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Localization of the Major NF-κB-activating Site and the Sole TRAF3 Binding Site of LMP-1 Defines Two Distinct Signaling Motifs*

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The TRAF3 molecule interacts with the cytoplasmic carboxyl terminus (COOH terminus) of the Epstein-Barr virus-encoded oncogene LMP-1. NF-κB activation is a downstream signaling event of tumor necrosis factor receptor-associated factor (TRAF) molecules in other signaling systems (CD40 for example) and is an event caused by LMP-1 expression. One region capable of TRAF3 interaction in LMP-1 is the membrane-proximal 45 amino acids (188–242) of the COOH terminus. We show that this region contains the only site for binding of TRAF3 in the 200-amino acid COOH terminus of LMP-1. The site also binds TRAF2 and TRAF5, but not TRAF6. TRAF3 binds to critical residues localized between amino acids 196 and 212 (HHDDSLPHPQQATDDSG), including the P*X*Q*X*(T/S) motif, that share limited identity to the CD40 receptor TRAF binding site (TAAPVQETL). Mutation of critical residues in the TRAF3 binding site of LMP-1 that prevents binding of TRAF2, TRAF3, and TRAF5 does not affect NF-κB-activating potential. Deletion mapping localized the major NF-κB activating region of LMP-1 to critical residues in the distal 4 amino acids of the COOH terminus (383–386). Therefore, TRAF3 binding and NF-κB activation occur through two separate motifs at opposite ends of the LMP-1 COOH-terminal sequence.

LMP-1 is an essential gene for the immortalization of B cells by Epstein-Barr virus *in vitro* (1) and functions as an oncogene in rodent transformation assays (2–4). With the exception of Burkitt's lymphoma, LMP-1 is also expressed in all EBV[1]-positive tumors where EBV is believed to play an etiologic role, including nasopharyngeal carcinomas and Hodgkin's disease (5–7). The apparent oncogenicity of the LMP-1 gene product has made this molecule the focus of intense interest in the field of EBV biology.

LMP-1 is a short lived protein with a half-life of between 2 and 4 h (8, 9). This rapid turnover occurs due to the specific cleavage in the COOH terminus at amino acid 242 resulting in the release of a 144-amino acid 25-kDa fragment that is itself rapidly degraded (10). LMP-1 is also selectively phosphorylated in the released 25-kDa fragment at serine 311 and threonine 324 (11). The role of these modifications of the LMP-1 carboxyl terminus are uncertain, although Thr-324 dephosphorylation is required for the transformation of Rat-1 cells (11). Additionally, immunohistochemical analysis of LMP-1 indicated that it aggregates in the plasma membrane and co-patches with the cytoskeletal protein vimentin (12). Patching of LMP-1 with cytoskeletal components occurs through the cytoplasmic amino-terminal residues (13). LMP-1 has thus been divided into three separate domains, the cytoplasmic amino terminus, the transmembrane, and the cytoplasmic carboxyl terminus.

All three domains of LMP-1 have been shown to be critical for transformation in focus-forming assays with the rodent cell line Rat-1 (14). The Rat-1 transformation assays were the first formal demonstration that the COOH-terminal domain of LMP-1 was critical to its function. These experiments delineated critical residues for transformation to be present in the last 23 COOH-terminal amino acids of LMP-1 (14), and additional unpublished studies suggested that the critical motif includes the last 4 COOH-terminal amino acids. Subsequent studies with recombinant virus confirmed the requirement for COOH-terminal components of LMP-1 in the EBV immortalization process during *in vitro* infection (15). The Rat-1 focus-forming assay, therefore, has good predictive value for LMP-1 function.

LMP-1 has been the focus of recent intensive study due to its ability to bind the family of adaptor molecules known as TRAFs (TNF receptor-associated factors; reviewed in Ref. 16), specifically to TRAF3 (also known as LAP1, CRAF1, CD40bp, and CAP1) (17–19). This is a significant finding, since it is known that the phenotypic changes and activation state of an EBV-infected cell is similar to that seen with CD40 receptor activation of B cells (20). CD40 receptor also binds to TRAF molecules, and its functions are mediated through these molecules (17, 18, 21). This is indirect evidence that EBV, specifically LMP-1, may subvert normal cellular pathways to establish and maintain the latent state of infection in B cells, as has been hypothesized previously (22).

LMP-1 expression has been linked to many of the cellular changes induced by EBV infection of B cells *in vitro* (reviewed in Ref. 20). These include induction of activation and adhesion markers (23–27) and Bcl-2 (28–30), changes in cellular proliferation (31), and NF-κB activation (30, 32–37). Most of these changes occur following stable or transient expression of LMP-1 into tumor cell lines. However, it has been shown recently that these changes, with the exception of NF-κB activation, are a consequence of LMP-1 overexpression in cell lines.[2] NF-κB activation is therefore likely to represent the most relevant measure of LMP-1 function.

The TRAF molecules are an expanding family of proteins

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[1] The abbreviations used are: EBV, Epstein-Barr virus; TRAF, TNF receptor-associated factor; TNF, tumor necrosis factor; GST, glutathione *S*-transferase; PCR, polymerase chain reaction; HA, hemagglutinin; CMV, cytomegalovirus.

[2] S. R. Brodeur and D. A. Thorley-Lawson, submitted for publication.

(TRAF1–TRAF6) that share a conserved COOH-terminal TRAF domain (reviewed in Ref. 16). Most of these molecules were identified due to their interaction with the TNF or CD40 receptors (17, 18, 38) but have been recently shown to also interact with other TNF receptor family members such as CD30 (39). TRAF2, TRAF5, and TRAF6 are the only members able to directly cause activation of NF-κB through transient overexpression (21, 40–42). TRAF2, TRAF3, and TRAF6 appear to be ubiquitously expressed, implying a general regulatory role, whereas TRAF1, TRAF4, and TRAF5 appear to be more tissue-specific. Multimerization of the TRAF molecules through the TRAF domain appears to be important for the function of all of the TRAFs identified to date (21). A molecule known as TANK or I-TRAF has also been identified that can bind to TRAF molecules and appears to be a negative regulator of the TRAF2, TRAF5, and TRAF6 NF-κB-activating function (41, 43).

Analysis of the LMP-1 carboxyl terminus has identified two regions that are potentially responsible for NF-κB activation. One of these sites maps to the first 40–45 amino acids (188–232) of the LMP-1 COOH terminus (33, 35), the same region shown to be important in TRAF3 binding activity (19). A second site maps to the last 35–45 amino acids (351–386) of the COOH terminus of LMP-1 (33, 35). This site is dispensable for B lymphocyte transformation (15) but correlates with sequences that are essential for oncogenic transformation of Rat-1 cells (14).

