LMP1 PROTEIN FROM THE EPSTEIN BARR VIRUS IS A STRUCTURAL CD40 DECOY IN B LYMPHOCYTES FOR BINDING TO TRAF3*

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Running Title: LMP1 Protein Binds TRAF3 as a Structural CD40 Decoy

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ABSTRACT

Epstein-Barr virus is a human herpes-virus that causes infectious mononucleosis and lymphoproliferative malignancies. The latent membrane protein1 (LMP1) encoded by this virus, which is essential for transformation of B lymphocytes, acts as a constitutively active mimic of the tumor necrosis factor (TNF) receptor CD40. LMP1 is an integral membrane protein containing six transmembrane segments and a cytoplasmic domain at the C-terminus that binds to intracellular TNF receptor-associated factors (TRAFs). TRAFs are intracellular co-inducers of downstream signaling from CD40 and other TNFRs, and TRAF3 is required for activation of B lymphocytes by LMP1. The CTAR1 cytoplasmic region of LMP1 bears a motif PQQAT that conforms to the TRAF recognition motif PVQET in CD40. In this study, we report the crystal structure of this portion of LMP1 CTAR1, residues 204-PQQATDD210, bound in complex with TRAF3. The PQQAT motif is bound in the same binding crevice on TRAF3 where CD40 is bound, providing a molecular mechanism for LMP1 to act as a CD40 decoy for TRAF3. The LMP1 motif is presented in the TRAF3 crevice as a close structural mimic of the PVQET motif in CD40 and the intermolecular contacts are similar. However, the viral protein makes a unique contact: a hydrogen bond network formed between Asp210 in LMP1 and Tyr395 and Arg393 in TRAF3. This intermolecular contact is not made in the CD40/TRAF3 complex. The additional hydrogen bonds may stabilize the complex and strengthen the binding to permit LMP1 to compete with CD40 for binding to the TRAF3 crevice, influencing downstream signaling to B lymphocytes, and contributing to dysregulated signaling by LMP1.

INTRODUCTION

The success of viral infection depends on effective evasion of the cell death machinery of the host. This is a formidable task for the pathogen because the response to infection is complex in mammals. The immune response to viruses may involve apoptosis or, in some cases, the host defense may incorporate the expression of survival and pro-inflammatory genes to avoid the serious side effects associated with the apoptotic response. One evasion tactic used by the oncogenic herpes viruses is a virally encoded ortholog of the anti-apoptotic regulator Bcl-2. For example, Epstein-Barr virus encodes two Bcl-2 orthologs (BHRF1 and BALF1; (1,2)) and Kaposi’s sarcoma-associated γ-herpesvirus MHV-8 (KSHV) expresses KSbcl-2 (3,4). These viral proteins block release of cytochrome c from mitochondria, an early and essential step in the apoptotic cascade. Viral mimicry is also evident when mammalian viruses express orthologs of the IAP inhibitors of apoptosis proteins that regulate caspase enzymes to initiate and effect protein degradation and ultimately cell death. African swine fever virus encodes the vIAP ortholog (5), and serpin CrmA derived from poxviruses (6-8) inhibits several caspases.

The tumor necrosis factor (TNF) family of cytokines and their receptors play a key role in regulating both the innate and adaptive immune responses to viral pathogens. Not surprisingly, therefore, the regulatory elements in cell death or TNF pathways are frequently targeted by viruses in death escape strategies (reviewed in (9,10)). Viral proteins have evolved that modulate the TNF receptors or in some cases even mimic the receptors or associated signaling molecules.

Epstein-Barr virus (EBV) is a human herpesvirus that causes infectious mononucleosis and lymphoproliferative malignancies such as AIDS-related lymphoma, Burkitt’s lymphoma, Hodgkin’s disease and nasopharyngeal carcinoma (11-15). The latent membrane protein1 (LMP1) encoded by this virus is essential for transformation of B lymphocytes (16). This protein acts as a constitutively active mimic of the TNF-receptor CD40 (17-19). CD40 is expressed on B lymphocytes and after ligation, activates B cells by interaction with intracellular TNF receptor-associated factors called TRAFs (20). This association leads to signaling through NF-κB and c-Jun N-terminal kinase (JNK) pathways to activate expression of anti-apoptotic genes.
There are six characterized TRAF proteins numbered sequentially TRAF1-6. In TRAFs 2-6 two N-terminal zinc-binding domains that bear a ring finger and five zinc finger motifs are followed by a conserved TRAF domain at the C-terminus. The TRAF domain mediates binding to the cytoplasmic portion of TNF receptors (TNFRs) or signaling activators. TRAFs are co-inducers of downstream signaling with a range of binding affinities for various TNFRs (21). For example, CD40 binds to TRAF2, TRAF3 and TRAF6 to control B cell proliferation growth and differentiation (22-25); reviewed in (26). Binding recognition is mediated by specific contacts of a recognition sequence by residues in a hydrophobic crevice on the TRAF domain. The specific contacts of three TNFRs have been defined in crystal structures of CD40, LTβR and BAFF-R in complex with TRAF3 (27-29) or with peptides of the motifs in complex with TRAF2 (30). From these structural studies, a recognition motif has been revealed in a shared consensus sequence PxQxT or (P/S/T/A)x(Q/E)E. The binding motifs from CD40, LTβR, BAFF-R as well as the downstream regulator TANK (31) are each accommodated in the same binding crevice on TRAF3, and this binding interface is structurally and functionally adaptive (32).

