Latent Membrane Protein 1 of Epstein-Barr Virus Stimulates Processing of NF-κB2 p100 to p52*

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Epstein-Barr virus (EBV) is a ubiquitous human herpes virus that infects B-lymphocytes and certain epithelial cells (1–9). EBV is the causative agent of infectious mononucleosis and is also implicated in the etiology of both epithelial (nasopharyngeal carcinoma) and lymphoid malignancies (Burkitt’s lymphoma and Hodgkin’s disease). Infection of primary human B lymphocytes in vitro with EBV leads to their immortalization and the establishment of lymphoblastoid cell lines (LCLs). Latent membrane protein (LMP) 1 is one of five latent genes shown to be essential for EBV-induced transformation of B cells (4). LMP1 is required for both LCL establishment and continued proliferation (4–6). The transforming potential of LMP1 has been confirmed both in vitro in fibroblast transformation assays (7) and in vivo in transgenic mice (8). Significantly, LMP1 is expressed in the majority of EBV-associated human malignancies (1).

LMP1 is an integral membrane protein with six hydrophobic transmembrane domains that mediate its constitutive oligomerization and targeting to plasma membrane lipid rafts (9). Constitutive oligomerization allows LMP1 to function as a ligand-independent receptor and is essential for its transforming potential in both fibroblasts and B cells. The 200-amino acid cytoplasmic C terminus of LMP1 is also required for cell transformation (10). This contains two subdomains, termed C-terminal-activating regions (CTARs) 1 and 2, which act as docking sites for complexes of signaling proteins that trigger activation of the transcription factors NF-κB and activator protein-1 (10). NF-κB activation is absolutely required to block apoptosis of EBV-transformed LCLs (11) and is also required for LMP1 transformation of fibroblasts (12).

Mammalian cells express five NF-κB proteins: RelA, RelB, c-Rel, NF-κB1 p50, and NF-κB2 p52, which combine to form homo- and heterodimers that regulate genes involved in immune responses, apoptosis, and development (13). NF-κB dimers are retained in the cytoplasm of unstimulated cells by interaction with a family of inhibitory proteins (IκBα), which includes IκBα. Activation of the canonical NF-κB-signaling pathway by agonists such as tumor necrosis factor (TNF) α and interleukin 1 induces IκBα phosphorylation, ubiquitination, and subsequent proteolysis by the proteasome. NF-κB dimers, which are predominantly p50-RelA heterodimers, are thereby released to translocate into the nucleus and modulate gene expression. Signal-induced phosphorylation of IκBα is mediated by the IκB kinase (IKK) complex, which comprises two catalytic subunits, IKK1 (Iκκα) and IKK2 (Iκκβ), and a structural subunit NEMO (IKKγ) (14, 15). Genetic studies indicate that IKK2 is essential for IκBα phosphorylation triggered by TNFα or interleukin 1, whereas IKK1 is largely dispensable (15).

Recently, an “alternative” NF-κB activation pathway has been described (16). This pathway triggers proteasome-mediated processing of the NF-κB2 precursor p100 to produce p52. In contrast to the canonical NF-κB pathway, which is triggered by multiple different stimuli (15), only three cellular receptors have so far been shown to stimulate p100 processing; namely, B cell-activating factor receptor (17, 18), lymphotoxin β receptor (LTβ-R), and CD40 (19). Receptor activation of this pathway is critically dependent on the mitogen-activated protein 3-kinase NIK and IKK1 (21, 22).

Functionally, LMP1 resembles an activated CD40 receptor, with both molecules promoting B cell survival and proliferation.
and regulating a highly overlapping spectrum of activation markers (23). Indeed, activation of CD40 can compensate for the lack of LMP1 expression and promote short term growth of EBV-transformed LCLs (6, 24). It is firmly established that LMP1 expresses the canonical NF-κB pathway that regulates IkBα proteolysis (9). In the present study evidence is presented that in B cells and 293 epithelial cells LMP1 also induces NF-κB p100 processing to p52, similar to CD40 (20). The potential importance of the alternative NF-κB signaling pathway for cell transformation by LMP1 is discussed.

