The C-terminal Activating Region 2 of the Epstein-Barr Virus-encoded Latent Membrane Protein 1 Activates NF-κB through TRAF6 and TAK1*

Received for publication, May 31, 2005, and in revised form, September 28, 2005 Published, JBC Papers in Press, November 8, 2005, DOI 10.1074/jbc.M505905200
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Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) is oncogenic and indispensable for EBV-mediated B cell transformation. LMP1 is capable of activating several intracellular signaling pathways including the NF-κB pathway, which contributes to the EBV-mediated cell transformation. Two regions in the cytoplasmic carboxyl tail of LMP1, namely C-terminal activating regions 1 and 2 (CTAR1 and CTAR2), are responsible for NF-κB activation, with CTAR2 being the main NF-κB activator. Although the CTAR1-mediated NF-κB activation was previously shown to be TRAF3-dependent, we showed here that the CTAR2-mediated NF-κB activation is mainly TRAF6-dependent but TRAF2/5-independent. In contrast to the interleukin-1 receptor/toll-like receptor-mediated NF-κB pathways, the CTAR2-mediated NF-κB pathway does not require MyD88, IRAK1, or IRAK4 for TRAF6 engagement. Furthermore, we showed that TAK1 is required for NF-κB activation by LMP1. Thus, LMP1 utilizes two distinct pathways to activate NF-κB: a major one through CTAR2/TRAF6/TAK1/IKKβ (canonical pathway) and a minor one through CTAR1/TRAF3/NIK/IKKα (noncanonical pathway).

Various mammalian cell viruses exert their cytotoxic effects by interfering with the function of specific host factors and the cellular signaling pathways associated with these host factors, eventually leading to abnormal cellular processes. Thus, elucidation of the molecular mechanisms by which viral proteins engage specific host factors and modulate cellular signaling pathways is the key to the understanding of viral-mediated diseases.

Epstein-Barr virus (EBV)† is known to be causally linked to a number of human malignancies including Burkitt’s lymphoma, Hodgkin disease, and nasopharyngeal carcinoma (NPC) (1). The incidence of NPC in southern China including Guangdong Province and Hong Kong is among the highest in the world (2). EBV mainly infects two cell types: peripheral B cells and nasopharyngeal epithelial cells (1, 3). Although it is difficult for EBV to infect nasopharyngeal epithelial cells in vitro, EBV readily infects resting human B cells in vitro and converts them to immortalized lymphoblastoid cell lines (1). Three forms of latency have been recognized for various EBV-associated diseases (1, 4). In latency I, as exemplified by endemic Burkitt’s lymphoma, only one EBV-encoded protein, EBNA1, is expressed. In latency II as exemplified by NPC, three EBV-encoded proteins, namely EBNA1, LMP1, and LMP2, are expressed. In latency III, as exemplified by lymphoblastoid cell lines and infectious mononucleosis, a total of nine EBV-encoded proteins are expressed.

Among all of the latent viral proteins expressed in the three different latencies, LMP1 is the only one known to be capable of transforming rodent fibroblasts in vitro (5). It is also indispensable for EBV-mediated B cell transformation (6). In addition, transgenic mice expressing LMP1 under a lymphocyte-specific promoter display a much higher incidence of lymphoma (7). LMP1 is a 386-amino acid membrane-localized viral protein with six transmembrane domains (Fig. 1A). Two subdomains in the 200-amino acid cytoplasmic carboxyl tail of LMP1, namely CTAR1 (amino acids 194–232) and CTAR2 (amino acids 351–386), are crucial for interacting with specific cellular factors and for engaging key cellular signaling pathways. Among them are the NF-κB pathway and two mitogen-activated protein kinase (MAPK) pathways (i.e. c-Jun N-terminal kinase (JNK) and p38 pathways) (8, 9). Although CTAR2 is solely responsible for the LMP1-induced JNK pathway, both CTAR1 and CTAR2 contribute to NF-κB activation, with CTAR2 accounting for 70% of the total NF-κB activity induced by LMP1 (8, 9).