In this study we have dissected the cytoplasmic COOH terminus of LMP-1 to attempt to understand the role of TRAF binding in LMP-1 signaling functions. Preliminary sequence analysis suggested three or four potential TRAF binding sites in the COOH terminus of LMP-1. However, we localized the sole recognition site for binding TRAF2, TRAF3, and TRAF5 to the membrane-proximal region of the COOH terminus. Loss of TRAF binding to this site does not affect LMP-1 activation of NF-κB. A second distinct region, representing the major NF-κB-activating sequence, is composed of critical residues that include the last 4 amino acids of the LMP-1 COOH terminus. These critical residues of LMP-1 are part of a sequence that resembles a TRAF binding site, although TRAF2, TRAF3, TRAF5, and TRAF6 do not bind, and correlate perfectly with critical sequences shown previously to be essential in Rat-1 transformation assays.

EXPERIMENTAL PROCEDURES

Cell Lines—BJAB is a human EBV-negative lymphoblastoid B cell line, and IB4 is an EBV-positive lymphoblastoid B cell line. Both were maintained in RPMI 1640 and 10% fetal bovine serum (Sigma). 293 cells are a human embryonic kidney cell line and were maintained in Dulbecco's modified Eagle's medium and 10% fetal bovine serum.

Plasmids and Constructs—GST fusion constructs were made by inserting PCR products of the *LMP-1* COOH terminus into the *Eco*RI-*Bam*HI site of the pGEX2T (Pharmacia) multicloning site. For the LMPC200 (from pB3LMP-1) and LMPN55 (from pB3LMP-1 del242–386) fragments, engineered in frame 5′ *Eco*RI and 3′ *Bam*HI sites were added to the ends of the *LMP-1* sequences 5′-TATTACCATGGACAACGACAC-3′ and 5′-GCCTATGACATGGTAATGCC-3′, respectively. The LMPC35 fusion used an engineered in frame 5′ *Eco*RI site added to the *LMP-1* sequence 5′-CATAGTCATGATTCCGGCCAT-3′. The LMPC140 fusion was generated by cutting the LMPN55 PCR product with *Bgl*II and *Bam*HI and inserting this piece into the *Bam*HI site of pGEX2T. The *Bgl*II site was introduced into the LMPEnd (del242–386) construct as described previously and cut to allow for an in frame sequence starting at amino acid 245 of LMP-1. All PCR products were confirmed by sequencing.

PCR mutagenesis was used to generate the LMPM2 and LMPEndM2 double point mutant constructs. The mutagenic oligonucleotides, 5′-CCTCAACAAGCTGCCGCGGATTCTGGCCAT-3′ and its corresponding opposite strand, were used with the above 5′ *Eco*RI and 3′ *Bam*HI primers by sequential PCR steps as described previously (48). The *Nco*I fragment of the LMPM2 and LMPEndM2 PCR products were cloned into the pB3LMP-1 construct to generate full-length coding sequence containing the mutation.

MTLM (4) and the derivatives End (del242–386) and 4Stop (del364–386) were described previously (14). LMP5Stop (del377–386) and LMP6Stop (del383–386) deletions were created in the same way by replacing coding sequence with stop codons using the Amersham *in vitro* mutagenesis kit in the pB3LMP-1 vector as described previously (14).

CMV LMP-1 constructs were derived by inserting the *Eco*RI (from pB3LMP-1 MCS)-*Pvu*II (LMP-1 noncoding) fragment of the various LMP-1 mutants into the *Eco*RI-*Sma*I multicloning site of pCI-Neo (Promega).

NF-κB transient transfections utilized the pIFNLuc and pIFN(kB)$_3$Luc (44). pCMVLuc was used as a positive control for luciferase activity (gift of Dr. David Livingston), and pCMVβgal was used as an internal control to normalize for transfection efficiency.

The vector for expressing FLAG-tagged human TRAF6 (pRK5FLAGTRAF6) (40) was a gift from Dr. David Goeddel (Tularik Inc.). To produce human HA-tagged TRAF5, a cDNA for human *TRAF5* was cloned into the vector pEBD (41). Oligonucleotides encoding the HA tag were then cloned in frame at the 3′-end of the *TRAF5* cDNA sequence.

Transfections—Transient transfection experiments in BJAB were performed by electroporation of 5×10^6 cells at 300V/500 microfarads at room temperature in the Bio-Rad Gene Pulser with 1–5 μg of the appropriate vectors. Cells were harvested 24–48 h post-transfection in 300–400 μl of Reporter lysis buffer (Promega). All experiments represent three separate transfections with results done in duplicate.

GST Fusion Purification—GST fusion proteins were purified on glutathione-Sepharose-4B beads (Pharmacia Biotech Inc.) as per the manufacturer's protocol from 1 liter of isopropyl-1-thio-β-D-galactopyranoside-induced culture. Following sonication of the cells to release the GST proteins, 0.25 ml of washed packed bead volume was used for purification. The coupled beads were then stored at 4 °C in phosphate-buffered saline until they were used for *in vitro* binding assays.

In Vitro Binding Assays—Binding assays were done essentially as described previously (45). In short, 293 cells, either stably or transiently transfected with HA-tagged human TRAF2 (TRAF2/HA), TRAF3 (TRAF3/HA), or TRAF5 (TRAF5/HA) or FLAG-tagged TRAF6 (FLAG/TRAF6) expression vectors, were lysed in Nonidet P-40 lysis buffer. Lysates were incubated with appropriate GST fusion proteins coupled to glutathione beads and washed four times with Nonidet P-40 lysis buffer. Samples were then boiled in SDS sample buffer with 2-mercaptoethanol and analyzed by SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred to nitrocellulose and probed with anti-HA monoclonal antibody (Berkeley antibodies) or anti-FLAG M2 monoclonal antibody (Eastman Kodak Co., IBI) followed by a goat-anti mouse immunoglobulin antibody coupled to horseradish peroxidase (Sigma). Bound antibodies were detected with the ECL kit (Amersham Corp.).

Luciferase Assays—50–200 μl of each extract (approximately $1–2 \times 10^5$ relative light units for pCMVLuc) was assayed using the Promega luciferase assay system and a luminometer for detection. All extracts were normalized for protein content using the Bradford assay (Bio-Rad) and transfection efficiency by β-galactosidase activity (exogenous pCMVβgal).

RESULTS

Identification of a TRAF3 Recognition Site in the LMP-1 COOH Terminus between Amino Acids 196 and 212—LMP-1 has been shown to interact with TRAF3 through its carboxyl-terminal cytoplasmic tail. Previous studies (19) have shown that TRAF3 binds to recombinant fusion proteins containing either the full-length (200 amino acids from 188 to 386) or the membrane proximal 44 amino acids (188–232) of the COOH terminus of LMP-1. The number and specific sequences of TRAF binding sites in the entire LMP-1 COOH terminus remains to be elucidated.