MATERIALS AND METHODS

cDNA Constructs, Antibodies, and Reagents—The following plasmids have been described previously: pSG5-CD2 (tCD2, extracellular and transmembrane domains of rat CD2), pSG5-CD2-LMP1 (25), pCDNA3-Oct1, pCDNA3-p100N, pCDNA3-Myc-p100SS/AA (residues 346–377 deleted), and pCDNA3-Myc-NIKSLN (residues 624–947 of NIK) (20). The tetracycline-regulated tEF vector constructs encoding EBNA1, EBNA2, LMP1 (26), and EBNA LP (27) have been described elsewhere. The following plasmids were generous gifts from the originating laboratories: pSV-LMP1, pSV-LMP1_TAD1, pSV-LMP1 AAA (residues P245A,Q260A,T280A), pSV-LMP1 ΔAAA (25), pCMV HA-ubiquitin (29), pCR-3-FLAG IKK1/S32A,S36A (IKK1-dn), pcDNA3-FLAG IKK2/L33A (IKK2-dn) (31), pSG5-Tax (32). pSG5-Tax (32).

Preparation of cytoplasmic and nuclear fractions was performed as previously described (23), which was supplemented with 0.5% deoxycholate and 0.1% CHAPS (RIPA buffer). p100 ubiquitination experiments were performed as previously described (20). Equal protein loading of whole cell lysates was confirmed by Western blotting for tubulin.

Preparation of cytoplasmic and nuclear fractions was performed as described (44). Fractionation efficiency and protein loading were controlled by Western blotting for cytoplasmic (tubulin) and nuclear (SAM68) markers. To facilitate NF-κB subunit detection in Western blots, ~5-fold more cell equivalents of nuclear extract (10–25 μg) was loaded relative to cytoplasmic extract (30 μg). For immunoprecipitation experiments, nuclear and cytoplasmic extracts were diluted in buffer A (43) and adjusted to 10% (v/v) glycerol and 150 mM NaCl before incubation with anti-p100N antibody.

RESULTS

LMP1 Induces NF-κB2 p52 Production in Transfected 293 Epithelial Cells—It has previously been reported that LMP1 expression in epithelial cells is associated with increases in p100 and p52 levels (45). However, it is unclear from this study whether other EBV-encoded latent proteins can also induce such changes. To address this question, LMP1, LMP2A, EBNA1, EBNA2, EBNA LP (Fig. 1A) and EBNA 3C (data not shown) were individually expressed in 293 epithelial cells. Western blotting of cell lysates indicated that none of the EBNA proteins or LMP2A had any effect on endogenous p100/p52 levels compared with empty vector (EV) control. In contrast, LMP1 expression stimulated a dramatic increase in steady state levels of p52 together with a more modest increase in p100. Therefore, of the panel of latent proteins tested, LMP1 was unique in its ability to increase p52 levels.

Previous studies have indicated that signaling from the LMP1 cytoplasmic tail can be induced by cross-linking of chimeric molecules comprising tCD2 fused to the C-terminal 192 amino acids of LMP1 (CD2-LMP1-192; Ref. 25). CD2-LMP1-192, transiently expressed in 293 cells, induced an increase in endogenous p52 levels after 3–6 h of extensive cross-linking with anti-CD2 mAb, whereas p100 levels were largely unaffected (Fig. 1B). The time course of the p52 increase was similar to that previously observed with cross-linked CD40 (20) and was markedly slower than IkBα proteolysis induced by CD2-LMP1-192, which was detectable at 30 min. (Fig. 1B). Cross-linked tCD2 failed to induce any alteration in p52 levels, whereas tCD2 fused to full-length wild type LMP1 (CD2-LMP1), which signals constitutively (25), increased p52 levels without anti-CD2 mAb cross-linking. These data suggest that stimulation of p52 levels by the LMP1 requires aggregation of its cytoplasmic tail.

