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Association of TRAF1, TRAF2, and TRAF3 with an Epstein-Barr Virus LMP1 Domain Important for B-Lymphocyte Transformation: Role in NF-κB Activation

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The Epstein-Barr virus (EBV) transforming protein LMP1 appears to be a constitutively activated tumor necrosis factor receptor (TNFR) on the basis of an intrinsic ability to aggregate in the plasma membrane and an association of its cytoplasmic carboxyl terminus (CT) with TNFR-associated factors (TRAFs). We now show that in EBV-transformed B lymphocytes most of TRAF1 or TRAF3 and 5% of TRAF2 are associated with LMP1 and that most of LMP1 is associated with TRAF1 or TRAF3. TRAF1, TRAF2, and TRAF3 bind to a single site in the LMP1 CT corresponding to amino acids (aa) 199 to 214, within a domain which is important for B-lymphocyte growth transformation (aa 187 to 231). Further deletional and alanine mutagenesis analyses and comparison with TRAF binding sequences in CD40, in CD30, and in the LMP1 of other lymphycryptoviruses provide the first evidence that PXQXT/S is a core TRAF binding motif. The negative effects of point mutations in the LMP1(1-231) core TRAF binding motif on TRAF binding and NF-κB activation genetically link the TRAFs to LMP1(1-231)-mediated NF-κB activation. NF-κB activation by LMP1(1-231) is likely to be mediated by TRAF1/TRAF2 heteroaggregates since TRAF1 is unique among the TRAFs in coactivating NF-κB with LMP1(1-231), a TRAF2 dominant-negative mutant can block LMP1(1-231)-mediated NF-κB activation as well as TRAF1 coactivation, and 30% of TRAF2 is associated with TRAF1 in EBV-transformed B cells. TRAF3 is a negative modulator of LMP1(1-231)-mediated NF-κB activation. Surprisingly, TRAF1, -2, or -3 does not interact with the terminal LMP1 CT aa 333 to 386 which can independently mediate NF-κB activation. The constitutive association of TRAFs with LMP1 through the aa 187 to 231 domain which is important in NF-κB activation and primary B-lymphocyte growth transformation implicates TRAF aggregation in LMP1 signaling.

Latent-infection membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) is essential for the ability of EBV to induce continuous proliferation of primary human B lymphocytes (20). LMP1 comprises an N-terminal cytoplasmic domain (amino acids [aa] 1 to 24), six markedly hydrophobic transmembrane domains separated by short reverse turns (aa 25 to 186), and a long cytoplasmic carboxyl terminus (CT) (aa 187 to 386) (6) (Fig. 1). Recombinant EBV genetic analyses indicate that the six transmembrane domains and the CT first 45 aa are sufficient for primary B-lymphocyte growth transformation in the context of a specifically mutated EBV recombinant (18, 20, 21, 23).

Several lines of evidence indicate that LMP1 is a constitutively activated tumor necrosis factor family receptor (TNFR) similar to activated CD40. First, expression of LMP1 in B lymphoblasts or in primary B lymphocytes results in NF-κB activation and expression of Bcl-2, activation markers, adhesion molecules, and autocrine growth factors, all of which are induced in normal B lymphocytes after stimulation by CD40 ligand and interleukin-4 or -10 (1, 11, 16, 24, 26, 30, 33, 41, 42). Second, a significant fraction of LMP1 constitutively aggregates in a patch in the plasma membrane of lymphoblastoid cell lines (LCLs) (12, 26, 27, 29). The aggregation is dependent on the six hydrophobic transmembrane domains and is essential for transformation. Thus, LMP1 comprising only the last five transmembrane domains and the entire CT, i.e., LMP1 aa 44 to 386, diffusely distributes in the plasma membrane and is nontransforming (20, 26), whereas LMP1 comprising the last two transmembrane domains and the CT, i.e., LMP1 aa 129 to 386, diffusely distributes in all cytoplasmic membranes and has no effect on lymphoblasts (16, 26, 41). Third, the LMP1 CT aa 187 to 231, which are sufficient for transformation when linked to the six transmembrane domains [LMP1(1-231)] (23), interact with a protein that also interacts with the CD40 cytoplasmic domain (3, 15, 31, 39); a CD40 cytoplasmic domain nonsignaling mutant fails to interact with this protein (15). This protein (previously called LAP1, CD40bp, CRAF1, or CAP1) is now designated TRAF3 because of its extensive C-terminal "TRAF domain" homology to TNFR-associated factor 2 (TRAF2) and TRAF1 (also called EBI6) (36, 37). The LMP1 CT can also bind to TRAF2 in vitro and associates with TRAF1, TRAF2, and TRAF3 in the plasma membrane of transiently transfected cells (22, 31). TRAF2 has been implicated in CD40-, p80 TNFR-, and p60 TNFR-mediated NF-κB activation (14, 36). The LMP1 CT has two domains (aa 187 to 231 and aa 332 to 386) that can transmit NF-κB-activating signals. LMP1 aa 1 to 386 with aa 188 to 331 deleted (LMPΔ188-331) activates NF-κB better than LMP1(1-231) (16, 30). LMP1(1-231)-mediated NF-κB activation can be inhibited by a dominant-negative TRAF2 mutant, implicating TRAF2 in NF-κB activation by LMP1(1-231). In contrast, LMPΔ188-331-mediated NF-κB activation was largely unaffected by dominant-negative TRAF2 overexpression (22).

The experiments reported here were designed to investigate

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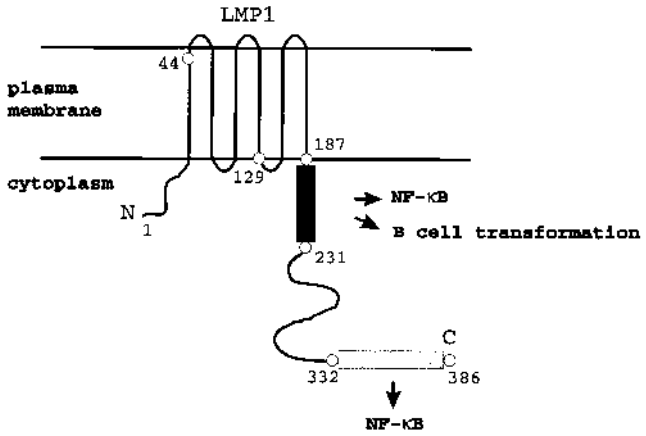

FIG. 1. Schematic representation of LMP1. LMP1 consists of a 25-aa N-terminal cytoplasmic domain, six hydrophobic transmembrane domains separated by short reverse turns, and a 200-aa C-terminal cytoplasmic domain. The LMP1 N-terminal cytoplasmic domain is not essential for growth transformation (18). Codon 44-initiated LMP1, expressed from the EBV genome, is unable to transform B lymphocytes and is diffusely distributed in the plasma membrane, illustrating the importance of plasma membrane patching in LMP1 effects on cell growth (20). LMP1 aa 1 to 231 [LMP1(1-231)] are sufficient for primary B-lymphocyte growth transformation (21, 23) and can activate NF-κB with 25% of the efficiency of wild-type LMP1 (16, 30). LMP1 aa 1 to 386 with a deletion of aa 188 to 331 (LMPΔ188-331) can activate NF-κB with about 75% of the efficiency of wild-type LMP1 (16, 30).

the interaction of TRAF1, -2, and -3 with LMP1 in LCLs, identify key residues in the LMP1 CT that interact with TRAF1, -2, and -3, and evaluate the relative roles of TRAF1, -2, and -3 in NF-κB activation by LMP1.

MATERIALS AND METHODS

Cell lines. IB4 is an EBV-transformed LCL. BJAB is an EBV-negative Burkitt lymphoma cell line. 293 is a human embryonal kidney cell line. An LCL transformed by recombinant EBV carrying an N-terminal FLAG-tagged LMP1 (FLAG-LMP1 LCL) was derived by transfecting *Eco*RI-A, *Sal*IEC, and a mutated B230 cosmid DNAs into P3HR-1 cells. B230 was derived by replacing codons 2 to 4 in the LMP1 gene in S-wt (18) with a double-stranded oligonucleotide that encodes the FLAG monoclonal antibody epitope (DYKDDDD KV). A *Not*I site was then inserted at a *Hin*dIII site (nucleotide [nt] 166480 of the EBV genome sequence) in the FLAG-oligonucleotide-containing DNA fragment, and a *Pac*I site was inserted into a *Bgl*II site (nt 169032), resulting in clone B220. Cosmid B230 was then formed from the *Eco*RI (nt 95239) to *Not*I (nt 117614) fragment from *Eco*RI-B, the *Not*I (nt 166480) to *Sal*I (nt 643) fragment from B220, the *Sal*I (nt 643) to *Sna*BI (13219) fragment from *Sna*BI, and pDVcosPENBSP vector DNAs (34). Cell lines were grown in RPMI (B-cell lines) or Dulbecco modified Eagle medium (293) supplemented with 10% fetal calf serum.

