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Characterization of LMP-1's Association with TRAF1, TRAF2, and TRAF3

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The latent membrane protein 1 (LMP-1) of Epstein-Barr virus (EBV) contributes to the immortalizing activity of EBV in primary, human B lymphocytes. LMP-1 is targeted to the plasma membrane, where it influences signaling pathways of infected cells. LMP-1 has been found to associate with members of the tumor necrosis factor receptor-associated factor (TRAF) family of proteins. As with LMP-1, the TRAF molecules have been shown to participate in cell signaling pathways. We have characterized and mapped in detail a region of LMP-1 that associates with TRAF1, TRAF2, and TRAF3. TRAF3 alone associates with LMP-1 in a yeast two-hybrid assay, whereas all three TRAF molecules associate with LMP-1 under various conditions when they are assayed in extracts of human cells. TRAF1, TRAF2, and TRAF3 appear to associate independently with LMP-1 but bind an overlapping target site. TRAF3 associates with LMP-1 most avidly and can compete with TRAF1 and TRAF2 for binding to LMP-1. TRAF2 associates with truncated derivatives of the carboxy terminus of LMP-1 more efficiently than with the intact terminus, indicating that LMP-1's conformation may regulate its association with TRAF2. Finally, point mutations that decrease LMP-1's association with the three TRAF molecules to 3 to 20% of wild-type levels do not detectably affect otherwise intact LMP-1's induction of NF-κB activity. Therefore, these associations are not necessary for the majority of intact LMP-1's induction of this signaling pathway.

Epstein-Barr virus (EBV) establishes a latent infection in resting human B lymphocytes by initiating and maintaining the proliferation of the infected cells, many of which become immortalized (33, 40). Latent membrane protein 1 (LMP-1) of EBV contributes to this immortalization (21), but the mechanism by which it does so is unknown. Studies aimed at elucidating LMP-1's contribution to EBV's immortalization of B lymphocytes have identified many changes associated with its expression in cells. LMP-1 can affect the growth properties of some cell lines, which identifies it as an oncoprotein (2, 5, 31, 37, 41). It can modify expression of a variety of cellular genes in lymphoblastoid cells as well as inhibit differentiation or induce expression of the epidermal growth factor receptor in epithelial cell lines (11, 18, 27, 42). Its effects in epithelial cells in culture may reflect its frequent expression in nasopharyngeal carcinoma tumor cells (4, 10). Finally, LMP-1 is cytotoxic when expressed at high levels in both lymphoid and epithelial cell lines, a shared phenotype which indicates that one or more of its activities or its level of expression must be regulated for survival of the host cell (15).

Several characteristics of LMP-1 are consistent with its stimulating signal-transducing pathways. LMP-1 is an integral membrane protein with an intracellular amino terminus of 25 amino acids, six hydrophobic membrane-spanning domains, and an intracellular carboxy terminus of 200 amino acids (3, 12, 24). LMP-1's oncogenic activity (26) correlates with its ability to attach to the cytoskeleton, localize in patches in the plasma membrane, and turn over rapidly (1, 24, 25). These properties are shared with activated growth factor receptors. LMP-1 can also induce the activity of the NF-κB/Rel family of transcription factors soon after LMP-1 is expressed in some cell lines (14, 22, 29, 37). However, LMP-1's ability to induce NF-κB efficiently is not required for it to be oncogenic (19, 29).

One path by which LMP-1 may signal was found when LMP-1 was shown to associate with a tumor necrosis factor receptor (TNFR)-associated factor (TRAF) molecule in the yeast *Saccharomyces cerevisiae* (32). TRAF1 or TRAF2 and derivatives of LMP-1 were also found to associate with each other (8, 20). TRAF molecules were originally named for a member found to associate with TNFR2; they all have a conserved domain at their carboxy termini, the TRAF domain, which is hypothesized to mediate signaling (35). The TRAF domain is known to mediate homodimer formation in all family members tested and heterodimer formation of TRAF1 and TRAF2 (34–36). The TRAF domain is also needed for interactions with the TNFR family of proteins (6, 17).

Amino acids 186 to 231 of LMP-1 are in its carboxy terminus and interact with the TRAF molecule in yeast (32). The last 53 amino acids (residues 334 to 386) of LMP-1 comprise a nonredundant domain that is required for efficient induction of NF-κB in 293 cells (19, 29). Because these domains do not overlap, we have characterized a binding site within LMP-1 for TRAF molecules to determine if this binding is necessary for the stimulation of NF-κB activity and to characterize the association of different TRAF molecules with LMP-1. CD30 binds TRAF1, TRAF2, and TRAF3 proteins, and CD40 binds TRAF2 and TRAF3 (13, 36). An alignment of the binding regions of CD30, CD40, and LMP-1 for TRAF1, TRAF2, and TRAF3 identifies conserved sequences in CD30, CD40, and LMP-1 that are candidates for mediating their associations with TRAF molecules (Fig. 1). Four adjacent mutants of LMP-1 which changed all of the charged and histidine residues to alanines in this region of LMP-1 (Fig. 1) were constructed and studied. Mutants of LMP-1 with reduced or undetectable binding for TRAF molecules stimulate the activity of NF-κB as

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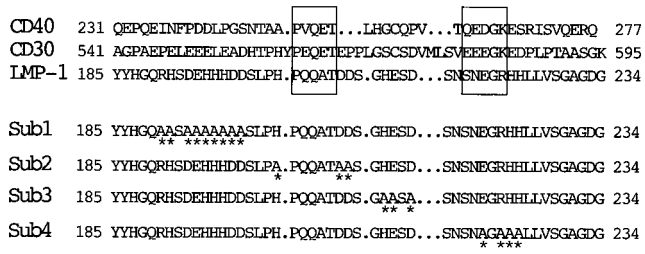

FIG. 1. Computer alignment of the carboxy-terminal regions of CD30, CD40, and LMP-1, each of which associates with TRAF2 and TRAF3. CD30 and LMP-1 but not CD40 associate with TRAF1. The two sets of boxed residues represent the regions that are conserved in CD30 and CD40 and are known to play a role in their association with TRAF molecules. Below the alignment is the amino acid sequence of each of the four substitution mutants characterized in this study, with an asterisk below each alanine that was introduced as a substitute for charged and histidine residues.

does wild-type LMP-1. Our studies with three TRAF members, TRAF1, TRAF2, and TRAF3, indicate that all can associate with LMP-1 but do so under different conditions with different apparent affinities. Only TRAF3 detectably associates with LMP-1 in yeast. TRAF3 expressed in mammalian cells also binds to LMP-1 in higher concentrations of salt than do TRAF1 and TRAF2. TRAF2 binds poorly to the intact carboxy terminus of LMP-1 relative to its binding to truncated derivatives of LMP-1, consistent with a model that TRAF2's association with LMP-1 is affected by LMP-1's conformation. TRAF3 competes equally for association of both TRAF1 and TRAF2 with LMP-1.

