Latent membrane protein-1 (LMP1) is a signaling molecule expressed by Epstein-Barr virus during latency. LMP1 is essential for B-cell immortalization by Epstein-Barr virus and transforms rodent fibroblasts. It activates many distinct signaling pathways including the transcription factors NFκB and AP1. We have generated a mutant of LMP1 with four point mutations: amino acids 204, 206, and 208 were mutated to alanine, and amino acid 384 was mutated to glycine. This mutant, termed LMP1AAAG, is not only unable to activate nuclear signaling pathways, but also inhibits signaling from wild type LMP1. We have demonstrated the effectiveness, selectivity, and mechanism of this inhibitory molecule. It inhibits LMP1-stimulated NFκB, STAT, and Jun transcriptional activity. It is selective, as it does not inhibit TNF or interleukin-2 signaling. We have demonstrated that it does not sequester the downstream signaling molecule, TRAF2, but instead binds LMP1 and interferes with its ability to bind TRAF2. This demonstrates the importance of the interplay between the signaling domains of LMP1 and the oligomeric structure of LMP1 for effective signaling. It identifies a tool that will be useful to probe LMP1 function in disease.

Epstein-Barr virus is found in a latent state following infection and growth transformation of B-lymphocytes (1). One of the limited number of genes expressed during this state is latent membrane protein-1 (LMP1), which has been shown to be essential for B-cell immortalization by EBV (2). LMP1 has also been shown to transform rodent fibroblasts (3, 4) and cause lymphomas in transgenic mice (5, 6). Cell transformation by LMP1 is at least in part due to the up-regulation of various anti-apoptotic proteins such as bcl-2, A20, mcl-1, and bfl-1 (7–10). LMP1 also plays a role in the immunogenicity of EBV by up-regulating a number of proteins involved in immune regulation such as the TAP-1 and TAP-2 peptide transporter components of the endogenous antigen processing pathway, major histocompatibility complex class I, and the intercellular adhesion molecules, ICAM-1 and LFA-3 (11, 12).

LMP1 is a 63-kDa integral membrane protein in which three signaling domains have been characterized (Fig. 1A). The protein has six hydrophobic transmembrane segments and is thought to spontaneously oligomerize to stimulate intracellular pathways. The three signaling domains are entitled C-terminal activating region-1 (CTAR1), CTAR2, and CTAR3 (13–15). CTAR1 has been shown to bind the complex of cellular proteins belonging to the family of tumor necrosis factor receptor-binding proteins (TRAFs) (16–18). CTAR2 binds TRADD and other signaling molecules (19, 20). Both of these domains can activate NFκB transcriptional activity independently but they function optimally together (21). Likewise, activation of the p38 kinase pathway is also mediated via both CTAR1 and CTAR2 (22). In contrast, LMP1 activates the JNK kinase pathways via CTAR2 (23, 24). The activation of NFκB and p38 have been shown to be required for a number of LMP1-regulated genes, demonstrating the importance of these pathways (22, 25). Recently a third signaling domain of LMP1 was described and termed CTAR3 (14). This has been suggested to bind Jak3 and activate the DNA binding of STAT1 (14). No function has been ascribed to the activation of the Jak-STAT pathway by LMP1, and the interaction of CTAR3 with the other signaling domains of LMP1 remains to be elucidated.

The functional domains of LMP1 were initially identified by deletional analysis. Recently, more detailed analysis has identified point mutations of critical residues that can abolish functions. By comparison of the LMP1 sequence with the functional domains of CD40, a PXQXT motif in CTAR1 was identified and substitution of the critical Pro204/Gln206/Thr208 residues to alanines abolished TRAF binding and NFκB activation from the CTAR1 of LMP1 (16, 26, 27). In the CTAR2 region, substitution of a critical Tyr584 residue completely abolished TRADD binding, and the activation of NFκB and AP1 (24, 28, 29). The critical residues in CTAR3 for Jak3 binding have not been identified.