Plasmids. pSG5TRAF1 was constructed by inserting the TRAF1 cDNA (EBI6 cDNA) (31) into the *Eco*RI site of pSG5 (Stratagene Corp.). pSG5FLAGTR AF1(184-416) was constructed by inserting the *Hin*dIII-*Bgl*II fragment of pSG5 TRAF1 into the *Eco*72I site of pSG5 FLAG (10). pSG5FLAGTRAF3(345-568) contains a *Bgl*II cDNA fragment encoding TRAF3 aa 345 to 568 (31) cloned into the *Hpa*I site of pSG5FLAG. pSG5FLAG-LMP1 was constructed by inserting an *Mlu*I fragment that contains the FLAG-LMP1 gene fusion from B220 into pSG5. pSG5FLAG-LMP1(1-231) contains an *Xba*I linker (CTAGGTCTAGACTAG) (New England Biolabs) inserted at an *Nae*I site (nt 168627) (21) and encodes a FLAG LMP1 to residue 231 plus an additional Leu-Val before termination. Plasmid pSG5LMP1(1-231) was constructed by PCR amplification of the cDNA encoding LMP1 aa 170 to 231, using the 5′ primer 170F (5′-GATCTCCTTTG GCTCCTCCTG-3′) and the 3′ primer 231R (5′-CCTAGGCATGCCATGGTT AGGCTCCACTCACGAGCAGGT-3′). The downstream primer places a stop codon followed by a *Nco*I site after Ala-231. The amplified fragment was digested with *Nco*I and substituted for a *Nco*I fragment of pBSLMP1 (26). An *Eco*RI fragment of the resulting plasmid was then subcloned in the *Eco*RI site of pSG5 to generate pSG5LMP1(1-231). pSG5LMP1(1-231)P204A, pSG5LMP1(1-231)Q206A, and pSG5LMP1(P204A,Q206A) were constructed in a similar manner, using PCR-mutated *Nco*I fragments. The outside primers used were 170F and 231R for the P-204 to A or Q-206 to A mutation and 170F and L1-4 PCR (31) for the P-204 to A, Q-206 to A mutation; the inside primers were 5′-GGT AGCTTGTTGAGCGTGCGGGAGGGAGTCATCGT-3′ and 5′-TCCCTCCC GCACGCTCAACAAGCTACCGATGATTC-3′ for the P-204 to A mutation, 5′-TCATCGGTAGCTGCTTGAGGGTGCGGGAGGGAGT-3′ and 5′-CCGC ACCCTCAAGCAGCTACCGATGATTCTGGCC-3′ for the Q-206 to A mutation, and 5′-TCCCTCCCGCACGCTCAAGCAGCTACCGATGATTCTGGCC -3′ and 5′-CTATCGGTAGCTGCTTGAGCGTGCGGGAGGGAGTCATCGT -3′ for the P-204 to A, Q-206 to A mutation. pSG5 vectors encoding FLAG-LMP1(1-231) with a P-204 to A or a Q-206 to A mutation, or FLAG-LMP1 with P-204 to A and Q-206 to A mutations, were constructed by inserting a *Mam*I-*Bpu*1102 I fragment of pSG5LMP1(1-231)P204A, pSG5LMP1(1-231)Q206A, or pSG5LMP1(P204A,Q206) between the *Mam*I and *Bpu*1102 I sites of pSG5FL AG-LMP1. Plasmids pGEXLMP1(187-386), pGEXLMP1(187-212), pGEXLM P1(201-231), pGEXLMP1(240-386), pGEXLMP1(333-386), and pGEXLMP1 (240-386) were constructed by PCR amplification of the corresponding LMP1 cDNA fragments, using primers having a 5′ *Bam*HI site to facilitate cloning in the *Bam*HI site of the pGEX-2TK vector. Plasmids expressing wild-type or mutant aa 199 to 214 or aa 199 to 210 LMP1 domains fused to glutathione *S*-transferase (GST) were made by inserting synthetic double-stranded oligonucleotides containing a 5′ *Bam*HI site and a 3′ *Eco*RI site preceded by a stop codon into the *Bam*HI and *Eco*RI sites of the pGEX-2TK vector. PCR-derived *Nco*I fragments encoding wild-type or mutant LMP1(187-231) were also inserted in the *Nco*I site of pGEXLMP1(187-212) to generate the pGEXLMP1(187-231), pGEXLMP1(187-231)P204A, and pGEXLMP1(187-231)Q206A plasmids. The Gal4 DNA binding domain fusions of LMP1 aa 187 to 212 (G4DBD-LMP1 187-212) and aa 201 to 231 (G4DBD-LMP1 201-231) were made by PCR amplification of the corresponding LMP1 cDNA fragments, using primers having a 5′ *Bam*HI site, followed by cloning into the *Bam*HI site of the yeast vector pAS2 (31). G4TAD-TRAF2 was constructed by fusing FLAG-TRAF2 (22) in frame to the transactivating domain of Gal4 (G4TAD) in the pACTII vector (31). Sequencing of G4TADLAP1(183-568) (31) revealed that this clone has codons 218 to 242 of TRAF3 deleted and is referred to in this paper as G4TAD-TRAF3(183-568). Vent polymerase was used in most PCRs, and the sequences of all PCR- or oligonucleotide-derived constructs were verified by DNA sequencing of the final clone (Sequenase; Amersham).

GST fusion protein binding assays and BJAB transfections. In vitro-translated proteins from 6 to 10 μl of reaction mix (TNT system; Promega Corp.) were diluted in 0.3 ml of binding buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40 [NP-40], 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and were precleared with glutathione-Sepharose beads (Pharmacia) for 1 h at 4°C. GST or GST fusion proteins bound to glutathione beads (5 to 10 μg) (40) were then incubated with in vitro-translated protein for 1 to 2 h at 4°C. The beads were washed 5 times with 1 ml of binding buffer. Bound proteins were recovered by boiling in sodium dodecyl sulfate (SDS) sample buffer and were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Gels were stained with Coomassie blue to verify the presence of equivalent amounts of the various fusion proteins and then processed for autoradiography and PhosphorImager analysis. For GST binding assays using cell extracts, 10^7 BJAB cells were transfected by electroporation at 210 V and 960 μF in 400 μl of RPMI medium containing 10% fetal calf serum. Eighteen hours after transfection, the cells were washed in phosphate-buffered saline (PBS) and lysed for 30 min on ice in 1% NP-40 lysis buffer (1% NP-40, 50 mM Tris [pH 7.4], 150 mM NaCl, 3% glycerol, 1.5 mM EDTA) containing protease inhibitors (1 mM PMSF, 1 mg of leupeptin per ml, and 1 mg of pepstatin per ml). Cell lysates were centrifuged at 14,000 × *g* for 15 min at 4°C and precleared with glutathione beads for 1 h at 4°C. Cleared cell lysates were then incubated for 2 h at 4°C with approximately 5 μg of GST control or GST fusion proteins. The beads were washed three times with 1 ml of 0.5% NP-40 lysis buffer, and precipitated material was analyzed by SDS-PAGE and Western immunoblotting. Alternatively, 1% NP-40 extracts from 2×10^7 cells from an LCL were incubated with approximately 24 μg of GST or GST fusion proteins and processed as described above.

Immunoprecipitation and immunoblotting. LCLs or transfected BJAB cells obtained 18 h posttransfection were washed in ice-cold PBS and lysed for 30 to 60 min on ice in 0.5% NP-40 lysis buffer containing protease inhibitors. Cell lysates were prepared by extensive homogenization by Dounce. Lysates were centrifuged for 15 min at 14,000 × *g* and precleared with protein G- or protein A-Sepharose beads (Pharmacia) for 1 to 2 h. Cleared lysates were incubated with anti-FLAG M2 affinity gel (International Biotechnologies Inc.) for 2 h at 4°C or with rabbit polyclonal antibodies recognizing TRAF1 or TRAF3 (TRAF1 N19 or CRAF1 H20; Santa Cruz Biotechnology) for 2 h at 4°C followed by incubation with protein A-Sepharose beads for 1 h at 4°C. Beads were then washed five times with 1 ml of 0.5% NP-40 lysis buffer, and bound proteins were recovered by boiling in SDS sample buffer or by elution with FLAG peptide. For peptide elution, M2 beads were further washed with 1 ml of TBS (10 mM Tris [pH 7.4], 150 mM NaCl) and immunoprecipitated proteins were eluted twice with 50 μl of FLAG peptide (500 mM in TBS). Eluted proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting. TRAFs were detected using rabbit polyclonal antisera (Santa Cruz Biotechnology) recognizing TRAF1 (TRAF1 S19), TRAF2 (TRAF2 C20), or TRAF3 (CRAF1 H20 and CRAF1 H122) or both TRAF1 and TRAF3 (CRAF1 C20), at 1 μg/ml. Binding of TRAF antisera was detected using horseradish peroxidase-conjugated protein A (1:7,500 dilution) and ECL reagents (Amersham). LMP1 or FLAG-tagged LMP1 was detected by using S12 anti-LMP1 (27) or M5 anti-FLAG monoclonal