MATERIALS AND METHODS

Construction of vectors. Different portions of the carboxy terminus of LMP-1 were introduced into the pASI vector (9) to test their association with TRAF molecules fused to the GAL4 activation domain (36) in yeast.

Four substitution mutants in which charged and histidine residues between amino acids 181 and 231 were altered to alanines were introduced into LMP-1. These were generated by first annealing two oligonucleotides and repairing their ends with the Klenow fragment of DNA polymerase I. The oligonucleotides used were as follows (their complementary sequences are underlined): Sub 1, 5′ TAT TACCATGGACAAGCAGCCAGTGC TGCAGCCGCCGCCGCTGCCT CCCTCCCG 3′ and 5′ CCAGAATCATCGGTAGCTTGTTGAGGGTGCGG GAGGGAG 3′; Sub 2, 5′ TATTACCATGGACAACGACACAGTGATGAAC ACCACCACGATGACTCCCTCCCG 3′ and 5′ CCAGAAGCAGCGGTAGC TTGTTGAGGGGCCGGGAGGGAG 3′; Sub 3, 5′ CGCCGCATCTGCCTCT AACT 3′ and 5′ CCGTCGCCGGCTCCACTCACGAGCAGGTGGTGTCTG CCCTCGTTGGAGTTAGAGGCAGATGCGGCG 3′; and Sub 4, 5′ GCATG AATCTGACTCTAACTCCAAC 3′ and 5′ CCGTCGCCGGCTCCACTCACG AGCAGGGCGGCTGCGCCCGCGTTGGAGTTAGA 3′.

After annealing and repair synthesis, Sub 1 and Sub 2 were digested with *Nco*I and introduced into LMP-1 between *Nco*I and *Msc*I sites. After annealing and repair synthesis, Sub 3 and Sub 4 were digested with *Ngo*MI and introduced into LMP-1 between *Msc*I and *Ngo*MI sites. The sequences of all derivatives of LMP-1 were verified. These substitutions in LMP-1 were introduced into the pASI vector containing the carboxy terminus of LMP-1 (residues 181 to 386) as well as into a mammalian vector, using the simian virus 40 early promoter to express full-length LMP-1 (29). The carboxy terminus of LMP-1 (residues 181 to 386), its derivatives containing the four substitution mutations, and two truncated derivatives [LMP-1(181-331) and LMP-1(181-231)] were fused to glutathione *S*-transferase (GST) by cloning the appropriate DNAs between the *Bam*HI and *Eco*RI sites of the bacterial vector pGEX-2T (Pharmacia). GST–LMP-1(181-331) contains amino acids 181 to 333 of LMP-1 but is referred to as GST–LMP-1(181-331) to be consistent with previous studies using this derivative (26, 29).

Yeast two-hybrid TRAF expression vectors were obtained from M. Rothe and are described elsewhere (36).

Mammalian TRAF expression vectors for hemagglutinin (HA)-human TRAF2 (hTRAF2) and HA-hTRAF3 are expressed from the cytomegalovirus immediate-early (CMV IE) promoter in pCDNA 3 (Invitrogen) with an HA epitope upstream of hTRAF2 or hTRAF3 (17). HA-hTRAF1 was cloned by digesting HA-hTRAF2 with *Bam*HI and *Xba*I (removing hTRAF2) and inserting a hTRAF1 PCR product digested with *Bam*HI and *Xba*I.

Assays in yeast. *S. cerevisiae* Y190 was transformed by a protocol using lithium acetate (39). Assays for β-galactosidase activity in colonies of yeast on lifts were performed as described previously (9).

Assays for β-galactosidase activity of yeast grown in liquid media were performed with GALacto-Light (Tropix). Yeast containing plasmids of interest were patched on minimal medium plates lacking leucine or tryptophan and grown overnight at 30°C. Patches of yeast were inoculated into YPAD medium (39) and grown to an optical density at 600 nm of ~0.1. Yeast cells were shaken for 5.5 h at 30°C and harvested. Yeast cells (5×10^5) were collected, and the remaining yeast cells were stored for analysis by Western blotting. Then 5×10^5 cells were resuspended in 100 μl of lysis buffer (100 mM KH_2PO_4 [pH 7.8], 0.2% Triton X-100, 5 μl of 0.1% sodium dodecyl sulfate [SDS], 1-μl of 0.1 M dithiothreitol). The cells were vortexed briefly, and 15 μl of $CHCl_3$ was added. The cells were vortexed again, and 10 μl of the suspension was added to 67 μl of 1× GALacton-100 (Tropix) and incubated in the dark for 1 h at room temperature; 100 μl of light emission accelerator was added, and the relative light units were read for 5 s on a Monolight 2010 luminometer.

The expression of derivatives of LMP-1 in yeast was determined by Western blotting. Yeast cells were washed in water and resuspended in 200 μl of buffer C without NaCl (20 mM HEPES, 2 mM EDTA, 10% glycerol, 0.1% Nonidet P-40 [NP-40]); ~100 mg of acid-washed glass beads was added, and the mixture was vortexed for 2 min on high. Debris and beads were spun out, and the supernatant was removed. The concentration of protein in the supernatant was measured by a Bradford assay (Bio-Rad). Ten micrograms of protein was loaded per lane and separated on a 10% polyacrylamide gel with SDS. The separated proteins were transferred to nitrocellulose and blocked in 1× phosphate-buffered saline (PBS)–1% nonfat dried milk–0.05% Tween 20. The derivatives were detected with a rabbit polyclonal antibody to LMP-1's carboxy terminus (1) and an anti-rabbit antibody conjugated to alkaline phosphatase.