LMP1 closely mimics signaling events and effector functions of CD40 in B lymphocytes, including activation of NF-κB and JNK, modulation of adhesion and co-stimulatory molecules, and secretion of antibodies and cytokines (reviewed in (22,33)). LMP1 is an integral membrane protein (386 residues) that contains a short N-terminal cytoplasmic region (residues 1-24), six transmembrane segments (residues 25-186) and a cytoplasmic domain (CCT; residues 187-386) at the carboxyl-terminus (34). The N-terminal region is essential for insertion into the membrane. The transmembrane segments oligomerize within the membrane and mediate constitutive activation. CCT contains two subdomains that have been implicated in LMP1 signaling: CTAR1 (residues 194-232) and CTAR2 (residues 351-386) (35), and these two regions play distinct yet overlapping roles in EBV-associated lymphoproliferation (36). The CCT domain of LMP1 binds to TRAFs 1, 2, 3, and 5 by recognition of a sequence in the CTAR1 segment, PQQAT, that conforms to the consensus PxQxT for TRAF recognition. In contrast, the CTAR2 segment has been proposed as a site for binding the death domain proteins TRADD or RIP (37,38). In addition, the CTAR2 region influences the recruitment of TRAFs to membrane rafts by the CTAR1 region (36). Interestingly, we have shown previously that TRAF3 mediates signaling through direct interactions with CTAR1 and may also play a role in the interactions between CTAR1 and CTAR2. Moreover, our data indicate that TRAF3 is required and essential for certain CTAR1-mediated effects in B cells, the principal target of EBV.

LMP1-mediated signaling is critical for EBV-associated pathogenesis and we have shown recently that TRAF3 is required for LMP1 activation of B cells (39). While this activation mimics that effected by CD40, and LMP1 and CD40 both bind to TRAF3, experiments in B cell lines deficient in TRAF3 have shown that LMP1 and CD40 differentially use TRAF3 and that TRAF3 is required for LMP1 signaling. LMP1 signaling remains intact in TRAF2 deficient B cells (36,39). In order to understand the molecular basis for binding of LMP1 to TRAF3, we crystallized a peptide representing part of the CTAR1 region of LMP1, and bearing the TRAF recognition motif, in complex with TRAF3. The structure reveals the intermolecular contacts and here we report the direct comparison of these contacts with those seen in a crystal structure of the CD40/TRAF3 complex (27).

**EXPERIMENTAL PROCEDURES**

*Crystallographic analysis* - For co-crystallization of LMP1 with TRAF3, crystals of TRAF3 were grown as described previously (40) after tryptic digestion of the protein to shorten the long N-terminal helix. Crystals formed in space group P321 in hanging drops from solutions of 0.1 M MES, pH 6.5 containing 15% polyethylene glycol 4000. The crystals grew to a size of 500 x 500 x 25 μm at room temperature and diffracted to 2.7 Å resolution.

To form the complex, synthetic peptides of various lengths representing the minimal region in the CTAR1 subdomain of LMP1 implicated for TRAF3 recognition were soaked into TRAF3 crystals. Each peptide contained the PQQAT TRAF recognition motif of LMP1, but differed in
the number of flanking residues. The peptides were:

\[ \text{202PHPQQATDDSGHESDSNSNEGRHH}^{225}, \]

\[ \text{202PHPQQATDDSGHESDSNSN}^{220}, \]

\[ \text{203HPQQATDD}^{210}. \]

Each peptide was tested in separate experiments. Peptides were dissolved in water and soaked into TRAF3 crystals, and then the crystals were cryoprotected with 2.5% polyethylene glycol and 20% glycerol and flash frozen for data collection. Diffraction data were collected at the Stanford Synchrotron Radiation Laboratory beamline 11-3 at -175°C using a Q4 image plate detector. The data were processed using DENZO and SCALEPACK (41). The data collection statistics are summarized in Table 1.