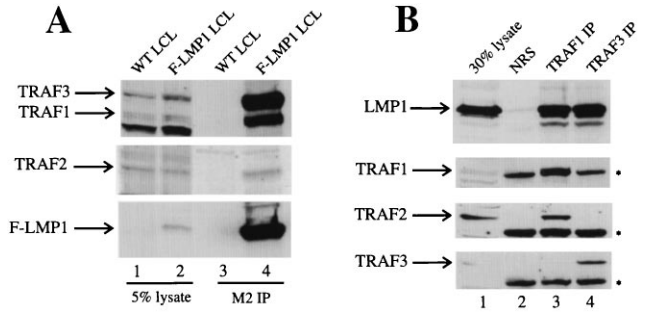

FIG. 2. In vivo association of TRAF1, TRAF2, and TRAF3 with LMP1 in LCLs. (A) NP-40 cell extracts from 40×10^6 cells of a recombinant LCL expressing an N-terminal FLAG-tagged LMP1 protein (F-LMP1 LCL) or of the control IB4 LCL (WT LCL) were subjected to immunoprecipitation with anti-FLAG M2 affinity gel, and coimmunoprecipitated proteins were eluted by competition with FLAG peptide. Cell lysates (5% of the total before immunoprecipitation) and the eluted proteins were analyzed by SDS-PAGE on an 8% gel and subjected to Western blot analysis with rabbit polyclonal antibodies recognizing both TRAF1 and TRAF3 (CRAF1 C20) or TRAF2 (TRAF2 C20) or with M5 anti-FLAG monoclonal antibody. The positions of the TRAF1 doublet and of TRAF2, TRAF3, and FLAG-LMP1 are indicated. (B) NP-40 cell extracts from the IB4 LCL (5×10^6 cells per lane) were subjected to immunoprecipitation with 2 μg of rabbit polyclonal antibodies recognizing TRAF1 (TRAF1 N19) or TRAF3 (CRAF1 H20) or with 4 μg of normal rabbit serum (NRS) as a control. Of the total cell lysate and the eluted proteins, 30% was analyzed by SDS-PAGE on an 8% gel and subjected to Western blot analysis with S12 anti-LMP1 monoclonal antibody or with rabbit polyclonal antibodies recognizing TRAF1 (TRAF1 S19), TRAF2 (TRAF2 C20), or TRAF3 (CRAF1 H122). At a higher magnification, the TRAF1 protein (arrow) is detected as a doublet. The lower TRAF1 band comigrates with the immunoglobulin heavy chain (asterisk) in the TRAF1 immunoprecipitate, while the upper TRAF1 band migrates just above it, resulting in the thicker band observed.

antibody (IBI), respectively, followed by sheep anti-mouse antibodies conjugated to horseradish peroxidase (1:5,000 dilution; Amersham).

NF-κB-dependent reporter assays in 293 cells. The day before transfection, 4×10^5 293 cells were plated in 35-mm-diameter dishes. The following day the 50% confluent cells were fed with fresh medium and were transfected using the calcium phosphate method (9). Transfections included pGKβgal (10), a phosphoglucokinase promoter-driven β-galactosidase expression plasmid to normalize for transfection efficiency, together with a reporter plasmid and effector expression plasmids. Two reporter plasmids were used, the 3X-κB-L plasmid, which has three repeats of the NF-κB site from the murine major histocompatibility complex class I promoter upstream of a minimal *fos* promoter and a luciferase reporter gene, or the plasmid 3X-mutκ-L, which contains mutated NF-κB sites (30). After 14 to 16 h, the cells were rinsed and fresh medium was added. After an additional 24 h, the cells were harvested in PBS and lysed in luciferase lysis buffer (Promega). The lysates were assayed for luciferase and β-galactosidase activities using an OPTOCOMP I Luminometer (MGM Instruments). ANOVA (13) was used for statistical analysis.

RESULTS

TRAF1, TRAF2, and TRAF3 associate in vivo with LMP1 in LCLs. Although TRAF1, TRAF2, or TRAF3 can associate with LMP1 when a TRAF and LMP1 are transiently overexpressed by gene transfer (22, 31), an association in EBV-transformed B lymphocytes has not been previously investigated. Such an investigation has been hindered by the fact that TRAFs and immunoprecipitating antibodies for LMP1 both bind to the LMP1 CT. We therefore first derived an EBV recombinant encoding a FLAG-epitope tag in the LMP1 N-terminal cytoplasmic domain, a domain previously shown to be nonessential for transformation (18). Most of the soluble FLAG-LMP1 was immunoprecipitated with anti-FLAG antibody in lysates from an LCL transformed by this EBV recombinant (Fig. 2A; compare the amount of FLAG-LMP1 in lane 4 with 5% of the lysate in lane 2 with the negative control anti-FLAG immunoprecipitate from a [non-FLAG] LMP1 wild-type-expressing LCL in lane 3). Under the extraction conditions used for these experiments, from 70 to 80% of LMP1 and more than 90% of TRAF1, TRAF2, and TRAF3 are in the soluble fraction (data not shown). Comparison of the amount of TRAF1, TRAF2, and TRAF3 in the FLAG-LMP1 immunoprecipitate with the amount in 5% of the cell extract revealed that most of the TRAF3 and TRAF1 had precipitated with FLAG-LMP1, while only 5% of the TRAF2 had precipitated with FLAG-LMP1.

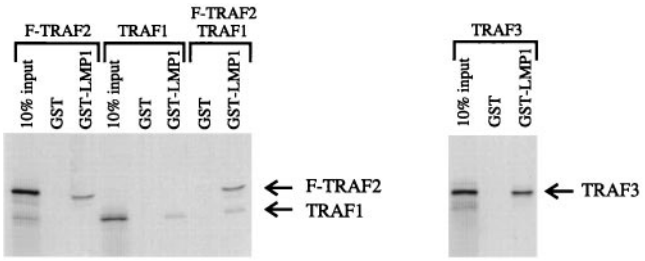

FIG. 3. In vitro binding of TRAF1, TRAF2, or TRAF3 to the LMP1 CT. TRAF1, FLAG-tagged TRAF2 (F-TRAF2) (21), or TRAF3 (29) was translated in vitro in a wheat germ extract in the presence of [^{35}S]methionine, and the ^{35}S-labeled proteins (10-μl reaction mix), alone or in combination, were incubated with Sepharose bead-bound GST or GST-LMP1 CT fusion protein. Precipitated proteins were analyzed on an 8% SDS-PAGE, followed by autoradiography and PhosphorImager analysis.

The extent of LMP1 association with TRAF1 or TRAF3 was also investigated by immunoprecipitating TRAF1 or TRAF3 from LCLs (Fig. 2B). An immunoprecipitating TRAF2 antibody was not available to assess the extent of LMP1 association with TRAF2. TRAF3 or TRAF1 antibody immunoprecipitated most of the TRAF3 or TRAF1 from the lysate (Fig. 2B). Approximately 30% of the LMP1 coimmunoprecipitated with TRAF3 or TRAF1 (Fig. 2B). No significant amount of TRAF1 or TRAF2 was present in the TRAF3 immunoprecipitate, while 30% of TRAF2 and no TRAF3 was in the TRAF1 immunoprecipitate (TRAF1 antibody was verified not to immunoprecipitate TRAF2 [data not shown]). These data indicate that about 30% of the LMP1 is associated with TRAF3 and that another 30% of LMP1 is associated with TRAF1 or TRAF1-TRAF2 complexes, while most of the TRAF3 and TRAF1 in LCLs is bound to LMP1.

In vitro binding of TRAF1, TRAF2, and TRAF3 to the LMP1 CT. Previous experiments performed with yeast two-hybrid liquid β-galactosidase assays demonstrated that TRAF3 (aa 346 to 568) interacts with LMP1 CT aa 187 to 386 or 187 to 231, while no interaction between TRAF1 (aa 53 to 416) and LMP1 CT aa 187 to 386 could be evidenced (31). Also, TRAF2 does not interact with the LMP1 CT in a yeast two-hybrid assay (data not shown) but shows specific binding to a GST-LMP1 CT fusion protein when translated in vitro in a wheat germ extract (22). To further explore the potential binding of TRAF1 to the LMP1 CT in the absence of animal cell TRAFs, TRAF1, TRAF2, or TRAF3 was translated in a wheat germ extract and incubated with a GST-LMP1 CT fusion protein or with the GST control (Fig. 3). TRAF1 and TRAF2 bound to the GST-LMP1 CT with similar efficiency, the average efficiency was approximately 4-fold less than TRAF3 and more than 100-fold above the GST background (Fig. 3 and replicate experiments not shown). Mixing in vitro-translated TRAF1 and TRAF2 did not increase their binding (Fig. 3). These results indicate that TRAF1, TRAF2, and TRAF3 can independently bind to the LMP1 CT in the absence of animal cell TRAFs.

