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Elucidation of the c-Jun N-Terminal Kinase Pathway Mediated by Epstein-Barr Virus-Encoded Latent Membrane Protein 1

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Epstein-Barr virus (EBV) is associated with several human diseases including infectious mononucleosis and nasopharyngeal carcinoma. EBV-encoded latent membrane protein 1 (LMP1) is oncogenic and indispensable for cellular transformation caused by EBV. Expression of LMP1 in host cells constitutively activates both the c-Jun N-terminal kinase (JNK) and NF-κB pathways, which contributes to the oncogenic effect of LMP1. However, the underlying signaling mechanisms are not very well understood. Based mainly on overexpression studies with various dominant-negative constructs, LMP1 was generally thought to functionally mimic members of the tumor necrosis factor (TNF) receptor superfamily in signaling. In contrast to the prevailing paradigm, using embryonic fibroblasts from different knockout mice and the small interfering RNA technique, we find that the LMP1-mediated JNK pathway is distinct from those mediated by either TNF-α or interleukin-1. Moreover, we have further elucidated the LMP1-mediated JNK pathway by demonstrating that LMP1 selectively utilizes TNF receptor-associated factor 6, TAK1/TAB1, and c-Jun N-terminal kinase kinases 1 and 2 to activate JNK.

Epstein-Barr virus (EBV) is a human γ-herpesvirus causally linked with several different human diseases including nasopharyngeal carcinoma (NPC), Hodgkin's lymphoma, Burkitt's lymphoma, and infectious mononucleosis (15, 31). The incidence of NPC in southern China, including Guangdong province and Hong Kong, is among the highest in the world (28). A clear understanding of the molecular mechanisms underlying EBV-associated pathogenesis is of paramount importance in formulating an effective therapy.

EBV can readily transform primary resting B lymphocytes into immortalized lymphoblastoid cell lines (LCLs) (15, 31). EBV-encoded latent membrane protein 1 (LMP1) is indispensable for establishment of lymphoblastoid cell lines by EBV (23). Among several latent viral genes expressed in NPC and EBV-positive Hodgkin's disease, LMP1 is the only one with oncogenic properties (31). When introduced into rodent fibroblasts, LMP1 causes oncogenic transformation (43). LMP1 also promotes a higher incidence of lymphoma in transgenic mice when specifically introduced into lymphocytes (27). These results establish a critical role for LMP1 in EBV-associated malignances.

LMP1 is a membrane protein of 386 amino acids containing six transmembrane domains. Both the amino (amino acids [aa] 1 to 24) and carboxyl (aa 186 to 386) termini of LMP1 are located in the cytoplasm (Fig. 1A). While the short amino terminus of LMP1 is implicated in anchoring LMP1 on the plasma membrane, its carboxyl terminus is implicated in both cellular transformation and activation of intracellular signaling pathways. Two subregions in the carboxyl tail of LMP1 are critical in cell transformation and signaling: carboxyl-terminal activating region 1 (CTAR1, aa 194 to 231) and CTAR2 (aa 351 to 386) (Fig. 1A). CTAR1 is capable of binding several tumor necrosis factor (TNF) receptor-associated factors (TRAFs) and activating the NF-κB pathway, while CTAR2 was shown to bind TNF receptor-associated death domain protein (TRADD) and receptor-interacting protein (RIP) and activate both the NF-κB and c-Jun N-terminal kinase (JNK) pathways (15, 31). Although CTAR1 can independently activate the NF-κB pathway, it is CTAR2 that is mainly responsible for activating both the NF-κB (accounting for ~70% of total NF-κB activity induced by LMP1) and JNK (100% of LMP1-mediated JNK activation) pathways (15, 31).

LMP1 has generally been thought to functionally mimic members of the TNF receptor (TNFR) superfamily in signaling, as it was shown to constitutively oligomerize on the plasma membrane, interact with TRADD, RIP and several TRAFs, including TRAF2, and activate both the JNK and NF-κB pathways in host cells (15, 31). As both TRADD and TRAF2 are known to be involved in the TNF-α-mediated JNK and NF-κB pathways (5, 30, 32), they are also widely thought to be involved in LMP1-mediated JNK and NF-κB activation (15, 31). Overexpression of truncated TRADD and TRAF2 mutants were employed by earlier studies, which generated conflicting

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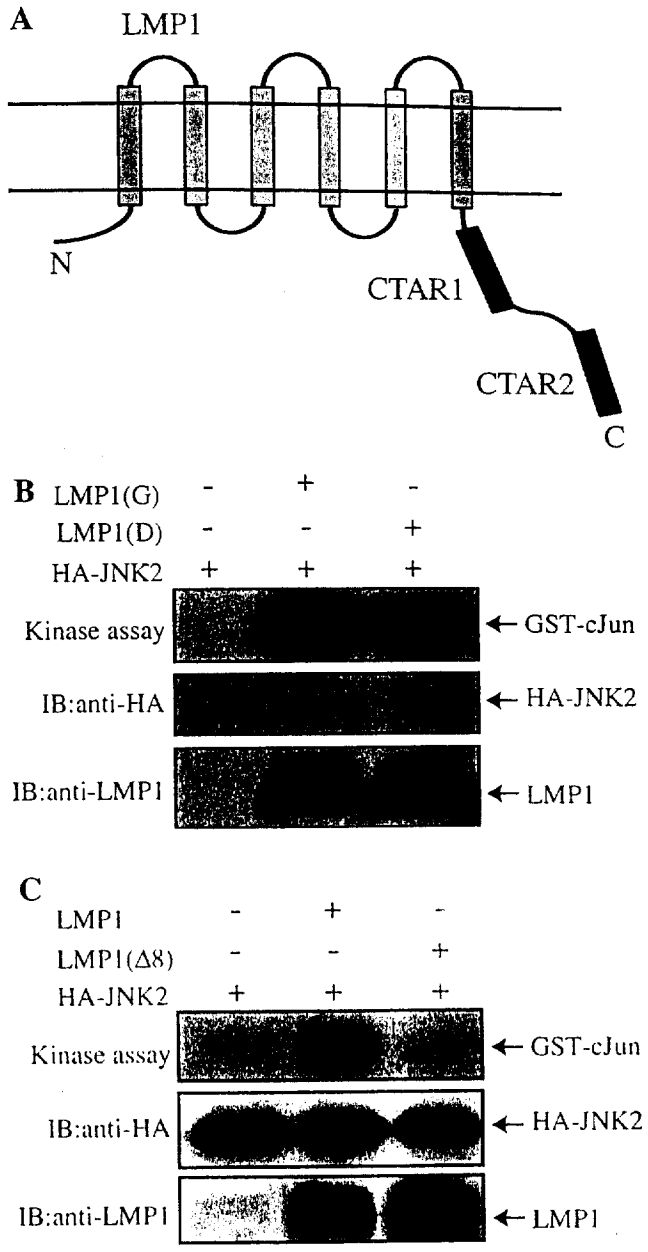