NF-κB, a dimeric transcription factor, plays a key role in activation of certain viral genes during viral infection and in host immune response to various microbial infections (10–12). NF-κB is normally sequestered in cytosol by the IκB family proteins. In response to cytokine stimulation or microbial infection, IκB is first phosphorylated by the IκB kinase (IKK) complex, which consists of three core members IκKα, IκKβ, and IκKγ, and subsequently degraded in a ubiquitin/proteasome-dependent manner (10, 11). Two distinct NF-κB pathways have been recognized; one is the canonical pathway, which mainly utilizes the catalytic activity of IκKβ to phosphorylate IκBα and induces its degradation resulting in generation of free p50/p65 dimer and their translocation into the nucleus, and the other is the noncanonical (or alternative) pathway, which mainly involves IκKα leading to processing of p100 (i.e. NF-κB2) and generation of free p52/RelB dimer (10, 11, 13). Both IκKα and β need to be further phosphorylated by upstream kinases to achieve maximal activities. Based on genetic and biochemical evidence, NF-κB-inducing kinase (NIK), a member of the MAP3K superfamily, was found to function mainly in the noncanonical pathway (14), whereas a number

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*This work was supported by Hong Kong Research Grant Council Grants HKUST6129/04 M and HKUST3/03C and a 973 Key Project 2002CB513005 from the Ministry of Science and Technology of China (to Z. Wu). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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‡The abbreviations used are: EBV, Epstein-Barr virus; MEF, mouse embryonic fibroblast; TNF, tumor necrosis factor; LMP, latent membrane protein; CTAR, C-terminal activating region; NPC, nasopharyngeal carcinoma; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; IKK, IκB kinase; NIK, NF-κB-inducing kinase; MAP3K, MAPK kinase kinase; MEKK, MAPK/extracellular signal-regulated kinase kinase kinase; TRAF, TNF receptor-associated factor; IL, interleukin; IL-1R, IL-1 receptor; TLR, toll-like receptor; TRADD, TNF receptor-associated death domain; MyD88, myeloid differentiation factor 88; IRAK, IL-1R-associated kinase; GFP, green fluorescent protein; siRNA, small interfering RNA; WCE, whole cell extract(s); HA, hemagglutinin; GST, glutathione S-transferase.
of MAP3Ks including MEKK1, MEKK3, and TAK1, have been implicated in the canonical pathway (see “Discussion”).

A major breakthrough in our understanding of the molecular mechanisms underlying the LMP1-induced NF-κB activation came after the discovery that LMP1 specifically recruits tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) through a conserved TRAF-binding motif (i.e. PQQAT in CTAR1 (Fig. 1A) (15–17). The TRAF family proteins consist of six members (i.e. TRAF1–6) and mainly function as adaptors in various cytokine-mediated NF-κB and MAPK pathways (18, 19). Among them, TRAF2/5 mainly function in the TNFα-mediated NF-κB pathway, whereas TRAF6 preferentially acts in the interleukin-1β receptor (IL-1R)/toll-like receptor (TLR)-mediated NF-κB pathway (18, 19). Interestingly, different TRAFs are selectively recruited to distinct cytokine receptors through unique adaptor molecules. Whereas TNF receptor-associated death domain (TRADD) protein specifically recruits TRAF2/5 and receptor-interacting kinase 1 to TNF receptor, myeloid differentiation factor 88 (MyD88) selectively links IL-1R-associated kinases 1 and 4 (IRAK1/4) and TRAF6 to IL-1R/TLR (18–20). Recent studies by several groups showed that the CTAR1 domain of LMP1 activates NF-κB mainly through the noncanonical pathway (21–24). It remains unclear how CTAR2 activates NF-κB. Although several earlier reports suggested that CTAR2 utilizes TRADD and TRAF2 to activate NF-κB based on overexpression studies (25–27), a few recent reports raised doubts about their involvement (27–29).

We showed here that CTAR2 preferentially recruits TRAF6 and TAK1 to activate IKKβ and NF-κB. TRADD, MyD88, TRAF2/5, IRAK1/4, TAB2, and MEKK1 are not essential in the LMP1-mediated NF-κB pathway.