LMP1 Induces p52 as a Consequence of Proteasome-mediated p100 Proteolysis—To determine whether NF-κB-dependent gene expression is required for LMP1-induced p52 production, 293 cells were co-transfected with LMP1 vector together with an expression vector encoding a super-repressor mutant of IkBα (IxBαSS/AA), which blocks NF-κB activation (31, or EV). Expression of IxBαSS/AA on its own reduced the basal levels of endogenous p100 expression compared with EV alone and blocked the ability of LMP1 to increase the steady state levels of p100. However, LMP1 still clearly induced p52 levels when co-expressed with IxBαSS/AA while concomitantly reducing levels of p100 (Fig. 2A). Thus, LMP1 induces p52 production independently of NF-κB activity.

To investigate the role of the proteasome in LMP1-mediated p52 production, 293 cells were transfected with a vector encoding CD2-LMP1-192 to facilitate analysis of the acute up-regulation of p52 by LMP1. Increases in p52 induced by cross-linked CD2-LMP1-192 were blocked by pretreatment of cells with the proteasome inhibitors MG132 (Fig. 2B) or N-acetyl-leu-leu-norleucinal (data not shown), demonstrating a requirement for proteasome activity for LMP1 induction of p52.

Protein ubiquitination is often a prerequisite for proteasome action. Therefore, experiments were conducted to determine...
whether LMP1 induced the ubiquitination of p100. 293 cells were co-transfected with plasmids encoding Myc-p100 and HA-ubiquitin together with LMP1 or EV. LMP1-induced polyubiquitination of Myc-p100 was clearly demonstrated by the appearance of high molecular weight bands in Western blots of immunoprecipitated p100 probed for HA-ubiquitin (Fig. 2C). NIK expression also induced Myc-p100 ubiquitination (Fig. 2C), as reported previously (22). Thus, proteasome-mediated production of p52 induced by LMP1 involves p100 ubiquitination. Furthermore, the observation that LMP induces p52 levels concomitantly with decreases in p100 levels when NF-κB activity is blocked (Fig. 2A) strongly suggests that LMP1 induces p52 production via proteasome-mediated proteolysis of p100.

Two motifs in p100 have been previously shown to be required for CD40-induced processing of p100 to p52 (20); namely the glycine-rich region (GRR; residues 346–377) (46) and two serines (Ser-866 and Ser-870) in the C-terminal PEST region of p100 that are thought to be phosphorylated by IKK1 (21). To investigate whether the GRR and serines 866/870 are important in LMP1-triggered p52 production, 293 cells were co-transfected with plasmids encoding LMP1 or EV and either wild type Myc-p100, Myc-p100<sub>GRR</sub>, or Myc-p100<sub>S866A,S870A</sub>. LMP1 co-transfection with wild type Myc-p100 significantly increased Myc-p52 levels compared with EV control (Fig. 2D). However, LMP1 failed to induce Myc-p52 from either Myc-p100<sub>GRR</sub> or Myc-p100<sub>S866A,S870A</sub> (Fig. 2D). Therefore, both the GRR and serines 866 and 870 in the PEST region of p100 are required for LMP1-induced p52 production from p100. These results further support the conclusion that LMP1 induces p52 as a consequence of p100 processing.

**LMP1 Induction of p52 Requires Protein Synthesis**—Both CD40 (20) and B cell-activating factor receptor (17) have a requirement for de novo protein synthesis to trigger p100 processing. The requirement for protein synthesis in CD2-LMP1 192-induced p52 production, which occurs over several hours (Fig. 1B), was therefore investigated. 293 cells were transfected with an expression vector encoding CD2-LMP 192 and cycloheximide was added 30 min before CD2-LMP 192 cross-linking with anti-CD2 mAb. Cycloheximide treatment inhibited p52 production induced by cross-linked CD2-LMP 192 (Fig. 2B). Cycloheximide also blocked CD2-LMP 192 induction of p52 when added simultaneously with anti-CD2 mAb but not when added 2 h after CD2 cross-linking (data not shown). These results, which show a striking similarity to those reported for CD40 (20), demonstrate that de novo protein synthesis is required during the first 2 h of cross-linked CD2-LMP 192 signaling to induce p100 processing efficiently.