FIG. 1. LMP1(G335) and LMP1(D335) activate JNK equally well. (A) Schematic representation of LMP1. Two horizontal straight lines represent the plasma membrane. The six grey bars represent transmembrane domains, and the two black bars represent CTAR1 and CTAR2. (B and C) HeLa cells were cotransfected with HA-JNK2 and different forms of LMP1. HA-JNK2 was subjected to immune-complex kinase assays. IB, immunoblot; N, amino terminus; C, carboxyl terminus.

results (12, 20, 24). In order to clarify the confusion mainly arising from the use of various dominant-negative mutants and further elucidate the LMP1-mediated signaling pathways, we resorted to mouse embryonic fibroblasts (MEFs) derived from different knockout mice whenever possible. In cases where no knockout MEFs were available, the small interfering (siRNA) technique was used to silence the expression of endogenous genes in order to determine their involvement in LMP1-mediated signaling. In contrast to the prevailing paradigm, we demonstrate in this report that TRADD, TRAF2, RIP, TAB2, myeloid differentiation factor 88 (MyD88), and interleukin-1 (IL-1) receptor-associated kinases 1 and 4 are not essential for LMP1-mediated JNK activation. Instead, LMP1 activates JNK through sequential activation of TRAF6, TAK1/TAB1, and c-Jun N-terminal kinase kinases 1 and 2 (JNKK1/2).

MATERIALS AND METHODS

Cell lines, DNA constructs, and reagents. 293T, HeLa, RIP1$^{-/-}$, TRAF2$^{-/-}$, TRAF6$^{-/-}$, MyD88$^{-/-}$, IRAK4$^{-/-}$, and TAB2$^{-/-}$ MEFs were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml in a 37°C incubator with 5% CO_2. Hemagglutinin (HA)-MEKK1-C(KM), HA-MEKK2(KM), HA-MEKK3(KM), Myc-ASK1(KR), and HA-ASK1(KR) were gifts from Shengcai Lin (Hong Kong University of Science & Technology). LMP1(G335), LMP1(D335), Myc-TAB1, and HA-TAK1(KW) were described previously (4, 38). GFP-TRAF6 and xp-TRAF6 were constructed by inserting the cDNA fragments into pEGFP-C1 and pcDNA3.1c, respectively. To generate a bacterial expression vector encoding His-thioredoxin-MKK6(KM), Lys 82 of human MKK6 was first mutated to Met and the cDNA was then inserted into pET32 M. IL-1β and TNF-α were purchased from R & D Systems.

Transfection and cell lysis. Cells were transfected with various plasmids, using either Lipofectamine Plus reagents (for 293T and HeLa cells) or Lipofectamine 2000 (Invitrogen) (for MEF cells) according to the manufacturer's instruction. Twenty-four to thirty-six hours after transfection, the cells were lysed in the lysis buffer (50 mM HEPES at pH 7.6, 10% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 20 mM *p*-nitrophenyl phosphate, 20 mM β-glycerol phosphate, 2 mM dithiothreitol, 50 μM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, 0.7 μg of pepstatin/ml), followed by removal of insoluble debris with a benchtop centrifuge at 15,000 × *g* for 2 min to obtain whole-cell extracts (WCEs). All siRNAs were purchased from Dharmacon Inc. (Lafayette, Colo.): TAK1 siRNA, 5′-(AA)GAGAUCGACUACAAGGAGA; TRADD siRNA, 5′-(AA)CUGGCUGAGCUGGAGGAUG; interleukin-1 receptor-associated kinase 1 (IRAK1) siRNA, 5′-(AA)GUUGCCAUCCUCAGCCUCC. siRNAs were transfected to 293T or HeLa cells twice at a 24-hour interval using either Oligofectamine or Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after the last transfection.

Antibodies and Western blot analysis. Mouse monoclonal antibodies to HA (Santa Cruz), Flag (M2) (Sigma), β-actin (Sigma), β-tubulin (Sigma), Myc (9E10), and TRAF6 (Santa Cruz), rabbit polyclonal antibodies to phospho-p38 (Cell Signaling), RIP (Pharmingen), Xpress tag (Omni-probe), and TRAF2 and IRAK1 (Santa Cruz), and goat polyclonal antibodies to TAB1 (Santa Cruz) and TRADD (Santa Cruz) were used in this study. Monoclonal anti-LMP1, anti-JNK, anti-TAK1, and anti-JNKK1 antibodies and polyclonal JNKK2 antibody were described previously (4, 42, 44). Twenty or thirty micrograms of WCEs was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinlidene difluoride membrane (Immun-Blot PVDF; Bio-Rad), and probed with various antibodies. Proteins were visualized with the enhanced chemiluminescence kit (Amersham Biosciences).