The structure of the complex was refined using the atomic coordinates of the native truncated TRAF3 (Ni et al., 2002) implementing simulated annealing in CNS (Brünger et al., 1998). An iterative process of refinement in CNS and model-building in O (42) was used to construct the model of the complex. After refinement, difference maps \( (Fo - Fc) \) and \( 2(Fo - Fc) \) and OMITMAPS (43) were used to fit the peptide. Clear electron density was visible only for the shortest peptide, in the TRAF3 binding crevice for backbone atoms. After several rounds of refinement, annealing and model adjustment, all atoms were clearly placed in density for residues 204-210. These residues were included in the final model. Refinement statistics for the complex are presented in Table 1. For the final structure the \( R \) factor and \( R_{free} \) values were 20.6% and 25.6% respectively. Graphic images and electrostatic surfaces presented in the figures were prepared with MOLMOL (44) and SPOCK (45).

**Peptide synthesis** - Peptides for the complex were designed to correspond to the sequence in LMP1 that contains the binding site for TRAF3. Peptides acetyl-HPQQATDD-amide, acetyl-PHPQQATDDSGHESDSNSN-amide and acetyl-PHPQQATDDSGHESDSNSNEGRHH-amide were synthesized using Fmoc chemistry with diisopropylcarbodiimide/hydroxybenzotriazole (DIC/HOBt) coupling on Rink’s amide (MBHA) resin with an Advanced ChemTech 350 multiple peptide synthesizer. The peptides were cleaved from the resin and deprotected by treatment with trifluoroacetic acid:water:triisopropylsilane (95:2.5:2.5) for 2 hours at room temperature. The cleaved peptides were precipitated and washed with cold diethyl ether. After drying, the peptides were dissolved in aqueous acetonitrile and purified on a preparative C18 column (Cosmosil 5C18-AR, 20 x 250 mm; Phenomenex (Torrance, CA)) with detection at 210 nm using a Gilson HPLC apparatus (Middleton, WI). The peptides were separated from impurities using a linear gradient of 0-40% B over 40 min (solvent A:0.1% trifluoroacetic acid in water, solvent B:0.1% trifluoroacetic acid in 90% acetonitrile) at a flow rate of 8 ml/min. Pure peptides, as judged by their elution as single peaks by HPLC on analytical C18 columns (Vydac 218TP54 and 238TP54, each 5 um, 4.6 mm x 250 mm), were confirmed by MALDI-TOF mass spectrometric analysis with an Applied Biosystems Voyager System 6264 (Foster City, CA).

**DNA constructs** - DNA oligonucleotide primers were obtained from Integrated DNA Technologies (Coralville, IA). The DNA construct used to stably express hCD40LMP1 has been described previously (46). The hCD40LMP1 mutants P204A, Q206A, T208A, D209A, and D210A were generated by PCR SOEing (47) using hCD40LMP1 as template, and hCD40-5' (5'-AAG TCG ACG CCT CGC TCG GGC GCC A - 3') as 5' primer, 3'-A2LMP1 (5'-AAT CTA GAA AGC CTA TGA CAT GGT AAT GCC - 3') as 3' primer, and different SOEing primers. The SOEing primers include P204A (5'-GAT GAC TCC CTC CCG CAC GCT CAA CAA GCT ACC GAT GAT TCT GG - 3'), Q206A (5'-GAC TCC CTC CCG CAC CCT CAA GCA GCT ACC TCT GGC CAT GAA TCT GAC - 3'), T208A (5'-CTC CCG CAC CCT CAA CAA GCT GCT ACC GAT TCT GC T - 3'), T209A (5'-CAC CCT CAA CAA GCT ACC GAT TCG TGC CAT GAA TCT GAC - 3'), and D210A (5'-CTA CAA CAA GCT ACC GAT GCA TCT GGC CAT GAA TCT GAC - 3'). The PCR products were verified by sequencing and cloned into pRSV.5 (neo) expression vector for stable transfection in M12.4.1 mouse B cells.

