Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11085–11090, October 1996
Medical Sciences

Tumor necrosis factor receptor associated factor 2 is a mediator of NF-κB activation by latent infection membrane protein 1, the Epstein–Barr virus transforming protein

KENNETH M. KAYE*†, ODILE DEVERGNE‡, JOSEPHINE N. HARADA*, KENNETH M. IZUMI‡, RAMANA YALAMANCHILI‡, ELLIOTT KIEFF‡, AND GEORGE MOSIALOS‡

*Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street; and ‡Departments of Medicine, Microbiology, and Molecular Genetics, Harvard Medical School, Brigham and Women's Hospital, Boston, MA 02115

Contributed by Elliott Kieff, July 26, 1996

ABSTRACT **Latent infection membrane protein 1 (LMP1), the Epstein–Barr virus transforming protein, associates with tumor necrosis factor receptor (TNFR) associated factor 1 (TRAF1) and TRAF3. Since TRAF2 has been implicated in TNFR-mediated NF-κB activation, we have evaluated the role of TRAF2 in LMP1-mediated NF-κB activation. TRAF2 binds *in vitro* to the LMP1 carboxyl-terminal cytoplasmic domain (CT), coprecipitates with LMP1 in B lymphoblasts, and relocalizes to LMP1 plasma membrane patches. A dominant negative TRAF2 deletion mutant that lacks amino acids 6–86 (TRAF2Δ6-86) inhibits NF-κB activation from the LMP1 CT and competes with TRAF2 for LMP1 binding. TRAF2Δ6-86 inhibits NF-κB activation mediated by the first 45 amino acids of the LMP1 CT by more than 75% but inhibits NF-κB activation through the last 55 amino acids of the CT by less than 40%. A TRAF interacting protein, TANK, inhibits NF-κB activation by more than 70% from both LMP1 CT domains. These data implicate TRAF2 aggregation in NF-κB activation by the first 45 amino acids of the LMP1 CT and suggest that a different TRAF-related pathway may be involved in NF-κB activation by the last 55 amino acids of the LMP1 CT.**

The Epstein–Barr virus (EBV)-encoded latent infection membrane protein 1 (LMP1) has a significant role in EBV-associated lymphoproliferative disease, Hodgkin disease, and anaplastic nasopharyngeal carcinoma (for review, see ref.1). In experimental models, LMP1 transforms immortalized rodent fibroblast cell lines (2–4), alters epithelial cell differentiation (5–7), induces the expression of lymphocyte activation markers and adhesion molecules (8–12), and is essential for EBV-mediated primary B-lymphocyte transformation (13).

LMP1 (depicted in Fig. 1) aggregates in the plasma membrane of latently infected cells and appears to mimic an activated tumor necrosis factor receptor (TNFR). The cytoplasmic TNFR-associated factor 1 (TRAF1) (16) and TRAF3 (also known as LAP1, CRAF1, CD40bp, or CAP1) (17–20) associate with LMP1 (17). TRAF3 binds directly to the first 45 amino acids of the 200-amino acid LMP1 carboxyl-terminal cytoplasmic domain (CT) (17). LMP1 amino acids 1–231 include only these first 45 amino acids of the CT and are sufficient for EBV-mediated primary B-lymphocyte growth transformation (ref. 21 and Fig. 1). The 45 amino acids are a necessary component of this LMP1 mutant for B-cell transformation (21). An important role for TRAFs in LMP1 signaling is consistent with the similarity of LMP1's effects on lymphocytes to those of CD40 activation (22); CD40 is a TNFR that associates with TRAF2 and TRAF3 (17–20, 23). Genetic and biochemical evidence link TRAF2 to NF-κB activation and TRAF3 to CD23 induction by CD40 (19, 23). The six hydrophobic transmembrane domains of LMP1 enable it to aggregate in the plasma membrane similar to receptor aggregates that would form in response to a multivalent ligand (24–26). Aggregation is likely to be essential for transformation since an LMP1 mutant that has the last five transmembrane domains and the full CT diffusely distributes in the plasma membrane and EBV recombinants that express this LMP1 mutant cannot transform primary B lymphocytes (13, 27). Further, LMP1 expression in cells causes TRAF1 or TRAF3 to colocalize with LMP1 aggregates in the cell plasma membrane, consistent with a role for aggregation and TRAF binding in LMP1 effects (17). In contrast to the LMP1 CT, no component of the amino-terminal cytoplasmic domain is essential for primary B-lymphocyte growth transformation (28).

Among the important similarities between LMP1 and CD40 in their effects on cells is NF-κB activation (10, 11, 22). The LMP1 CT has two domains that activate NF-κB. The first 45 amino acids of the LMP1 CT, which are critical for primary B lymphocyte growth transformation (21), mediate only 25% of the NF-κB activation, whereas the last 35 or 55 amino acids mediate 75% of the NF-κB activation (14, 29). TRAF2 has recently been implicated in NF-κB activation by CD40, TNFRI, and TNFR2 (23, 30). Overexpression of TRAF2 activates NF-κB, whereas overexpression of TRAF1 or TRAF3 does not activate NF-κB. Also, an amino-terminal RING finger deletion mutant of TRAF2 blocks CD40- and TNFRII-induced NF-κB activation (23). We have therefore investigated whether TRAF2 associates with LMP1 and whether TRAF2 has a role in LMP1-mediated NF-κB activation. During the course of these investigations, a TRAF-associated protein, TANK, was implicated in NF-κB activation (31), and we have also evaluated its role in LMP1 mediated NF-κB activation.

MATERIALS AND METHODS

Plasmids. Human TRAF2 was cloned using a mouse TRAF2 probe. Mouse cDNA was reverse-transcribed from kidney RNA (a gift of Arlene Sharpe, Brigham and Women's Hospital). DNA corresponding to nt 304-1042 of the murine TRAF2 cDNA (16) was PCR-amplified and used to retrieve a human TRAF2 cDNA from a B-cell cDNA library (32). The cDNA lacked the 64 nt of the human TRAF2 ORF that are 3′ to an *Eco*RI site and this was reconstructed with an oligonucleotide linker based on the human TRAF2 sequence (33). TRAF2 deleted for codons 6–86 (TRAF2Δ6-86) was constructed by digesting TRAF2 with *Nhe*I and *Eag*I and replacing

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: LMP1, latent infection membrane protein 1; EBV, Epstein–Barr virus; TNFR, tumor necrosis factor receptor; TRAF, TNFR-associated factor; CT, carboxyl-terminal cytoplasmic domain; TANK, TRAF associated NF-κB; GST, glutathione *S*-transferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base [accession no. U63830 (TANK)].