Coimmunoprecipitation assays. 293T cells were cotransfected with various plasmids. Thirty-six hours after transfection, the cells were cross-linked with 20 μg of dithiobis(succinimidylpropionate) (Pierce) per ml for 10 min followed by lysis in RIPA buffer (25 mM HEPES at pH 7.4, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg of aprotinin/ml, 0.5 μg of leupeptin/ml, 0.7 μg of pepstatin/ml). Protein A-Sepharose beads were incubated with 400 μg of extracts and 2 μg of appropriate antibodies for 2 h at 4°C. After extensive washing with the RIPA buffer, bound proteins were eluted out by boiling and subjected to SDS-PAGE and immunoblotting.

Protein kinase assays. For all JNK assays and the coupled kinase assays for JNKKs, we followed the protocols described previously (44). For TAK1 kinase assay, TAK1 was immunoprecipitated from 300 μg of WCEs with 2 μg of TAK1 antibody. The immunoprecipitates were analyzed for TAK1 kinase activity with His-MKK6(KM) as a substrate (40). Kinase reactions were separated by SDS-PAGE, and protein bands were visualized by autoradiography.

Fluorescence microscopy studies. HeLa cells were transfected as described above. Twenty-four hours after transfection, cells were first fixed in methanol for 15 min and then permeabilized in 0.2% Triton X-100 for 15 min and blocked in

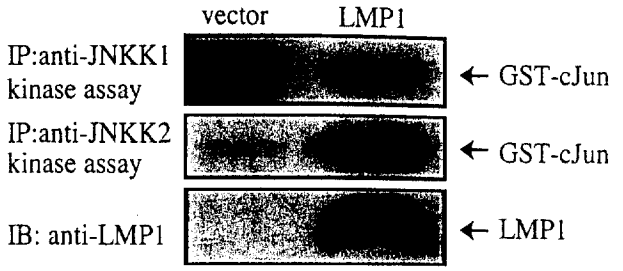

FIG. 2. Both the endogenous JNKK1 and JNKK2 can be activated by LMP1. 293T cells were transfected with either an empty vector or LMP1. The endogenous JNKK1 and JNKK2 were separately immunoprecipitated and subjected to the coupled kinase assays. IP, immunoprecipitation.

5% bovine serum albumin–phosphate-buffered saline (PBS) (pH 7.4) for 1 h. Cells were then incubated with the mouse anti-LMP1 antibody for 1 h, washed three times with PBS, and reincubated with Rhodamine-conjugated donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories Inc.) for another hour. Fluorescence microscopy was performed using an Olympus IX70 microscope linked to a charge-coupled device digital camera (Spot RT; Diagnostic Instruments Inc., Sterling Heights, Mich.).

RESULTS

LMP1(D335) activates JNK as well as its normal counterpart, LMP1(G335). It is already well established that LMP1 is capable of activating JNK (14, 17, 25). While a majority (73%) of healthy EBV carriers have a glycine (G) present at position 335 in the LMP1 protein, around 90% of NPC patients in Hong Kong have an aspartic acid (D) substituting for G335 in LMP1 (7, 8). To test whether this mutation leads to enhanced JNK activation by LMP1, we cotransfected HeLa cells with HA-JNK2 along with either form of LMP1. As shown in Fig. 1B, both forms of LMP1 activated JNK to the same level. A detailed dose-dependent study also failed to reveal any significant difference between the two forms of LMP1 in their ability to activate JNK (unpublished data). This indicated that LMP1(D335) does not cause enhanced JNK activation in host cells. For simplicity, we only used LMP1(D335) in subsequent experiments. Consistent with a previous report (12), we found that a truncated LMP1 mutant without the last eight amino acids at the carboxyl terminus (LMP1Δ8) completely failed to activate JNK (Fig. 1C), suggesting that the carboxyl-terminal eight amino acids are essential for LMP1 to engage the JNK pathway.

Endogenous JNKK1 and JNKK2 can both be activated by LMP1. As only two homologous mitogen-activated protein kinase kinases (MAP2Ks), namely JNKK1 (or MKK4) and JNKK2 (or MKK7), are known to act specifically upstream of JNK (10), we asked whether LMP1 preferentially activates one of them. 293T cells were transfected with either an empty vector or LMP1, and the endogenous JNKK1 and JNKK2 were separately immunoprecipitated and subjected to a coupled kinase assay (44). As shown in Fig. 2, the endogenous JNKK1 and JNKK2 could both be efficiently activated by LMP1, indicating that both JNKK1 and JNKK2 can mediate the stimulatory effect of LMP1 on JNK.