Isolation of fusions between GST and LMP-1. The GST–LMP-1 fusions in pGEX-2T were expressed in the *Escherichia coli* DH5α. Colonies of these cells were grown in 5 ml of Terrific broth with 100 μg of ampicillin per ml overnight at 37°C. These cultures were added to 50 ml of Terrific broth with 100 μg of ampicillin per ml and grown for 3 h at 37°C. Isopropylthiogalactopyranoside was added to a final concentration of 0.4 mM, and the cultures were shaken at 25°C. The cells were harvested after 1 h by centrifugation, two washes with H_2O, and resuspension in 1 ml of NETN (20 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg of benzamidine per ml, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml). The resuspended cells were sonicated with two 6-s pulses and centrifuged at 12,000 rpm in a microcentrifuge to remove debris. The supernatant was removed and stored at −70°C for use later in binding assays.

Generation of 293 cell lysates expressing TRAF molecules. 293 cells were transfected with 20 μg of each hTRAF expression vector per 15-cm-diameter dish with calcium chloride and HEPES-buffered saline. The transfected cells were harvested after 48 h and washed with PBS. Then 10^7 cells were resuspended in buffer A (0.1% NP-40, 50 mM HEPES [pH 7.2], 150 mM NaCl, 10% glycerol, 2 mM EDTA, protease inhibitors). The resuspended, lysed cells were centrifuged for 10 min at 10,000 rpm at 4°C in a microcentrifuge, and the supernatant was further centrifuged for 20 min at 25,000 rpm at 4°C in an ultracentrifuge. The final supernatant was collected and stored at −70°C for later use.

Detection of the binding of GST–LMP-1 to TRAF molecules in extracts of 293 cells. Glutathione-Sepharose beads (50 μl; Pharmacia) were washed twice with NETN and incubated with 25 μl of extracts of *E. coli* containing GST–LMP-1 or its derivatives and 125 μl of NETN at 4°C for 30 min. The beads bound to the fusion proteins were washed twice with buffer A. Typically 50 μl of a human TRAF lysate and 200 μl of buffer A and GST–LMP-1-bound beads were incubated for 2 to 3 h at 4°C. The beads were then washed four times with buffer A containing 350 mM NaCl; 25 μl of SDS sample buffer was added to each pellet of beads and boiled for 15 min. Ten microliters (~1/4 of the total reaction) was resolved by SDS-polyacrylamide gel electrophoresis on a 10% gel. The resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked with 1% dried nonfat milk–0.2% Tween 20 and then incubated sequentially with 12CA5 anti-HA monoclonal antibody, affinity-purified rabbit anti-mouse immunoglobulin G (IgG) antibody (Serotech), and ^{35}S-labeled donkey anti-rabbit IgG antibody (Amersham). The amount of TRAF was determined by PhosphorImager (Molecular Dynamics) analysis.

Measurement of the induction of NF-κB activity by LMP-1 and its derivatives. 293 cells were transfected with expression vectors for LMP-1 and its derivatives with calcium chloride and HEPES-buffered saline. A derivative of LMP-1 that lacks the first 43 amino acids (NΔ43) expressed from the CMV IE promoter was used as a negative control (29). An NF-κB-dependent luciferase reporter described previously (29) was cotransfected with the expression vectors for LMP-1 to measure their stimulation of NF-κB activity. The transfected cells were harvested after 48 h for measurement of LMP-1 and of luciferase; 4×10^5 cells were assayed for luciferase as described previously (29). Between 10^6 and 2×10^6 cells were harvested for Western blotting for detection of LMP-1 levels. Samples were washed once with PBS and resuspended with SDS sample buffer at 8×10^3 cells/μl. Samples were sonicated with two 6-s pulses, separated on an SDS–10% polyacrylamide gel, and transferred to nitrocellulose membranes, which were blocked as described above. The levels of expression of LMP-1 and its derivatives were measured by incubating the nitrocellulose sequentially with affinity-purified

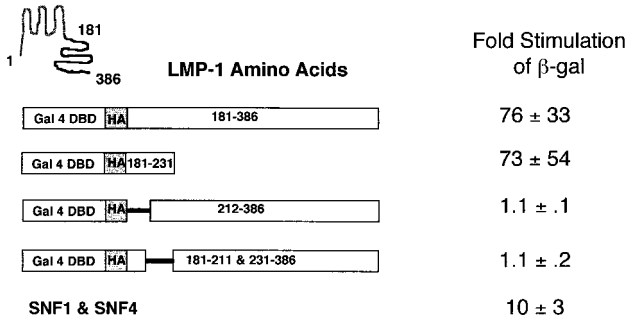

FIG. 2. Stimulation of β-galactosidase activity by derivatives of LMP-1 associating with hTRAF3 in yeast. LMP-1 is represented at top; the darkened stretch represents the residues of LMP-1 analyzed in these assays. At the left is a diagram of each derivative of LMP-1 used. The GAL4 DNA-binding domain (DBD) was fused to an epitope tag (HA) which was ligated in frame to the amino acids of LMP-1 shown. At the right is shown the fold stimulation calculated from assays of yeast transformed with the designated LMP-1 derivative (1 μg) and pACT or pGAD-hTRAF3 (1 μg). SNF1 and SNF4 are yeast proteins that interact in yeast two-hybrid and represent positive controls for the assay (9). β-Galactosidase (β-gal) levels were measured in three independent yeast colonies transformed with pASI-LMP and pACT (empty vector) or pGAD-hTRAF3, and each colony was assayed three times. The fold stimulation of β-galactosidase was calculated by dividing the β-galactosidase activity detected from yeast expressing LMP-1 and hTRAF3 by that in yeast expressing LMP-1 and pACT; ± represents 1 standard deviation from the mean.

rabbit anti-LMP-1 antibody and ^{35}S-labeled donkey anti-rabbit IgG antibody. The proteins were quantified by PhosphorImager analysis.

RESULTS

LMP-1's carboxy terminus specifically interacts with TRAF3 and not TRAF1 or TRAF2 in yeast. We tested whether LMP-1's last 205 amino acids could interact with TRAF1, TRAF2, or TRAF3 in a yeast two-hybrid assay. It has been shown previously that hTRAF3 interacts with the last 200 amino acids of LMP-1 in yeast, whereas human TRAF1 does not (32). The last 205 amino acids of LMP-1 were tested for interaction with murine TRAF1 (mTRAF1), mTRAF2, hTRAF2, or hTRAF3, each fused to the GAL4 activation domain in pGAD. Expression of each TRAF fusion was confirmed by detecting dimerization of each TRAF molecule fused to the DNA-binding and activation domains of GAL4. Expression of LMP-1 was verified by Western blotting using a polyclonal antibody to the C terminus of LMP-1. LMP-1 did not associate with mTRAF1, mTRAF2, or hTRAF2 but did associate with hTRAF3, as measured by production of β-galactosidase and growth of cells on minimal plates lacking histidine.