EXPERIMENTAL PROCEDURES

Cell Lines, DNA Constructs, and Reagents—HEK293, HEK293T, MEKK1−/−, TAB2−/−, TRAF6−/−, TRAF2/5−/−, IRAK4−/−, MyD88−/−, and the wild type MEF cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 unit/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in 5% CO2. LMP1(D335), LMP1(G335), HA-IKKβ, HA-TRAF6, and LMP1(ΔC8) were described previously (30, 31). LMP1(3A) mutant (i.e. Pro204Glu206Thr208 → Ala204Ala206Ala208) was generated by PCR-based mutagenesis using LMP1(D335) as the template. LMP1(3A/ΔC8) was generated from LMP1(3A) by deleting the last 8 amino acids at the C terminus. GFP-p65 was generated by inserting human p65 gene into pEGFP-C1 between XhoI and SmaI sites (a generous gift from G. Natoli). IL-1β and TNFα were purchased from R & D Systems. d(−)−Luciferin was purchased from Roche Applied Science. Puromycin, Polybrene, and tetracycline were purchased from Sigma.

Plasmid and siRNA Transfection—For plasmid transfection, either Lipofectamine Plus reagents (for HEK293 cells) or Lipofectamine 2000 (for MEFs) (Invitrogen) were used following the manufacturer’s instructions. For siRNA transfection, Lipofectamine 2000 was routinely used. In transfection experiments involving both siRNA and plasmids, the cells were first transfected twice with siRNA. 15 h later, the cells were harvested for transfection. For siRNA transfection, Lipofectamine Plus reagents (for HEK293 cells) or Lipofectamine 2000 (for MEFs) (Invitrogen) were used following the manufacturer’s instructions.
buffer and once with the kinase buffer (20 mM HEPES, pH 7.6, 20 mM dithiothreitol, and 50 mM sodium vanadate). The kinase reaction was reconstituted in 20 μl of the kinase buffer containing 1 μg of GST-IκB (1-54), 20 mM ATP, and 3 μCi of \([\gamma-32P]\)ATP (3,000 Ci/mmol) and incubated at 30 °C for 30 min. The reaction mixtures were separated by SDS-PAGE, and the protein bands were visualized by autoradiography.

**Generation of the Wild Type and IRAK4−/− MEFs Stably Expressing LMP1 by Retroviral Infection**—The full-length LMP1 cDNA fragment was reconstituted in 20 μl of the kinase buffer containing 1 μg of GST-IκB (1-54), 20 mM ATP, and 3 μCi of \([\gamma-32P]\)ATP (3,000 Ci/mmol) and incubated at 30 °C for 30 min. The reaction mixtures were separated by SDS-PAGE, and the protein bands were visualized by autoradiography.

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puromycin (1.5 μg/ml) were split into several 10-cm dishes in selection medium containing tetracycline (1.5 μg/ml). After culturing for another 48 h, the cells were replaced with fresh medium. After culturing for another 48 h, the cells were then harvested and subjected to IKK kinase assays. The expression levels of HA-IKKβ and LMP1 were examined by immunoblot (IB). B. TRAF6+/− MEFs were co-transfected with HA-IKKβ together with either an empty vector or LMP1 as indicated. WCE were harvested and subjected to the IKK kinase assays. C. TRAF2/5−/− and TRAF6−/− MEFs were separately transfected with GFP-p65 together with either an empty vector or LMP1 as indicated. 24 h after transfection, the cells were subjected to microscopic imaging, and p65 was visualized by autofluorescence of GFP. Vec, vector. D. The percentage of cells containing the nuclear GFP-p65 over the total GFP-positive cells from the experiment in C was calculated and presented. A total of 200 GFP-positive cells were counted for TRAF2/5−/− MEFs, and 90 GFP-positive cells were counted for TRAF6−/− MEFs (because of lower transfection efficiency in TRAF6−/− MEFs).

STR1 and pCLeco (a packaging vector) were then co-transfected into HEK293T cells for virus production (35). The supernatant containing retrovirus was collected 48 h after transfection, filtered through a 0.45-μm filter (Millipore, Bedford, MA), and stored at −80 °C freezer. The wild type and IRAK4−/− MEFs (50% confluent) were separately infected with the viruses in the presence of polybrene (8 μg/ml) and tetracycline (1.5 μg/ml) for 8 h. The medium was then aspirated and replaced with fresh medium. After culturing for another 48 h, the cells were split into several 10-cm dishes in selection medium containing puromycin (1.5 μg/ml) and tetracycline (2.0 μg/ml). After 7–10 days, the well isolated individual clones were picked and expanded. To induce LMP1 expression, stable cells were grown in 10% fetal bovine serum with puromycin but without tetracycline.