**Stable transfection of mouse B cell line** - The mouse B cell line M12.4.1 has been described previously (48,49). Cells were cultured in RPMI supplemented with 10% FCS, 10 \( \mu \)M \( \beta \)-mercaptoethanol, and antibiotics (BCM-10) as described (18). M12.4.1 cells were stably transfected using electroporation as described (48).
Cell lines transfected with hCD40LMP1 or its mutants were selected and maintained with 400 μg/ml geneticin (Life Technologies, Grand Island, NY). Surface expression of hCD40LMP1 and its mutants was determined by immunofluorescence flow cytometry and expression matched clones were selected for experiments as previously described (46).

**Antibodies (Ab)** - An anti-human CD40 hybridoma (clone G28-5, mouse IgG1) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Purified MOPC-21 (isotype control of mouse IgG1) was from Sigma (St. Louis, MO). Anti-LMP1 hybridoma (clone S12, mouse IgG2a) was a generous gift from Dr. Frederick Wang (Harvard University, Boston, MA). mAbs were purified from hybridoma supernatants by ammonium sulfate precipitation, and quantified by isotype-specific ELISAs. Polyclonal rabbit Ab to TRAF3 (H122) was from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal rabbit Ab to TRAF2 was from Medical and Biological Laboratories (Nagoya, Japan). HRP-labeled secondary Abs were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

**TRAF recruitment to hCD40LMP1 and its mutants in detergent-insoluble microdomains (rafts), and co-immunoprecipitation.** Binding of LMP1 to TRAF3 was tested in cell-based assays, but was too weak to permit measurements by isothermal titration calorimetry, as we have seen with other TRAF3 complexes (LTβR or BAFF-R; (28,29)). Instead, assays were developed that mimic the in vivo binding situation and provide the capability to measure significant differences in binding. Stably transfected M12.4.1 subclones (2 x 10^7 cells) were stimulated in a total volume of 1 ml with 10 μg of anti-human CD40 (G28-5, to trigger signaling through hCD40LMP1) or isotype control mAbs for 10 min at 37°C to induce recruitment of TRAFs to membrane rafts and allow formation of LMP1 signaling complexes. Detergent-soluble and insoluble raft lysates were prepared as previously described (50). The lysates were immunoprecipitated with protein G-sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) pre-armed with anti-hCD40 (G28-5) as previously described (36). Aliquots of the immunoprecipitates were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (ProTran, Schleicher & Schuell Biotechnology, Keene, NH). Immunoblot analysis was performed as previously described (36). Generally, blocking and secondary Ab incubations were done at room temperature for 1h, while primary Ab incubation was done at 4°C overnight. A chemiluminescent substrate (Pierce, Rockford, IL) was used to detect HRP-labeled Abs on Western blots. The same protein blot was stripped and re-immunoblotted with different antibodies sequentially. Bands of immunoblots were quantitated using a low-light imaging system (LAS-1000, FUJIFILM Medical Systems USA, Inc., Stamford, CT).

**RESULTS AND DISCUSSION**

TRAF adaptor proteins are trimeric assemblies that are stabilized by coiled-coil interactions of elongated N-terminal α-helices. At the end of these helices, a conserved C-terminal TRAF domain exists with a folding pattern that is structurally maintained in TRAF3 (27), TRAF2 (51,52) and TRAF6 (53). This independently folded domain is an eight-stranded β-sandwich formed by two layers of β-sheet that each contain four antiparallel strands, and enclose a hydrophobic core (Fig. 1). In the TRAF3 crystals, one monomer is the asymmetric subunit, and the three structurally identical subunits are related by crystallographic three-fold symmetry.

Residues 348-504 in TRAF3 form the TRAF domain. In the mushroom-shaped molecule, intermolecular contacts are made between the C-terminal TRAF domain at one end of the trimer, and by typical coiled-coil interactions at the other end. Because of the shape of the molecule, there are large solvent channels in the crystal lattice along the length of the extended helices. To form the complex, synthetic peptides corresponding to the TRAF-binding region of LMP1 were soaked into existing TRAF3 crystals. These peptides varied in length from 24 to 8 residues, but each contained the PQQAT motif for TRAF3 binding: ^202^PHPQQATDD^207^, ^202^PHPQQATDD^207^, ^203^HPQQATDD^210^ . The structure of each complex was solved but density for the peptide was strong and clearly defined only for the short 8 residue fragment. In the case of the longer peptides, some density was apparent in the TRAF3 binding...
crevice, but there was no evidence of ordered peptide and residues could not be placed with confidence (data not shown). Furthermore, there was no extra non-protein density anywhere around the TRAF3 surface. This was surprising since we have shown in several previous studies that peptides as long as 24 residues in length can be accommodated in TRAF3 crystals in a restricted solvent ‘cave’ located at the binding crevice on the TRAF domain (27,28,31). The structure presented here is the complex with the short peptide representing residues 203-208.