The LMP1 CT has a single TRAF binding site within aa 201 to 210. Since both LMP1 CT aa 187 to 231 and aa 332 to 386

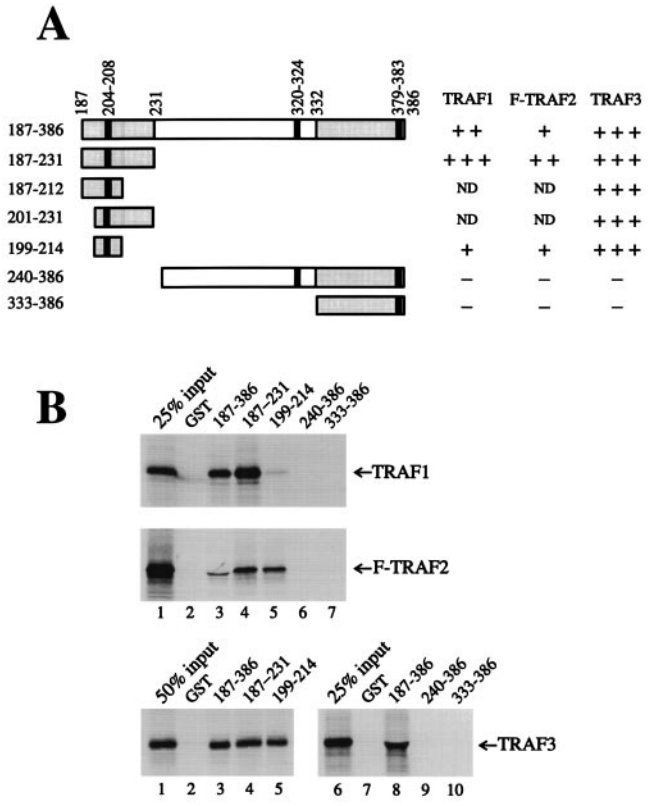

FIG. 4. Mapping of the TRAF1, TRAF2, and TRAF3 binding sites in the LMP1 CT. (A) Schematic representation of the LMP1 CT deletion mutant GST fusion proteins. Domains shown to mediate NF-κB induction (30) are identified by grey boxes, and domains containing a **PXQXT/S** motif (see Discussion) by black boxes. Average binding of total input in vitro-translated TRAFs from multiple experiments is indicated by +++ (>20%), ++ (5 to 15%), + (1%), and − (no binding above background). (B) Binding of in vitro-translated TRAF1, TRAF2, or TRAF3 to deletion mutants of LMP1 CT GST fusion proteins. Sepharose beads bound to GST control protein or GST fusion proteins containing the full-length LMP1 CT or various deletion mutants were incubated in vitro with ^{35}S-labeled TRAF1, FLAG-TRAF2 (F-TRAF2) (22), or TRAF3 (31) translated in vitro in rabbit reticulocyte lysate. Precipitated proteins were analyzed on a 10% SDS-PAGE followed by autoradiography and PhosphorImager analysis.

have been implicated in NF-κB activation, TRAF binding to both sites was further investigated in yeast two-hybrid assays and in in vitro GST pull-down experiments. As previously observed, in yeast two-hybrid liquid β-galactosidase assays, a Gal4 DNA binding domain (G4DBD) fusion with LMP1 aa 187 to 231, G4DBD-LMP1(187-231), interacted strongly with G4TAD-TRAF3(183-568) (31). In contrast, G4DBD-LMP1 (240-386) did not interact with G4TAD-TRAF3(183-568) either in liquid or in more sensitive colony-based β-galactosidase assays (data not shown). Expression of G4DBD-LMP1(240-386) was confirmed by LMP1 immunoblot and by interaction with a specific G4TAD-cDNA gene fusion (data not shown). Further, TRAF3, translated in vitro in a reticulocyte lysate, did not bind to GST-LMP1(333-386) or GST-LMP1(240-386) but did bind strongly to GST-LMP1(187-386) or GST-LMP1(187-231) (Fig. 4). Thus, TRAF3 binds only to a site(s) within LMP1 CT aa 187 to 231.

Similarly, TRAF1 or FLAG-TRAF2, translated in a reticulocyte lysate, did not bind to GST-LMP1(333-386) or GST-LMP1(240-386) but did bind to GST-LMP1(187-386) or GST-LMP1(187-231) (Fig. 4). FLAG-TRAF2 was used in most experiments because the efficiency of in vitro translation was higher than with native TRAF2. Surprisingly, both TRAF1 and FLAG-TRAF2 bound better to GST-LMP1(187-231) than to GST-LMP1(187-386). On average, more than 50% of TRAF1, 10% of FLAG-TRAF2, and 25% of TRAF3 bound to GST-LMP1(187-231), while 15% of TRAF1, 1% of FLAG-TRAF2, and 38% of TRAF3 bound to GST-LMP1(187-386) (Fig. 4). Since TRAF1 and FLAG-TRAF2 bound more strongly to GST-LMP1(187-231) than to GST-LMP1(187-386), G4TAD-TRAF2 or G4TAD-TRAF1(53-416) was tested against a G4DBD-LMP1(187-231) in a yeast two-hybrid β-galactosidase filter assay to determine whether any interaction could be detected in this assay. While cells with G4DBD-LMP1(187-231) that were transfected with G4TAD-TRAF3(183-568) became blue in 30 min, the same cells transfected with G4TAD-TRAF1(53-416) turned blue at 2 to 3 h and with G4TAD-TRAF2 or G4TAD failed to turn blue even after overnight incubation (data not shown). Thus, as observed for TRAF3, TRAF1 and TRAF2 bind only to a site(s) within LMP1 CT aa 187 to 231; TRAF1 and TRAF3 binding to this site can be detected both in yeast two-hybrid assays and in GST fusion protein binding assays, while TRAF2 binding, which is weaker than TRAF1 or TRAF3 binding, can only be detected in GST fusion protein binding assays.

The TRAF3 binding site within LMP1 aa 187 to 231 was further defined. Both G4DBD-LMP1(187-212) or G4DBD-LMP1(201-231) interacted with G4TAD-TRAF3(183-568) (data not shown). Also, in vitro-translated TRAF3 bound nearly as well to GST-LMP1(187-212) or GST-LMP1(201-231) as to GST-LMP1(187-231) (Fig. 4A). Therefore, the TRAF3 binding site is within aa 201 to 212 (or there are two separate, nonoverlapping binding sites). In vitro-translated TRAF3 bound nearly as well to GST-LMP1(199-214) as to GST-LMP1(187-231) or to GST-LMP1(187-386) [on average, 20% of input TRAF3 bound to GST-LMP1(199-214) versus 25% for GST-LMP1(187-231) and 38% for GST-LMP1(187-386)] (Fig. 4). Thus, LMP1 aa 199 to 214 are highly competent for TRAF3 binding, and the binding site is almost certainly within aa 201 to 212.

TRAF1 and TRAF2 also bound to the LMP1 aa 199 to 214 site. In vitro-translated TRAF1 or FLAG-TRAF2 bound specifically to GST-LMP1(199-214), although less efficiently than TRAF3 (average binding of approximately 1% for TRAF1 or FLAG-TRAF2 versus 20% for TRAF3) (Fig. 4). In contrast to the situation with TRAF3, both TRAF1 and FLAG-TRAF2 bound less efficiently to GST-LMP1(199-214) than to GST-LMP1(187-231). On average, 1% of TRAF1 or FLAG-TRAF2 bound to GST-LMP1(199-214), while more than 50% of TRAF1 and 10% of FLAG-TRAF2 bound to GST-LMP1(187-231).

To test whether posttranslational modifications or other cellular proteins might affect the TRAF binding to the LMP1 CT, GST-LMP1 CT fusion proteins were incubated with extracts from an EBV-negative Burkitt lymphoma cell line (BJAB) transiently overexpressing TRAF1, FLAG-TRAF2, or TRAF3 (Fig. 5A). As observed with in vitro-translated TRAFs, FLAG-TRAF2 and to a lesser extent TRAF1 overexpressed in lymphoblasts bound to GST-LMP1(187-231) better than to GST-LMP1(187-386) or to GST-LMP1(199-214) and TRAF3 bound similarly to all three GST-LMP1 fusion proteins. The binding of in vivo-expressed TRAF2 to GST-LMP1(187-231) and GST-LMP1(187-386) was similar to that of FLAG-TRAF2 (data not shown). Also, consistent with the lack of binding of in vitro-translated TRAFs to GST-LMP1(240-386), in vivo-expressed TRAF1, FLAG-TRAF2, or TRAF3 did not bind to GST-LMP1(240-386) (Fig. 5A). Thus, in vitro- and in vivo-

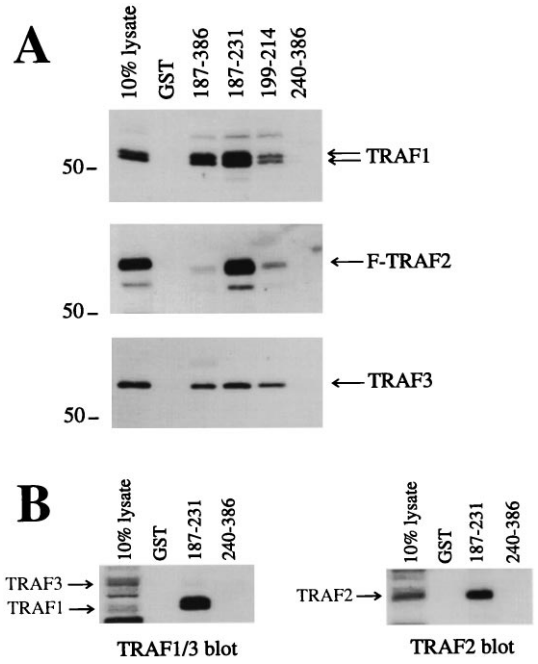

FIG. 5. GST binding assay using cell extracts from BJAB cells overexpressing TRAF1, TRAF2, or TRAF3. (A) BJAB cells (10^7 per transfection) were electroporated with 30 μg of pSG5 vector expressing TRAF1, FLAG-TRAF2 (F-TRAF2) (22), or TRAF3 (31) and lysed 18 h posttransfection in 1% NP-40 lysis buffer. Cleared lysates (2×10^6 cells per pull down) were incubated with Sepharose beads bound to GST control or GST fusion proteins containing various deletion mutants of LMP1 CT. Cell lysates (10% obtained before the GST binding assay) and coprecipitated material were separated by 8% SDS-PAGE. Coprecipitating TRAF proteins were detected by immunoblot analysis using rabbit polyclonal antisera recognizing both TRAF1 and TRAF3 (CRAF1 C20) or TRAF2 (TRAF2 C20). The positions of prestained molecular markers (in kilodaltons) are indicated on the left. (B) GST binding assay using LCL extracts. NP-40 extracts from IB4 cells (20×10^6 cells per pull down) were incubated with Sepharose bead-bound GST or GST-LMP1 CT deletion mutant fusion proteins. The binding of endogenous TRAFs was analyzed by SDS-PAGE and Western blotting as described in Materials and Methods.

expressed TRAFs are similar in their binding to GST-LMP1 CT fusion proteins.