We used the known TRAF3 binding sequence of the CD40 receptor COOH terminus to identify, by inspection, potential TRAF3 binding sites in the COOH-terminal domain of LMP-1. We found several likely regions with homology (Fig. 1*A*). Overall, the analysis revealed three potential TRAF3 binding sites, designated LMP-1 A, B, and C, including one in the 44-amino acid membrane proximal region (LMP-1 A). All three contained

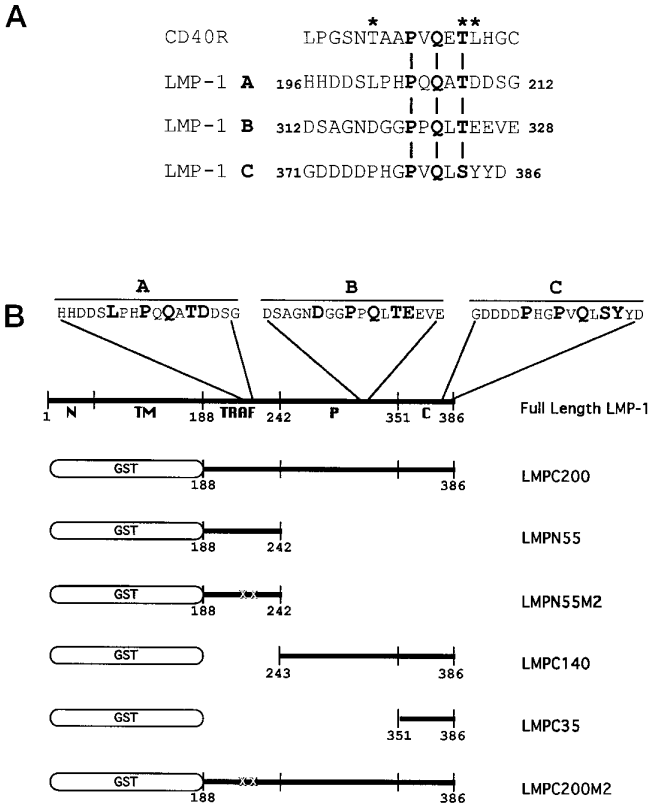

FIG. 1. **Identification of Potential TRAF3 binding sites in LMP-1.** *A*, the alignment of the putative 17-amino acid TRAF3 binding sequences of LMP-1 with the known binding region of the CD40 receptor. The LMP-1 A (amino acids 196–212), B (amino acids 312–328), and C (amino acids 371–386) peptides shown, contain putative TRAF3 binding sites as defined by the highlighted P*X*Q*X*(T/S) motif. This motif was determined by visual inspection and comparison with the known TRAF3 binding region of the CD40 receptor (*CD40R*) sequence. *Asterisks* label the critical residues in the CD40 receptor sequence as determined by alanine substitution experiments.[3] *B*, the GST fusion proteins modeled on the COOH-terminal regions of LMP-1. The full-length 386 amino acid LMP-1 sequence is shown (not to true scale) with the relative positions of the three putative TRAF3 binding sites (A, B and C peptides at the *top*). The GST portion of the fusions is linked to the amino-terminal end of the LMP-1 fragments. The LMPC200 fragment represents the full cytoplasmic COOH terminus of LMP-1 and contains all three putative TRAF3 binding sites. The LMPN55 fragment contains only one TRAF3 binding site (A peptide) and represents the portion of LMP-1 remaining following cleavage. The LMPC140 and LMPC35 fragments contain the other two putative TRAF3 binding sites (B and C peptides). Highlighted residues in the peptide sequences at the *top* of *panel B* show the positions of putative critical residues and the P*X*Q*X*(T/S) motif. LMPC140M2 and LMPN55M2 are the same as LMPC140 and LMPN55 with the exception that two point mutations, Thr-234 and Leu-235 to alanine, have been introduced into the LMP-1A TRAF site. These mutations are sufficient to destroy the TRAF binding capacity of this site. Abbreviations *below* the full-length LMP-1 are as follows. *N*, the cytoplasmic N terminus; *TM*, the transmembrane region; *TRAF*, the previously identified TRAF binding region; *P*, the sites of phosphorylation; *C*, the COOH-terminal region previously shown to be essential for Rat-1 transformation and NF-κB activation.

a conserved P*X*Q*X*(T/S) motif in common with the CD40 receptor. The sequences are shown aligned with the CD40 receptor motif (in Fig. 1*A*), and their location in the LMP-1 COOH terminus is shown at the *top* of Fig. 1*B*. To test the potential TRAF3 binding capacity of each of these sites, we made a number of GST fusion proteins containing various portions of the LMP-1 sequence (Fig. 1*B*). The ability of these fusion proteins to bind a recombinant TRAF3 protein fused to a hemagglutinin tag of influenza (TRAF3/HA) was then assessed using an *in vitro* binding assay (see "Experimental Procedures"). The results are shown in Fig. 2*A*. From these results, it is clear that

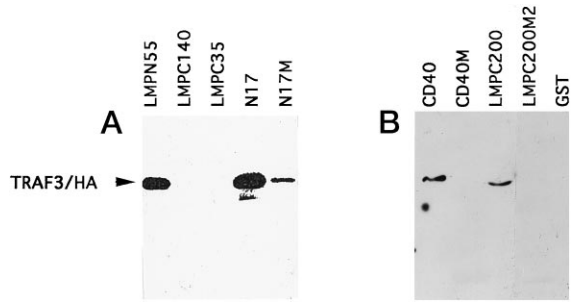

FIG. 2. ***In vitro* binding assays of the GST-LMP-1 fusion proteins with the TRAF3 molecule.** *A*, *in vitro* binding assays define the sole TRAF3 binding site in the LMP-1 COOH terminus. Various GST-LMP fusion proteins were tested for binding to HA-tagged TRAF3 that was stably transfected into the human embryonic kidney cell line 293. Shown is an anti-HA Western blot to identify the ability of GST-LMP fusion proteins to precipitate TRAF3 (TRAF3/HA) from a Nonidet P-40 lysate. LMPN55, LMPC140, and LMPC35 are described in the legend to Fig. 1*B* and represent various fragments of the cytoplasmic COOH terminus of LMP-1. The *N17* and *N17M lanes* show the wild type 17 amino acid LMP-1 peptide A (residues 196–212) and the corresponding peptide containing the mutation of threonine 208 to alanine, respectively. *B*, *in vitro* binding assays show that point mutations in the TRAF3 binding region preclude TRAF3 binding to the full COOH-terminal sequences of LMP-1. Shown is the ability of CD40 receptor cytoplasmic tail (CD40) to precipitate TRAF3 (TRAF3/HA) from Nonidet P-40 lysates of the 293 cell line. The single point mutation of the CD40 receptor at amino acid threonine 234 (CD40M) prevents TRAF3 binding. The full COOH terminus of LMP-1 (LMPC200) also binds TRAF3, which can be abolished by the double point mutation at threonine 208 and aspartic acid 209 (LMPC200M2).

the only sites for TRAF3 binding in LMP-1 are present in the first 55 amino acids (N55; residues 188–242), since no detectable binding to the C140 (residues 243–386) and C35 (residues 351–386) fusion proteins was seen. This indicates that the LMP-1 B and C sites are not functional TRAF3 binding sites, leaving LMP-1 A or other unspecified regions in N55 as candidate binding sites.