**Structure of the LMP1/TRAF3 complex.**
The structure of the LMP1/TRAF3 complex at 2.8-Å resolution is presented in Fig. 1A and B. One LMP1 peptide was bound to each of the three subunits in TRAF3, and the structure of the peptide was identical at each of the three sites in the trimer, related by strict crystallographic three-fold symmetry. The binding site for LMP1 is located in the same crevice on TRAF3 that accommodates other TNFRs including CD40, LTβR and BAFF-R (27-29). There was clear density for the polypeptide backbone atoms of the LMP1 peptide 204PQQATDD210 and residues in the PQQAT motif could be positioned unambiguously in the electron density (see Fig. 1C). Density for the N-terminal histidine was weak and fragmented so this residue was omitted from the model. Density was clear and continuous for the rest of the peptide, except for the side chain of Asp209, where the density was broken and poorly defined, probably because this residue does not make contact with TRAF3 and is flexible in the complex. In contrast, the density for the side chain of the adjacent aspartic acid, Asp210, was strong and clearly defined.

**LMP1/TRAF3 intermolecular contacts.**
The detailed intermolecular contacts that mediate LMP1/TRAF3 recognition are shown in Fig. 1D. In the complex Pro204 is within van der Waal’s distance of the phenyl rings of Phe448 and Phe457 in TRAF3. The side chain of Gln206 forms a hydrogen bond with the hydroxyl group of Ser454 and is within hydrogen-bonding distance of the hydroxyls of Ser455 and Ser456. Thr208 participates in a hydrogen-bonded network. The side chain hydroxyl forms a hydrogen bond with OD1 of Asp399 in TRAF3, while the main chain imino group of the threonine is hydrogen-bonded to OD2 of Asp399. In addition to intermolecular interactions, Thr208 also participates in an intramolecular hydrogen bond between the threonine hydroxyl and OD2 of Asp210 (2.87 Å), two residues away in LMP1. The last contact residue in LMP1, Asp210, also participates in a hydrogen-bonded network forming hydrogen bonds with two side chains in TRAF3: between OD2 and the phenolic hydroxyl of Tyr395 (2.98 Å), and between OD1 and NH2 in Arg393 (2.78 Å). No intermolecular contacts are observed involving Gln205, Ala207 or Asp209.

The intermolecular contacts between TRAF3 and LMP1 observed in the crystal structure were further examined by site-directed mutagenesis. LMP1 self-aggregates through its 6 transmembrane domains and thus is constitutively active when it is expressed on cells (15,33,54). It has been shown previously that only the cytoplasmic carboxyl-terminal tail (CCT) of LMP1 is required for post-aggregation delivery of signals (18,22,33,54). To better determine the recruitment and binding of TRAF molecules by LMP1 signaling in B cells, we have previously generated a chimeric molecule (hCD40-LMP1) composed of the extracellular and transmembrane domains of human CD40 and the CCT of LMP1. This chimeric molecule signals indistinguishably from LMP1 but with controllable initiation, and like wild type LMP1, its aggregation localizes the hybrid receptor to plasma membrane rafts (36,46,55). To evaluate the individual contributions of residues of the ‘PQQATDD’ motif of LMP1 in TRAF3 binding, we have mutated each contact residue seen in the crystal structure to alanine within the context of hCD40-LMP1 chimeric molecule. These chimeric molecules were stably transfected into the M12.4.1 mouse B cell line, and expression matched clones were selected by immunofluorescence flow cytometry and used in the co-immunoprecipitation study.