†To whom reprint requests should be addressed.

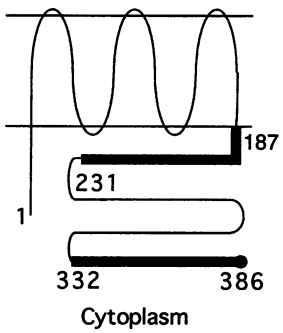

FIG. 1. NF-κB-activating domains of LMP1. The first 45 amino acids of the LMP1 CT (amino acids 187–231; shown by a thickened line) mediate ≈25% of the LMP1 NF-κB activation. The last 55 amino acids of the CT (amino acids 332–386; also shown by a thickened line) mediate ≈75% of the LMP1 NF-κB activation (14, 15) .

the DNA between these sites with the annealed oligonucleotides CTAGCAGCAGTTCGGCCTTCCCAGATAATGCTGCCCGCAGGGAGGTGGAGAGCCTGCC and GGCCGGCAGGCTCTCCACCTCCCTGCGGGCAGCATTATCTGGGAAGGCCGAACTGCTG. The DNA sequences at this site and at the 3′ oligonucleotide reconstitution site were confirmed by sequencing. Both TRAF2 and TRAF2Δ6-86 were cloned into pSG5 (Stratagene) under control of a simian virus 40 promoter for expression in BJAB cells or into pcDNA3 (Invitrogen) under the control of a cytomegalovirus promoter for expression in 293 cells. FLAG-epitope-tagged TRAF2 (F-TRAF2) was cloned by digesting the pSG5TRAF2 DNA with *Bss*HII (upstream of the TRAF2 ORF), blunting the ends with T4 DNA polymerase, releasing TRAF2 by digestion with *Bam*HI, and ligating the resulting fragment into *Eco*RV/*Bam*HI-digested pSG5FLAG (17). pASTRAF2 contains F-TRAF2 fused to the DNA binding domain of GAL4. pSG5 LMP(1–231) expresses only the first 231 amino acids of LMP1 (termed LMP231) (15). FLAG-epitope-tagged LMPΔ188-331 (pSG5-FLAG LMPΔ188-331) was constructed by replacing the *Bgl*II–*Nhe*I fragment of pSG5-FLAG LMP1 (15) with that of SV_2LMPΔ188-331 (14). FLAG-epitope-tagged LMP231 (pSG5-FLAG LMP1-231) has a stop codon after LMP1 codon 231 in pSG5-FLAG LMP1 (15). DNA sequencing analysis was done by automated sequencing.

Yeast Two-Hybrid Screening. TRAF3 codons 12–568 fused to the DNA binding domain of GAL4 [G4DBDLAP1(12–568)] (17) were transformed into yeast strain Y190 and used as bait to identify TRAF3-interacting proteins encoded by a cDNA library constructed from an EBV-transformed lymphoblastoid cell line (a gift of S. Elledge, Baylor College of Medicine). Of 5×10^6 yeast transformants, 148 clones grew in Trp−/Leu−/His− medium, 51 clones were positive for β-galactosidase expression, and about 30% of these were cDNA that encode significant parts of the TANK (31) ORF. The full-length human TANK sequence was obtained from a human cDNA library (32). FLAG-epitope-tagged TANK (F-TANK) was constructed with codons for MDYKDDDDKLIWNSDPRGHEGQ replacing the first three codons of TANK. F-TANK was cloned into pSG5 for expression in BJAB cells or into pcDNA3 for expression in 293 cells.

Transfections and NF-κB Assays. Approximately, 10×10^6 BJAB, EBV-negative Burkitt lymphoma, cells were electroporated at 960 μF and 200 V and were harvested after 18–24 h (17). Human embryonic kidney 293 cells, 4×10^5 cells per well in six-well plates, were transfected by the calcium phosphate DNA coprecipitation method (35). All transfections included 0.5 μg of pGKβgal, which expresses β-galactosidase from a phosphoglucokinase promoter (E. Hatzivassiliou, P. Cardot, V. Zannis, and A. Mitsialis, unpublished results), and 0.5 μg of 3X-κB-L, which has three copies of the NF-κB binding site from the murine major histocompatibility complex class I promoter upstream of a minimal fos promoter and a luciferase gene (14). At 40–48 h after transfection, cells were washed once in PBS and lysed in 200 μl of reporter lysis buffer (Promega). Assays for luciferase or β-galactosidase activity were done with an Optocomp I Luminometer (MGM Instruments, Hamden, CT) using luciferase assay reagent (Promega) or Galacto-Light Plus (Tropix, Bedford, MA), respectively. Values were normalized by β-galactosidase activity.

Glutathione *S*-Transferase (GST) Binding Assays. F-TRAF2 was translated *in vitro* using wheat germ extract (Promega). *In vitro* translated protein (≈10 μl of reaction mixture) was diluted in 0.3 ml of binding buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/10% glycerol/0.1% Nonidet P-40/0.5 mM DTT/1 mM phenylmethylsulfonyl fluoride) and precleared with glutathione-Sepharose beads (Pharmacia) for 1 h at 4°C. GST or GST fusion proteins (37) (≈10 μg) bound to glutathione-Sepharose beads (Pharmacia) were incubated with the *in vitro*-translated protein for 1–2 h at 4°C and then washed five times with 1 ml of binding buffer. Bound proteins were recovered by boiling in SDS sample buffer and analyzed by SDS/PAGE. Gels were stained with Coomassie blue to ensure that comparable amounts of fusion proteins were used and then analyzed by autoradiography with a Molecular Dynamics PhosphorImager. For GST binding assays with cell extracts, BJAB cells were lysed 18–24 h after transfection in lysis buffer containing 50 mM Hepes (pH 7.2), 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (1 μg/ml). For binding competition, cells were lysed in 50 mM Tris·HCl, pH 7.4/0.5% Nonidet P-40/150 mM NaCl/3% glycerol/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/1 μg/ml leupeptin/1 μg/ml pepstatin), lysates were precleared by centrifugation at 14,000 × *g* for 15 min and by incubation with glutathione beads for 1 h at 4°C. Lysates were then incubated for 2 h at 4°C with ≈10 μg of GST or GST fusion protein bound to beads. After washing the beads, bound proteins were assayed by immunoblot. In the competition experiment, 1–2 μg of GST and GST fusion protein was used.