TAK1 is the specific mitogen-activated protein kinase kinase kinase (MAP3K) involved in LMP1-mediated JNK activation. As there are a total of 14 MAP3Ks in the human genome, we set out to test which MAP3K specifically mediates JNK activation by LMP1. We started out with several kinase-dead MAP3Ks in our collection. 293T cells were cotransfected with HA-JNK2 and LMP1 with or without the kinase-dead form of MEKK1, -2, and -3, ASK1, and TAK1. While the kinase-dead MEKK1, -2, and -3 had no obvious effect, the kinase-dead TAK1 significantly inhibited LMP1-mediated JNK activation (Fig. 3A). Unexpectedly, two differently tagged (i.e., HA and Myc) kinase-dead ASK1s further enhanced JNK activation by LMP1 (Fig. 3A), in contrast to its reported effect in the TNF-α-mediated JNK pathway (34). To further confirm a role for TAK1 in the LMP1-mediated JNK pathway, we resorted to the siRNA technique to suppress the expression of the endogenous TAK1. 293T cells were cotransfected with HA-JNK2 and LMP1 with or without siRNA. While a scrambled 21-nucleotide control siRNA had no effect, the TAK1-specific siRNA both reduced the amount of the endogenous TAK1 and significantly inhibited LMP1-mediated JNK activation (Fig. 3B). As LMP1 was also shown to activate p38 mitogen-activated protein kinase (MAPK) (13, 36), we asked whether TAK1 is also required for LMP1-mediated p38 MAPK activation. 293T cells were transfected with LMP1 with or without siRNA, and the activation status of the endogenous p38 MAPKs was monitored by a specific antibody recognizing only the active form (i.e., dually phosphorylated) of the p38 MAPKs. Although the control siRNA had no effect, the TAK1-specific siRNA significantly reduced activation of the p38 MAPKs by LMP1 (Fig. 3C). In addition, we further tested whether LMP1 could directly activate the endogenous TAK1. An empty vector and LMP1 were separately transfected into 293T cells, and the endogenous TAK1 was immunoprecipitated for kinase assays. As shown in Fig. 3D, LMP1 could indeed enhance the activity of the endogenous TAK1. Thus, our data indicate that TAK1 is the MAP3K specifically involved in the LMP1-mediated JNK pathway.

TAB2 is not essential in the LMP1-mediated JNK pathway. As TAB2 directly binds TAK1 and was shown to link TAK1 to upstream signaling molecules (22, 40, 42), we first tested whether TAB2 was also involved in the LMP1-mediated JNK pathway. Taking advantage of recently generated TAB2 knockout mice (35), we tested whether LMP1 could activate JNK in $TAB2^{-/-}$ MEFs. Both the wild-type and $TAB2^{-/-}$ MEFs were separately transfected with HA-JNK2 together with either an empty vector or LMP1. IL-1β treatment was included as a control. Like IL-1β (35), LMP1 still activated JNK in $TAB2^{-/-}$ MEFs, and it did so even better than in wild-type MEFs (Fig. 4A). As TAB1 also binds TAK1 and is the only known direct TAK1 activator (26, 38), we then tested whether TAB1 and LMP1 could coexist in the same complex. 293T cells were cotransfected with TAB1 together with either LMP1 or an empty vector. Indeed, TAB1 was found to be specifically coprecipitated with LMP1 (Fig. 4B). Thus, although TAB2 is not critically required, our results suggest that TAB1 is a component involved in the LMP1-mediated JNK pathway.

TRAF6, but not TRAF2 or RIP, mediates the stimulatory effect of LMP1 on JNK. Upstream of the TAK1/TAB1 complex, we focused on RIP and TRAFs which were found to associate with LMP1 and contribute to LMP1-mediated signaling (15, 31). To minimize the ambiguity mainly caused by