This project was instigated to test the hypothesis that mutation of LMP1 could yield not only a nonsignaling form of the protein but also generate a dominant inhibitor. We have generated such a mutant, LMP1AAAG, in which both CTAR1 and CTAR2 were inactivated by four point mutations: P204A, Q206A, T208A, and Y384G. This report describes the key features of the LMP1AAAG mutant which enhance our understanding of the mechanism of LMP1 signaling. First, this mutant is unexpectedly defective in the ability to activate a STAT-regulated reporter, a function that was shown to be regulated by CTAR3. Second, the LMP1AAAG mutant was found to function
as a dominant inhibitory molecule when coexpressed with wild type LMP1. The efficiency, specificity and mechanism of the dominant negative molecule have been examined.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat is a cell line derived from an EBV negative T cell lymphoma (30). EL-Bl is an EBV-positive B cell line established from a Burkitt’s lymphoma, and it displays a latency I form of infection in which Epstein-Barr virus nuclear antigen I is the only viral protein detected (31). DG75 is an EBV-negative Burkitt’s lymphoma B cell line. KI225 is a human leukemic cell line (32) that is dependent upon IL-2 for growth. The KI225 cells were deprived of IL-2 for 24 h prior to transfection by washing the cells twice in phosphate-buffered saline. All the lymphoid cell lines were grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (200 units/ml penicillin and 200 μg/ml streptomycin), and were maintained at 37 °C in a humidified atmosphere with 5% CO2.

Plasmids—Plasmid pSG5-LMP1 expresses a wild type LMP1 cDNA derived from the B95.8 strain of EBV, under the control of the SV-40 promoter of the SG5 vector. The pSG5-LMP1AAAG mutant was generated by site-directed mutagenesis, (23), to mutate codon 384 from (GAAGATCTTTAGTCATAGCCGCTTAGC) to mutate codon 384 from the LMP1 promoter of the SG5 vector. The pSG5-LMP1AAAG mutant was constructed in two steps. First, a pSG5-LMP1 AAA mutant was generated by site-directed mutagenesis, (23), to mutate the codons 204–208 from P to A, thereby deleting for the first 128 amino acids and corresponding to the truncated from of LMP1 (tr.LMP1) expressed during lytic cycle in B95.8 cells (13). The pSG5-LMP1AAA mutant was constructed in two steps. First, the pSG5-LMP1AAA mutant was generated by site-directed mutagenesis, (23), to mutate the codons 204–208 from P to A, thereby deleting for the first 128 amino acids and corresponding to the truncated from of LMP1 (tr.LMP1) expressed during lytic cycle in B95.8 cells (13).

Generation of a Nonfunctional LMP1 Mutant, LMP1AAAG—To create a nonnonsignaling LMP1, a form of LMP1 was generated in which four of critical residues in CTAR1 and CTAR2 are mutated. A schematic of this mutant, called LMP1AAAG, is shown in Fig. 1. This point mutations in LMP1AAAG are indicated. B, Western blotting using wild type LMP1, LMP1AAAG, and tr.LMP1 following transfection of expression vectors in DG75 cells. The products of all three constructs were detected with an anti-LMP1 antibody (CS.1–4) following SDS-PAGE and transfer onto polyvinylidene difluoride membrane.

For each immunoprecipitation and Western Blotting—For each immunoprecipitation, 15 × 10^6 cells of the DG75 line in 0.5 ml of growth medium were electroporated at 270 V and 950 microfarads at room temperature in 500 μl of fresh growth medium and were then incubated under normal conditions.

For the induction of ICAM-1 protein in transfected cells was routinely assayed by immunofluorescence staining of viable cells, followed by flow cytometry using a Becton Dickinson FACSCalibur analyzer as described previously (21). Briefly, at 48 h post-transfection the cells were stained and with a phycoerythrin-conjugated monoclonal antibody to human CD54 (MCA675PE; Serotec) at 4 °C for 60 min. The transfected population was marked by the expression of co-transfected EGFP-C1 plasmid (CLONTECH), and the GFP-positive population was assayed by immunofluorescence staining of viable cells, followed by flow cytometry using a Becton Dickinson FACSCalibur analyzer as described previously (21). The four point mutations in LMP1AAAG are indicated. B, Western blotting using wild type LMP1, LMP1AAAG, and tr.LMP1 following transfection of expression vectors in DG75 cells. The products of all three constructs were detected with an anti-LMP1 antibody (CS.1–4) following SDS-PAGE and transfer onto polyvinylidene difluoride membrane.

Fig. 1. Essential features of some LMP1 molecules used in this study. A, schematic showing the structure of wild type LMP1, dominant negative LMP1 (LMP1AAAG) and a naturally occurring truncated LMP1 mutant (tr.LMP1). Functional C-terminal signaling domains are illustrated (gray boxes). The four point mutations in LMP1AAAG are indicated. B, Western blotting using wild type LMP1, LMP1AAAG, and tr.LMP1 following transfection of expression vectors in DG75 cells. The products of all three constructs were detected with an anti-LMP1 antibody (CS.1–4) following SDS-PAGE and transfer onto polyvinylidene difluoride membrane.