**LMP1 Induces the Nuclear Translocation of p52 and RelB in 293 Epithelial Cells—**p100 is the major IκB for RelB in the cell (47). Therefore, one predicted outcome of LMP1-mediated p100 processing is the nuclear translocation of p52/RelB complexes. To initially investigate this, cytoplasmic/nuclear fractions were prepared from 293 cells transfected with expression vectors encoding CD2-LMP 192 or CD2, stimulated with anti-CD2 mAb, or left unstimulated. Efficient separation of the cytoplasmic (tubulin) and nuclear (SAM68) markers. In unstimulated or control tCD2-transfected cells, very little endogenous p52 or RelB was detected in the nuclear fraction (Fig. 3A). Cross-linking of CD2-LMP 192 for 30 min resulted in a small increase in nuclear p52 but had no effect on nuclear RelB levels. This small increase in nuclear p52 at 30 min is likely to be due to the release of pre-existing p52 bound to small IκBs, including IκBα (Fig. 1B; Ref. 20). However, marked increases in both nuclear p52 and RelB were detected after 6 h of cross-linking of CD2-LMP 192, although cytoplasmic RelB levels were unaffected. Moreover, immunoprecipitation of nuclear extracts with antip100N antibody demonstrated that the translocated nuclear p52 and RelB are associated with one another (Fig. 3B). These experiments indicate that cross-linked CD2-LMP 192 stimu-
NF-κB de novo activity and de novo protein synthesis but is independent of NF-κB activity. A, 293 cells were transfected with 200 ng of vectors encoding LMP1 or EV together with 800 ng of plasmid encoding IxBαR00A, or EV. After 48 h cells were lysed in RIPA buffer and Western-blotted. IxBαR00A was expressed at a similar level in all transfections as determined by Western blot analysis (data not shown). B, 293 cells were transfected with CD2-LMP1 192 vector (200 ng). Cells were cultured for 42 h, preincubated for 20 min with MG132, cycloheximide (CHX), or vehicle control, and then stimulated for the indicated times with cross-linked anti-CD2 mAb (αCD2/GaM). Western blots of RIPA cell lysates were probed as shown. C, 293 cells were co-transfected as indicated with 500 ng of vector encoding Myc-p100 or EV control (−) together with 1000 ng of plasmid encoding HA-ubiquitin (HA-Ubi) or EV (−) and 500 ng of NIK or LMP1 plasmid or EV control. After 24 h culture, cells were incubated for 3 h with MG132 (50 μM) and lysed in buffer A, and p100 was immunoprecipitated. Immunoprecipitates (Ip) were resolved on a 6% SDS-polyacrylamide gel and Western-blotted. D, 293 cells were co-transfected with 200 ng of plasmid encoding LMP1 or EV together with 200 ng of plasmids encoding Myc-p100 (WT), Myc-p100GRR (ΔGRR), or Myc-p100SSR63A,SS70A (SS/AA). After 48 h cells were lysed in RIPA buffer and Western-blotted. NS, non-specific.

lates p100 processing, resulting in the release of RelB, which translocates to the nucleus in a complex with p52.

Importantly, expression of wild type LMP1 in 293 cells also resulted in nuclear translocation of both p52 and RelB (Fig. 3C). Unlike the CD2-LMP192 construct, wild type LMP1 caused a concomitant increase in the steady state levels of cytoplasmic RelB (Fig. 3C). However, p100 depletion experiments using a p100 C-terminal directed antiserum indicated that essentially all the cytoplasmic RelB was associated with p100 in LMP1-transfected 293 cells (Fig. 3D). This strongly suggests that LMP1 promotes RelB nuclear translocation as a result of p100 processing rather than simply through an increase in steady state levels of RelB protein.