Endogenous TRAF1, TRAF2, and TRAF3 from an EBV-transformed LCL and overexpressed TRAFs bound similarly to GST-LMP1 fusion proteins except that endogenous TRAF3 did not bind to GST-LMP1(187-231) (Fig. 5B), although it was readily detected in the lysate. Thus, in LCLs there appears to be very little free TRAF3; TRAF3 is probably stably associated with LMP1 and other proteins.

A core TRAF binding motif. A point mutation that changes Thr-234 to Ala in the CD40 cytoplasmic tail prevents TRAF3 binding to CD40 and results in a signaling-defective phenotype (15, 17). Alignment of the CD40 site around Thr-234 with the LMP1 aa 199 to 214 TRAF binding site reveals a common PXQXT sequence and little other similarity (Fig. 6A). To test the significance of this similarity, LMP1 aa 199 to 210 were systematically mutated to alanine and the effects on TRAF binding were evaluated (Fig. 6A and B). TRAF3 bound as well to GST-LMP1(199-210) with D-199 or S-200 mutated to A as to GST-LMP1(199-214). This indicates that LMP1 aa 199 to 210 act as a sufficient TRAF3 binding site and that D-199 and S-200 are not critical in this minimal binding site. Mutation of L-201 reduced TRAF3 binding by 94%, while mutation of P-202 or H-203 decreased TRAF3 binding by 40 and 84%, respectively. In contrast, mutation of P-204, Q-205, or Q-206 to alanine decreased TRAF3 binding by more than 99% and mutation of T-208 decreased TRAF3 binding more than 95%. Mutation of D-209 resulted in only a 40% decrease in TRAF3 binding, but simultaneous mutation of D-209 and D-210 resulted in a 95% decrease in TRAF3 binding (Fig. 6A and B). Thus L-201, P-204, Q-205, Q-206, T-208 and DD(209,210) appear to be important in binding TRAF3, while P-202 and H-203 may be less important and A-207 has not been evaluated because of the nature of the test. TRAF1 and TRAF2 binding to the set of LMP1 mutants paralleled TRAF3 binding. P-204, Q-206, T-208, and DD(209,210) mutations abolished most of the TRAF1 or TRAF2 binding, while the D-209 mutation had little effect (Fig. 6A). These data are further evidence that TRAF1, TRAF2, and TRAF3 recognize the same core site.

Analysis of the effect of the most disruptive mutations, i.e., P-204 to A or Q-206 to A, in the context of GST-LMP1(187-231) confirmed the role of these residues in TRAF binding and revealed a significant role for surrounding residues. TRAF2 binding was almost completely abrogated by either mutation, and TRAF1 binding was decreased more than 60% by the P-204 to A or the Q-206 to A mutation. Surprisingly, TRAF3 binding was largely unaffected by either mutation (Fig. 6C). Thus, surrounding residues in the LMP1 CT can support high-level interaction of a significantly mutated core site with TRAF3 and to a lesser extent with TRAF1.

To further evaluate the importance of the core binding site in TRAF1, TRAF2, and TRAF3 association with LMP1 in vivo, a FLAG-tagged LMP1 mutant consisting of aa 1 to 231 [FLAG-LMP1(1-231)], either wild-type or with a P-204 to A or Q-206 to A mutation, was expressed in BJAB cells and the binding of endogenous TRAFs was assessed in coimmunoprecipitation experiments. As expected from the GST binding data, FLAG-LMP1(1-231) bound efficiently TRAF1, -2, and -3, while FLAG-LMP1(1-231) with P-204 to A or Q-206 to A mutations retained substantial TRAF3 binding but was reduced in TRAF1 binding and did not bind TRAF2 (data not shown). In addition, full-length FLAG-LMP1 with both P-204 and Q-206 mutated to A did not bind TRAF1 or TRAF2 and showed minimal or no TRAF3 binding (data not shown). Thus, LMP1 residues around the core TRAF binding site can support continued interaction with TRAF3 in the face of the mutation of one key residue but not with two mutations. These data confirm the importance of the PXQXT motif for TRAF1, TRAF2, and TRAF3 binding and further indicate that there is no other TRAF1, TRAF2, or TRAF3 binding site in LMP1.

Mutation of LMP1(1-231) P-204 or Q-206 to A affects NF-κB activation. The effect of the core TRAF binding site mutations on NF-κB activation was assessed by transfection of 293 cells with a wild-type or mutant FLAG-LMP1(1-231) expression vector and a NF-κB-dependent promoter luciferase reporter plasmid. FLAG-LMP1(1-231) induced greater than 20-fold higher luciferase activity than reporter plasmid alone (Fig. 7), and the effect was dependent on the NF-κB sites in the reporter plasmid (data not shown). FLAG-LMP1(1-231) with P-204 mutated to A had 10% of the wild-type activity and was expressed at a level similar to that of wild-type LMP1(1-231) as assessed by immunoblotting. FLAG-LMP1(1-231) with Q-206 mutated to A had from 15 to 49% of the wild-type activity when expressed at similar levels to at least twofold higher levels than wild type, respectively (Fig. 7 and data not shown). These results link TRAF binding to NF-κB activation by LMP1(1-231), favoring a role for TRAF1 or TRAF2 since TRAF3 binding was only slightly affected by the P-204 or Q-206 mutation.

TRAF3 binds to LMP1 and represses LMP1(1-231)-mediated NF-κB activation. To investigate the role of TRAF3 in