**Gel Mobility Shift Assays**—A double-stranded oligonucleotide probe containing a consensus NF-κB site, 5′-AGTTGAGGGGACTTTC-3′ (sense) (Promega, WI), was end-labeled with T4 polynucleotide kinase and [γ-32P]ATP. For each binding reaction, 15 μg of WCE, 1 μg of poly(dI-dC) (Amersham Biosciences), and 20 μg of bovine serum albumin were mixed in the binding buffer (15 mM HEPES, pH 7.6, 40 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol) and preincubated on ice for 30 min. The binding reaction was initiated upon addition of the probe (~20,000 cpm) and incubated at room temperature for another 30 min. The samples were then separated on a 5% native polyacrylamide gel. After electrophoresis, the gel was subsequently dried and visualized by autoradiography.

**RESULTS**

An NPC-associated LMP1 Variant, LMP1(D335), Activates NF-κB as Well as the Prototypic LMP1 Does—Many different LMP1 variants have been found over the years (36). One of them, LMP1(D335), was frequently found in biopsies from NPC patients in Hong Kong (36). To gain insight into the potential pathological impact of this particular LMP1 variant, we first compared it with its prototypic counterpart (i.e. LMP1(G335)) for their ability to activate NF-κB. When separately co-transfected with an NF-κB-dependent reporter gene into 293T cells, both forms of LMP1 significantly activated the reporter to similar extent (Fig. 1A), in agreement with the reporter assay above. Our data suggested that LMP1(D335) behaves similarly to its prototypic counterpart in terms of its impact on the NF-κB pathway.
CTAR2 Is the Main Contributor to the LMP1-mediated NF-κB Activation—Both CTAR1 and CTAR2 have previously been implicated in NF-κB activation (37, 38). To reassess their individual contribution to the LMP1-mediated NF-κB activation, we generated three LMP1 mutants: one (3A) with a triple mutation in the conserved TRAF3-binding motif in CTAR1 (i.e. P204QQAT208 to AQAAA), another (ΔC8) with an 8-amino acid deletion at the C terminus of CTAR2, and the third one (3A/ΔC8) with both types of mutations combined. Previous studies have shown that the first two mutations described above abolish the functions of CTAR1 and CTAR2, respectively, whereas the last one inactivates both (15, 16, 30, 39, 40). When co-transfected into HEK293T cells together with an NF-κB reporter, the wild type LMP1 potently activated the reporter activity, whereas LMP1(3A) and LMP1(ΔC8) were about 65 and 29% as active as the wild type, respectively (Fig. 2A). In contrast, LMP1(3A/ΔC8) was almost completely inactive. Using immune complex kinase assays, we also compared LMP1 with its mutant derivatives for their ability to activate the endogenous IKK (Fig. 3). As shown in Fig. 2B, LMP1(3A), which retains a functional IKKβ-activating activity of these LMP1 constructs was found to be in the following order: wild type > 3A > ΔC8, whereas the double mutant (3A/ΔC8) was completely inactive (Fig. 2B).