Immunoprecipitation and Immunoblot Analysis. Cells were washed in ice-cold PBS and lysed in buffer containing 50 mM Hepes·NaOH (pH 7.2), 0.5% Nonidet P-40, 250 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (1 μg/ml). Lysates were precleared by centrifugation and by incubation with protein G-coated beads (Pharmacia) for 1 h at 4°C. Lysates were then incubated with 10 μg of anti-FLAG M2 antibody (IBI) at 4°C for ≈12 h and with protein G beads for 1 h at 4°C. After the beads were washed five times immunoprecipitated proteins were analyzed by SDS/PAGE in 8% gels and immunoblot analysis. Alternatively, cleared lysates were incubated with M2 antibody affinity gel (IBI) for 2 h at 4°C and the beads were washed. Bound proteins were eluted with FLAG peptide (IBI) or by boiling in SDS sample buffer. S12 monoclonal antibody (26) was used to detect LMP1. TRAF2 was detected with rabbit anti-TRAF2 polyclonal antibody at 1 μg/ml (TRAF2 C20, Santa Cruz Biotechnology). F-TANK was detected with M5 (IBI) anti-FLAG antibody. Secondary antibody conjugated to horseradish peroxidase was used in conjunction with reagents for chemiluminescence (Amersham) to detect signal.

RESULTS

TRAF2 Binds to the LMP1 CT. To investigate whether TRAF2 can associate with the LMP1 CT, the binding of *in vitro*-translated FLAG-epitope-tagged TRAF2 (F-TRAF2) to a GST–LMP1 CT fusion protein (15) was determined. The GST–LMP1 CT fusion protein specifically bound 0.6% of the F-TRAF2 and the GST control bound less than 0.01% (Fig. 2*A*). TRAF2 probably binds directly to the LMP1 CT since F-TRAF2 was translated in a wheat germ extract and wheat

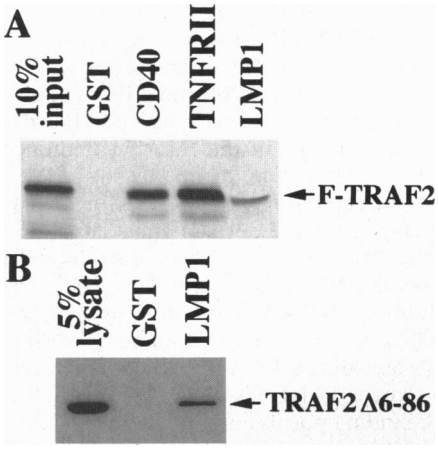

FIG. 2. TRAF2 associates with the LMP1 CT. (*A*) GST–LMP1 specifically precipitates *in vitro*-translated F-TRAF2 in a binding assay. F-TRAF2 was translated *in vitro* in a wheat germ extract in the presence of [^{35}S]methionine and was precipitated with GST–CD40 CT, GST–TNFRII CT (17), or GST–LMP1 CT (15). Precipitated proteins were analyzed by autoradiography. The F-TRAF2 band in the LMP1 lane is distorted due to comigrating GST–LMP1 CT fusion. (*B*) GST–LMP1 specifically precipitates TRAF2Δ6-86. After expression in BJAB cells, TRAF2Δ6-86 was precipitated from cell extracts by GST–LMP1 CT and detected by TRAF2 immunoblot analysis.

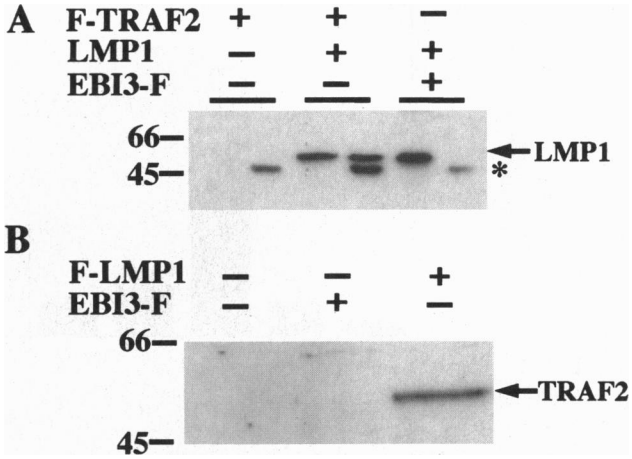

FIG. 3. LMP1 and TRAF2 coimmunoprecipitate from cells. (*A*) LMP1 coimmunoprecipitates F-TRAF2 from BJAB cells. After cotransfection of BJAB cells with F-TRAF2, EBI3-F, or LMP1 expression vectors as indicated above, extracts were immunoprecipitated with M2 anti-FLAG antibody and protein G beads. LMP1 was detected by S12 anti-LMP1. The left lane of each pair is 5% of the total cell lysate. The asterisk indicates immunoglobulin heavy chain. (*B*) TRAF2 coimmunoprecipitates with F-LMP1 from BJAB cells. F-LMP1 or EBI3-F were expressed in BJAB cells, and extracts were immunoprecipitated with M2 anti-FLAG affinity gel. Immunoprecipitates only are shown in each of the three lanes. TRAF2 was detected by immunoblot analysis. Size markers (kDa) are shown at the left.