Alanine substitution studies of the TRAF binding site in the CD40 cytoplasmic tail[3] showed that substitution of any one of three critical residues (shown in *boldface type* in Fig. 1*A*) in the 17-amino acid sequence of the CD40 receptor resulted in significant loss of TRAF3 binding. Specifically, mutation of the threonine residue at position 234 to alanine in the CD40 receptor blocks both TRAF3 binding and CD40 functions (18, 46). Use of a 17-amino acid synthetic peptide (N17) equivalent to the LMP-1 A site of LMP-1 (residues 196–212) showed that this region bound the TRAF3 protein (Fig. 2*A*) as well as or better than N55. Introduction of a point mutation (equivalent to Thr-234 in the CD40 receptor) into the 17-amino acid LMP-1 peptide (N17M) by substitution of threonine 208 with alanine caused an extensive reduction in TRAF3 binding (Fig. 2*A*). This establishes that the LMP-1 A site, initially identified by sequence homology, is capable of binding to TRAF3.

The Amino Acids 196–212 Site Is the Only TRAF3 Recognition Site in the LMP-1 COOH Terminus—Having identified a short peptide sequence between amino acids 196 and 212 that was sufficient to bind TRAF3, we wished to establish if this was the sole TRAF3 binding site in the entire LMP-1 COOH-terminal sequence. This was done by testing if mutation of the motif was able to block binding of TRAF3 to the intact COOH-terminal domain. Based on mutagenesis studies of the CD40 receptor, the introduction of two point mutations converting Thr-234 and Leu-235 to alanine was sufficient to completely inhibit TRAF binding and CD40 functions (Fig. 2*B*). Therefore, we introduced the equivalent mutations into the LMP-1 A motif in

[3] G. Cheng and D. Baltimore, unpublished results.

