLEDGMENTS

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