We mapped the site in LMP-1 to which TRAF3 binds in yeast. Deletion mutants were constructed based on the findings of Mosialos et al. (32), and each was tested for its ability to associate with TRAF3 in three independent yeast colonies. Deletion of residues 181 to 211 and 212 to 230 of LMP-1 abrogated LMP-1's association with TRAF3. LMP-1 residues 181 to 231 were sufficient to associate with TRAF3 as effectively as LMP-1's intact carboxy terminus (Fig. 2).

The deletional mapping indicated that residues 181 to 231 of LMP-1 included all residues that were necessary and sufficient to bind hTRAF3 in yeast. To identify the residues critical for this binding, we constructed four mutants in which the charged amino acids and histidines were substituted with alanines (Fig. 1 and 3A). These four substitution mutants spanned residues 181 to 231 within the full-length carboxy terminus of LMP-1 and were expressed in the yeast vector (Fig. 3A). Sub 1 associated with hTRAF3 as effectively as did wild-type LMP-1, Sub 2 did not associate, the association of Sub 3 was reduced by approximately 20%, and that of Sub 4 was reduced to 10% of the wild-type level (Fig. 3B). Clearly, substituting alanines for histidine and charged amino acids throughout a stretch of 25 amino acids of LMP-1 disrupts its association with hTRAF3 in yeast.

Association of hTRAF1, hTRAF2, and hTRAF3 in extracts of mammalian cells with LMP-1 in vitro. The failure of TRAF1 and TRAF2 to associate with LMP-1 in yeast may arise for a variety of reasons. To test if these TRAF molecules could associate with LMP-1 in a cytoplasmic extract, expression vectors for HA-tagged hTRAF molecules were introduced into 293 cells and cytoplasmic extracts were tested for an association of the HA-tagged moieties with a GST–LMP-1 fusion. In these experiments, the amount of GST–LMP-1 used

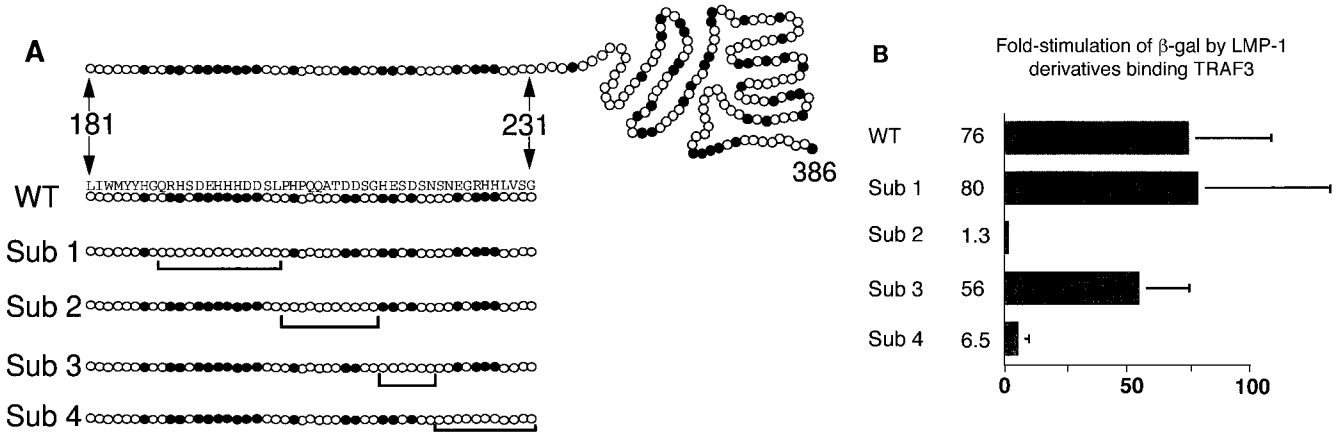

FIG. 3. Stimulation of β-galactosidase activity by LMP-1 and its substitution mutants associating with hTRAF3 in yeast. (A) At the top are depicted the carboxy-terminal amino acids of LMP-1(181-386), with the charged (K, D, E, and R) and H residues in black. LMP-1 residues 181 to 231 are labeled with the appropriate amino acid above each position. Below are diagrams of residues 181 to 231 of wild-type LMP-1 (WT) and four substitution mutants. Underlined in each mutant is the stretch of amino acids in which charged and H residues were substituted with A. Not shown in the diagrams but present in each derivative is the GAL4 DNA-binding domain, HA tag, and the remainder of LMP-1's C terminus. (B) Fold stimulations of β-galactosidase activity assayed in yeast cotransformed with the LMP-1 derivatives in pAS1 and pGAD-TRAF3. Three independent colonies of yeast were assayed three times as described in the legend to Fig. 1. Error bars represent 1 standard deviation from the mean.