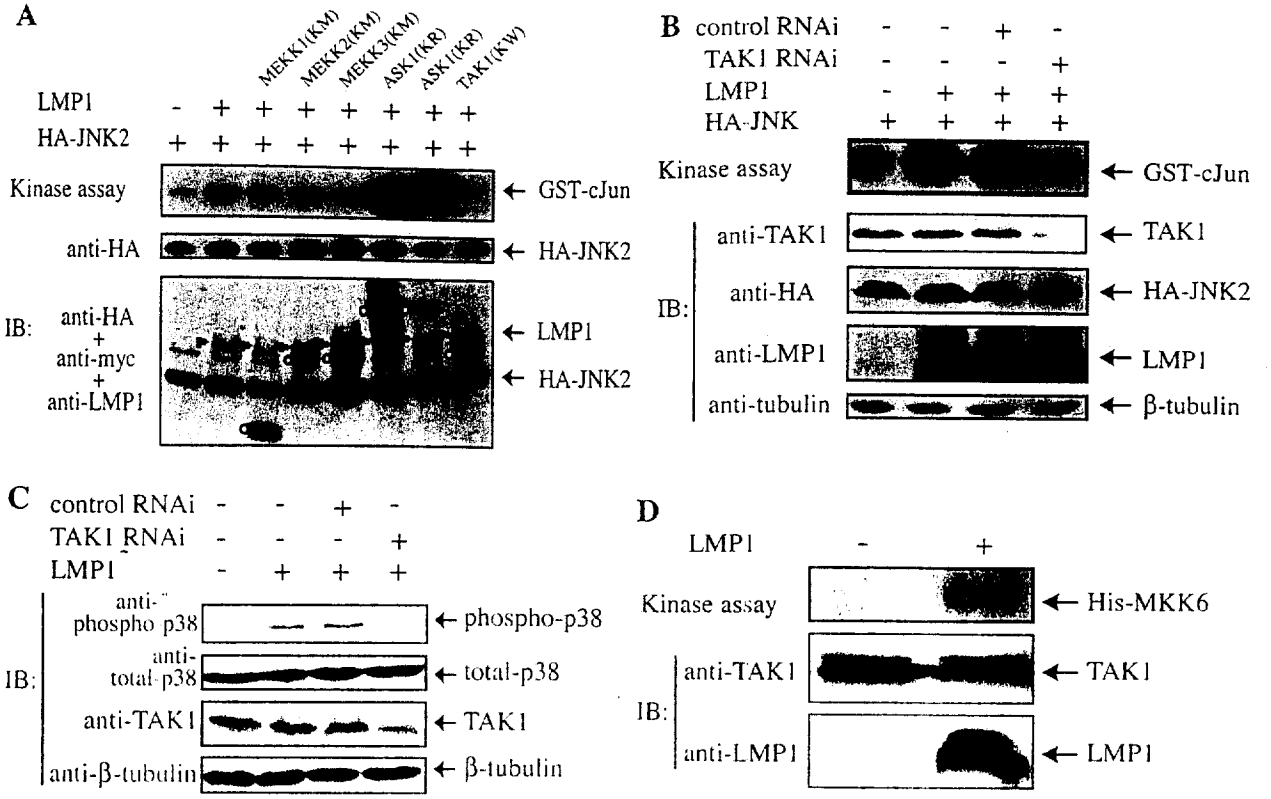

FIG. 3. TAK1 functions in the LMP1-mediated JNK pathway. (A) 293T cells were separately transfected with HA-JNK2 and LMP1 with or without the kinase-dead form of various MAP3Ks. Expression of LMP1 and various MAP3K mutants is indicated by arrowheads and open circles, respectively. (B and C) 293 T cells were transfected with various plasmids and siRNAs as indicated. HA-JNK2 from panels A and B was subjected to immune-complex kinase assays. WCEs from panel C were subjected to immunoblotting with anti-phospho-p38. (D) 293T cells were separately transfected with either an empty vector or LMP1. The endogenous TAK1 was subjected to immune-complex kinase assays using His-MKK6(KM) as a substrate.

overexpression of dominant-negative mutants (12, 20, 24), we carried out our experiments with MEFs derived from different knockout mice. The wild-type, TRAF2 $^{-/-}$, RIP $^{-/-}$, and TRAF6 $^{-/-}$ MEF cells were separately cotransfected with HA-JNK together with either an empty vector or LMP1 with or without TNF-α treatment. While TNF-α-mediated JNK activation was nearly abolished in both TRAF2 $^{-/-}$ and RIP $^{-/-}$ cells (11, 46), LMP1-mediated JNK activation was unaffected in these two knockout cell lines (Fig. 5A). In contrast, in TRAF6 $^{-/-}$ MEFs, while TNF-α-mediated JNK activation was unaffected (3, 37), LMP1-mediatd JNK activation was largely abolished (Fig. 5B). To further confirm a role for TRAF6 in the LMP1-mediated JNK pathway, we transfected either a TRAF2 or a TRAF6 expression vector back into the TRAF6 $^{-/-}$ cells. While LMP1, TRAF2, or TRAF6 alone barely activated JNK, addition of both LMP1 and TRAF6, but not LMP1 and TRAF2, significantly reactivated JNK in TRAF6 $^{-/-}$ cells (Fig. 5C). In addition, we found that the endogenous TRAF6 was specifically coprecipitated with LMP1 (Fig. 5D). Furthermore, by attaching green fluorescent protein (GFP) to TRAF6, we found that GFP-TRAF6 alone was pancytoplasmic (Fig. 5E, top panels). However, when cotransfected with LMP1, both TRAF6-GFP and LMP1 colocalized in the clustered patches on the plasma membrane (Fig. 5E, bottom panels), a localization pattern typical for LMP1 (29). To further understand how TRAF6 transmits signals to the TAK1

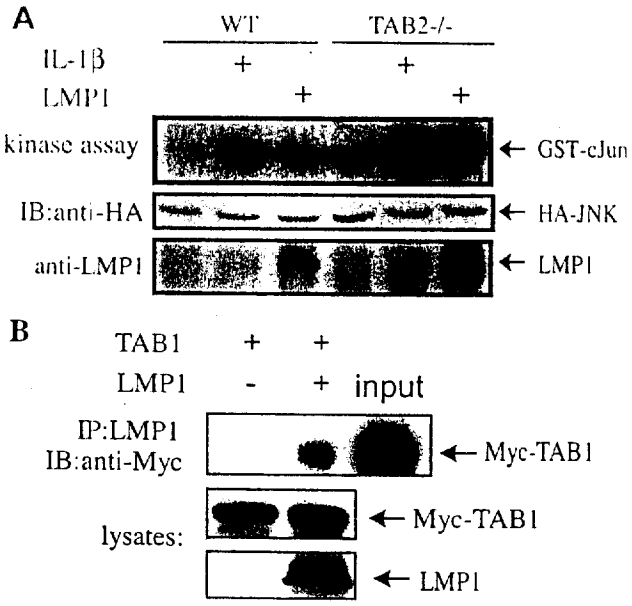

FIG. 4. TAB2 is not essential in the LMP1-mediated JNK pathway. (A) Wild-type or TAB2 $^{-/-}$ MEFs were cotransfected with HA-JNK2 and either an empty vector or LMP1 with or without IL-1β treatment (10 ng/ml for 10 min). HA-JNK2 was then subjected to immune-complex kinase assays. (B) 293T cells were cotransfected with TAB1 and either an empty vector or LMP1. LMP1 was immunoprecipitated, and the coprecipitated TAB1 was detected by immunoblotting