germ extract is unlikely to have a homologue of a putative mammalian protein that could mediate the binding of TRAF2 to LMP1. Although presumably direct, the binding of TRAF2 to the LMP1 CT was much lower than to the CD40 or TNFRII CTs (Fig. 2*A*). Consistent with previous results (16, 23, 33), 5.6% of F-TRAF2 bound to a GST–CD40 CT fusion protein and 12% of F-TRAF2 bound to a GST–TNFRII CT fusion protein. TRAFs have highly homologous carboxyl-terminal TRAF-C domains that have so far been found to mediate their interaction with TNFRs and with LMP1 (16–20, 30, 31, 38). Consistent with this previous experience, a mutated form of TRAF2, lacking the TRAF-C domain after codon 380, did not bind to GST–LMP1 CT (data not shown) but an amino-terminal RING-finger-deleted TRAF2, TRAF2Δ6-86, did bind to GST–LMP1 CT (Fig. 2*B*). Therefore, TRAF2 binding to the LMP1 CT requires the TRAF-C domain but not the amino-terminal RING finger. Subsequent experiments revealed that TRAF2 binds only to the first 45 amino acids of the LMP1 CT and not to the rest of the LMP1 CT (see Fig. 8 and ref. 15).

TRAF2 Associates with LMP1 in B Lymphoblasts. Approximately 5% of LMP1 specifically coimmunoprecipitated with F-TRAF2 but not with FLAG-epitope-tagged EBV-induced gene 3 (EBI3-F) (36) when LMP1 was expressed with F-TRAF2 or EBI3-F in EBV-negative B-lymphoma cells (Fig. 3*A*). As expected from the GST–LMP1 CT binding data, LMP1 did not coimmunoprecipitate with a FLAG-epitope-tagged TRAF2 that lacks the TRAF-C domain (data not shown). In the reciprocal experiment, at least 10% of endogenous TRAF2 specifically coimmunoprecipitated with FLAG-epitope-tagged LMP1 (F-LMP1) when F-LMP1 was expressed in BJAB cells. (Fig. 3*B* and data not shown; see also Fig. 6, lanes 2 and 5). Endogenous TRAF2 did not coimmunoprecipitate with a control FLAG protein, EBI3-F (Figs. 3*B* and 6). These results demonstrate that TRAF2 and LMP1 can specifically associate in B-lymphoma cells.

LMP1 and TRAF2 Colocalize in B Lymphoblasts. When expressed in BJAB cells without LMP1, F-TRAF2 localizes to small vesicle-like structures in the cytoplasm, similar to the localization of overexpressed TRAF1 or TRAF3 (17) (Fig. 4). As with TRAF1 or TRAF3, LMP1 coexpression with F-TRAF2 results in localization of F-TRAF2 to LMP1 patches in the plasma membrane (Fig. 4).

A TRAF2 Dominant Negative Mutant Inhibits LMP1 NF-κB Activation. The role of TRAF2 in LMP1-mediated NF-κB activation was evaluated using a TRAF2 dominant negative mutant in NF-κB activation [TRAF2Δ6-86 (23, 30)]. As reported (14, 29), an LMP1 mutant consisting of amino acids 1–231 (LMP231) activated NF-κB 3- to 4-fold less efficiently than a mutant LMP1 deleted for most of the LMP1 CT except for the last 55 amino acids (LMPΔ188-331) (Fig. 5). As little as 200 ng of TRAF2Δ6-86 expression vector inhibited more than 75% of the LMP231-mediated NF-κB activation (Fig. 5*A*); 1 μg inhibited more than 80% of the NF-κB activation. In contrast, 200 ng of TRAF2Δ6-86 inhibited less than 20% of the LMPΔ188-331-mediated NF-κB activation and 1 μg inhibited less than 40% (Fig. 5*B*).

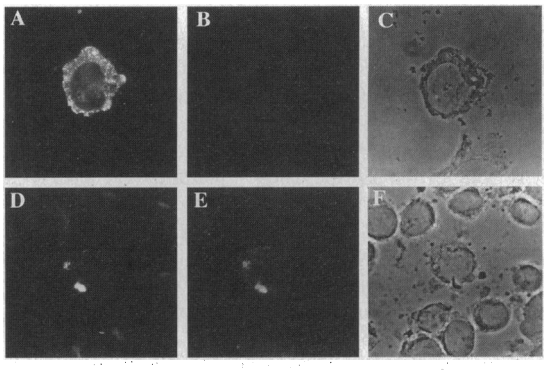

FIG. 4. LMP1 causes F-TRAF2 to relocalize in cells. BJAB cells were transfected with expression vectors for F-TRAF2 (*A–C*) or with F-TRAF2 and LMP1 (*D–F*). Cells were fixed and incubated with rabbit anti-FLAG polyclonal antibody (Santa Cruz Biotechnology) and S12 anti-LMP1 monoclonal antibody. Secondary anti-rabbit antibody tagged with fluorescein isothiocyanate (FITC) and anti-mouse antibody tagged with Texas Red were used to detect the anti-FLAG antibody and S12 antibody, respectively. Fluorescent microscopy was performed with a FITC filter (*A* and *D*) or a Texas Red filter (*B* and *E*). Phase-contrast images are also shown (*C* and *F*). (×1000.)