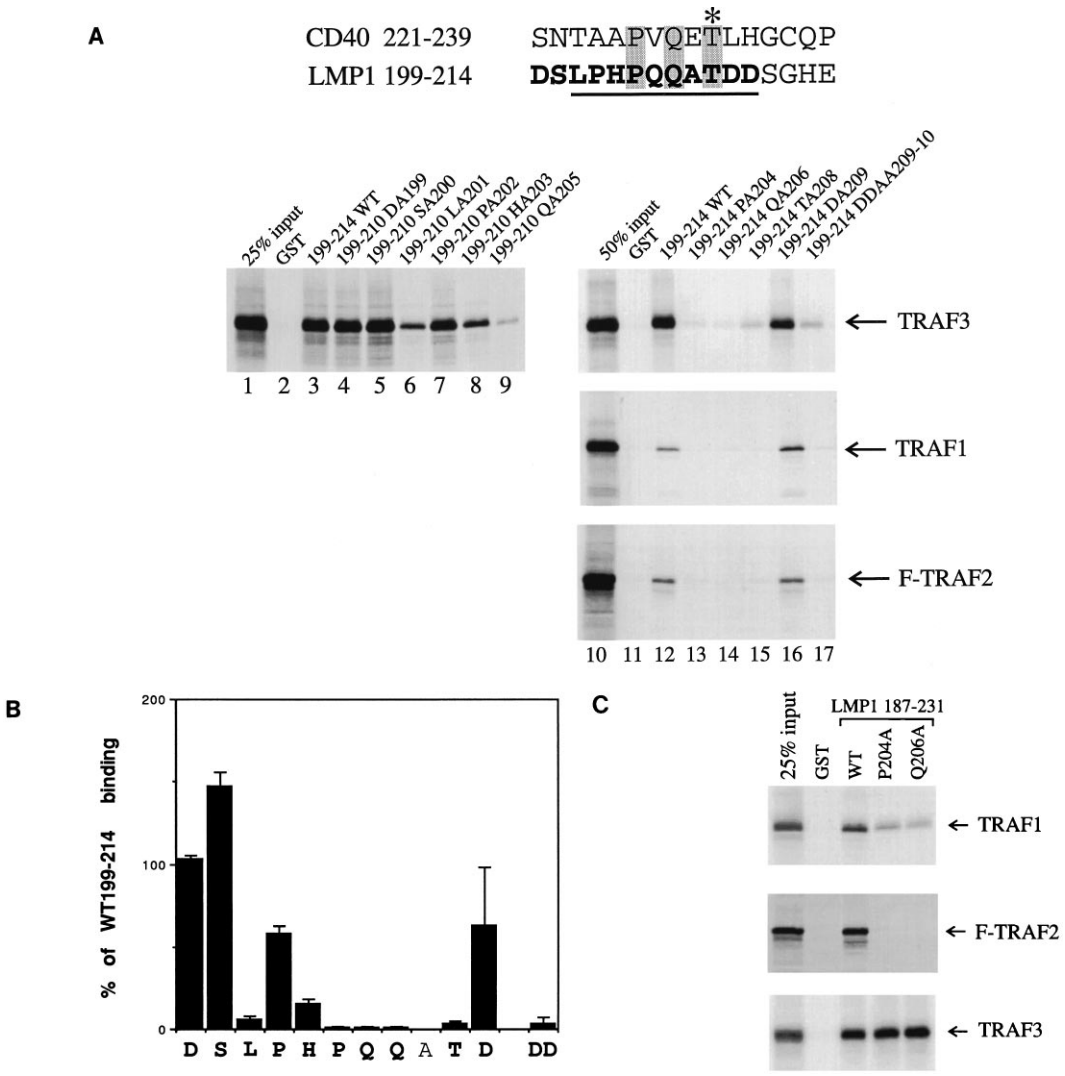

FIG. 6. Mutagenesis analysis of the minimal TRAF binding site on the LMP1 CT. (A) GST binding assay with GST fusion proteins containing point mutations in the TRAF binding site. Sequence alignment between the CD40 and LMP1 CT TRAF binding sites is shown at the top. The conserved PXQXT motif is identified by grey boxes. CD40 Thr-234 shown to be critical for TRAF3 binding and for signaling is indicated by an asterisk (15, 17). The LMP1 amino acids which have been tested by alanine mutagenesis are in bold characters. The core sequence for TRAF binding to LMP1 is underlined. ^{35}S-labeled TRAF1, FLAG-TRAF2 (F-TRAF2) (22), or TRAF3 (31) translated in vitro in rabbit reticulocyte lysate was incubated with Sepharose bead-bound GST or GST-LMP1(199-214) or -(199-210) with specific amino acids (identified by sequence number) mutated to alanine. Precipitated material was analyzed by 10% SDS-PAGE followed by autoradiography and PhosphorImager analysis. (B) Histogram analysis of TRAF3 binding to LMP1 mutant peptides. The average (mean ± standard deviation) of TRAF3 binding to the mutant relative to wild-type LMP1 aa 199 to 214 from replicate experiments is represented. The amino acids which were mutated to alanine are in bold. (C) Binding of TRAF1, TRAF2, and TRAF3 to GST LMP1 187 to 231 fusion proteins with a P-204 to A or Q-206 to A mutation. Sepharose bead-bound GST or GST-LMP1(187-231), either wild-type (WT) or with a P-204 to A or Q-206 to A mutation, was incubated in vitro with ^{35}S-labeled TRAF1, FLAG-TRAF2 (F-TRAF2) (22), or TRAF3 (31) translated in vitro in rabbit reticulocyte lysate. Precipitated material was analyzed by 10% SDS-PAGE followed by autoradiography and PhosphorImager analysis.

LMP1(1-231)-mediated NF-κB activation, 293 cells were transfected with a TRAF3 expression vector, a FLAG-LMP1 (1-231) expression vector, and an NF-κB-dependent promoter luciferase reporter plasmid. Cotransfection of TRAF3 expression vector (1 or 4 μg) resulted in a substantial decrease of the LMP1(1-231)-mediated NF-κB activation (Fig. 8A). The effect on NF-κB activation was not due to an effect of TRAF3 on LMP1(1-231) expression. Although TRAF3 overexpression caused a decrease in some experiments, the decrease was minor (no more than twofold) and did not correlate with the decrease in NF-κB activation (data not shown). A similar negative effect of TRAF3 overexpression on NF-κB activation has been observed with CD40 or p80 TNFR overexpression in 293 cells (36).

The TRAF domain of TRAF3 (aa 345 to 568), which has high affinity for the LMP1 CT in the yeast two-hybrid assay (31), also significantly decreased LMP1(1-231)-mediated activation without affecting LMP1(1-231) expression (Fig. 8B). These results are consistent with the effect being due to TRAF3 occupancy of the LMP1 TRAF binding site and displacement of other NF-κB activating TRAFs. As expected from the lack of TRAF3 binding to the LMP1 CT outside of the aa 187 to 231 binding site, TRAF3 coexpression had no significant effects on NF-κB induction mediated by LMPΔ188-331 (data not shown).

To directly evaluate whether TRAF3 overexpression displaced other TRAFs from LMP1, FLAG-tagged LMP1 or a FLAG-tagged control protein (FLAG-tagged EBI3) (4) was

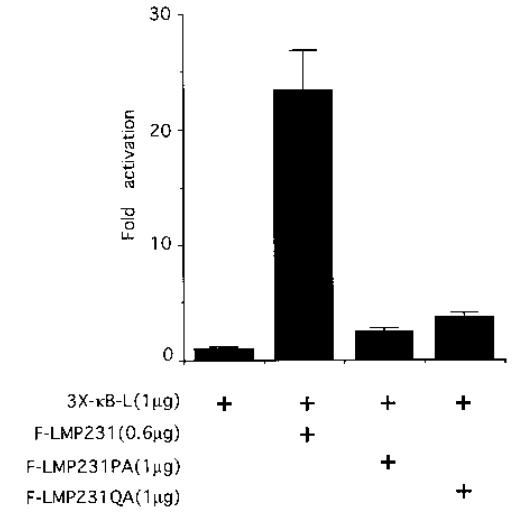

FIG. 7. Effect of the P-204 to A and Q-206 to A mutations on LMP1(1-231)-mediated NF-κB induction. 293 cells were transfected with 1 μg of a reporter gene containing three NF-κB sites (3X-κB-L), 0.5 μg of pGKβgal, and the indicated amount (in micrograms) of pSG5 vector expressing wild-type FLAG-LMP1(1-231) (F-LMP231) or FLAG-LMP1(1-231) with a P-204 to A (F-LMP231PA) or Q-206 to A (F-LMP231QA) mutation. Reporter luciferase values were normalized for transfection efficiency as determined by β-galactosidase expression under control of a constitutive promoter in a cotransfected plasmid. The results of one experiment are shown of two in which each transfection was performed in duplicate or triplicate. Values are shown as the mean (± standard deviation) of relative luciferase activity. In this experiment, mutant LMP1(1-231) proteins were expressed at levels similar to that of the wild type, as assessed by immunoblotting with anti-FLAG antibody (data not shown).

expressed in a non-EBV-infected Burkitt lymphoma cell line (BJAB) along with TRAF3 or TRAF1 or an unrelated protein and the effect on the association of endogenous heterologous TRAFs with LMP1 was evaluated. Immunoblots of cell lysates indicated that TRAF1 levels are low in BJAB cells and increased with LMP1 expression, while TRAF2 and TRAF3 are constitutively expressed in BJAB cells. In each instance, the

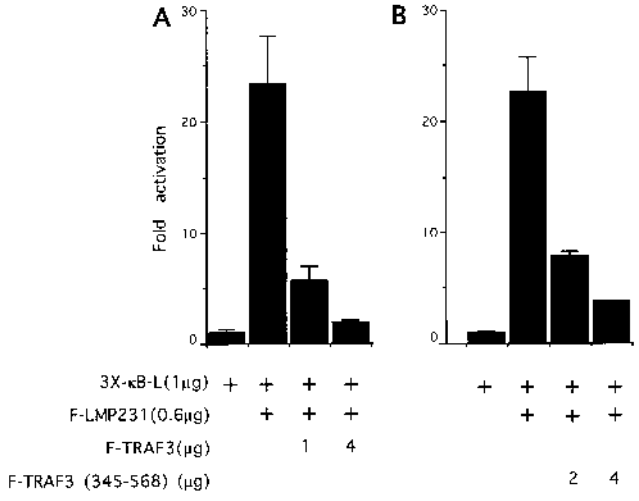

FIG. 8. TRAF3 or TRAF3(345-568) repress LMP1(1-231)-mediated NF-κB induction. 293 cells were cotransfected with 1 μg of 3X-κB-L reporter plasmid, 0.5 μg of pGKβgal, and the indicated amount of pSG5 vector expressing FLAG-LMP1(1-231) (F-LMP231), FLAG-TRAF3 (A), or FLAG-TRAF3(345-568) (B). Values are shown as the mean ± standard deviation of relative luciferase activity from one representative experiment of three, in which the transfections were performed in triplicate or duplicate.

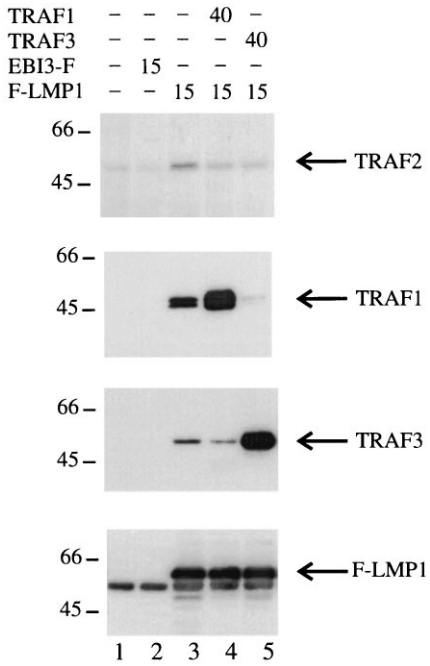

FIG. 9. In vivo competition between overexpressed TRAF1 or TRAF3 and endogenous TRAFs for binding to LMP1. BJAB cells were transfected with the indicated amount (in micrograms) of pSG5 FLAG-LMP1 or FLAG-EBI3 (4) together with TRAF1 or TRAF3 or an unrelated protein (data not shown). Eighteen hours posttransfection, the cells were lysed in 0.5% NP-40 lysis buffer and cleared lysates were immunoprecipitated with M2 anti-FLAG affinity gel. Coprecipitating TRAF proteins were detected with rabbit anti-TRAF polyclonal antibodies. Blotting with M5 anti-FLAG antibody showed that a similar amount of FLAG-LMP1 was precipitated in each lane. The lower amount of TRAF3 coprecipitated with FLAG-LMP1 in the presence of overexpressed TRAF1 was not observed in other experiments. The band present just below TRAF2 in the TRAF2 blot corresponds to immunoglobulins which cross-react with TRAF2 antibody.

levels of endogenous TRAFs were unaffected by expression of a heterologous TRAF (data not shown). Endogenous TRAF1, TRAF2, and TRAF3 coprecipitated with FLAG-LMP1 but not with control FLAG-tagged EBI3 (Fig. 9). TRAF3 overexpression resulted in more TRAF3 and less TRAF1 or TRAF2 associated with LMP1, while TRAF1 overexpression resulted in more TRAF1 and less TRAF2 associated with LMP1 (Fig. 9). In most experiments TRAF3 association with LMP1 was not affected by TRAF1 overexpression. This is compatible with the TRAFs competing for the same site and with a binding affinity of TRAF3 > TRAF1 > TRAF2, as was indicated by the GST-LMP1(187-386) binding data. The negative effect of overexpression of TRAF3 on LMP1(1-231)-mediated NF-κB activation is therefore likely due to TRAF3 displacement of endogenous TRAF2 and/or TRAF1. Competition experiments with TRAF2 overexpression are not reported because high-level TRAF2 overexpression in BJAB cells resulted in a marked decrease in LMP1 expression (data not shown).