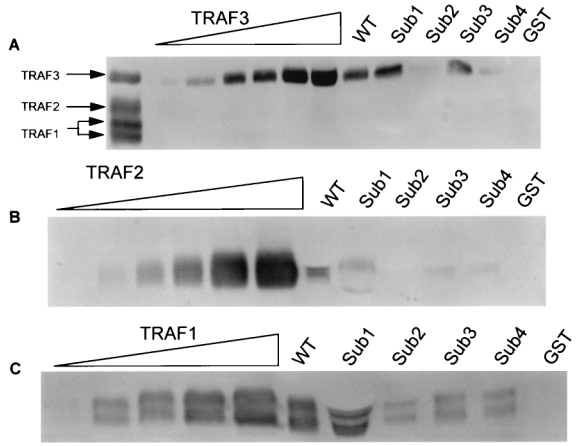

FIG. 4. Association of hTRAF1, hTRAF2, and hTRAF3 with wild-type GST–LMP-1 (WT) and its substituted mutants. HA-tagged TRAF molecules were expressed in 293 cells, and cell extracts containing approximately equal amounts of hTRAF1, hTRAF2, or hTRAF3 were incubated for 2 h with GST–LMP-1 or mutant derivatives bound to 50 μl of glutathione-Sepharose beads. The beads were washed extensively and boiled in 25 μl of SDS sample buffer; an aliquot was separated in an SDS–10% polyacrylamide gel and transferred to nitrocellulose for detection by Western blotting. Increasing amounts of hTRAF lysate were loaded to generate a standard curve for measurement of the amount of hTRAF detected in each sample. (A) hTRAF3's association with GST–LMP-1(181-386) and its substitution mutants is shown; 12.5 μl of a lysate of GST–LMP-1 and 12.5 μl of a lysate of hTRAF3 were used in each incubation; 0.02, 0.05, 0.2, 0.5, 2, and 5 μl of the hTRAF3 lysate were loaded left to right to generate the standard curve. Lanes with 12.5% of the reactions of GST–LMP-1(181-386), Sub 1, Sub 2, Sub 3, Sub 4, and GST with TRAF3 were loaded as indicated. (B) LMP-1's association with hTRAF2 is shown; 40 μl of 293 cell lysate expressing hTRAF2 was assayed, and 0.02, 0.05, 0.2, 0.5, 2, and 5 μl of the hTRAF2 lysate were used to generate the standard curve. The migration of hTRAF2 in several lanes is altered because of the interfering migration of derivatives of the GST–LMP-1. Lanes with 25% of the reactions of GST–LMP-1(181-386), Sub 1, Sub 2, Sub 3, Sub 4, and GST with hTRAF3 were loaded as indicated. (C) LMP-1's association with hTRAF1 is shown; 12.5 μl of 293 cell lysate expressing hTRAF1 was assayed, and 0.02, 0.05, 0.2, 0.5, 2 μl of hTRAF1 lysate were used to generate the standard curve. The migration of associated hTRAF1 in the Sub 1 lane is altered because of the interfering migration of the Sub 1 GST fusion protein. See Fig. 5A for Sub migration patterns. Lanes with 25% of the reactions of GST–LMP-1(181-386), Sub 1, Sub 2, Sub 3, Sub 4, and GST with hTRAF3 were loaded as indicated.

was in excess such that increasing amounts of input cytoplasmic extracts resulted in increasing amounts of hTRAF molecules associating with the GST–LMP-1 (data not shown). Cytoplasmic extracts which individually contained HA-tagged hTRAF1, hTRAF2, or hTRAF3 were tested for association of the TRAF molecules with GST fused to the carboxy terminus of LMP-1 and its substitution mutants. hTRAF3 associated with wild-type LMP-1 and Sub 1 similarly; it bound Sub 2 with 3%, Sub 3 with 50%, and Sub 4 with 10% of the signal of its binding to the wild-type fusion of LMP-1 (Fig. 4A). These findings paralleled those for the yeast two-hybrid assay. However, hTRAF1 and hTRAF2 expressed in 293 cells did associate with LMP-1 and some of its mutants. hTRAF2 associated with the wild-type fusion of LMP-1, Sub 1, Sub 3, and Sub 4 barely detectably (Fig. 4B). hTRAF1 associated with wild-type LMP-1 and Sub 1 similarly; it associated with Sub 2 to 10%, with Sub 3 to 25%, and with Sub 4 to 10% of the level that it did to the wild-type fusion of LMP-1 (Fig. 4C; Table 1).

hTRAF1, hTRAF2, and hTRAF3 present in extracts of 293 cells associate with different apparent affinities to LMP-1's carboxy terminus. The associations shown in Fig. 4 occurred at 0.35 M NaCl. Incubations with 0.5 M NaCl eliminated the association of hTRAF1 and hTRAF2 with GST–LMP-1 but did not affect that of hTRAF3 with the LMP-1 fusion (data not shown). The associations of the different hTRAF molecules with the different mutants of LMP-1 indicate that the regions of LMP-1 required for these associations are similar.

TABLE 1. Percentages of the input TRAF molecules associating with derivatives of LMP-1's carboxy terminus fused to GST

TRAF family member	% Associating with indicated GST–LMP-1 derivative[a]				
	WT[b]	Sub 1	Sub 2	Sub 3	Sub 4
TRAF3	30	35	1	15	2
TRAF2	2	1	<0.2	0.4	0.4
TRAF1	20	20	3	5	3

[a] Values are derived from PhosphorImager analysis of the data in Fig. 3. The data are averages of two independent experiments.
[b] WT, wild-type LMP-1.

Association of hTRAF molecules with truncated derivatives of LMP-1's carboxy terminus. The association of HA-tagged hTRAF molecules with two truncated derivatives of LMP-1's carboxy terminus was assayed such that each derivative of LMP-1 was present in excess. Derivatives of LMP-1 containing amino acids 181 to 333 or 181 to 231 fused to GST were tested for their association with hTRAF1, hTRAF2, and hTRAF3 in extracts of 293 cells that coexpressed them. The intact carboxy terminus GST–LMP-1(181-386) was not used in this experiment because its migration in gels alters the migration of hTRAF2 such that signals from hTRAF1 and hTRAF2 cannot be individually quantified. GST–LMP-1(181-331) and GST–LMP-1(181-231) associate with all three human TRAFs. It is striking that the shorter derivative, GST–LMP-1(181-231), associates with approximately 3-fold more hTRAF1 and 10-fold more hTRAF2 than does GST–LMP-1(181-331) (Fig. 5A).

The association of hTRAF2 with the intact carboxy terminus of LMP-1, GST–LMP-1(181-386), GST–LMP-1(181-331), and