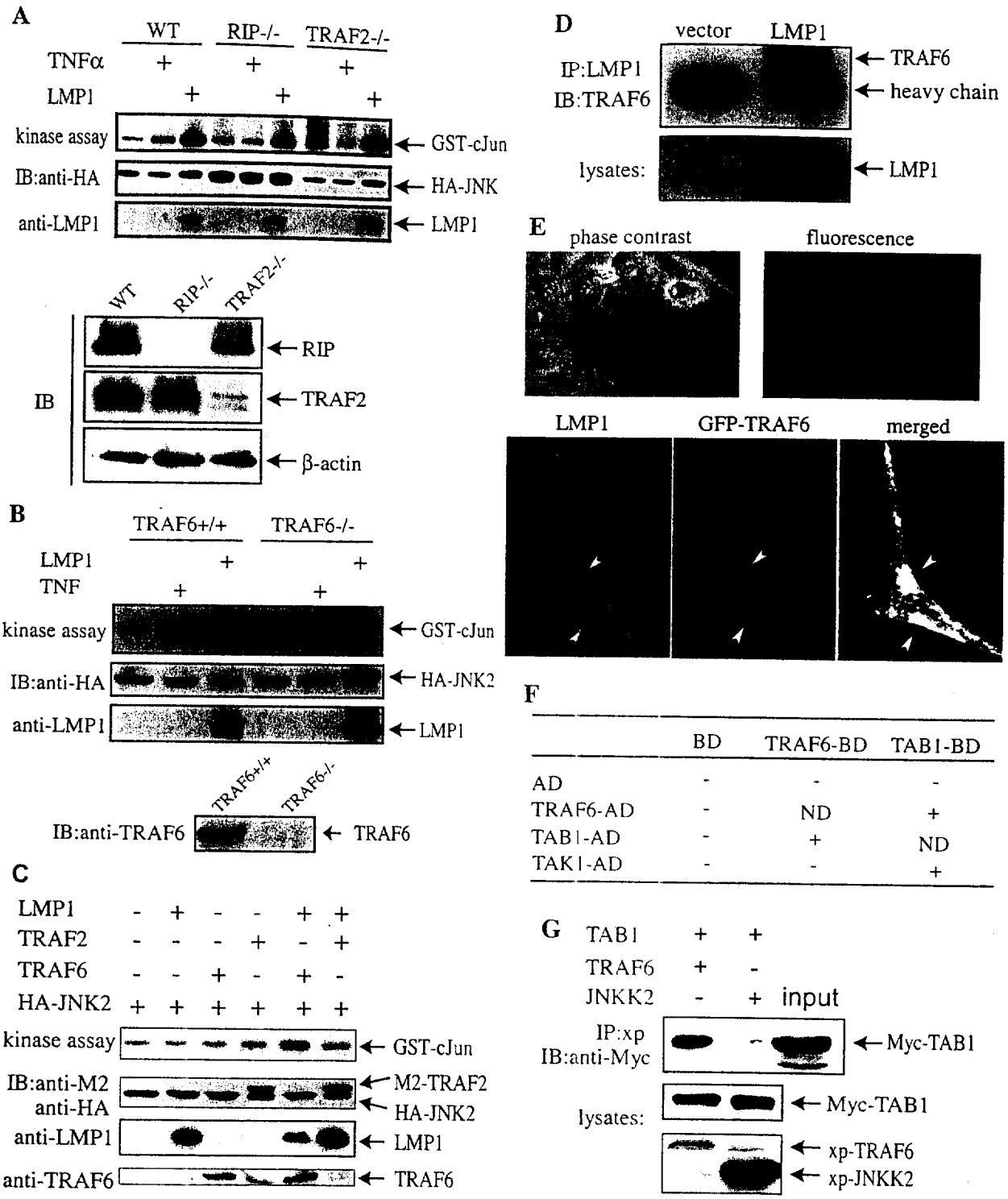

FIG. 5. TRAF6 is required in the LMP1-mediated JNK pathway. (A and B) HA-JNK along with either an empty vector or LMP1 were cotransfected into either the wild-type, $RIP^{-/-}$, or $TRAF2^{-/-}$ (A) or $TRAF6^{-/-}$ (B) MEFs with or without TNF-α (10 ng/ml for 10 min) treatment. (C) $TRAF6^{-/-}$ cells were cotransfected with plasmids as indicated. HA-JNK2 from panels A, B, and C was subjected to immune-complex kinase assays. (D) 293T cells were separately transfected with either an empty vector or LMP1. LMP1 was immunoprecipitated with anti-LMP1, and the coprecipitated TRAF6 was detected by immnoblotting. (E) HeLa cells were transfected with either GFP-TRAF6 alone (top panels) or LMP1 and GFP-TRAF6 together (bottom panels). LMP1 was visualized by indirect immunofluorescence (red), and GFP-TRAF6 was visualized by autofluorescence (green). The white arrowheads indicate patches on the membrane. The images of LMP1 and GFP-TAB2 were superimposed (yellow) using Spot RT software v3.4. (F) Summary of protein-protein interaction in yeast two-hybrid assays. AD, yeast pGADT7 vector; BD, yeast pGBKT7 vector. The plus and minus signs denote growth and no growth, respectively, of yeast clones on synthetic dropout plates lacking leucine, tryptophan, histidine, and adenine. ND, not determined. (G) 293T cells were cotransfected with Myc-TAB1 and either xp-TRAF6 or xp-JNKK2. TRAF6 and JNKK2 were immunoprecipitated with the anti-Xpress antibody, and the coprecipitated TAB1 was detected by immunoblotting.