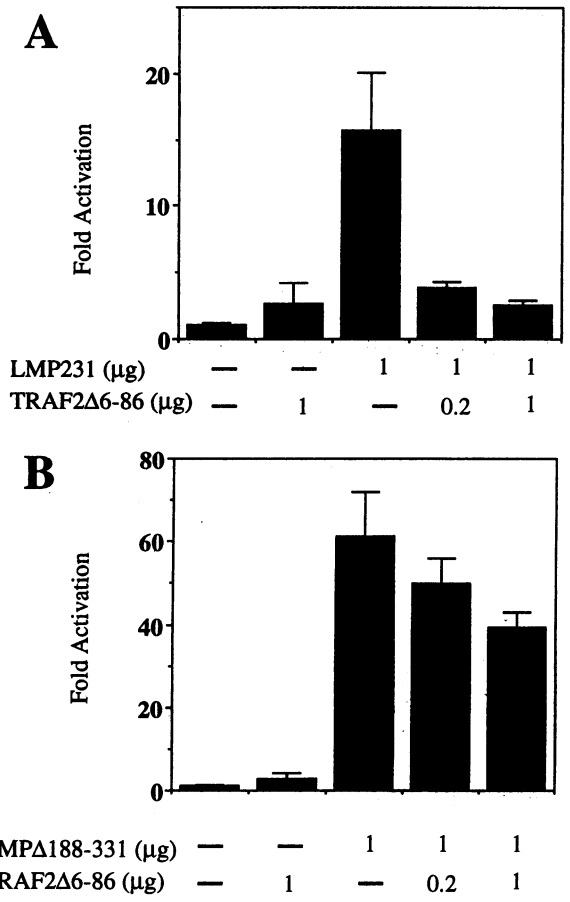

FIG. 5. TRAF2Δ6-86 inhibits LMP1 induction of NF-κB. The 293 cells were transfected with the indicated amounts of expression vectors for TRAF2Δ6-86 and LMP231 (*A*) or TRAF2Δ6-86 and LMPΔ188-331 (*B*). Fold activation was determined relative to reporter construct alone. Standard deviations are shown. The experiment shown was done in triplicate and the result is representative of more than six other experiments.

Similar results were obtained with FLAG-epitope-tagged LMP231 or LMPΔ188-331 (data not shown); these constructs could, therefore, be used in the important control experiments to determine whether the inhibition of NF-κB activation by TRAF2Δ6-86 was due to decreased LMP1 mutant expression. The inhibition of LMP1-induced NF-κB activation was not due to decreased LMP1 expression since F-LMP231 and F-LMPΔ188-331 levels as monitored by immunoblot analysis were not diminished by TRAF2Δ6-86 cotransfection (data not shown). The lower inhibitory effect of TRAF2Δ6-86 on LMPΔ188-331 as compared with LMP231 was also not due to a differential effect of the LMP1 mutants on TRAF2Δ6-86 levels as monitored by immunoblot analysis since expression of TRAF2Δ6-86 as monitored by immunoblot analysis was not affected by either LMP1 mutant (data not shown). Thus, TRAF2Δ6-86 has a strong inhibitory effect on NF-κB induction by LMP231 and only a partial inhibitory effect on NF-κB activation by LMPΔ188-331.

When cotransfected with full-length LMP1, TRAF2Δ6-86 inhibited NF-κB activation by ≈50% (data not shown). This is the expected result since LMP231 contributes ≈25% of full-length LMP1 NF-κB activity (14, 29) and is inhibited more than 80%, while LMPΔ188-331 contributes ≈75% of full-length LMP1 NF-κB activity (14, 29) and is inhibited less than 40% by TRAF2Δ6-86. As was previously found in studies with TRAF2 and CD40 or TNFRII (23), high-level expression of TRAF2 alone induced ≈20-fold NF-κB activation (data not shown).

We next investigated whether TRAF2Δ6-86 binding to the LMP1 CT inhibits NF-κB activation by blocking the association of TRAF2 with LMP1. Cotransfection of TRAF2Δ6-86 expression vector with amino-terminal FLAG-epitope-tagged LMP1 (F-LMP1) expression vector into BJAB cells resulted in less endogenous TRAF2 in the F-LMP1 immunoprecipitate than when F-LMP1 was transfected alone (Fig. 6). TRAF2Δ6-86 competition with TRAF2 for LMP1 binding is thereby expected to decrease LMP1-mediated NF-κB activation since the TRAF2 RING finger domain is required for TRAF2-mediated NF-κB activation (23, 30).

TANK Inhibits NF-κB Activation by LMP231 and by LMPΔ188-332. We had identified human TANK (31) cDNA as commonly encoding a TRAF3-interacting protein in a yeast two-hybrid screen. TANK also interacted with TRAF2 fused to the DNA binding portion of GAL4 (pASTRAF2). Human TANK has 81% amino acid identity to murine TANK (31) and contains a conserved Zn finger at the carboxyl terminus. TANK is ubiquitously expressed as a 2.3-kb transcript in human tissues (data not shown).

Since TANK can affect NF-κB activation (31), the effect of TANK on LMP1-mediated NF-κB activation was investigated. LMP231 or LMPΔ188-331 were expressed independently or were coexpressed with FLAG-epitope-tagged TANK (F-TANK) in 293 cells. LMP231 induction of NF-κB was inhibited ≈50% by transfection with 0.35 μg of F-TANK expression vector and more than 80% by 1 μg of F-TANK (Fig. 7A). The inhibitory effect was not due to lower LMP1 expression since F-TANK did not diminish F-LMP231 expression as monitored by immunoblot analysis (data not shown). F-LMP231 did not affect F-TANK expression as monitored by immunoblot analysis (data not shown). LMPΔ188-331 induction of NF-κB was inhibited ≈25% by 0.35 μg of F-TANK and more than 70% by 1 μg of F-TANK (Fig. 7*B*). However, F-LMPΔ188-331 increased F-TANK expression by ≈2-fold and F-TANK increased F-LMPΔ188-331 levels at least 3-fold as monitored by immunoblot analysis (data not shown). Therefore, the magnitude of the negative effect of F-TANK on LMPΔ188-331-mediated NF-κB activation may be somewhat different than the apparent result. As expected from these results, F-TANK inhibited full-length LMP1 activation of NF-κB by ≈70% (data not shown). Based on these overexpression experiments,

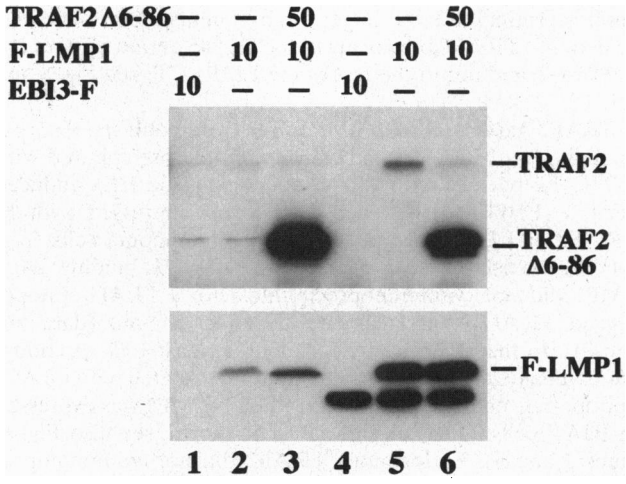

FIG. 6. TRAF2Δ6-86 competes with TRAF2 for LMP1 binding. After transfection of BJAB cells with F-LMP1, F-LMP1, and TRAF2Δ6-86 or EBI3-F expression vectors, extracts were immunoprecipitated with M2 anti-FLAG affinity gel. F-LMP1 was detected by M5 anti-FLAG immunoblot and TRAF2 was detected with anti-TRAF2 antibody. Numbers at the top indicate μg of DNA transfected. Lanes 1–3 are 10% of cell lysate and lanes 4–6 are immunoprecipitates.