EMSA demonstrated that LMP1 expression induced marked activation of NF-κB binding activity, which was of a similar magnitude to that detected in cells stimulated for 20 min with TNFα (Fig. 4A). Supershift EMSA analyses indicated that the majority of DNA binding activity induced by LMP1 comprised p50 and p65 NF-κB subunits (Fig. 4B, left panel). However, a p52-containing NF-κB complex was also clearly detectable in LMP1-transfected cells (Fig. 4B, right panel). Thus, LMP1-induced nuclear p52 can bind to specific xB sites in target DNA.

LMP1 Induces Nuclear Translocation of p52 and RelB in Ramos B Cells—Although EBV can infect epithelial cells, the major cell type targeted in EBV infection is the B cell (3). Furthermore, LMP1 plays an essential role in maintaining the transformed phenotype of EBV-infected B lymphoblastoid cells (48). It was, therefore, important to determine whether LMP1 could induce p100 processing to p52 in B cells as well as epithelial cells. To address this question, an EBV-negative Ramos B cell line was used that had been stably transfected with a vector system which allows LMP1 expression to be inducibly regulated by tetracycline (Tet) (26). In this system, when Tet is removed from growth medium, the constitutively expressed tTA can bind to sites in the vector promoter, resulting in LMP1 gene transcription.

Removal of Tet from the growth medium resulted in a clear increase in steady state levels of LMP1 protein in whole cell
extracts from tTA-LMP1-transfected Ramos cells, which paralleled a dramatic increase in p52 levels (Fig. 5A). In contrast, no change in p52 levels was detected in control Ramos tTA cells upon removal of Tet. LMP1 expression also resulted in increased steady state levels of p100 in addition to increased p52 levels (Fig. 5A). Because transcription of p100 is an NF-κB-regulated event (49), increases in p100 presumably arise due to LMP1-induced NF-κB activation. Even in the presence of Tet, Ramos tTA-LMP1-transfected cells contained increased steady state levels of p100 and p52 compared with control Ramos tTA-transfected cells (Fig. 5A). This is probably due to low levels of LMP1 transcription in the absence of promoter-bound tTA.

To determine whether increased levels of p52 induced by LMP1 expression contribute to NF-κB activation, nuclear extracts were prepared from Ramos tTA-LMP1 B cells in the presence and absence of Tet. Nuclear p52 was barely detectable by Western blot analysis in the presence of Tet. However, upon LMP1 induction, nuclear p52 levels markedly increased (Fig. 5B). In control tTA cells, nuclear p52 was not detectable in the presence or absence of Tet (Fig. 5B). Supershift EMSAs confirmed that LMP1-induced p52 in Ramos tTA-LMP1 cells was able to bind to target DNA containing an NF-κB binding site (Fig. 5C). These p52-containing complexes only represented a small fraction of NF-κB binding activity induced, which was predominantly composed of p50 and RelA (data not shown).

In Ramos B cells, as in other cell types (47), p100 is the only cytoplasmic IκB that binds to RelB, as determined by co-immunoprecipitation experiments (data not shown). Therefore, any nuclear translocation of RelB upon NF-κB activation is likely to arise from p100 processing. Blotting nuclear extracts from Ramos tTA-LMP cells revealed that RelB levels increased in parallel with p52 after LMP1 induction (Fig. 5B). Furthermore, immunoprecipitation of nuclear extracts with anti-p100N antibody clearly showed that nuclear RelB is associated with p52 (Fig. 5D). These data suggest that LMP1 expression induces p100 processing in Ramos B cells.
EBV Infection Results in Nuclear Translocation of p52-RelB Heterodimers—It was important to determine whether EBV infection of B cells, which involves expression of LMP1 (23), also induces p100 processing to p52. To investigate this question, cell extracts were prepared from BL41 Burkitt lymphoma cells and their B95.8 EBV-infected derivative, BL41+B95 (39). Western blotting demonstrated a clear increase in both p100 and p52 after EBV infection (Fig. 6A). Subcellular fractionation of the two cell lines revealed a dramatic increase in nuclear p52 induced by EBV infection (Fig. 6B). Immunoprecipitation of nuclear lysates with anti-p100N antibody confirmed that nuclear p52 was associated with RelB (Fig. 6C). These data suggest that the alternative NF-κB-signaling pathway which triggers p100 processing is activated as a consequence of EBV infection of B cells.