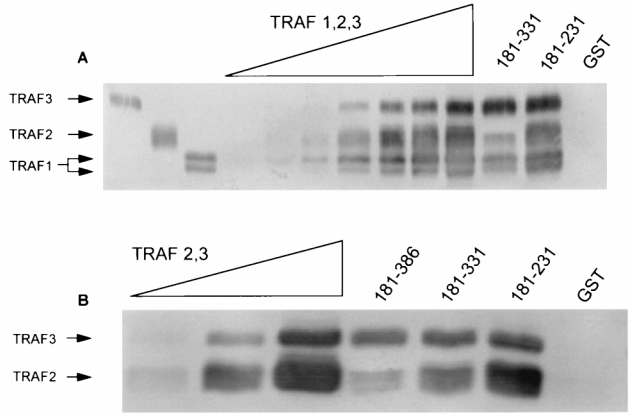

FIG. 5. Association of hTRAF1, hTRAF2, and hTRAF3 with truncated derivatives of LMP-1. (A) Western blot of hTRAF1, hTRAF2, and hTRAF3 associated with GST–LMP-1(181-331) and GST–LMP-1(181-231). The assays were performed as described in the legend to Fig. 3; 25 μl of an extract of 293 cells cotransfected with all three TRAF expression vectors was incubated with the GST fusions; 0.5-μl aliquots of 293 cell lysates individually transfected with each hTRAF vector were loaded as markers for identification of each hTRAF molecule; 0.05, 0.1, 0.2, 0.5, 1, 1.5, and 2 μl of 293 cell lysate cotransfected with hTRAF1, hTRAF2, and hTRAF3 were loaded left to right to generate the standard curve. (B) Western blot of hTRAF2 and hTRAF3 associated with GST–LMP-1(181-386) (intact carboxy terminus), GST–LMP-1(181-331), and GST–LMP-1(181-231). Assays were performed as described in the legend to Fig. 3; 12.5 μl of an extract of 293 cells cotransfected with vectors for hTRAF2 and hTRAF3 was incubated with the glutathione-Sepharose beads; 0.02, 0.2, and 2 μl of the hTRAF2/hTRAF3-containing lysate were used to generate the standard curve.

TABLE 2. Percentages of the input TRAF molecules associating with LMP-1's intact carboxy terminus or its truncated derivatives

TRAF family member	% Associating with indicated GST–LMP-1 derivative[a]		
	181-386	181-331	181-231
TRAF1	ND[b]	10	30
TRAF2	1	6	>60
TRAF3	30	35	40

[a] Values are derived from PhosphorImager analysis of the data in Fig. 4. The data are averages of two independent experiments. The TRAF1 percentages were generated from Fig. 4A. In the case of GST–LMP-1(181-331) associating with TRAF1, extrapolation was used to estimate levels of association. The TRAF2 and TRAF3 percentages were generated from Fig. 4B.

[b] ND, not determined. In comparable experiments, the amount of TRAF1 associated with GST–LMP-1(181-386) was indistinguishable from the amount associated with GST–LMP-1(181-331).

GST–LMP-1(181-231), was assayed with extracts of cells in which hTRAF2 was coexpressed with hTRAF3. hTRAF1 was omitted from this assay to permit measurement of hTRAF2 without the confounding signal of hTRAF1. GST–LMP-1(181-386) associated with approximately 30% of input hTRAF3 and only 1% of input hTRAF2 when the levels of these two human TRAF molecules were similar in the extract. GST–LMP-1(181-331) associated with slightly more input hTRAF3 (35%) and sixfold more of the input hTRAF2 (6%). GST–LMP-1(181-231) associated with 40% of the input hTRAF3 and over 60% of the input hTRAF2 (Fig. 5B; Table 2).

The finding that hTRAF2's association to LMP-1 is enhanced by deleting the last 53 and 155 amino acids of LMP-1's carboxy terminus whereas these truncations do not greatly alter the associations with hTRAF1 or hTRAF3 indicates that LMP-1's structure peculiarly affects its association with hTRAF2. These findings also indicate that hTRAF2's association with LMP-1 can be independent of that of hTRAF1 and hTRAF3 with LMP-1.

hTRAF3 can compete with hTRAF1 and hTRAF2 for association with LMP-1. Because hTRAF1, hTRAF2, and hTRAF3 associate with the same region of LMP-1, we determined if hTRAF3 could compete with hTRAF1 and hTRAF2 for association with LMP-1. The hTRAF1 and hTRAF2 expression vectors were cotransfected into 293 cells, while the expression vector for hTRAF3 was transfected into 293 cells separately. A limiting amount of GST–LMP-1(181-331) was bound to glutathione-Sepharose beads and washed extensively. GST–LMP-1(181-331) was used in this assay in place of the intact carboxy-terminus fusion, GST–LMP-1(181-386), to permit simultaneous detection of both hTRAF1 and hTRAF2. A constant amount of the extract with hTRAF1 and hTRAF2 was mixed with incrementally increasing amounts of hTRAF3-containing extract. After incubation with GST–LMP-1(181-331) bound to beads, the reaction mixtures were washed extensively and assayed by Western blotting. The association of hTRAF1, hTRAF2, and hTRAF3 appears to be biphasic. When small amounts of hTRAF3 are added, a 50 to 75% increase in the association of hTRAF1 and hTRAF2 to LMP-1 was observed (Fig. 6A). When higher amounts of hTRAF3 were added to the reactions, the amounts of associated hTRAF1 and hTRAF2 decreased proportionately. At the highest level tested, competition by hTRAF3 reduced the association of hTRAF1 and hTRAF2 to 10 to 15% of their highest levels (Fig. 6B). hTRAF3 competed with hTRAF1's and hTRAF2's association with the GST–LMP-1(181-331) derivative similarly (Fig. 6B).