Our laboratory has recently developed an approach to better detect the recruitment and binding of endogenous TRAFs to rafts upon LMP1 signaling in B cells (36, 39). In this method, non-ionic detergent-soluble proteins are first extracted with 1% Brij, which does not disrupt rafts, then Brij-58-insoluble proteins assembled in rafts are resolubilized with 1% NP-40 supplemented with 60 mM octylglucopyranoside and 0.1% SDS (both
octylglucopyranoside and SDS ensure the solubilization of rafts). Brij 58-soluble and -insoluble (raft) lysates are subsequently analyzed by co-immunoprecipitation and immunoblotting. Using this approach, we have determined the association of TRAF3 by the above hCD40LMP1 mutants in comparison with wild type (WT) hCD40LMP1 in B cells. After stimulation with anti-hCD40 Ab to trigger signaling through hCD40LMP1, most cellular TRAF3 was recruited by WT hCD40LMP1 to detergent insoluble membrane rafts (Fig. 2) (36,39). Interestingly, we consistently observed that a significant amount of TRAF3 was also co-immunoprecipitated with WT hCD40LMP1 in the detergent soluble fraction stimulated with an isotype control Ab (Fig. 2) (36,39). One possibility is that TRAF3 may be able to bind to un-aggregated (monomeric) hCD40LMP1, due to its particularly high avidity for the cytoplasmic tail of LMP1. During the immunoprecipitation procedure, all samples were incubated with protein G beads pre-armed with anti-hCD40 Ab G28-5, which would aggregate or crosslink hCD40LMP1 in the lysates onto the beads. Hence, another possibility is that the aggregated hCD40LMP1 on protein G-G28-5 beads may mimic the clustering of hCD40LMP1 triggered by anti-hCD40 Ab stimulation in live cells, and thus may recruit TRAF3 in the lysates during the immunoprecipitation procedure. Therefore, the amount of TRAF3 co-immunoprecipitated with each mutant in both lanes of Brij-58-soluble fraction stimulated with the isotype control Ab (iso, S) and Brij-58-insoluble raft fraction stimulated with anti-hCD40 Ab (α-h, I) shown in Fig. 2 reflects the ability of this mutant to bind to TRAF3. Our results demonstrated that in M12.4.1 mouse B cells, recruitment and binding of TRAF3 was dramatically diminished by substitution of alanine for Pro204, Gln206 and Thr208, moderately decreased by substitution of alanine for Asp210, but not affected by the Asp209Ala mutation (Figure 2). Corroborating with our co-immunoprecipitation data, a previous study using in vitro pull-down experiments with GST fusion proteins also showed that mutations of Pro204, Gln206, Thr208 and Asp210 have important effects in dampening TRAF3 association (59). These findings indicate that Pro204, Gln206, Thr208 and Asp210, but not Asp209, of LMP1 are critical for binding TRAF3. Gln206, Thr208 and Asp210 participate in hydrogen bonded interactions with TRAF3, while Pro204 participates in van der Waal’s interaction in a hydrophobic pocket in the TRAF3 binding crevice. Substitution of alanine for the three polar residues in the LMP1 motif would prevent formation of key hydrogen bonds. Substitution of alanine for Pro204 apparently affects the strength of the hydrophobic interactions at that site in the motif, or perhaps may affect the folding pattern of the LMP1 motif in a more general manner that diminishes binding.

Comparison of LMP1 versus CD40 contacts with TRAF3. LMP1 mimics signaling events and effector functions of CD40 in B lymphocytes (18,19,46,54) and TRAF3 appears to be a major adaptor protein required to transmit LMP1 signals, while acting as a negative regulator for CD40 signals (56,57). Recently we have demonstrated that TRAF3 is actually required for activation of B cells by LMP1, and that thus CD40 and LMP1 use TRAF3 in different ways (39). The carboxyl-terminal cytoplasmic regions of CD40 and LMP1 each bear a TRAF recognition motif PxQxT that binds in the same binding crevice on the surface of TRAF3. The sequences are closely similar: PVQET in CD40 and PQQAT in LMP1 and the structural features of the pentapeptide motifs are also similar (Fig. 3). LMP1 and CD40 bind in the same crevice on TRAF3 and the intermolecular interactions involving proline and glutamine in the motif are the same. While the recognition motifs are accommodated in a similar mode, there is a molecular adaptation of the TRAF3 surface illustrated by changes in the electrostatic surface of TRAF3 calculated when bound to LMP1 versus CD40 (Fig. 3). This is consistent with our previous observations that the TRAF3 binding crevice contains structurally adaptive ‘hot spots’ that undergo conformational adjustments in the binding crevice to provide a unique shape and electrostatic character for each binding partner.

The overall structural similarity of the recognition motifs in LMP1 and CD40 facilitate docking in the binding crevice, but there are also distinct differences in the binding patterns that must be considered in light of the functional differences that are known to result from binding events with these two proteins. It should be noted
that extensive comparisons are not possible because the segment of LMP1 that has been structurally defined is short (eight residues) compared to the portion of the cytoplasmic domain of CD40 (twenty residues) that was determined in the complex with TRAF3 (27). With the longer segment from CD40, as well as similar fragments from LTβR (28) or BAFF-R (29), secondary structural features of the peptides were determined to define binding of the receptors either in the context of a hairpin or extended ‘boomerang’ conformation. CD40 binds to TRAF3 in a hairpin or reverse turn configuration. The hairpin is stabilized by an intramolecular contact made by the threonine in the consensus motif (27). In LMP1, the equivalent threonine, Thr208, does make an intramolecular contact with the side chain of neighboring residue Asp210. It is not possible with the present data to predict whether LMP1 also assumes a hairpin/reverse turn configuration upon binding TRAF3, as we observed for CD40 (27). Both CD40 and LTβR bind TRAF3 in reverse turn configurations and each of these receptors bears one or two prolines at the turn. LMP1 does not have proline(s) at the equivalent sequence, and in this respect is more like the sequence pattern seen in BAFF-R or the downstream regulator TANK, which bind to TRAF3 as extended chains. This is of particular interest, given the recent report that BAFF-R, like LMP1, utilizes TRAF3 as a positive signal regulator and shows unique binding features (58).