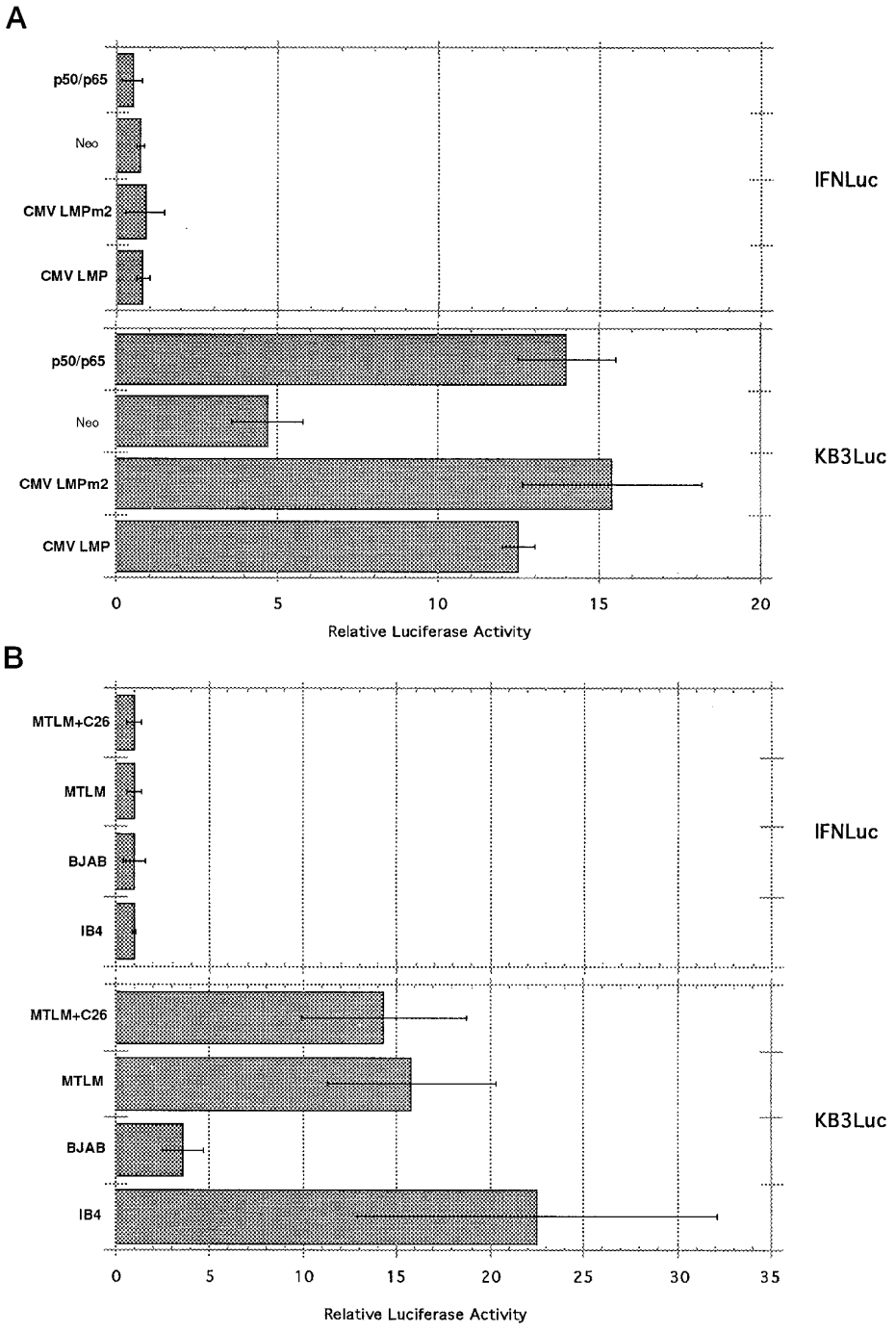

FIG. 3. **TRAF3 binding to LMP-1 is not sufficient to cause activation of NF-κB.** *A*, mutation of the sole TRAF3 binding site in the LMP-1 COOH terminus does not block NF-κB activation. Transient transfection of BJAB cells with wild type full-length LMP-1 expressed under the control of a strong promoter (CMV LMP) causes NF-κB activation as detected by luciferase reporter activity with the IFN(kB_3)Luc construct. The TRAF3 site double point mutant of LMP-1 (CMV LMPm2) does not prevent the activation of NF-κB. The activating heterodimer NF-κB pair of p50 and p65 (p50/p65) is a positive control for NF-κB activation, and mock-transfected (Neo) BJAB cells are a negative control to show endogenous NF-κB activation. Luciferase activity measured with the IFN promoter construct in the absence of NF-κB enhancer sequences (IFNLuc) is also shown to illustrate background promoter activity. NF-κB activity is reported as the normalized luciferase activity of the NF-κB reporter construct (IFN(kB_3)Luc) *versus* the normalized luciferase activity of the promoter construct (IFNLuc) under the same conditions (relative luciferase activity). *B*, binding of dominant negative TRAF3 to the sole TRAF3 binding site of LMP-1 does not block NF-κB activation. Transient transfection of BJAB cells with LMP-1 under the control of a weak promoter (MTLM) activates NF-κB as measured with the IFN(kB_3)Luc reporter. Co-transfection of the dominant negative TRAF3 molecule (C26 CRAF1/TRAF3) under control of a strong promoter (overexpression with CMV promoter) with the LMP-1 expression vector (MTLM) does not affect NF-κB activity. The same C26 TRAF3 construct under similar conditions was previously shown to act as a dominant negative molecule (17). The EBV-positive lymphoblastoid cell line, IB4, has been previously shown (34) to be a good positive control for NF-κB activation. Mock-transfected BJAB acts as a negative control to show endogenous NF-κB activity of the cell line. All values are reported as relative luciferase activity as described in the legend to Fig. 3*A*.

the GSTLMPC200 vector, converting the T-208 and D-209 residues to alanine, generating the mutant GST fusion protein LMPC200M2. The wild type and mutant fusion proteins were then tested for binding to TRAF3 (Fig. 2*B*). The wild type COOH-terminal domain of LMP-1 (C200) bound TRAF3 as efficiently as the wild type CD40 receptor. However, the double point mutation (C200M2) of the full-length carboxyl terminus of LMP-1 completely ablates binding to TRAF3 (Fig. 2*B*). This mutation does not show the weak binding that is displayed by the single point mutation in the synthetic peptide (N17M) even upon longer exposure. This is the first demonstration of point mutations in the LMP-1 COOH-terminal cytoplasmic tail (C200M2) that effectively knock out TRAF3 binding, therefore confirming the presence of only one TRAF3 binding site (referred to as the TRAF3 recognition site).

Effect of TRAF3 Recognition Site Mutants on NF-κB Activation by LMP-1—Having identified the sole TRAF3 binding site in the LMP-1 COOH terminus, we asked if mutations in this site that prevent TRAF3 binding result in functional inactivation of LMP-1. Many functions have been attributed to ectopic expression of LMP-1 in cell lines including induction of cell surface activation and adhesion molecules, increased Bcl-2 expression, and NF-κB activation (reviewed in Ref. 20). Recent evidence, utilizing an LMP-1-inducible system in the BJAB cell line, showed that the only consequence of physiological levels of expression of LMP-1 is the activation of NF-κB; the other phenotypic effects occurred only at very high levels of LMP-1 expression that were also associated with the induction of apoptosis.[2] NF-κB activation is also a major function associated with TRAF binding to TNF receptor family members including CD40. We therefore used transient transfection studies in the BJAB cell line to test for the functional role of the LMP-1

TRAF3 binding site in the activation of NF-κB.

Transient expression of the entire LMP-1 coding sequence (CMVLMP) showed about a 15-fold induction of NF-κB from the IFN(kB)3Luc construct (Fig. 3*A*) *versus* the IFNLuc construct. This induction of NF-κB activity was equal to or perhaps even higher than the activity produced by direct expression of the p50 and p65 components of NF-κB. Transient expression of the TRAF3 recognition site mutant (CMVLMPM2) produced NF-κB activity comparable to that of wild type LMP-1. Repeat experiments performed after different times of culture (24 and 36 h) gave comparable results, and overall, no significant difference in NF-κB activation was ever observed between the wild type LMP-1 expression vector and the expression vector expressing the mutant LMP-1 that could no longer bind TRAF3. Therefore, the TRAF3 recognition site makes no detectable contribution to the NF-κB activation associated with LMP-1.

Truncated TRAF3 (C26 CRAF1; Ref. 17) is able to bind to its recognition site and allows for homodimerization of the TRAF3 molecules, but it is no longer able to generate downstream signals when receptors are stimulated. Loss of this activity allows truncated TRAF3 to act as a dominant negative molecule that, when highly overexpressed, will block the NF-κB- and CD23-activating function of the CD40 receptor (17). To further test the role of the LMP-1 TRAF3 site in NF-κB activation, LMP-1 was transiently expressed from the MTLM expression plasmid in the presence of co-transfected C26 at levels previously shown to block CD40 signaling. Expression of LMP-1 from the MTLM vector alone or in the presence of the dominant negative TRAF3 produced comparable levels of NF-κB, similar to those obtained with the EBV-positive lymphoblastoid cell line IB4. This result confirms that the NF-κB-activating function of LMP-1 does not require the TRAF3 recognition site.

Deletion Mapping Studies of the LMP-1 Sequence Required for NF-κB Activation—Deletion mapping studies of LMP-1 using Rat-1 focus-forming assays provided the first demonstrations that the COOH terminus of LMP-1 was required for transformation (14). These studies mapped an essential domain for transformation by LMP-1 to the last 23 amino acids (LMP del364–386-4 stop; Fig. 4*A*). Finer deletion mutants of LMP-1 were also made (del377–386-5 stop and del383–386-6 stop), and these mutants were also unable to cause LMP-1-induced transformation of Rat-1 cells.[4] Subsequently, it was demonstrated that the last 35–45 amino acids were also essential for NF-κB activation, raising the possibility that the COOH-terminal motif identified in the Rat-1 assays was also responsible for NF-κB activation. Therefore, we tested the non-transforming deletion mutants in transient NF-κB assays to see how closely the transforming ability of LMP-1 in Rat-1 cells mapped to the site of NF-κB activation.

For these experiments a set of deletion mutants previously constructed in the MTLM vector was used. Results of transient transfection experiments in BJAB cells confirmed that the lower levels of LMP-1 expression obtained with the MTLM promoter nevertheless resulted in levels of NF-κB induction similar to those obtained with the stronger CMV promoter (Figs. 3*A* and 5*A* and data not shown).