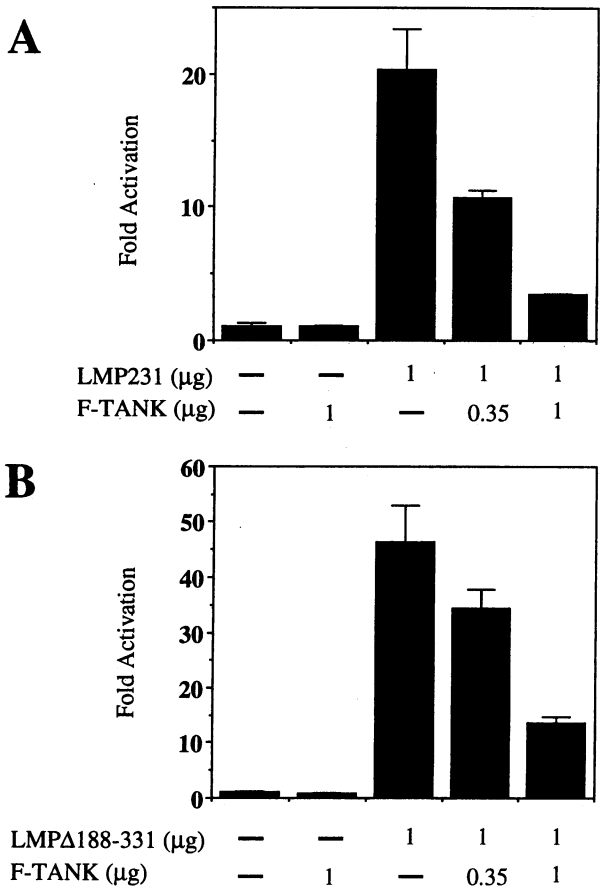

FIG. 7. TANK inhibits LMP1 induction of NF-κB. The 293 cells were transfected with the indicated amounts of expression vectors for F-TANK and LMP231 (*A*) or F-TANK and LMPΔ188-331 (*B*). Standard deviations are shown. The experiment shown was done in triplicate and the result is representative of more than six other experiments.

TANK appears to be a negative modulator of NF-κB activation by both LMP1 effector domains.

Double staining immunofluorescent analysis of 293 cells transfected with expression vectors for LMP231, LMPΔ188-331, or F-TANK alone or with one of these LMP1 constructs and F-TANK revealed both LMP1 proteins localized in plasma membrane patches and F-TANK did not affect their distribution. LMP1 was detected with an antibody to the amino-terminal cytoplasmic domain (15). F-TANK, expressed alone in 293 cells, localized diffusely throughout the cytoplasm in vesicle-like structures and LMP1 expression did not alter the distribution of F-TANK (data not shown).

F-TANK Competes with LMP1 for TRAF2. Since TANK binds to TRAF2 and competes with CD40 for TRAF2 binding (31), we investigated whether F-TANK inhibition of LMP1 NF-κB activation could be due to competition between TANK and LMP1 for TRAF2 binding. When F-TANK was expressed in BJAB cells, it did not bind to GST–LMP1 CT (data not shown) or to GST–LMP187-231 (Fig. 8). At low-level F-TANK expression, TRAF2 binding to GST–LMP187-231 decreased slightly (Fig. 8). At high-level F-TANK expression, TRAF2 binding to GST–LMP187-231 was almost completely blocked (Fig. 8). Similar results were obtained with GST–LMP1 CT (data not shown). These data indicate that TANK binding to TRAF2 prevents TRAF2 from associating with LMP1. TANK probably inhibits LMP1-mediated NF-κB activation by preventing TRAF2 association with aggregated LMP1.

DISCUSSION

These results indicate that TRAF2 is a mediator of NF-κB activation by LMP1. TRAF2 has been shown to bind specifically to the LMP1 CT *in vitro* and to associate with LMP1 in cells as determined by coimmunoprecipitation. LMP1 also caused TRAF2 to relocalize from a diffuse cytoplasmic distribution to LMP1 patches at the plasma membrane. Furthermore, a TRAF2 binding protein, TANK (31), blocked TRAF2 from binding to LMP1 and inhibited LMP1-mediated NF-κB activation. Importantly, a dominant negative TRAF2 mutant (23, 30), TRAF2Δ6-86, still bound to LMP1, competed with TRAF2 for LMP1 binding, and inhibited LMP1-mediated NF-κB activation. The failure of TRAF2Δ6-86 to transmit an NF-κB activation signal implicates the TRAF2 amino terminus as being important in transducing NF-κB activation from LMP1.