TAK1 Is a Specific MAP3K Involved in the CTAR2-mediated NF-κB Pathway—Several MAP3Ks including MEKK1 and TAK1 have been shown to act upstream of IKK in various cytokine-mediated NF-κB pathways (see “Discussion”). To find out which MAP3K specifically acts in the LMP1-mediated NF-κB pathway, we first co-transfected LMP1 and HA-IKKβ into MEFs derived from either the wild type or MEKK1 knock-out mice (41). As shown in Fig. 2A, LMP1 was still able to activate IKKβ in MEKK1−/− MEFs as well as in the wild type MEFs, suggesting that MEKK1 is less likely to be involved in the LMP1-mediated NF-κB pathway. We then turned to TAK1, which was recently shown by us as a key MAP3K involved in the LMP1-mediated NF-κB pathway (30). HEK293T cells were co-transfected with LMP1 and HA-IKKβ in the presence of either a control or TAK1-specific siRNA. Whereas the control siRNA had no obvious effect, the TAK1-specific siRNA significantly knocked down the expression of the endogenous TAK1 and reduced the LMP1-mediated IKKβ activation (Fig. 3B, lane 3). To find out which CTAR domain preferentially utilizes TAK1, we also tested two CTAR mutants of LMP1, LMP1(3A) and LMP1(ΔC8) (see previous section). In agreement with results in Fig. 2, LMP1(3A), which retains a functional CTAR2, was still able to significantly activate IKK, and this activation could be largely abolished by the TAK1-specific siRNA (Fig. 3B, lanes 4 and 5). In contrast, LMP1(ΔC8), which retains a functional CTAR1, poorly activated IKK (Fig. 3B, lane 6). Importantly, the TAK1-specific siRNA had little effect on the LMP1(ΔC8)-induced IKK activation (Fig. 3B, lane 7). This result suggested that TAK1 is mainly involved in the CTAR2-mediated NF-κB pathway. To further substantiate our claim, we also tested the involvement of TAK1 in the LMP1-induced p100 processing, a process known to be mediated by CTAR1 (21–24). The whole cell extracts from the above experiment were subjected to immunoblot to detect the levels of the endogenous p100 and its processed product p52. As shown in Fig. 3C, LMP1 clearly induced increased p52 production in the presence of the control siRNA. Importantly, the TAK1-siRNA had no obvious effect on LMP1-induced p52 production, suggesting that TAK1 is not involved in the CTAR1-mediated p100 processing.

Loss of TAB2 Does Not Affect the LMP1-induced IKK Activity—TAB2 is a TAK1-binding protein and is involved in IL-1β-mediated NF-κB activation (42, 43). To test whether TAB2 is absolutely required in the LMP1-mediated NF-κB pathway, we compared the LMP1-induced IKK activity in both the wild type and TAB2−/− MEFs (44). As shown in Fig. 4, LMP1 activated IKK in TAB2−/− MEFs as well as in the wild type MEFs. This suggested that TAB2 is not essential in the LMP1-mediated NF-κB pathway.

CTAR2 Specifically Utilizes TRAF6 for NF-κB Activation—Although it has been convincingly shown that CTAR1 specifically recruits TRAF3 to activate NF-κB (28, 29), it is less clear which molecule mediates the effect of CTAR2 on NF-κB activation. TRAF2 was previously implicated in the LMP1-mediated NF-κB pathway (26). To find out whether CTAR2 utilizes TRAF2 to activate NF-κB, we separately transfected TRAF2−/−/TRAF5−/− double knock-out MEFs with the wild type LMP1 and its mutant derivatives together with HA-IKKβ and measured the IKK kinase activity (45). As a control, we first showed that IL-1β but not TNFα significantly activated IKKβ in the TRAF2/5 double knock-out cells (Fig. 5A, lanes 5 and 6), in agreement with the original report (45). Interestingly, in the TRAF2/5 double knock-out MEFs, both the
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wild type LMP1 and LMP1(3A) were equally effective in activating IKKβ, whereas LMP1(ΔC8) was as inactive as the vector control (Fig. 5A). This result showed that the CTAR2-mediated NF-κB activation is TRAF2/5-independent.

Recently, we showed that TRADD specifically recruits TRAF6 in the LMP1-mediated JNK pathway (30), suggesting that TRAF6 may mediate the effect of CTAR2 on NF-κB activation. To test this hypothesis, we co-transfected TRAF6−/− MEFs with LMP1, HA-IKKβ, and TRAF6 in various combinations. In the absence of TRAF6, LMP1 completely failed to activate IKKβ (Fig. 5B, lane 2). When we put TRAF6 back, it restored LMP1-mediated IKKβ activation (Fig. 5B, lane 4). This suggested that TRAF6, which is mainly engaged by TRADD, is the major signal mediator in the LMP1-mediated NF-κB pathway.

To further strengthen our results from IKK kinase assays, we also studied LMP1-induced GFP-p65 nuclear translocation in both TRAF2/5−/− and TRAF6−/− MEFs. As shown qualitatively in Fig. 5C and quantitatively in Fig. 5D, LMP1 significantly promoted GFP-p65 nuclear translocation only in TRAF2/5−/− cells but not in TRAF6−/− cells. This result demonstrated that TRAF6 is indispensable for LMP1-induced p65 nuclear translocation (i.e. NF-κB activation).