We first tested the MTLMEnd deletion construct, which expresses a truncated from of LMP-1 that lacks the COOH-terminal 143 amino acids that constitute the p25 fragment that is cleaved from LMP-1 at position 243 during the rapid turnover of LMP-1. This LMP-1 construct includes the TRAF3 binding site (Fig. 1*B* or 4*A*) but has been shown to be inactive

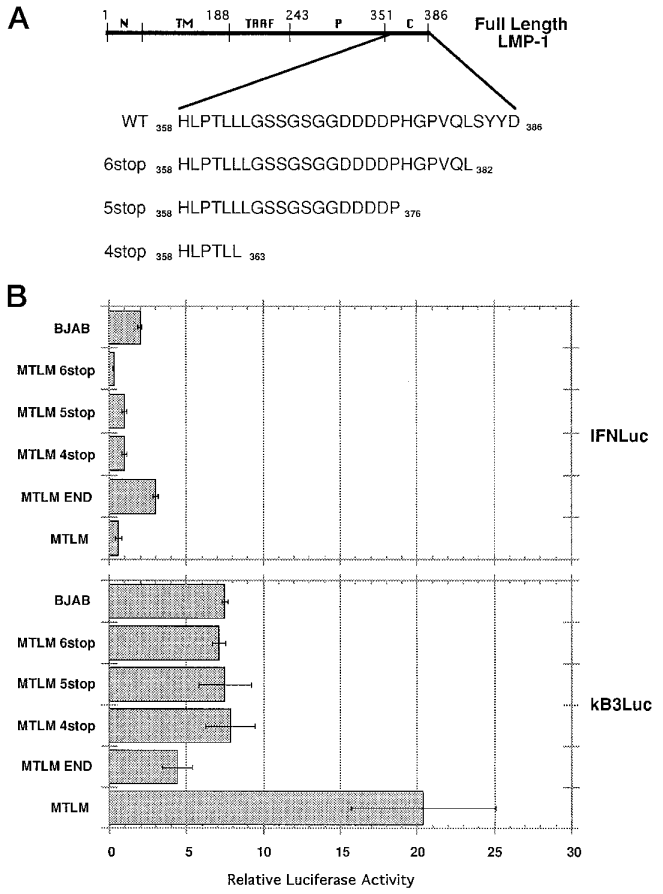

FIG. 4. **NF-κB activation is localized to critical amino acids at the distal COOH terminus of LMP-1.** *A*, a series of mutants are shown that delete 4 (6 stop), 10 (5 stop), or 23 (4 stop) amino acids from the distal COOH terminus of the LMP-1 sequence. The 4 stop mutant (del363–386) was previously shown to abolish the transforming ability of LMP-1 in RAT-1 assays (14). The 5 stop and 6 stop mutants were subsequently also shown to prevent transformation.[4] *B*, COOH-terminal deletions up to the last 4 amino acids of LMP-1 prevents the activation of NF-κB. Expression of various COOH-terminal deletion mutants under control of the weak MTLM promoter in a series of transient transfections in the BJAB cell line showed that NF-κB activation is prevented. Mock-transfected (BJAB) cells are used to show endogenous levels of NF-κB activity, and the full-length LMP-1 expression vector (MTLM) is used as a positive control. All values are reported as relative luciferase activity as described in the legend to Fig. 3*A*.

in the Rat-1 transformation assays. Therefore, the TRAF3 site is insufficient to cause oncogenic transformation. As can be seen from the *lower part* of Fig. 4*B*, the MTLMEnd construct is also unable to activate NF-κB to levels higher than those obtained from the endogenous NF-κB activity of BJAB cells. This result confirms previous studies that have demonstrated the requirement of the COOH-terminal 35–45 amino acids for NF-κB activation. We have tested for NF-κB activity the three most proximal COOH-terminal deletions (MTLM 4, 5, and 6 stop), all of which are inactive in RAT-1 transformation assays. All of the LMP-1 deletion constructs tested, including the 6 stop (del383–386) mutant, were unable to cause activation of NF-κB over background levels seen in BJAB cells alone (CMVNeo). The expression of LMP-1 protein from the MTLM 4, 5, and 6 stop constructs was confirmed by Western blot analysis (14);[5] however, we have no antibody to confirm the expression of MTLMEnd. It thus appears that constructs containing the TRAF3 site but lacking the last four amino acids are inactive in

[4] R. Moorthy and D. A. Thorley-Lawson, unpublished data.

[5] S. R. Brodeur, G. Cheng, D. Baltimore, and D. A. Thorley-Lawson, unpublished observations.

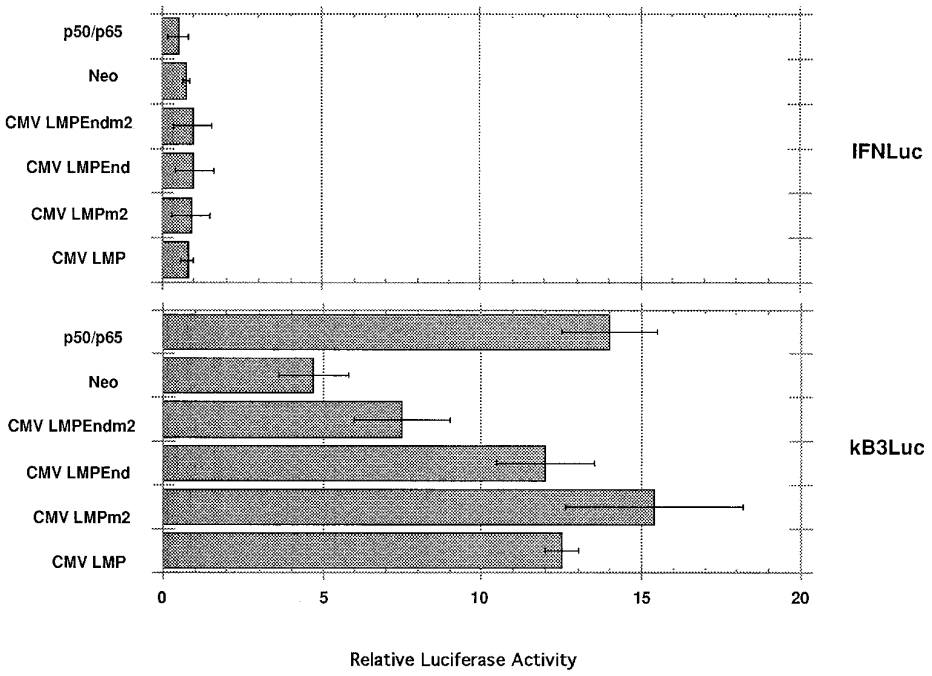

FIG. 5. **Overexpression of LMP-1 allows for activation through the TRAF3 binding site.** Overexpression of the natural truncation of LMP-1 is able to allow for activation of NF-κB through the TRAF3 site. Transient transfection of BJAB cells with LMP-1 expression vectors containing a strong promoter (CMV) showed that the TRAF3 site in the truncated LMP-1 construct (CMV LMPEnd) is able to stimulate NF-κB activity (36 h) as well as the wild type (CMV LMP). The LMPEnd stimulation of NF-κB activity could be diminished by mutation of the TRAF3 site (CMV LMPEndm2), whereas it had no effect on NF-κB activation by the full-length LMP-1 as shown in Fig. 3. The heterodimer pair of NF-κB molecules (p50 and p65) transfected into BJAB cells were used as a positive control, and mock-transfected (Neo) BJAB cells were used as a negative control for NF-κB activation. All values are reported as relative luciferase activity as described in the legend to Fig. 3*A*.

both NF-κB activation and Rat-1 transformation.