RESULTS

Generation of a Nonfunctional LMP1 Mutant, LMP1AAAG—To create a nonnonsignaling LMP1, a form of LMP1 was generated in which four of critical residues in CTAR1 and CTAR2 are mutated. A schematic of this mutant, called LMP1AAAG, is shown in Fig. 1A. Fig. 1A also shows a schematic of a rare but naturally occurring truncated LMP1 (tr.LMP1) that can be expressed in a limited number of strains of EBV, and is postulated to inhibit LMP1 func-
activate NFκB reporter activity. This experiment was also performed in two B-cell lines, Eli-BL cells and DG75, and an epithelial cell line, 293. LMP1\textsuperscript{AAAG} was not able to stimulate NFκB transcriptional activity in any of these lines (data not shown).

It was possible that mutation of CTAR1 and CTAR2 would only affect signaling to pathways activated by CTAR1 and CTAR2. For this reason we tested the ability of LMP1 to trigger STAT transcriptional activity as LMP1 effects on STAT signaling have recently been mapped to a new domain entitled CTAR3 (Fig. 1A). A GRR reporter assay was chosen to test for STAT transcriptional activity. This is a well characterized reporter construct based on the STAT-binding site from the FcγR promoter. It has been shown to bind various STATs (34, 40) and has been used as a reporter in many different studies (34, 38). Eli-BL cells were co-transfected with the GRR reporter (10 μg) together with various amounts of both wild type LMP1 and LMP1\textsuperscript{AAAG} expression vectors. Fig. 2B shows that while wild type LMP1 was able to stimulate GRR luciferase transcriptional activity, LMP1\textsuperscript{AAAG} showed no stimulatory capacity for this reporter. This was somewhat surprising and suggests that either CTAR1 or CTAR2 are required for the activation of STAT transcriptional activity.

Protein expression of LMP1\textsuperscript{AAAG} was examined to demonstrate that the mutant expressed at a similar level to wild type. Fig. 2C shows the results from the expression of wild type and LMP1\textsuperscript{AAAG} in Eli-BL cells. Cells were transfected with a range of amounts of an LMP1 expression vector, cells were lysed, and cellular proteins were resolved by SDS-PAGE. Western blot analysis was performed with an anti-LMP1 antibody (CS.1–4) and similar levels of both wt LMP1 and LMP1\textsuperscript{AAAG} were seen (Fig. 2C). Similar data were also obtained with DG75 and 293 cells (data not shown).

**LMP1\textsuperscript{AAAG} Acts as a Dominant Negative Molecule**—Since the LMP1\textsuperscript{AAAG} could not activate LMP1 signaling pathways, it was postulated that it may be able to inhibit wild type LMP1 activity by preventing the formation of a functional signaling complex. NFκB transcriptional activity was tested by co-transfection of the reporter with various amounts of an expression vector for wild type LMP1 (from 0.1 to 4 μg) in combination with a constant 5 μg of LMP1\textsuperscript{AAAG}. Fig. 3A shows that expression of LMP1\textsuperscript{AAAG} dramatically inhibits the activation of NFκB by wild type LMP1. A ratio of 1 μg of wild type LMP1 plasmid to 5 μg of LMP1\textsuperscript{AAAG} plasmid inhibited NFκB transcriptional activity by greater than 90%. Over a number of experiments, the expression of equal levels of LMP1\textsuperscript{AAAG} to wild type LMP1 inhibited signaling by 50–75%, demonstrating the efficiency of the mutant. A dose response of LMP1\textsuperscript{AAAG} (0–12 μg) was tested with a fixed amount of wild type LMP1 expression vector (2 μg) (Fig. 3B). This shows that the inhibition correlates with amount of LMP1\textsuperscript{AAAG}.

A naturally occurring truncated LMP1, tr.LMP1, which lacks amino acids 1–127, has been postulated to inhibit LMP1 signaling (39). We compared the LMP1\textsuperscript{AAAG} mutant with the putative inhibitory effects of the truncated from of LMP1 (Fig. 3C). A direct comparison of the two molecules suggests that a much lower level of expression of LMP1\textsuperscript{AAAG} is efficient relative to tr.LMP1 (compare 3rd bar with 5th bar and 4th bar with 6th bar). In this experiment, the co-transfection of 3 μg of LMP1\textsuperscript{AAAG} expression vector inhibited wild type LMP1 activation of NFκB by greater than 90%, whereas 3 μg of the expression vector for tr.LMP1 had very little effect.