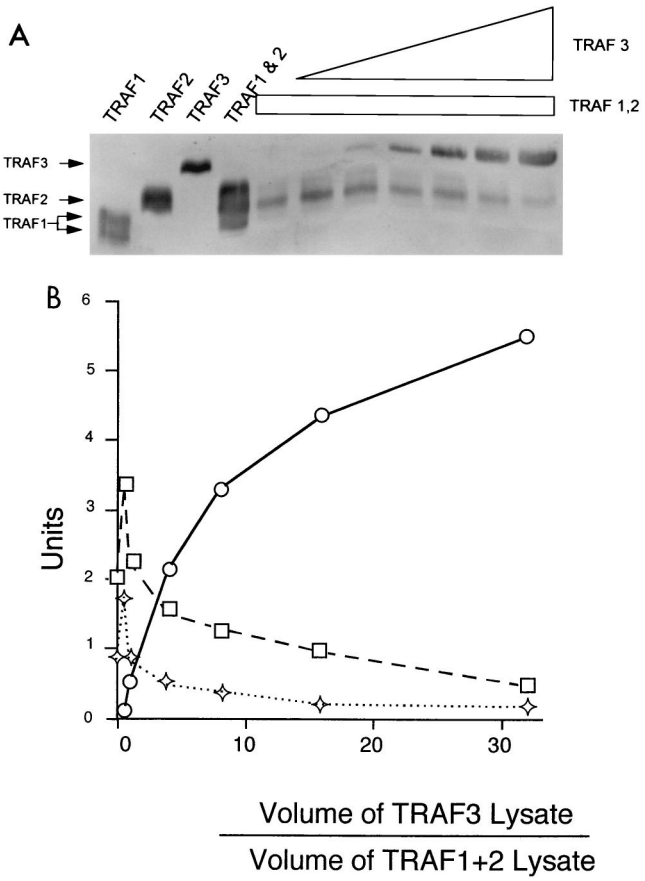

FIG. 6. hTRAF3 competes with hTRAF1 and hTRAF2 for binding to GST–LMP-1(181-331). The association of human TRAF molecules with a derivative of LMP-1 was assayed as described in the legend to Fig. 3 except that a reduced amount of GST–LMP-1(181-331) (25% of the amount used in Fig. 4) was used. GST–LMP-1(181-331) was used in place of GST–LMP-1(181-386) to avoid the distortion of the signal for hTRAF2 in the gel. (A) Six microliters of an hTRAF1/hTRAF2-containing cell lysate was incubated with 0, 3, 6, 24, 48, 96, and 192 μl of hTRAF3-containing lysate for 2 h at 4°C, washed extensively, and assayed. Loaded from left to right in the first four lanes are 293 cell extracts with hTRAF1, hTRAF2, hTRAF3, and hTRAF1 plus hTRAF2 to document the migration of the human TRAF molecules. The next seven lanes are loaded with the competition reaction mixtures with increasing quantities of hTRAF3 added from left to right. (B) Measurement of the association of hTRAF1, hTRAF2, and hTRAF3 with GST–LMP-1(181-331). The signal for each associated HA-tagged hTRAF molecule was quantified by PhosphorImager analysis and plotted on the *y* axis. The amount (microliters) of hTRAF3-containing extract added was divided by the 12.5 μl of hTRAF1/hTRAF2-containing lysate assayed and plotted on the *x* axis. □, hTRAF1; ◇, hTRAF2; ○, hTRAF3. The data are representative of two independent experiments.

LMP-1's association with hTRAF1, hTRAF2, and hTRAF3 is not required for its efficient induction of NF-κB. The expression of hTRAF2 at high levels in 293 cells can stimulate the activity of NF-κB (35, 36); similarly, the binding of hTRAF2 by CD40 and TNFR2 appears to be required for them to stimulate the activity of NF-κB (36). It was possible that the association of LMP-1 with hTRAF1, hTRAF2, or hTRAF3 could contribute to its efficient induction of NF-κB activity; however, the region of LMP-1 with which these hTRAF molecules associate was previously found not to be necessary for the majority of this induction (29). To address this possibility, LMP-1 and the four substitution mutants that affect binding of the hTRAF molecules were tested for their induction of NF-κB activity in 293 cells. The level of induction

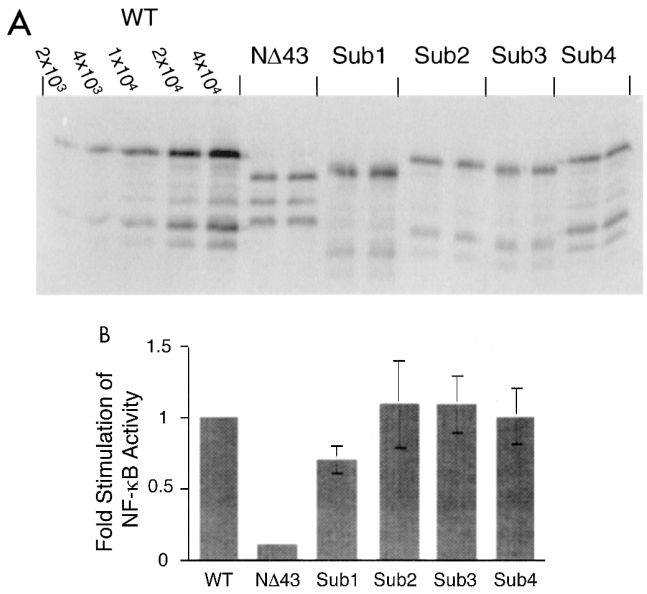

FIG. 7. Stimulation of NF-κB activity by LMP-1 and its derivatives in 293 cells. 293 cells were transfected with expression vectors and an NF-κB-responsive luciferase reporter (29). Cells were harvested after 48 h and assayed for the expression of the LMP-1 derivatives and luciferase activity. (A) Quantitative Western blot measuring expression of wild-type LMP-1 and its derivatives in 293 cells. All samples were run on an SDS–10% polyacrylamide gel and transferred to nitrocellulose for detection using a polyclonal antibody to LMP-1's carboxy terminus and an ^{35}S-radiolabeled anti-rabbit IgG antibody (Amersham). Increasing numbers (2×10^3, 4×10^3, 1×10^4, 2×10^4, and 4×10^4) of 293 cells expressing wild-type LMP-1 were used to generate a standard curve for interpolating expression the derivatives; 10^4 cells expressing mutant derivatives of LMP-1 were analyzed in duplicate. Expression of LMP-1 and its derivatives was quantified by using PhosphorImager analysis to measure the amount of the intact (slowest-migrating) species in each lane. The expression of each derivative was compared with that of the wild type (WT) by using the standard curve for interpolation. Wild-type LMP-1, Sub 1, Sub 2, Sub 3, and Sub 4 were expressed from the simian virus 40 early promoter. NΔ43 (LMP-1 that lacks the first 43 amino acids) is expressed from the CMV IE promoter. In the experiment shown, the numbers of cell equivalents relative to wild-type LMP-1 in the two lanes were 6×10^3 and 5×10^3 for NΔ43, 10×10^3 and 17×10^3 for Sub 1, 8×10^3 and 6×10^3 for Sub 2, 4×10^3 in both lanes for Sub 3, and 6×10^3 and 4×10^3 for Sub 4. (B) Fold stimulation of NF-κB activity by LMP-1 and its mutant derivatives. The level of luciferase activity induced by wild-type LMP-1 was measured and set at 1. The level of luciferase activity induced by each derivative was also measured. The level of expression of each LMP-1 protein relative to the wild type was measured in the quantitative Western blots. The fold stimulation of NF-κB activity was calculated by normalizing the luciferase activity to the relative level of expression for each mutant. Three independent experiments were performed, and the error bars represent 1 standard deviation of the mean.

by each was normalized to its own expression with quantitative Western blots. The LMP-1 proteins were detected with affinity-purified rabbit anti-LMP-1 antibodies that recognize epitopes common to all of the proteins examined (1). Three separate experiments were performed (Fig. 7). After normalization and averaging, Sub 1 induced 70% of the level of NF-κB as did wild-type LMP-1. Sub 2, Sub 3, and Sub 4 behaved similarly to wild-type LMP-1. Because some of these mutants associate with the TRAF molecules at only 3 to 20% of the level of wild-type LMP-1, yet we observe no reduction of the NF-κB activity produced by these mutants of LMP-1 in 293 cells, we conclude that these TRAF associations with LMP-1 do not mediate most of LMP-1's induction of NF-κB activity. Associations of TRAF molecules with LMP-1 that are indirect or are mediated by other regions of LMP-1 could mediate LMP-1's induction of NF-κB activity.