LMP1 makes unique contacts with TRAF3. LMP1 makes two additional key contacts at or near the recognition motif that are not made by CD40. Thr208 in the consensus pentapeptide forms a hydrogen bond with Asp399 in TRAF3. This hydrogen bond is also made by the conserved threonine in the motif in TANK (31). In CD40, the equivalent of this threonine is engaged in the stabilizing intramolecular interaction within the hairpin, and does not make any direct contacts with TRAF3 (27). Another interesting contact is made between LMP1 Asp210 and two residues, Tyr395 and Arg393, in TRAF3. The hydrogen bonds formed at this network apparently provide stability to the complex since substitution of alanine for Asp210 diminishes binding to TRAF3 (Fig. 2). In CD40, the equivalent residue, two residues downstream of the recognition motif, is histidine, which does not contact TRAF3. Interestingly, Asp209 does not make an intermolecular contact with TRAF3. This observation was confirmed by mutagenesis where substitution of alanine for Asp209 did not affect binding.

Our results contrast with studies for TRAF2 (30) where Asp209 in LMP1 was reported to be the contacting residue, while Asp210, which is in close proximity, did not form hydrogen bonds with residues 393 and 395. Although binding of LMP1 to TRAF2 is considerably weak, our Asp210Ala mutant LMP1 also showed reduced binding to TRAF2 while Asp209Ala retained binding (data not shown) consistent with our results for TRAF3, and implicating Asp210 as the key contact for both TRAF2 and TRAF3.

Stabilizing interactions with TRAF3. It is possible within the TRAF crevice for hydrogen bonds to form with an aspartic acid adjacent to the PxQxT motif, as we have shown in crystal structures of LTβR or TANK complexes with TRAF3 (28,31). In LTβR, within the context of the sequence IPEEGD, the aspartic acid participates as a sixth residue for recognition by forming a hydrogen bond with Tyr395 in TRAF3. In the complex of TANK with TRAF3, we noted that within the context of the sequence PIQCTD, the aspartic acid makes hydrogen bonds with Tyr395 and Arg393 in TRAF3, similar to the pattern we report here for Asp210 from LMP1. The additional hydrogen bonds provided by these aspartic acids may serve to strengthen binding over contacts made only by residues in the consensus motif, as seen with CD40. In the case of TANK, this downstream regulator competes with CD40 for binding to the same binding crevice on TRAF3. Stronger binding affinity was proposed to have important implications for release of TRAFs from CD40 or for modulation of TANK-mediated inhibition of NF-κB activation by CD40.

The question of whether LMP1 competes with CD40 for the TRAF3 binding crevice awaits future experiments in cell-based assays that are beyond the scope of this study. But our work to date has already given us some insight that stronger binding of LMP1 to TRAF3 than CD40 is critical for LMP1’s ability to transform B lymphocytes. We have demonstrated previously that LMP1 exhibits stronger binding to TRAF3 relative to CD40. In B lymphocytes, LMP1
recruits the majority (about 80%) of cellular TRAF3, while CD40 engagement recruits only about 30% of cellular TRAF3 (39,46,59). Furthermore, the amount of TRAF3 co-immunoprecipitated with CD40 was dramatically reduced by the presence of LMP1, suggesting that LMP1 may sequester cellular TRAF3 making it unavailable for CD40 (39). The additional hydrogen bonds provided by Asp210 in the LMP1/TRAF3 complex strengthen the binding and stabilize the complex, and thus may represent the molecular basis for understanding these binding properties.

In summary, here we have reported the structural details of the molecular interactions that are made when LMP1 binds to TRAF3 and shown that more hydrogen-bonded contacts are formed than exist in the TRAF3/CD40 complex. In particular, Asp210 forms key intermolecular hydrogen bonds that do not exist when CD40 binds to TRAFs. Stability of the LMP1/TRAF3 complex may play a role in the dysregulated signaling and sustained B cell activation caused by LMP1. Presentation of LMP1 as a structural mimic of CD40 may be an effective viral strategy to introduce a molecular decoy for the CD40 binding site on TRAF3, influencing downstream signaling in B lymphocytes.