Analysis of the deleted sequences (Fig. 4*A*) reveals that the distal COOH terminus of LMP-1 contained the putative TRAF recognition site designated LMP-1 C in Fig. 1*A* based on the presence of the P*X*Q*X*(T/S) motif originally found in the CD40 receptor motif. The residues deleted in the 6 stop (del383–386) mutant would delete two essential TRAF binding residues including the critical T/S and adjacent amino acids. To date we have tested TRAF2, TRAF3, TRAF5, and TRAF6 for their ability to bind either the C140 or C35 construct (Figs. 1*B*, 2*A*, and 6, Table I, and see below) and have consistently failed to observe an interaction that could be mediated through the LMP-1 C site. This site may therefore represent a binding region important for interaction with as yet uncharacterized TRAF molecules, or the presence of the motif may be fortuitous.

The TRAF3 Site Mediates NF-κB Activation When Overexpressed—The natural truncation of LMP-1 (MTLMEnd (del242–386)) and the 4, 5, and 6 stop deletions do not induce NF-κB (Fig. 4*B*) although they contain the TRAF3 binding site. This is consistent with the failure of the TRAF3 point mutant to block NF-κB activation and further supports the conclusion that the TRAF3 binding site is not involved in activation of NF-κB. This is contradictory to previous observations, however, which claimed that truncated LMP-1 could activate NF-κB (33, 35, 47). In these studies, strong promoters were used for LMP-1 expression. We have confirmed this as the explanation for the discrepancy by repeating our experiments using expression vectors with the strong CMV promoter in place of the weaker MTLM promoter. As shown in Fig. 5, expression of LMPEnd from a strong promoter can cause NF-κB activation (CMVLMPEnd (del243–386)) to levels close to those obtained with full-length LMP. This activity appears to involve the TRAF3 site, since the introduction of point mutations into the TRAF3 site of the LMPEnd construct (LMPEndm2) results in a diminution of NF-κB activation. The TRAF3 recognition site (exemplified by the LMPEnd constructs) can therefore only stimulate NF-κB activity when overexpressed (CMVLMPEnd).

TRAF2 and TRAF5 Bind to the TRAF3 Site—TRAF2, TRAF5, and TRAF6, unlike TRAF3, can directly mediate NF-κB activation. Therefore, we tested if any of these molecules could bind to the potential TRAF binding sites in the COOH terminus of LMP-1 thereby conferring NF-κB activation. Binding experiments with FLAG-tagged TRAF6 failed to demonstrate any association between this molecule and any portion of the LMP-1 COOH terminus (Table I and data not shown). However, HA-tagged TRAF2 and TRAF5 gave results essentially identical to those obtained with TRAF3 (Fig. 6 and Table I). Only the membrane-proximal region (N55) bound TRAF2 or TRAF5; there was no binding to the distal COOH-terminal sequences (C140 and C35). In both cases, binding was abolished upon mutation of the TRAF3 binding site (N17M, N55M2, and C200M2) (Table I), showing that TRAF2 and TRAF5 interact with this site. The only consistent difference observed was that TRAF2 exhibits weaker binding to the LMP-1 COOH terminus (C200 and N55) than TRAF3 and TRAF5. This difference was not apparent in binding studies to the CD40 receptor or to the isolated 17-amino acid LMP-1A site (N17), suggesting that constraints on TRAF2 binding are imposed by the sequences flanking the TRAF binding site. The binding of TRAF2 or TRAF5 probably accounts for the NF-κB activation observed through the LMP-1A TRAF binding site when expressed at high levels. However, neither TRAF2, TRAF5, nor TRAF6 can account for activation of NF-κB through the distal COOH terminus of LMP-1, since we have never observed binding of any of these molecules to this domain.

These results taken together are consistent with the idea that overexpressed truncated LMP-1 (LMPEnd) can recruit enough TRAF2 or TRAF5 to cause potentially artifactual activation of NF-κB. We conclude, therefore, that TRAF2, TRAF3, TRAF5, and TRAF6 play no significant role in the NF-κB-activating potential of LMP-1.

DISCUSSION

The COOH-terminal domain of LMP-1 is essential for its biological activity including immortalization of B cells, transformation of rodent fibroblasts (Rat-1), and activation of NF-κB. Deletion mapping studies using the Rat-1 focus-forming assays showed that critical residues in the COOH-terminal 23 amino acids were essential for transformation (14), thus mapping a critical domain for the oncogenic function of LMP-1.

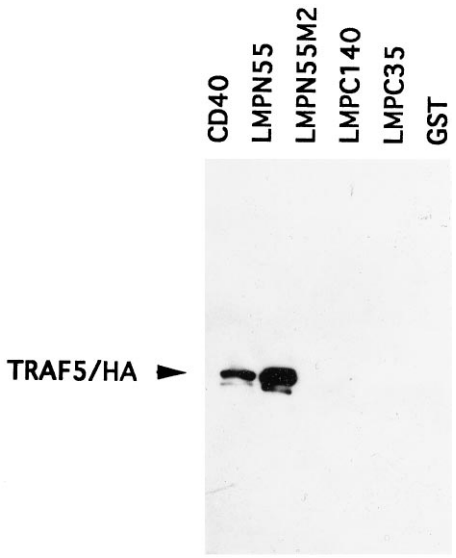

FIG. 6. ***In vitro* binding assays of the GST-LMP-1 fusion proteins with the TRAF5 molecule.** *In vitro* binding assays demonstrate that TRAF5 binds to the same site in the LMP-1 COOH terminus as TRAF3. Various GST-LMP fusion proteins were tested for binding to HA-tagged TRAF5 that was stably transfected into the human embryonic kidney cell line 293. Shown is an anti-HA Western blot to identify the ability of GST-LMP fusion proteins to precipitate TRAF5/HA from a Nonidet P-40 lysate. LMPN55, LMPN55M2, LMPC140, and LMPC35 are described in Fig. 1*B* and represent various fragments of the cytoplasmic COOH terminus of LMP-1. LMPN55M2 is the same as LMPN55 except that two critical residues in the TRAF3 binding site have been mutated so that it can no longer bind TRAF3. CD40 is the cytoplasmic tail of the human CD40 receptor.

TABLE I
Summary of in vitro binding assays with GST-LMP fusion proteins to the TRAF2 and TRAF3 molecules

Table gives summary of results of Western blots showing *in vitro* binding of TRAF2, TRAF3, TRAF5, and TRAF6 in Nonidet P-40 lysates of transfected 293 cells with LMP-1 fusion proteins. + indicates detectable binding of LMP-1 or CD40 receptor fusion peptides to TRAF molecules. The extent of TRAF binding is depicted by multiple + signs, with +++ being the highest binding detected and + being the lowest detectable binding. The relative binding indicated by the plus signs can be seen by comparing Table I and Fig. 2. The GST-LMP fusions are as described in Fig. 1*B*. ND, not detected.