DISCUSSION

LMP-1 associates with members of the TRAF family (20, 32). In this study, we have mapped and characterized a region of LMP-1 that is required for an association with hTRAF1, hTRAF2, and hTRAF3.

hTRAF3 has been shown to bind LMP-1 in a yeast two-hybrid assay, while hTRAF1 does not interact detectably with LMP-1 in this assay (32). We have confirmed these results and have also shown that both mTRAF2 and hTRAF2 do not interact detectably with LMP-1 in this assay. The interaction of LMP-1 and hTRAF3 has been characterized by making and testing substitution mutants within the minimal region of LMP-1 that is sufficient to interact with hTRAF3. This characterization has revealed two nonadjacent stretches of charged and histidine residues that are required for LMP-1 to interact efficiently with hTRAF3.

Although LMP-1 does not associate with hTRAF1 and hTRAF2 in yeast (32), it does associate with all three studied hTRAF molecules in mammalian cell extracts (20) (Fig. 4 and 5; Table 2). LMP-1 appears to associate with the hTRAF molecules independently. When each of the TRAF species is expressed efficiently from the CMV IE promoter and immunologically equivalent amounts of each are tested for binding to LMP-1, hTRAF3 associates most efficiently of the three with LMP-1's intact carboxy terminus (Fig. 4A; Table 1). hTRAF3 also associates with LMP-1 under conditions (0.5 M NaCl) in which hTRAF1 and hTRAF2 do not. Finally, hTRAF3 can compete for the association of both hTRAF1 and hTRAF2 with LMP-1 (Fig. 6). A greater portion of hTRAF1 in the 293 extracts associates with LMP-1 and its substitution mutants than does hTRAF2 when both are expressed equally, as judged by their HA epitopes (Fig. 4B and C). However, the association of hTRAF2 with LMP-1 is greatly enhanced relative to that of hTRAF1 and even that of hTRAF3 when LMP-1 is progressively truncated at the C terminus (Fig. 5; Table 2). In all of our experiments using extracts of 293 cells, it is likely that the hTRAF molecules expressed from the CMV IE promoter are more abundant than endogenous hTRAF molecules or other endogenous molecules that could mediate their association with the GST–LMP-1 fusions. However, only when the hTRAF molecules are purified to homogeneity can we be certain whether they bind directly to LMP-1.

Evidence consistent with LMP-1's binding hTRAF1, hTRAF2, and hTRAF3 directly has emerged from studies of these TRAF molecules' binding to CD30 and CD40 (7, 13, 23, 38). It is striking that sequences in LMP-1 which flank those shared with CD30 and CD40 are also essential for binding the three hTRAF molecules (Fig. 1). The alterations in the Sub 2 mutant, for example, which flank one conserved set of residues reduce binding of each TRAF molecule. The CD30 mutant corresponding to Sub 4 is important for hTRAF1 and hTRAF2 but not hTRAF3 association (13). We hypothesize that it is these flanking residues which mediate the differential binding of each TRAF molecule to LMP-1 along with possible conformational changes in the carboxy terminus of LMP-1 itself.

The Sub 2 derivative of LMP-1 in which one histidine and two aspartic acids are substituted with alanines reduces the association of LMP-1 with hTRAF1, hTRAF2, and hTRAF3 almost to background levels (Fig. 4; Table 1) without affecting LMP-1's capacity to induce the activity of NF-κB (Fig. 7). This region was found not to be necessary for most of LMP-1's ability to stimulate NF-κB in our previous work (29). hTRAF2 does efficiently associate with derivatives of LMP-1 lacking the last 53 or 155 amino acids (Table 2) (8). Binding of hTRAF2 to these deletion derivatives of LMP-1 contributes to their

induction of NF-κB activity, the level of which is approximately 25 to 30% of that of wild-type LMP-1. Recent studies indicate that the last 53 amino acids of LMP-1 do induce NF-κB by an hTRAF2-dependent mechanism even though no hTRAF2 association is directly observed with this region of LMP-1 (8, 20). This finding is consistent with a model in which intact LMP-1 can induce NF-κB activity indirectly by hTRAF2. A high level of expression of hTRAF3 inhibits truncated derivatives of LMP-1 but not wild-type LMP-1 from inducing the activity of NF-κB (20, 28). We interpret this inhibition to result from hTRAF3 competing with hTRAF2 for association with LMP-1 at residues 203 to 225. In this interpretation hTRAF3 fails to inhibit intact LMP-1 from inducing the activity of NF-κB because the carboxy-terminal 53 residues of intact LMP-1 induce NF-κB indirectly through hTRAF2. In all assays where NF-κB activity is normalized to levels of expression of LMP-1, the region of LMP-1 identified here as associating with hTRAF1, hTRAF2, and hTRAF3 does not contribute detectably to NF-κB activity mediated by the intact LMP-1 protein (29) (Fig. 7).

The association of LMP-1 with hTRAF1, hTRAF2, hTRAF3, and possibly the multiple additional newly identified and yet to be identified family members could mediate signaling by LMP-1 through unknown pathways. One insight into this complexity of possibilities is provided by the recognition that truncated derivatives of LMP-1's carboxy terminus associate with TRAF2 selectively more efficiently than with TRAF1 and TRAF3 (Fig. 5; Table 2). LMP-1 turns over rapidly (1, 25), and this turnover correlates with LMP-1's functions (26). One naturally occurring cleavage in LMP-1 removes approximately 120 residues from its carboxy terminus (30). Our findings indicate that this truncated version of LMP-1 would associate with hTRAF2 efficiently, perhaps sequestering it from cellular receptors such as CD30, CD40, and TNFR2 which need to associate with TRAF2 to signal. We speculate that LMP-1 may signal through TRAF molecules by indirectly modulating the signaling of cellular receptors that also associate with these TRAF molecules.

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