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ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Structure of the LMP1/TRAF3 complex. The complex is shown schematically with the TRAF3 trimer represented in a ribbon diagram, and each subunit colored separately. LMP1 is shown as a gray ball and stick model. One molecule of LMP1 binds to each TRAF3 subunit, in a crevice at the edge of the TRAF3 β-sandwich domain. The TRAF3 trimer is stabilized by coiled-coil interactions between long helices that are at the N-terminus of each TRAF3 monomer. In the view in panel A, the location of the cell membrane would be at the top of the image. In panel B, a view from the top of the trimer is shown. The three-fold symmetry is apparent illustrating that the TRAF3 subunits and the LMP1 molecule are identical and related by strict crystallographic symmetry. Panel C. The model of LMP1 is displayed in a 2Fo-Fc density map contoured at 2.8-Å resolution. Clear strong electron density was visible to define the polypeptide backbone and the orientation of the side chains for the residues labeled: PQQATDD. Panel D. Contacts for LMP1/TRAF3 recognition. This is a close-up view of the intermolecular contacts in the LMP1/TRAF3 complex, with TRAF3 shown as an orange ribbon and contact residues as gray ball and stick models. LMP1 is shown as a green ball and stick model. Critical contact residues are labeled and the label is underlined for residues from TRAF3. Intermolecular and intramolecular hydrogen bonds are drawn as red or green dotted line, respectively.

Fig. 2. Recruitment and binding of TRAF3 by the CCT of LMP1 with single amino acid mutations of the PQQATDD motif in B cells. (A) Co-immunoprecipitation assay of hCD40LMP1 mutants with TRAF3. M12.4.1 B cells stably transfected with hCD40LMP1 (WT), hCD40LMP1 P204A (P204A), hCD40LMP1 Q206A (Q206A), hCD40LMP1 T208A (T208A), hCD40LMP1 D209A (D209A), or hCD40LMP1 D210A (D210A) were stimulated with 10 μg/ml of anti-hCD40 Ab to trigger signaling through these chimeric receptors (α-h) or an isotype control Ab (iso) for 10 min. Detergent soluble (S) and insoluble raft (I) lysates were prepared. The lysates were incubated with anti-hCD40 Ab (G28-5) to immunoprecipitate the chimeric receptors. The immunoprecipitates were analyzed by immunoblotting for TRAF3 and LMP1. (B) Histogram analysis of TRAF3 binding to hCD40LMP1 mutants. TRAF3 and hCD40LMP1 bands on immunoblots were quantitated using a low-light imaging system. The amount of TRAF3 in each lane was normalized to the intensity of the corresponding hCD40LMP1 band. The graph depicts the results of 2 independent experiments (mean ± standard deviation).

Fig. 3. LMP1 and CD40 bind to the same binding crevice on TRAF3. The recognition motifs of LMP1 (PQQAT; left panel) and CD40 (PVQET; right panel) are shown in their bound configurations in the shallow binding crevice on the surface of TRAF3. The binding crevice is shown as a molecular surface
colored according to electrostatic characteristics, with blue corresponding to negative regions, red representing positive regions and white as the neutral regions. The view of the two complexes is in the same orientation for direct comparison. The surface residues of TRAF3 undergo conformational adjustments as a mechanism for molecular adaptation (32), and the effect of the adaptations can be seen in the differences in electrostatic patterns when the two receptors are bound. Overall, the conformation of the recognition motif in LMP1 mimics that of CD40.
TABLE 1

Summary of crystallographic data and refinement statistics

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<td>I/σ(I)</td>
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<td>Rmsd bond angles (°)</td>
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<sup>a</sup> values for the highest resolution shell (2.9-2.8 Å) are given in parentheses.

<sup>b</sup> Rmsd, root mean square deviation.
Fig. 1B
Fig. 1D
A

<table>
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</table>

TRAF3

hCD40LMP1

B

Fig. 2
LMP1 protein from the Epstein Barr virus is a structural CD40 decoy in B lymphocytes for binding to TRAF3
ShuangDing Wu, Ping Xie, Kate Welsh, Chenglong Li, Chao-Zhou Ni, Xiuwen Zhu, John C. Reed, Arnold C. Satterthwait, Gail A. Bishop and Kathryn R. Ely

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