TRADD Has Minimal Effect on the LMP1-mediated NF-κB Activation—Several previous reports implicated TRADD in the LMP1-mediated NF-κB pathway mainly based on studies employing overexpressed "dominant-negative" TRADD mutants (25, 27, 46). To rigorously reassess the role of TRADD in the LMP1-mediated NF-κB pathway, we first resorted to siRNA to knockdown the endogenous TRADD in HEK293 cells followed by transfection with LMP1 and HA-IKKβ. As shown in Fig. 6A, although a control siRNA had no obvious effect, the TRADD-specific siRNA slightly reduced LMP1-mediated IKK activation. In a separate NF-κB-dependent reporter assay, we also found that the LMP1-mediated NF-κB activation was slightly reduced by the TRADD-specific siRNA (Fig. 6B). Thus, our results suggested that TRADD is not essential in the LMP1-mediated NF-κB pathway.

Both IRAK1 and IRAK4 Are Not Essential in the LMP1-mediated NF-κB Pathway—Because both IRAK1 and IRAK4 act upstream of TRAF6 in the IL-1R/TLR-mediated NF-κB pathways (20), we next tested whether these two IRAKs are involved in the LMP1-mediated NF-κB pathway. We first transfected HEK293 cells with LMP1 and HA-IKKβ in the presence of either a control or the IRAK1-specific siRNA. We found that the knockdown of IRAK1 did not significantly affect the LMP1-induced IKK activation (Fig. 7A). We then measured the LMP1-induced NF-κB DNA binding activity with or without the IRAK1-siRNA. As shown in Fig. 7B, knockdown of the endogenous IRAK1 had no obvious effect on the LMP1-induced NF-κB DNA binding activity. To study the role of IRAK4, we stably transfected LMP1 into both the wild type and IRAK4−/− MEFs by retroviral infection and compared two independent clones from each stable cell line for their NF-κB DNA binding activity. As a control, we showed that TNFα but not IL-1β activated NF-κB in IRAK4−/− MEFs (Fig. 7C) (47). The expression of LMP1 in the wild type MEFs significantly enhanced the NF-κB DNA binding activity (Fig. 7D, lanes 1–3). Importantly, in IRAK4−/− MEFs, LMP1 was still able to activate NF-κB, and the extent of NF-κB activation was as good as that in the wild type MEFs (Fig. 7D, lanes 4–6). Our results showed that both IRAK1 and IRAK4 are less likely involved in the LMP1-mediated NF-κB pathway.

FIGURE 7. Neither IRAK1 nor IRAK4 is essential for the LMP1-induced NF-κB activation. A and B, HEK293 cells were either mock transfected or transfected twice with siRNA as indicated, followed by another transfection with various constructs as indicated. WCE were harvested and subjected to either the IKK kinase assays (A) or gel mobility shift assays (B). The expression level of HA-IKKβ, IRAK1, LMP1, and β-tubulin was examined by immunoblot (IB). C, IRAK4−/− MEFs were either left untreated or treated separately with IL-1β and TNFα (10 ng/ml) for 10 min. D, the wild type (wt) and IRAK4−/− cells stably expressing LMP1 were generated by retroviral infection as described under "Experimental Procedures." For both C and D, WCE were harvested and subjected to NF-κB gel shift assays. Lanes C, control MEF cells containing an empty vector. Lanes S2, S3, S5, and S8 are individual clones stably expressing LMP1.
**LMP1 CTAR2-mediated NF-κB Pathway**

**FIGURE 8. MyD88 is not essential for NF-κB activation by LMP1.** A, the wild type (wt) and MyD88 \(^{-/-}\) MEFs were separately transfected with expression vectors for HA-IKKβ together with either LMP1 or an empty vector. WCE were harvested and subjected to the IKK kinase assays. The expression level of HA-IKKβ and LMP1 was examined by immunoblot. B, a schematic representation of the LMP1-mediated NF-κB pathways. The CTAR1 and CTAR2 of LMP1 activate NF-κB through two distinct mechanisms. CTAR1 specifically recruits TRAF3, which may further recruit other TRAFs (e.g., TRAF2/5) to engage NIK and IKKα to induce p100 processing resulting in the formation of a specific NF-κB dimer: p52/RelB (noncanonical pathway). In contrast, CTAR2 preferentially recruits TRAF6 through a conserved TRAF-binding motif and directly recruits TRAF3 through a conserved TRAF-binding motif.