CTAR1 Is Required for LMP1 Stimulation of p100 Processing—Previous research has indicated that optimal induction of "global" NF-κB activity by LMP1 requires both CTAR1 and CTAR2, with CTAR2 having a dominant role (50–52). However, it is unclear to what extent CTAR1 and CTAR2 contribute to the activation of the canonical and alternative NF-κB pathways, which together generate the total NF-κB activity detected in EMSAs. To address this question, 293 epithelial cells were transfected with vectors encoding wild type or mutant versions of LMP1 with defective CTAR1 and/or CTAR2 signaling (Fig. 7, A–C). LMPAAA contains inactivating point mutations in CTAR1, LMPY384G contains an inactivating point mutation in CTAR2, and LMPAAA/Y384G is defective in signaling from both CTAR1 and CTAR2 (53, 54).

A Western blot assay for phosphorylation of IkBα on serine 32, which is directly phosphorylated by the IKK complex (15), was used as a readout for activation of the canonical NF-κB pathway. Wild type LMP1 expression resulted in robust IkBα phosphorylation of similar magnitude to that observed after 15 min of TNFα stimulation of EV-transfected cells (Fig. 7A). In contrast, expression of LMPY384G or LMPAAA/Y384G induced little detectable IkBα phosphorylation. LMPAAA was able to elicit marked IkBα phosphorylation, although not as great as with wild type LMP1 (Fig. 7A). Thus, activation of the canonical NF-κB pathway, which triggers IkBα phosphorylation, is primarily mediated via CTAR2.

The effect of each of the LMP1 mutants was also tested on activation of the alternative NF-κB pathway leading to p52 production. As noted previously (Figs. 1 and 2), wild type LMP1 expression increased levels of both p100 and p52 compared with EV (Fig. 7B). However, although expression of the LMPAAA increased p100 levels similar to wild type LMP1, this mutant had no effect on p52 levels. Conversely, LMPY384G expression induced steady state levels of p52 to a similar degree to wild type LMP1 but had little effect on p52 levels. In addition, LMPY384G and LMPAAA/Y384G induced the nuclear translocation of p52 and RelB (Fig. 7C). In contrast, LMPAAA, which can efficiently activate the canonical NF-κB pathway (Fig. 7A), did not induce increases in the cytoplasmic levels of p100 and RelB without coordinately stimulating their nuclear translocation (Fig. 7C). This suggests that transcriptional activation of the NF-κB-sensitive genes RelB and p100 (47, 55), requires signals from CTAR2, which activates the canonical NF-κB pathway. Maximal activation of p100 processing, which results in nuclear translocation of p52 and RelB, in contrast requires signals from CTAR1.

LMP1-mediated p100 Processing Is Dependent on NIK and IKK1 but Independent of IKK2—The mitogen-activated protein 3-kinase NIK is required for p100 processing induced by LTβ-R, B cell-activating factor receptor, and CD40 (17, 19, 20) but not by the HTLV-transforming protein, Tax (56). To investigate whether LMP1-induced processing of p100 requires NIK, 293 epithelial cells were co-transfected with expression vectors encoding wild type LMP1 or Tax and the C-terminal portion of NIK (NIKΔN; residues 624–947), which functions as a potent inhibitor of wild type NIK (57), or EV. LMP1-mediated increases in p52 but not p100 were clearly inhibited with the co-expression of NIKΔN (Fig. 8A). In contrast, NIKΔN expression had little effect upon Tax-induced p100 processing, indicating that NIKΔN was acting in a specific manner (Fig. 8A).
These results suggest that LMP1 induces p100 processing via a NIK-dependent pathway.