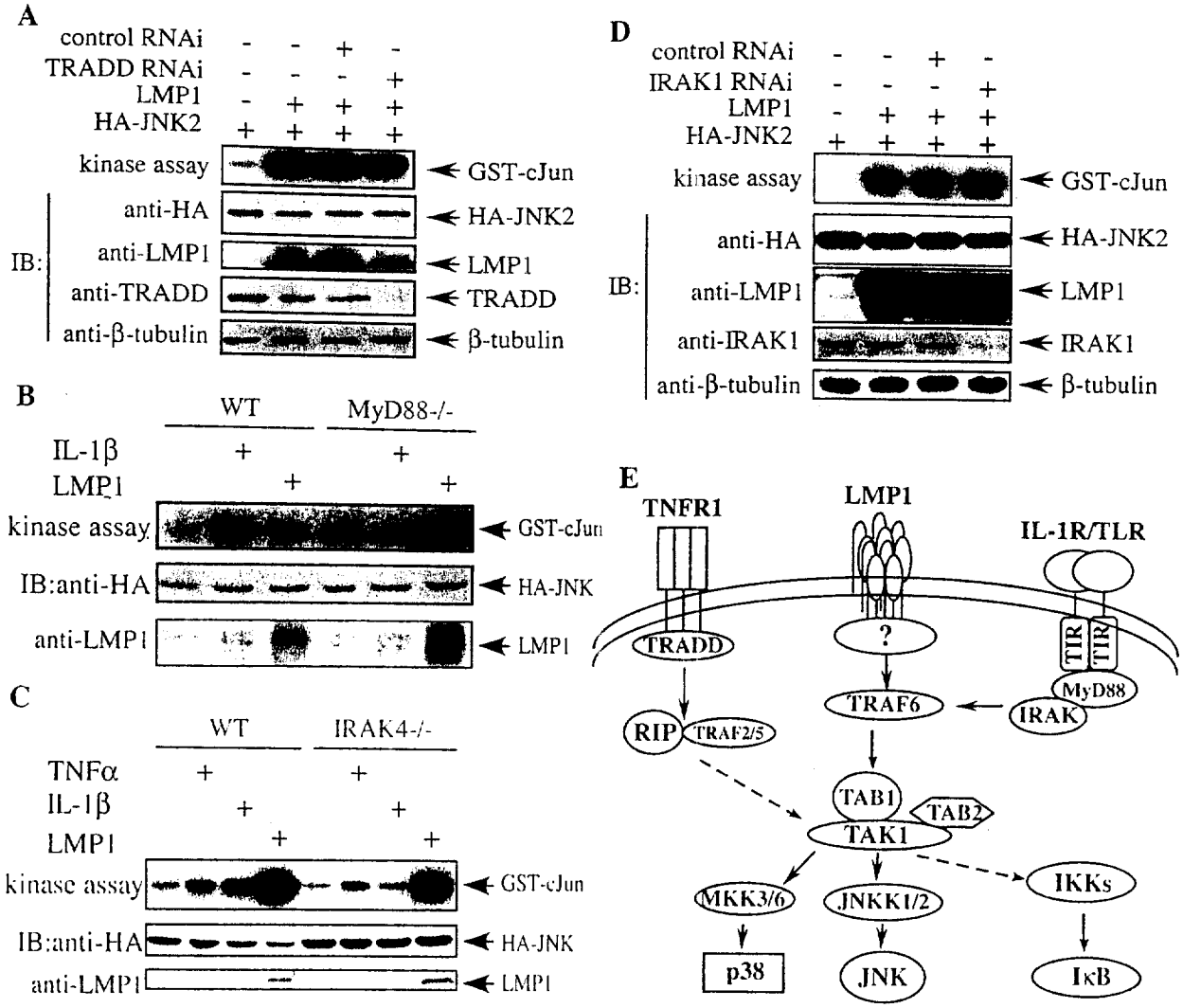

FIG. 6. TRADD, MyD88, IRAK1, and IRAK4 are not essential in the LMP1-mediated JNK pathway. (A) HeLa cells were cotransfected with various plasmids as indicated with or without a control or TRADD-specific siRNA. (B and C) The wild-type, MyD88$^{-/-}$, and IRAK4$^{-/-}$ MEFs were separately cotransfected with HA-JNK2 and either an empty vector or LMP1 with or without IL-1β treatment (10 ng/ml for 10 min). (D) 293T cells were cotransfected with various plasmids as indicated with or without a control or IRAK1-specific siRNA. HA-JNK2 from panels A through D was subjected to immune-complex kinase assays. (E) Summary of the LMP-mediated JNK pathway in comparison with the TNFR1- and IL-1R/TLR-mediated JNK pathways. Solid arrows connect molecules with an established direct physical interaction, while broken arrows connect molecules with or without direct interaction. TIR, TLR- and IL-1R-related domain.

complex, we asked whether TRAF6 directly interacts with TAB1 or TAK1. When cotransformed into yeast cells, TRAF6 readily interacted with TAB1 but not TAK1 in the yeast two-hybrid assays (Fig. 5F). Furthermore, when cotransfected into 293T cells, TAB1 was specifically coprecipitated with TRAF6 but not JNKK2 (a negative control) (Fig. 5G). Our data indicate that TRAF6 is specifically involved in the LMP1-mediated JNK pathway and serves to bridge LMP1 and TAB1.

TRADD, MyD88, IRAK1, and IRAK4 are not essential in the LMP1-mediated JNK pathway. Upstream of TRAF6, either TRADD or MyD88/IRAKs could potentially be involved (5, 16, 21). As no TRADD knockout mice were available, we again resorted to siRNA. HeLa or 293T cells were cotransfected with HA-JNK2 and LMP1 with or without siRNA. Although the endogenous TRADD expression was largely abolished by the TRADD-specific siRNA, LMP1-mediated JNK activation was not significantly affected in either HeLa (Fig. 6A) or 293T cells (data not shown). This result was in agreement with a previous report based on overexpression of a truncated TRADD (24). Similarly, in MyD88$^{-/-}$ and IRAK4$^{-/-}$ MEF cells, even though IL-1β failed to activate JNK (1, 39), LMP1-mediated JNK activation was unaffected (Fig. 6B and C). To further test whether IRAK1 could be involved, we assayed JNK activation by LMP1 in the presence of a control or the IRAK1-specific siRNA. As shown in Fig. 6D, even though the endogenous IRAK1 protein level was largely reduced by the IRAK1-specific siRNA, LMP1-mediated JNK activation was not significantly compromised. Our results suggest that neither TRADD nor MyD88/IRAKs are critically involved in the LMP1-mediated JNK pathway.