**DISCUSSION**

CTAR1 and CTAR2 Recruit Distinct TRAFs to Activate NF-κB—Both CTAR1 and CTAR2 are known to be responsible for the LMP1-mediated NF-κB activation (37, 38, 49). In agreement with previous reports (15, 37–39, 50), we confirmed that CTAR2 accounts for the majority (70%) of the NF-κB activation by LMP1 and HA-IKKβ. Furthermore, TRAF2 knock-out cells clearly show that TRAF2 is not essential for the LMP1-mediated JNK and NF-κB pathways (Fig. 5A). This indicates that TRAF2 and TRAF5 are not required in the CTAR2-mediated NF-κB pathway. Moreover, we previously showed that both IRAK1 and IRAK4 are not required in the LMP1-mediated JNK pathway (30). Thus, we think that IRAK1 is less likely involved in the LMP1-mediated NF-κB pathway. A potential problem for the IRAK1-defective HEK293 cells used by Luftig et al. (52) is that some unidentified molecules, in addition to IRAK1, could be affected by ICR191 (a DNA-intercalating mutagen), because the IRAK1-defective cells were generated and selected by ICR191-based random mutagenesis (53).

Taken together, we believe that the LMP1-mediated NF-κB activation is achieved mainly by the CTAR2 domain through its specific interaction with TRAF6, whereas the CTAR1 domain contributes to a lesser extent through its interaction with TRAF3 (Fig. 8B). Because LMP1 does not directly interact with TRAF6 (30), it remains unclear how CTAR2 specifically recruits TRAF6 to activate the NF-κB pathway.

**TAK1 Is a Key MAP3K Specifically Involved in the LMP1-mediated NF-κB Pathway**—It has been well established that IKKα/β needs to be phosphorylated by some upstream kinases to achieve maximum activity (10, 11, 54). In the noncanonical NF-κB pathway, NIK has been found to be the key kinase directly phosphorylating and activating IKKα (14, 55). In the canonical NF-κB pathway, several MAP3Ks and Ste20-like kinases including MEKK1, MEKK3, ASK1, TAK1, and GCK have been implicated as the kinase phosphorylating and activating IKKβ in various cytokine-mediated NF-κB pathways (11, 54). For MEKK1, ASK1, and GCK, their involvement in IKK activation was initially based on overexpression studies (11, 54). Subsequent studies in cells derived from knock-out mice do not support a critical role for these kinases in NF-κB activation (11, 54). For MEKK3, studies based on MEKK3 knock-out mice show that it is required for both the TNFα and IL-1β-mediated NF-κB activation (56, 57). It remains to be seen whether MEKK3 also plays a role in the LMP1-mediated NF-κB pathway. In addition, TAK1

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has also been shown to play a key role in the cytokine-mediated MAPK and NF-κB pathways (33, 58). It acts downstream of TRAFs and its activity depends on TRAF6-mediated ubiquitination (33, 58, 59). Importantly, the TAK1-specific siRNA significantly reduces the IL-1- and TNFα-induced IKK and NF-κB activation (58). Our present studies also implicate TAK1 as a key kinase acting upstream of IKKβ in the LMP1-mediated NF-κB pathway. This is consistent with its critical role in the LMP1-mediated JNK pathway (30).

In summary, our data and those by other groups show that LMP1 uses the CTAR2 domain by engaging TRAF6/TAK1/IKK (i.e., the canonical pathway) and a minor one through the CTAR1 domain by engaging TRAF3/NIK/IκKα (i.e., the noncanonical pathway) (Fig. 8B).

Acknowledgments—We thank Drs. Y. Xia and M. Karin for MEKK1 and NF-κB pathways (33, 58). It acts downstream of TRAFs and its activity depends on TRAF6/TAK1/IKK (i.e., the canonical pathway) and a minor one through the CTAR1 domain by engaging TRAF3/NIK/IκKα (i.e., the noncanonical pathway) (Fig. 8B).

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