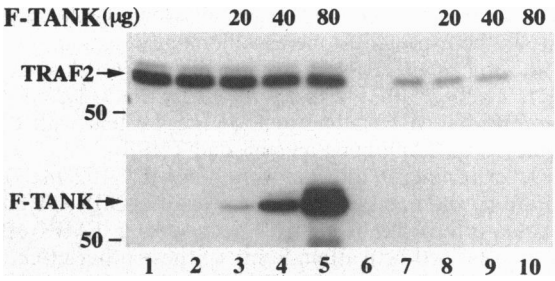

FIG. 8. TANK competes with the LMP1 CT for TRAF2 binding. BJAB cells were transfected with vector alone, with 10 μg of TRAF2 expression vector, or with 20, 40, or 80 μg of F-TANK expression vector. After lysis, the extract from TRAF2-expressing cells was divided equally and incubated with extract from vector- or F-TANK-expression-vector-transfected cells and precleared with glutathione beads for 14 h at 4°C. The cleared cell extracts were incubated with GST control or GST LMP187-231 (15) for 1.5 h at 4°C. The complexes were washed five times with 1 ml of 0.5% Nonidet P-40 lysis buffer. Lanes 1–5 contain 5% of total cell extract and lanes 6–10 contain precipitates. Lanes 1 and 2 contain lysate from TRAF2 expressing cells and no lysate from F-TANK-expressing cells. (*Upper*) Blot was probed with TRAF2 C-20 antibody. (*Lower*) Blot was reprobed with M5 anti-FLAG monoclonal antibody (IBI). Numbers above the lanes indicate μg of F-TANK transfected. Lane 6 was incubated with only GST and lanes 7–10 were incubated with the GST–LMP187-231 fusion protein. Size marker (kDa) is shown on the left.

TRAF2 is now directly implicated in NF-κB activation by the first 45 amino acids of the LMP1 CT. TRAF2 has a much less significant role in NF-κB activation by the last 55 amino acids of the LMP1 CT. Transfection of 0.2 μg of the TRAF2Δ6-86 expression vector blocked most of the NF-κB activation by LMP231, whereas transfection with 1 μg of the TRAF2Δ6-86 blocked less than 40% of the NF-κB-inducing activity of the last 55 amino acids of the LMP1 CT. Further, recent data indicate that TRAF2 binds only to the first 45 amino acids of the CT *in vitro* and associates with this domain *in vivo* (15).

These data further support the model that LMP1 mimics a constitutively activated TNFR. (*i*) TRAF2, a TNFR-associated protein implicated in CD40-, TNFRI-, and TNFRII-mediated NF-κB activation (23, 30), has been shown to associate with LMP1 and is now implicated in LMP1-mediated NF-κB activation. (*ii*) LMP1 forms constitutive patches in the plasma membrane that are similar to aggregates formed when receptors encounter ligand. Aggregation of LMP1 mediated by its six transmembrane domains is necessary for LMP1 function since mutations in these domains disrupt the ability of LMP1 to form plasma membrane patches and to alter cell growth (3, 8, 13, 27, 34) or activate NF-κB (14, 29). In contrast to the more transient signal from ligand–TNFR interaction, LMP1 has continuous effects on cell growth and NF-κB activation (2, 4, 11, 13). TRAF2 has been shown to relocalize to LMP1 aggregates when LMP1 is expressed in the cell; the aggregation of TRAF2 may be a key step in TRAF2-mediated NF-κB activation. This would be consistent with the previous finding that TRAF2 overexpression can induce NF-κB activation since

TRAF2 can bind to itself (16, 23) and high-level TRAF2 expression would be expected to increase homoaggregation. (*iii*) The TRAF-C domain of TRAF2, which mediates TRAF2 binding to TNFRII and the TNFRI adapter protein TRADD (30, 38) is also essential for TRAF2 binding to LMP1, consistent with LMP1 engaging TRAF2, imitating an activated TNFR.

These experiments indicate a role for TRAF2 in NF-κB activation by the first 45 amino acids of the LMP1 CT but do not evaluate the role of TRAF2 in mediating LMP1 effects other than NF-κB activation. Some of these other effects are likely to be mediated by TRAF2 or by TRAF1 or TRAF3, which associate with the LMP1 CT (15, 17). Furthermore, the relationship of TRAF1 and TRAF3 to TRAF2 in LMP1-mediated NF-κB activation has not been evaluated here and is the subject of another investigation (15).

TANK appears to be a negative regulator of LMP1-mediated NF-κB activation. TANK bound to TRAF2 (ref. 31 and results herein), and we now have shown that TANK directly competes with the LMP1 CT for TRAF2 binding. Even low-level TANK expression inhibited TRAF2 binding to the LMP1 CT and partially blocked LMP1-mediated NF-κB activation. The negative effect of low-level TANK expression contrasts with the stimulatory effect of low-level TANK overexpression on the NF-κB activation that was induced by low level TRAF2 overexpression (31). The failure to discern a positive effect of low-level TANK expression on LMP1-mediated NF-κB activation in our experiments may be due to LMP1's reorganization of TRAF2 into aggregates in the plasma membrane; the strong positive effect of this reorganization on NF-κB activation likely exceeds the positive effect of low-level TANK expression on low-level TRAF2 overexpression. In the absence of LMP1, TRAF2 is distributed throughout the cytoplasm, similar to the localization of TANK. The similar localization of TRAF2 and TANK is compatible with the possibility that TRAF2 and TANK may colocalize. If LMP1 activates NF-κB by inducing TRAF2 aggregation, TANK may have similar effects. Low-level TANK and TRAF2 overexpression may coactivate NF-κB by aggregating TRAF2 with TANK and TANK associated proteins. Alternatively, an NF-κB activation domain at the TANK animo terminus may be activated by TRAF2 (31).

While the negative effects of TANK overexpression on TRAF2 binding to LMP1 probably account for the TANK inhibition of NF-κB activation by the first 45 amino acids of the LMP1 CT, they are unlikely to account for the negative effects of TANK on NF-κB activation by the last 55 amino acids of the LMP1 CT. TRAF2 does not bind to or associate with the last 55 amino acids of the LMP1 CT (15). Also, while TRAF2Δ6-86 and TANK exert similar inhibitory effects on NF-κB activation by the first 45 amino acids of the CT, the inhibitory effect of TRAF2Δ6-86 is much weaker than that of TANK on the last 55 amino acids of the LMP1 CT. Both of these results are most compatible with the working hypothesis that the last 55 amino acids of the LMP1 CT induces NF-κB through a TRAF-interactive pathway that does not directly involve TRAF2. The negative effect of TANK on the last 55 amino acids of the LMP1 CT could be mediated by the carboxyl terminus of TANK that has a negative effect on NF-κB activation that is independent of the TRAF2 interacting domain of TANK (31).

Note Added in Proof. I-TRAF (TANK) inhibition of NF-KB activation has been described (39).