GST-FUSION CONSTRUCT	TRAF2	TRAF3	TRAF5	TRAF6
C200	++	+++	ND	-
C140	-	-	-	-
C35	-	-	-	-
N55	++	+++	+++	-
N55M2	-	-	-	-
N17	+++	+++	ND	ND
N17M	-	+/-	ND	ND
C200M2	-	+/-	ND	-
CD40 ct	+++	+++	+++	ND
CD40M ct	+/-	+/-	ND	ND

Subsequent studies showed that the COOH-terminal 35–45 amino acids were also required for NF-κB activation. Following the results of the Rat-1 studies, recombinant virus experiments confirmed the importance of the COOH-terminal domain of LMP-1 (15) by showing it was essential for B cell immortalization. The later studies additionally mapped a second essential site to the membrane-proximal 44 amino acids (188–232) that was subsequently shown to bind TRAF3 (19). In this present study, we have identified the critical residues for the TRAF3 binding site and a second, discrete, distal COOH-terminal site essential for inducing NF-κB activity. This demonstrates that the TRAF3 binding site does not play a critical role in NF-κB activation and that the LMP-1 COOH terminus has two domains that are both critical for its transforming potential. In particular, the NF-κB-activating site would appear to be essential for the oncogenic activity of LMP-1 based on Rat-1 transformation assays. It is thus surprising that recombinant virus studies have suggested that this domain is not essential for B cell immortalization (15). It seems more likely that both regions working in concert are responsible for the transforming or immortalizing activity of LMP-1.

The association of TRAF3 with the cytoplasmic COOH terminus of LMP-1 and the ability of LMP-1 to induce NF-κB activity in a large variety of cell lines has led to the suggestion that TRAF signaling can mediate this event. Work from a number of labs indicates that of the TRAF family members identified thus far, only the TRAF2, TRAF5, and TRAF6 molecules are capable of directly causing induction of NF-κB activity. TRAF3 would therefore not be expected to be an active participant in NF-κB induction through LMP-1. So how does LMP-1 effect induction of NF-κB activation?

There is the possibility that TRAF2 or TRAF5 could mediate NF-κB signaling through binding to the TRAF3 recognition site. This is unlikely, however, because we detect no activation of NF-κB through the TRAF3 recognition site unless LMP-1 is overexpressed. We have also shown that this is the only site for binding the known TRAF molecules in the entire COOH-terminal domain and that mutation of the binding site completely ablates TRAF binding without affecting NF-κB activation. Therefore, TRAF2 or TRAF5 binding to the TRAF3 recognition site in LMP-1 cannot account for a significant amount of NF-κB activation.

What is clear from our work is that the major NF-κB-activating potential is localized to critical residues in the last few amino acids of LMP-1, but how these residues achieve this is unknown. By inspection, the major NF-κB-activating sequence has similarity to the TRAF binding site, although it does not bind TRAF2, TRAF3, TRAF5, or TRAF6. It is possible that this site can bind as yet uncharacterized TRAF molecules. This is consistent with recent evidence (47) demonstrating that the general TRAF inhibitor molecule, TANK/I-TRAF can block induction of NF-κB and that the dominant negative TRAF2 del6–86 can partially inhibit NF-κB activation. How the TRAF2 dominant-negative molecule mediates this weak inhibition of LMP-1-induced NF-κB activity is unclear given the inability of TRAF2 to bind to this region. It is likely, therefore, that another TRAF molecule will be implicated as the responsible factor. We have identified critical amino acid residues for TRAF binding in the TRAF3 recognition site of LMP-1, and we are currently deriving point mutations of the distal COOH-terminal site. These mutations should help identify the critical residues and establish the relevance of this second putative TRAF binding site.

Finally, a number of laboratories have reported that the region of LMP-1 containing the TRAF3 recognition sequence can mediate induction of NF-κB activity. The data on the ability of the TRAF3 recognition site to cause NF-κB activation are conflicting. All reports to date (33, 35) suggest that activation of NF-κB with truncated forms of LMP-1 containing sequences up to the first 45 amino acids of the COOH terminus (equivalent to LMPEnd in our studies) occurs at 25–50% of the level of full-length LMP-1. Our results show this same effect, but only in the case where LMP-1 is overexpressed (CMV-driven constructs). In contrast to this, when lower expression levels are used, the induction of NF-κB activity is only detected with

full-length LMP-1. The likely explanation for this is that NF-κB induction through the TRAF3 site is an artifact induced by overexpression of truncated LMP-1. If the TRAF3 recognition site binds TRAF3 almost exclusively, NF-κB activity would not be expected to occur through this site, consistent with the TRAF3 site mutant being fully active in NF-κB activation. An artifact from the overexpression of truncated LMP-1 could occur if sufficient TRAF2 is bound such that a signal can be generated to activate NF-κB. Recent evidence (47) supports this interpretation, since a dominant negative TRAF2 (del6–86) completely blocks activation of NF-κB by truncated LMP-1 (LMP del232–386). The complete block of NF-κB activation by this TRAF2 mutant indicates that the signal generated is specifically due to the ability of TRAF2 to bind to the TRAF3 site.

The demonstration that high level expression of LMP-1 may lead to nonphysiologic activation of NF-κB through artifactual TRAF2 binding is also of considerable importance in providing a mechanism for understanding our recent observations that many of the phenotypic effects of LMP-1 expression detected by transfection studies in B cell tumor lines are only seen at high levels of LMP-1 expression. Only NF-κB activation was observed at the levels of expression found in lymphoblastoid cell lines. This is entirely consistent with our observation that NF-κB activation at moderate levels of LMP-1 expression appears to occur exclusively through the distal COOH-terminal site (amino acids 383–386). At high levels, the signaling through the TRAF3 site is probably corrupted, leading to other phenotypic effects. It is perhaps not surprising that one of these effects is the induction of apoptosis.

TRAF3 binding and the TRAF3 recognition site in LMP-1 therefore are unlikely to play a role in NF-κB activation. This does not rule out a potential role for TRAF3 binding in the transforming ability of LMP-1. This role is evidenced by recombinant virus studies (15), but the mechanism by which TRAF3 achieves immortalization through LMP-1 is unknown. Future experiments will focus on the transforming potential of TRAF3 binding site mutants using the ability of LMP-1 transfected epithelial cell lines to cause tumorigenesis in SCID mice (49).

To date, it has been shown that LMP-1 undergoes specific cleavage and phosphorylation and binds TRAF3, but no clear downstream consequences of these events have been demonstrated. At this time, there is no mechanism to explain how these modifications occur, but point mutation of critical signaling motifs may lead to answers. It will be interesting to see if biochemical modifications of the LMP-1 COOH terminus will be affected by loss of TRAF3 binding or loss of NF-κB-activating potential.

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