To initially investigate the role of IKK1 and IKK2 in LMP1-induced p100 processing, the effect of dominant negative (dn) versions of each of these kinases was investigated in 293 cells. These both contain two serine to alanine mutations in the activation loops of their kinase domains (30). IKK1 dn markedly reduced the level of p52 detected in LMP1 α-expressing cells compared with EV (Fig. 8B). However, IKK1 dn had little effect on the LMP1-mediated increase in p100. Conversely, expression of IKK2 dn blocked the LMP1-mediated increase of p100 levels but had little effect on p52 up-regulation (Fig. 8B). These data suggest that IKK1 is required for LMP1 triggering of the alternative NF-κB pathway regulating p100 processing to p52, whereas IKK2 is required for transcriptional up-regulation of p100.

The role of IKK1 and IKK2 was further investigated using knockout MEFs lacking individually IKK1 (40) or IKK2 (41). LMP1 was transiently expressed in WT, IKK1−/− and IKK2−/− MEFs, and p100 processing was monitored by assaying the nuclear translocation of p52 and RelB. LMP1 expression increased cytoplasmic p100 and RelB levels in both WT and IKK1−/− MEFs but only marginally in IKK2−/− MEFs (Fig. 8C). Furthermore, LMP1 expression in WT and IKK2−/− resulted in clear nuclear translocation of p52 and RelB (Fig. 8C). However, in IKK1−/− MEFs, LMP1 expression failed to induce nuclear translocation of either of these Rel subunits. LMP1 expression was also shown to stimulate the p38 mitogen-activated protein kinase phosphorylation to a similar degree in each of the MEFs (Fig. 8C), confirming that the transfection efficiency of LMP1 was similar in each of the transfected cell lines. Thus, there is a specific requirement for IKK1 in LMP1-induced p100 processing and for IKK2 in LMP1-induced p100 and RelB up-regulation.

Taken together these results demonstrate that LMP1-induced p100 processing requires NIK and IKK1 but not IKK2. However, LMP1 induced up-regulation of the p100 and RelB, which are both NF-κB-sensitive genes (47, 55), requires IKK2 but not IKK1. These data are consistent with recent published studies on LTβ-R signaling (19), in which it has been shown that NIK and IKK1 are required for p100 processing to p52 induced by LTβ-R, whereas IKK2 is required for LTβ-R up-regulation p100 expression.

**DISCUSSION**

Previous studies using an IκBα super-repressor mutant indicate that NF-κB activation is critical for transformation of fibroblasts by LMP1 (12) and for survival of EBV-transformed LCLs (11). Although these experiments have firmly established an important role for NF-κB in cell transformation by EBV, the potential role of the alternative NF-κB pathway in EBV-mediated cell transformation is not known. In the present study, LMP1 is shown to stimulate p100 processing to p52. This results in the nuclear translocation of p52-RelB heterodimers in epithelial cells and B cells, which are two cell types targeted by EBV infection. Thus, LMP1 simultaneously triggers both canonical and alternative NF-κB pathways. Interestingly, Tax, the transforming protein of HTLV type I, also activates both canonical and alternative NF-κB pathways, therefore, may facilitate viral propagation.

The activation of p100 processing by LMP1 extends the number of signaling pathways that it activates in common with CD40 (20, 23). Indeed, it is likely that both LMP1 and CD40 trigger p100 processing via a similar mechanism. Thus, a TRAF binding site in the cytoplasmic tail of both proteins is required to induce p52, and both proteins require NIK activity to trigger this pathway (Figs. 7B and 8A; Ref 20). The present
study further demonstrates that LMP1-mediated processing is a delayed event (Fig. 1B) that requires de novo protein synthesis but is independent of NF-κB activation (Fig. 2, A and B), similar to CD40 (20).