Genhong Cheng and David Baltimore shared their TANK sequence and results prior to publication. Tom Mitchell and Bill Sugden provided 3X-κB-L and SV2LMPΔ188-331 and Martin Rowe provided antibody that recognizes the amino terminus of LMP1. Eudoxia Hatzivassiliou provided advice and assistance with calcium phosphate transfections. Susannah Barbee assisted in doing the immunofluorescence staining for Fig. 4 and Qian Miao ably assisted in tissue culture. This research was supported by Grants CA67380-02 (K.M.K.) and CA47006 (E.K.) from the National Cancer Institute. K.M.K. is supported by National Institutes of Health Physician Scientist Award 1K11CA01568-04. K.M.I. is supported by The Medical Foundation/Charles A. King Trust. G.M. is a Fellow of the Leukemia Society of America.

1. Rickinson, A. B. & Kieff, E. (1996) in *Fields Virology*, eds. Fields, B. N., Knipe, D. M. & Howley, P. M. (Lippincott, New York), pp. 2397–2446.
2. Wang, D., Liebowitz, D. & Kieff, E. (1985) *Cell* **43,** 831–40.
3. Wang, D., Liebowitz, D. & Kieff, E. (1988) *J. Virol.* **62,** 2337–46.
4. Baichwal, V. R. & Sugden, B. (1988) *Oncogene* **2,** 461–7.
5. Dawson, C. W., Rickinson, A. B. & Young, L. S. (1990) *Nature (London)* **344,** 777–80.
6. Wilson, J. B., Weinberg, W., Johnson, R., Yuspa, S. & Levine, A. J. (1990) *Cell* **61,** 1315–27.
7. Fahraeus, R., Rymo, L., Rhim, J. S. & Klein, G. (1990) *Nature (London)* **345,** 447–9.
8. Wang, D., Liebowitz, D., Wang, F., Gregory, C., Rickinson, A., Larson, R., Springer, T. & Kieff, E. (1988) *J. Virol.* **62,** 4173–84.
9. Henderson, S., Rowe, M., Gregory, C., Croom, C. D., Wang, F., Longnecker, R., Kieff, E. & Rickinson, A. (1991) *Cell* **65,** 1107–15.
10. Hammarskjold, M.-L. & Simurda, M. C. (1992) *J. Virol.* **66,** 6496–6501.
11. Laherty, C. D., Hu, H. M., Opipari, A. W., Wang, F. & Dixit, V. M. (1992) *J. Biol. Chem.* **267,** 24157–60.
12. Rowe, M., Peng, P. M., Huen, D. S., Hardy, R., Croom, C. D., Lundgren, E. & Rickinson, A. B. (1994) *J. Virol.* **68,** 5602–12.
13. Kaye, K. M., Izumi, K. M. & Kieff, E. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9150–54.
14. Mitchell, T. & Sugden, B. (1995) *J. Virol.* **69,** 2968–76.
15. Devergne, O., Hatzivassiliou, E., Izumi, K. M., Kaye, K. M., Kleijner, M., Kieff, E. & Mosialos, G. (1996) *Mol. Cell. Biol.*, in press.
16. Rothe, M., Wong, S. C., Henzel, W. J. & Goeddel, D. V. (1994) *Cell* **78,** 681–92.
17. Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C. & Kieff, E. (1995) *Cell* **80,** 389–99.
18. Hu, H., O'Rourke, K., Boguski, M. S. & Dixit, V. M. (1994) *J. Biol. Chem.* **269,** 30069–30072.
19. Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S. & Baltimore, D. (1995) *Science* **267,** 1494–8.
20. Sato, T., Irie, S. & Reed, J. C. (1995) *FEBS Lett.* **358,** 113–8.
21. Kaye, K. M., Izumi, K. M., Mosialos, G. & Kieff, E. (1995) *J. Virol.* **69,** 675–83.
22. Banchereau, J., Bazan, F., Blanchard, D., Briere, F., Galizzi, J. P., van Kooten, C., Liu, Y. J., Rousset, F. & Saeland, S. (1994) *Annu. Rev. Immunol.* **12,** 881–922.
23. Rothe, M., Sarma, V., Dixit, V. M. & Goeddel, D. V. (1995) *Science* **269,** 1424–7.
24. Hennessy, K., Fennewald, S., Hummel, M., Cole, T. & Kieff, E. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7207–11.
25. Liebowitz, D., Wang, D. & Kieff, E. (1986) *J. Virol.* **58,** 233–7.
26. Mann, K. P., Staunton, D. & Thorley, L. D. (1985) *J. Virol.* **55,** 710–20.
27. Liebowitz, D., Mannick, J., Takada, K. & Kieff, E. (1992) *J. Virol.* **66,** 4612–6.
28. Izumi, K. M., Kaye, K. M. & Kieff, E. D. (1994) *J. Virol.* **68,** 4369–76.
29. Huen, D. S., Henderson, S. A., Croom, C. D. & Rowe, M. (1995) *Oncogene* **10,** 549–60.
30. Hsu, H., Shu, H. B., Pan, M. G. & Goeddel, D. V. (1996) *Cell* **84,** 299–308.
31. Cheng, G. & Baltimore, D. (1996) *Genes Dev.* **10,** 963–73.
32. Birkenbach, M., Josefsen, K., Yalamanchili, R., Lenoir, G. & Kieff, E. (1993) *J. Virol.* **67,** 2209–20.
33. Song, H. Y. & Donner, D. B. (1995) *Biochem. J.* **309,** 825–829.
34. Baichwal, V. R. & Sugden, B. (1989) *Oncogene* **4,** 67–74.
35. Graham, F. L. & van der Eb, A. J. (1973) *Virology* **52,** 456–467.
36. Devergne, O., Hummel, M., Koeppen, H., Le, B. M., Nathanson, E. C., Kieff, E. & Birkenbach, M. (1996) *J. Virol.* **70,** 1143–53.
37. Smith, C. A. & Johnson, K. S. (1988) *Gene* **67,** 31–40.
38. Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M. & Goeddel, D. V. (1995) *Cell* **83,** 1243–52.
39. Rothe, M., Xiong, J., Shu, H.-B., Williamson, B., Goddard, A. & Goeddel, D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 8241–8246.