TRAF1 (not TRAF2) augments LMP1(1-231)-mediated NF-κB activation; RING finger-deleted TRAF2 blocks NF-κB activation in the presence of TRAF1. Since TRAF1 is highly associated with the LMP1 CT in LCLs, we considered the possibility that TRAF1 might have a role in LMP1-mediated NF-κB activation. Transfection of 293 cells with a TRAF1 expression vector (0.25 or 1 μg) and increasing amounts of FLAG-LMP1(1-231) expression vector (0.3 to 3 μg) resulted in at least a threefold synergistic effect on NF-κB activation (Fig.

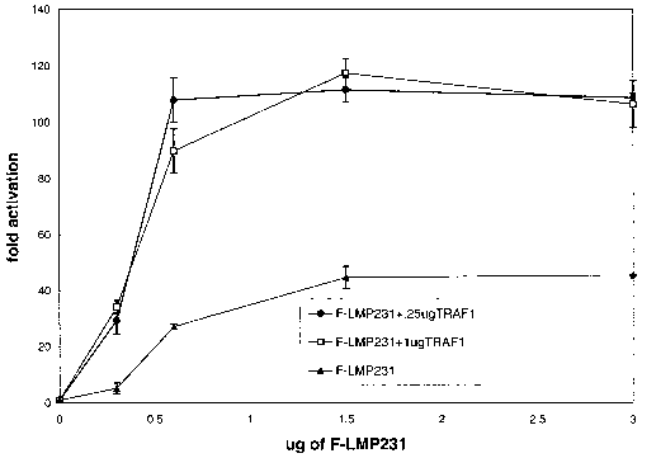

FIG. 10. TRAF1 acts synergistically with LMP1(1-231) to augment NF-κB transactivation. 293 cells were cotransfected with 1 μg of 3X-κB-L reporter plasmid, 0.5 μg of pGKβgal, and increasing concentrations (0.3 to 3 μg) of pSG5 FLAG-LMP1(1-231) (F-LMP231), alone or in combination with 0.25 or 1 μg of pSG5 TRAF1. Values shown represent the mean ± standard deviation of relative luciferase activity from one representative experiment of five, in which each transfection was performed in duplicate or triplicate.

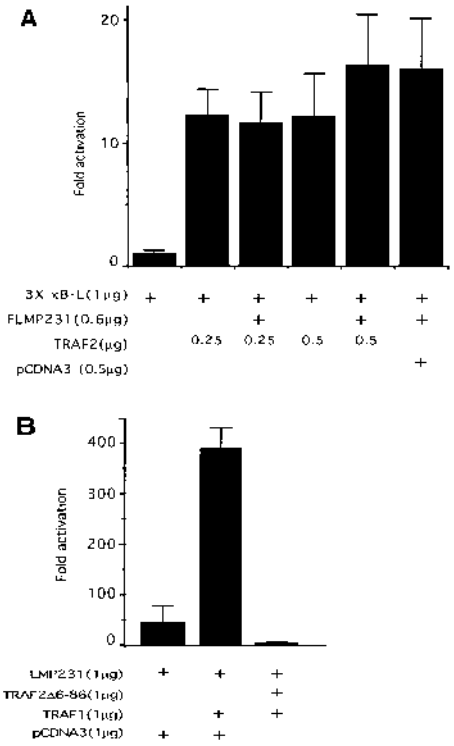

FIG. 11. (A) TRAF2 overexpression has no effect on LMP1(1-231)-mediated NF-κB induction. 293 cells were cotransfected with 1 μg of 3X-κB-L reporter plasmid, 0.5 μg of pGKβgal, and the indicated amount of pSG5 FLAG-LMP1(1-231) (F-LMP231), pcDNA3 TRAF2 (22), or pcDNA3 vector control. FLAG-LMP1(1-231) expression was slightly increased when TRAF2 was coexpressed, as assessed by Western blotting with anti-FLAG antibody (data not shown). Values are shown as the mean ± standard deviation of relative luciferase activity from one experiment of two, in which each transfection was performed in triplicate. (B) RING finger-deleted TRAF2 (TRAF2Δ6-86) blocks TRAF1 coactivation of LMP1(1-231)-mediated NF-κB induction. 293 cells were cotransfected with 1 μg of 3X-κB-L reporter plasmid, 1 μg of pGKβgal, and the indicated amount of pSG5 LMP1(1-231) or TRAF1 or of pcDNA3 TRAF2Δ6-86 (22). The amount of pcDNA3 plasmid was kept constant in each transfection. Values shown represent the mean ± standard deviation of relative luciferase activity from three experiments, in which each transfection was performed in triplicate.

10). The effect was dependent on NF-κB sites, and TRAF1 overexpression did not activate NF-κB in the absence of FLAG-LMP1(1-231) (data not shown). TRAF1 had no significant effect on NF-κB induction by LMPΔ188-331 (data not shown). Although TRAF1 coexpression was accompanied by an increase in LMP1 levels in some experiments, TRAF1 synergy with FLAG-LMP1(1-231) can only be due in small measure to an effect of TRAF1 on FLAG-LMP1(1-231) expression. Cotransfection of 0.6 or 1.5 μg of FLAG-LMP1(1-231) expression vector together with TRAF1 expression vector resulted in FLAG-LMP1(1-231) levels that were lower than those with 1.5 or 3 μg of FLAG-LMP1(1-231) expression vector, respectively. TRAF1 coexpression always increased NF-κB activation severalfold, while levels of LMP1 (1-231) expression above that achieved with 0.6 or 1.5 μg of LMP1 (1-231) expression vector alone resulted in little or no increase in NF-κB activation (Fig. 10).

TRAF1 coactivation required the TRAF1 N-terminal putative Zn finger domain. Although TRAF1 with aa 2 to 183 deleted [TRAF1(184-416)] is similar to TRAF1 in binding to GST-LMP1(187-231), TRAF1(184-416) expression did not enhance and in fact decreased LMP1(1-231)-mediated NF-κB activation ($P = 0.0026$; data not shown). The deletion may affect TRAF1 interaction with TRAF2 or with another protein in the NF-κB activation pathway.

In contrast to that with TRAF1, TRAF2 overexpression had almost no effect on LMP1(1-231)-mediated NF-κB activation. Expression of TRAF2 over a broad range under the control of a simian virus 40 or cytomegalovirus promoter did not result in increased LMP1(1-231)-mediated NF-κB activation (Fig. 11A and data not shown). FLAG-LMP1(1-231) levels were not reduced by TRAF2 expression as assessed by Western blotting (data not shown). Thus, TRAF2 does not coactivate NF-κB with LMP1(1-231) in 293 cells.

Despite the lack of synergy of TRAF2 with LMP1(1-231) in NF-κB activation, TRAF2 could still be a mediator of LMP1(1-231) activation of NF-κB since TRAF2 is constitutively expressed in cells, can bind weakly to the LMP1 TRAF binding site, can heterodimerize with TRAF1 which is highly associated with LMP1, and can activate NF-κB (36). In fact, expression of the dominant-negative RING finger deletion mutant of TRAF2 which was previously shown to inhibit p80 TNFR-, p60 TNFR-, and CD40-mediated NF-κB activation (14, 36) strongly blocked LMP1(1-231)-mediated NF-κB activation (22) as well as the TRAF1 synergistic effect on LMP1(1-231)-mediated NF-κB activation (Fig. 11B). The effect of the RING finger-deleted TRAF2 could be due to occupancy of the LMP1 TRAF binding site and displacement of TRAF1 from LMP1 since high-level overexpression of RING finger-deleted TRAF2 in BJAB cells resulted in decreased TRAF2 and also TRAF1 binding to the LMP1 CT in experiments similar to those shown in Fig. 8 (reference 22 and data not shown).

DISCUSSION

These data on the biochemical interactions of the LMP1 CT with each of the known TRAFs and on the role of these interactions in NF-κB activation confirm and extend previous evidence that LMP1 is a constitutively activated receptor of the TNFR family. In EBV-transformed LCLs, most of LMP1 is associated with TRAF3 or TRAF1, TRAF3 and TRAF1 are each extensively complexed with LMP1, and at least 5% of TRAF2 is associated with LMP1. TRAF1, TRAF2, and

TRAF3, together, constitutively occupy a single site in the aa 187 to 231 domain of the LMP1 CT. This domain is sufficient for transformation when expressed from the EBV genome in the natural context of the six hydrophobic transmembrane domains of the LMP1 which enable constitutive aggregation in the plasma membrane (23). The association of TRAF1, TRAF2, and TRAF3 with an NF-κB-activating and -transforming domain of LMP1 in EBV-transformed B lymphocytes implicates constitutive TRAF aggregation in NF-κB activation and transformation by LMP1.