Supershift EMSAs indicate that p52 containing NF-κB dimers make up only a minor component of total NF-κB DNA binding activity induced by either LMP1 or CD40 (Figs. 4B and 5C; Ref. 20). Nevertheless, analyses of NF-κB2 (60, 61) and RelB (62, 63) knockout mice reveal that p52 and RelB perform functions that cannot be compensated by other Rel subunits. Therefore, activation of the alternative NF-κB pathway by endogenous cell surface receptors is likely to be important for their functions (16). Consistent with this hypothesis, p100 processing has been implicated in B cell-activating factor receptor-mediated B cell development and survival (17, 18). Moreover, two subsets of NF-κB-regulated genes induced by LTβ-R have been described, one activated by the canonical pathway and the other by the alternative pathway (19). Notably, both RelB (64) and NF-κB2 (60) have been shown to be required for optimal CD40-induced B cell proliferation and are therefore, by analogy, most likely required for the proliferative signals generated by LMP1. Thus, activation of the alternative NF-κB pathway by LMP1 is likely to play an important role in its transforming function.

CTAR1 and CTAR2 have distinct functions in the activation of NF-κB by LMP1. Thus, CTAR1 has only a minor role in activating the canonical NF-κB pathway that controls IκBα phosphorylation (Fig. 7A) but is essential for maximal stimulation of p100 processing to p52 (Fig. 7B). In contrast, the majority of LMP1-triggered IκBα phosphorylation requires CTAR2 function, but this is dispensable for induction of p100 processing (Fig. 7, A and B). CTAR2 function is also required for LMP1-mediated increases in total protein levels of p100 and RelB. This suggests that activation of the canonical NF-κB pathway is responsible for transcriptional up-regulation of these genes but is not able to induce nuclear translocation of p52 and RelB, consistent with earlier studies on LTβ-R signaling (19). The two IKK subunits also have distinct functions in LMP-1 induction of NF-κB. Thus, IKK2 is required for up-regulation of p100 and RelB via the canonical NF-κB pathway, whereas IKK1 regulates the alternative pathway that triggers p52 production and p52/RelB nuclear translocation (Fig. 8, B and C). This implies that LMP1 CTAR1 predominantly triggers the IKK1-dependent alternative NF-κB pathway, whereas LMP1 CTAR2 triggers the IKK2-dependent canonical NF-κB pathway.

The critical role of NF-κB activation for EBV- or LMP1-mediated cell transformation is highlighted by studies showing that global inhibition of NF-κB activity triggers apoptosis of EBV-transformed LCLs (11) and blocks LMP1 transformation of Rat1 fibroblasts (12). However, the relative importance of the canonical and alternative pathways of NF-κB activation in EBV- and LMP1-induced transformation is not known. Nevertheless, it is intriguing that in EBV-infected B cells, CTAR1 is
required for the initial establishment of the transformed phenotype, whereas CTAR2 signals maintain long term cell proliferation (4, 65–67). It is possible, therefore, that the persistent activation by LMP1 CTAR1 of the alternative NF-κB pathway contributes to the transforming potential of this protein. Consistent with this hypothesis, p52 is generated constitutively in such lymphoma-associated NF-κB proteins can transform murine fibroblasts (69). The possible role of p100 processing in cell transformation (4, 65–67). It is possible, therefore, that the persistent activation by LMP1 CTAR1 of the alternative NF-κB pathway contributes to the transforming potential of this protein. Consistent with this hypothesis, p52 is generated constitutively in such lymphoma-associated NF-κB proteins can transform murine fibroblasts (69). The possible role of p100 processing in cell transformation by LMP1 is currently being investigated.

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