DISCUSSION

The LMP1-mediated JNK pathway is distinct from those utilized by members of the TNFR and TLR/IL-1R subfamilies. Although both TNFR1 and IL-1 receptor (IL-1R)/Toll-like receptor (TLR) can activate NF-κB and JNK when engaged by their respective ligands, the underlying signaling mechanisms are quite different. While TNFR1 utilizes both TRADD and TRAF2/5 to activate NF-κB and JNK, IL-1R/TLR selectively utilizes MyD88, IRAKs, and TRAF6 for signaling (Fig. 6E) (5, 16, 21). For the LMP1-mediated JNK pathway, in contrast to the prevailing paradigm (15, 31), we find that TRADD, TRAF2, and RIP are not essential; instead, TRAF6 is absolutely required. The latter result completely differs from a recent report in which a role for TRAF6 in the LMP1-mediated JNK pathway was ruled out solely based on the data derived from overexpression of a dominant-negative TRAF6 (36). Although both the LMP1- and IL-1R/TLR-mediated JNK pathways share some common intermediary signaling components (i.e., TRAF6 and TAK1), the LMP1-mediated pathway is unique in that it does not require MyD88, IRAK1, and IRAK4 (Fig. 6E). It remains unclear how exactly LMP1 interacts with TRAF6. Various TRAF proteins are known to interact with receptors through one of the following three mechanisms: direct interaction with receptors (e.g., CD40/TRAF6 and TNFR2/TRAF2), indirect receptor interaction through TRADD (e.g., TNFR1/TRAF2), and indirect receptor interaction through MyD88/IRAKs (e.g., TLR4/TRAF6 and IL-1R/TRAF6) (2, 9). In the LMP1-mediated JNK pathway, as MyD88, IRAK1/4, and TRADD are not required, we examined whether LMP1 directly interacts with TRAF6. Although a consensus TRAF6-binding motif (**P**-X-**E**-X-X-[aromatic/acidic residues]) defined from crystallographic studies is not present in LMP1 (45), a similar motif (^{379}**PVQLSY**^{384}) is present at the carboxyl terminus of LMP1, which may allow a weak but direct interaction between LMP1 and TRAF6. Nevertheless, we were unable to detect such a direct interaction in either the yeast two-hybrid assays or glutathione *S*-transferase pull-down assays (unpublished data). Thus, further investigation is needed to address the issue.

Downstream of TRAF6, TAB2 was thought to bridge TRAF6 and TAK1 in the IL-1-mediated JNK and NF-κB pathways (22, 40, 42). Unexpectedly, IL-1-mediated JNK and NF-κB activation was unaffected in TAB2$^{-/-}$ MEFs (35). Similarly, LMP1-mediated JNK activation was not affected in TAB2$^{-/-}$ cells either (Fig. 4A). As TAB1 is indispensable for TAK1 activation (26, 38), our findings that TRAF6 interacts with TAB1 in both yeast and mammalian cells and that both TRAF6 and TAB1 form complexes with LMP1 indicate that TAB1 bridges TRAF6 and TAK1.

TAK1 functions as one of the key signal hubs in the intracellular signaling network. Identification of a specific MAP3K in a particular biological process has been a difficult task owing to the presence of a large number of MAP3Ks and their promiscuous properties when overexpressed. Few pathway-specific MAP3Ks have been convincingly identified so far. Many previous studies relied on overexpression of either a constitutively active or a dominant-negative MAP3K, which often leads to confusing and conflicting results. With more knockout mice generated and the siRNA technique in widespread use, one should resort to them whenever possible as a complementary and more informative approach to avoid potential artifacts arising from overexpression of dominant-active/negative mutants. In this report, supported by experiments using either a kinase-dead form of TAK1 (classical dominant-negative approach) or the TAK1-specific siRNA, we reveal that TAK1 is the specific MAP3K involved in the LMP1-mediated JNK pathway. In addition, we also show that TAK1 is required for LMP1 to activate the p38 MAPK. A recent report based on the siRNA approach also convincingly demonstrated that TAK1 is specifically involved in the lipopolysaccharide (LPS)-mediated JNK pathway (6), which is in line with the fact that LPS selectively engages TLR4/MyD88/IRAKs/TRAF6 for downstream signaling (2, 9, 18, 19). Interestingly, TAK1 is also shown to be required for NF-κB activation in response to either IL-1β or TNF-α by acting upstream of IκB kinases (33, 41, 42). Moreover, our preliminary results indicate that TAK1 is also critically involved in LMP1-mediated NF-κB activation (L. Wu and Z. Wu, unpublished data). Thus, we propose that TAK1 functions as one of the important signal hubs in the interlaced signaling network in cells, as it serves as both a point of convergence for upstream signal inputs (e.g., TNF-α, IL-1, LPS, and LMP1, etc.) and a point of divergence for downstream signal outputs (e.g., activation of JNK, p38 MAPK, and NF-κB) (Fig. 6E).

Our current studies not only clarify the existing confusion in the field of LMP1 signaling but also completely change the prevailing paradigm on the LMP1-mediated JNK pathway. In the future, it will be important to define the exact roles played by the JNK and NF-κB pathways in LMP1-mediated cellular transformation. The intermediary signaling components we have identified could be used as potential drug targets to interfere with the LMP1-mediated JNK pathway and hence EBV-associated diseases.

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