In the broad context of TNFR signaling, the constitutive high-level association of TRAFs with aggregated LMP1 in LCLs and the continuous effects of LMP1 on cell growth and NF-κB activation are compatible with the notion that the TRAFs associate with and signal from aggregated TNFRs. The finding that high-level TRAF2 overexpression can independently activate NF-κB is also consistent with signaling being dependent on TRAF aggregation since TRAF2 can homoaggregate (36, 37). TNFRs differ from LMP1 in that aggregation and signaling from TNFRs are ligand dependent. An expectation from these findings is that TRAFs or a TRAF-interacting downstream effector protein will associate with and signal from TNFRs only in response to ligand.

These data also significantly extend our understanding of a core TRAF-interacting site and of its ability to directly interact with TRAF1, TRAF2, and TRAF3. Previous evidence indicated that the CD40 cytoplasmic domain directly interacts with TRAF3 and TRAF2, the p80 TNFR with TRAF2 and through TRAF2 with TRAF1, the p60 TNFR with TRADD and through TRADD with TRAF2 and TRAF1, and the LMP1 CT aa 187 to 231 with TRAF3 (3, 14, 15, 31, 36, 37, 39). While this report was in preparation, the CD30 cytoplasmic domain was shown to interact with TRAF1, TRAF2, and TRAF3 (7, 25), providing the first evidence that all three TRAFs can directly interact with a TNFR. We have now demonstrated an interaction between the LMP1 CT aa 187 to 231 and TRAF3 or TRAF1 in the yeast two-hybrid assay and between GST-LMP1 CT and TRAF1, TRAF2, or TRAF3 translated in wheat germ extracts. Thus, the LMP1 CT aa 187 to 231 site resembles the CD30 cytoplasmic domain in interacting directly with TRAF1, TRAF2, and TRAF3.

A core motif through which several TNFRs engage TRAFs had not been previously defined. LMP1 aa 199 to 214 are sufficient for high-level TRAF3 binding and for specific TRAF1 and TRAF2 binding. Mutagenesis analyses indicate that 10 aa, LPHPQQATDD, are a sufficient core TRAF3 binding site. Several similar sites in the LMP1s of the rhesus and baboon lymphycryptoviruses bind TRAF3. The minimal consensus binding site that emerges from comparison of the EBV and other lymphycryptovirus LMP1 TRAF binding sites is PXQXT/S (7). CD40 has a similar sequence, TAAPVQE**T** LHGC, and the boldface T is critical for TRAF3 binding and NF-κB activation (15, 39). Recently, a 17-aa sequence containing the PVQET motif has been defined as the minimum TRAF1 and TRAF2 binding site in CD40 (2). CD30 also has a similar sequence, ADHTPH**P**E**Q**E**T**EPPLG, which can mediate high-level TRAF3 binding (7). A yeast two-hybrid screen with a G4DBD-TRAF3 gene fusion as bait has identified a number of cDNAs that encode novel proteins that have PXQXT/S motifs (32). Among these proteins was TANK, a novel protein which was shown to interact with TRAF2 and TRAF3 through a 21-aa peptide centered around a PIQCT motif (2). Thus, PXQXT/S appears to be a frequent core TRAF binding site.

PXQXT/S is not the only sequence that can engage TRAF proteins, since the p80 TNFR cytoplasmic domain does not have a PXQXT/S motif. Furthermore, the surrounding context is important for the binding of TRAFs to the core residues as is evidenced by the finding that several PXQXT/S sites in the rhesus, baboon, or human LMP1 do not bind TRAF3 (reference 7 and this study) and by the alanine mutagenesis results that indicate that residues N terminal and CT to the core motif are important for high-level TRAF interaction. Moreover, differences in the extent of binding of TRAF1, -2, or -3 to the core site in the context of the LMP1 CT aa 199 to 214 versus the LMP1 CT aa 187 to 231 or the whole LMP1 CT illustrate the effects of broader sequence contexts on TRAF interaction.

A surprising aspect of these data is that TRAF1 and TRAF2 are both implicated in LMP1(1-231)-induced NF-κB activation. Mutation of the core TRAF binding site in LMP1(1-231) substantially reduces TRAF1 and TRAF2 binding and NF-κB activation, thereby linking the binding and aggregation of TRAF1 or TRAF2 to NF-κB activation. TRAF1 is induced by LMP1 and extensively associates with LMP1 in LCLs, while TRAF2 is constitutively expressed and is less extensively associated with LMP1. Most importantly, TRAF1 substantially coactivates NF-κB when expressed in cells along with LMP1(1-231) and TRAF2 does not. The failure of TRAF2 to coactivate NF-κB is not evidence against a role for TRAF2, since TRAF2 is constitutively expressed and may be already in excess. However, the unique coactivating effects of TRAF1 are evident despite the putative TRAF2 excess. Thus, TRAF1 has a role in LMP1-mediated NF-κB activation which cannot be filled by TRAF2. Further, TRAF1 and TRAF2 can heterodimerize in the yeast two-hybrid assay (36, 37), and we have now demonstrated that TRAF1 and TRAF2 complexes exist in vivo in LCLs. Moreover, a dominant-negative RING finger deletion mutant of TRAF2 can block LMP1(1-231)-mediated NF-κB activation (22) and TRAF1 coactivation. The simplest model to explain these effects is that TRAF1 binds preferentially to the LMP1 CT and recruits TRAF2 which in turn mediates NF-κB activation. TRAF1 may have a similar role in NF-κB activation by other TNFRs, to which it binds with higher affinity than TRAF2.

TRAF3 is extensively associated with the LMP1 TRAF binding site in LCLs, binds strongly to the site, and in gene transfer-mediated overexpression downregulates NF-κB activation from LMP1(1-231). Thus, the high level of TRAF3 binding to LMP1 may dampen NF-κB activation by LMP1 in EBV-transformed LCLs.

TRAF1, TRAF2, and TRAF3 did not bind to the LMP1 CT aa 333 to 386 domain which has a strong NF-κB activating effect and did not significantly affect NF-κB induction by this domain. Further, the dominant-negative RING finger TRAF2 has a much smaller inhibiting effect on LMPΔ188-331-mediated NF-κB activation than on LMP1(1-231)-mediated NF-κB activation (22). Together, these data indicate that induction of NF-κB by the LMP1 CT aa 332 to 386 domain is not mediated by direct interaction with TRAF1, TRAF2, or TRAF3.

The extensive association of LMP1 with TRAF3 and TRAF1 rather than with TRAF2 in LCLs is consistent with a model in which TRAF3 and TRAF1 dominate over TRAF2 for direct occupancy of the LMP1 CT TRAF binding site. The differential association of TRAFs with the LMP1 CT in LCLs may also be affected by their differential affinity for other proteins, including TNFRs. Several TNFRs are abundantly expressed in LCLs and in other cells in which LMP1 is expressed, including Hodgkin's disease Reed Sternberg cells and nasopharyngeal carcinoma cells. CD40 can bind TRAF3 and TRAF2, p80 TNFR can bind TRAF2, TRAF3, and TRAF1/TRAF2 heterodimers, LTβR can bind TRAF3, and CD30 can bind TRAF1, TRAF2, and TRAF3. Different TRAF complexes

forming at aggregated TNFR cytoplasmic tails may be the basis for signaling specificity from these receptors. Continued signaling through p80 TNFR appears to contribute to the growth of EBV-transformed B lymphocytes (5, 8) and may affect LMP1 signaling. Thus, the differential association of TRAFs with TNFRs in LCLs may affect their individual association with the LMP1 CT, and the extensive association of TRAF1 and TRAF3 with the LMP1 CT almost certainly affects their availability for signaling from TNFRs.

NF-κB activation through the TRAF binding site is probably only important in primary B-lymphocyte transformation by the LMP1(1-231) recombinant EBV since this recombinant lacks the LMP1 CT 332 to 386 domain that independently conveys 70 to 80% of the NF-κB activity. However this domain must be important in mediating other LMP1 effects, including lymphocyte activation, adhesion, growth, or survival. In fact, LMP1 with the TRAF binding site deleted induces near wild-type NF-κB activation, but recombinant EBV genomes with this deletion are unable to transform primary B lymphocytes (19). Together with the previous data that LMP1(1-231) is sufficient for primary B-lymphocyte growth transformation (21), these data indicate that the TRAF binding domain has essential transforming role(s) distinct from NF-κB activation. TRAF3, TRAF1, and TRAF2 are the only proteins that have been identified to interact with the transformation-sufficient aa 187 to 231 domain. Thus, TRAF3, TRAF1, and TRAF1/TRAF2 heteroaggregates almost certainly mediate LMP1 activation, adhesion, growth-enhancing, and survival effects in addition to low-level NF-κB activation. TRAF3, TRAF1, or TRAF1/TRAF2 heteroaggregates are likely to mediate similar effects from activated TNFRs. One potentially unique effect of TRAF1/TRAF2 heteroaggregates is in recruiting inhibitor of apoptosis proteins (35) which may enhance cell survival under conditions that would otherwise result in apoptosis (28). The mechanisms of these and other cell growth and survival effects remain to be elucidated.

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