**LMP1\textsuperscript{AAAG} Inhibits STAT Transcriptional Activity and Jun Transactivation**—Two other nuclear signals stimulated by LMP1 were also tested. The STAT reporter (GRR luciferase) was tested in Eli-BL cells (Fig. 4A). This showed that...
LMP1AAAG effectively inhibited STAT transcriptional activity stimulated by LMP1. A Jun-transactivation assay was also performed (Fig. 4B). Jurkat cells were co-transfected with a mammalian expression vector for a chimeric protein with the DNA-binding domain from the bacterial lex protein and the transactivation domain of c-Jun (amino acids 1–194) (41) together with a reporter that contains two binding sites for lex-protein upstream of the CAT gene (35). This reporter therefore gives a measurement of Jun phosphorylation in vivo, which requires the activity of Jun N-terminal kinase (JNK) or p38 stress activated protein kinase. When co-transfected with this reporter, LMP1 could induce Jun transactivation, and this activation was inhibited by coexpression of the LMP1AAAG (Fig. 4B).

LMP1AAAG Inhibits Induction of ICAM-1 by LMP1—It is important to demonstrate that the LMP1AAAG can also block downstream biological functions of LMP1, i.e., endogenous protein changes, and not just affect reporter constructs. Fig. 5 demonstrates that LMP1AAAG efficiently inhibits the LMP1-mediated induction of the ICAM-1 (CD54) adhesion molecule. Jurkat cells were co-transfected with a GFP vector and a wild type LMP1 plasmid. The GFP vector allows the identification of transfected cells by flow cytometry. The cells were left for 48 h and expression of ICAM-1 was determined using phycoerythrin-conjugated CD54 antibodies and flow cytometric analysis. Fig. 5A (top panel) shows the expression of ICAM-1 in Jurkat cells transfected with EGFP and control vector DNA. When Jurkat cells were transfected with the GFP-LMP1AAAG expression vector no increase in ICAM-1 levels was detected demonstrating that LMP1AAAG could not signal effectively (Fig. 5A, 2nd panel). In contrast, cells transfected with wild type LMP1 and EGFP expression vectors showed a dramatic increase in ICAM-1 expression (Fig. 5A, 3rd panel).
arbitrary threshold gate. In the example shown in Fig. 5A, expression of wt LMP1 caused an increase from 2.7 to 34.2% of ICAM-1 positive cells. The effects upon ICAM-1 can also be determined by measuring the increase in mean fluorescence intensity which, in Fig. 5A, shows a 3-fold increase in the level of expression of ICAM-1 caused by expression of wt LMP1 (control transfectant, mean fluorescence intensity of 68; wt LMP1 transfectant, mean fluorescence intensity 195). When wild type LMP1 and GFP-LMP1AAAG expression vectors were co-transfected, no increase in either the percentage of positive cells or the mean fluorescence intensity was detected (Fig. 5A, compare top and bottom panels). A dose response of LMP1 plasmid was performed in the presence and absence of a constant amount of LMP1AAAG. Whether the data are expressed as mean fluorescence intensity (Fig. 5B) or as a percentage of ICAM-1 positive cells (not shown), LMP1AAAG dramatically inhibits LMP1 signaling. These results demonstrate that LMP1AAAG can inhibit endogenous cellular gene expression in addition to synthetic reporter genes.

**LMP1AAAG Is Selective as It Does Not Inhibit TNF or Interleukin-2 Signaling**—The usefulness of a dominant-negative molecule is enhanced if it is also specific for its intended target. We therefore examined whether LMP1AAAG might interfere with the signaling pathways activated by other receptor molecules. The selectivity of the LMP1AAAG inhibitor was tested in two different systems. Jurkat cells were transfected with the 3enh (NFκB) luciferase reporter and increasing amounts of the LMP1AAAG vector. The cells were left overnight to ensure expression, and were then stimulated with TNF. The results in Fig. 6A show that doses of LMP1AAAG which inhibit wt LMP1 signaling by more than 75% had no effect on TNF activation of NFκB. The LMP1AAAG molecule retains the putative Jak3-binding site (CTAR3) and while it appears unable to signal to STAT transcriptional activity, it could theoretically act to sequester Jak3 and thus inhibit Jak 3-dependent signaling from other receptors. This possibility was tested in Kit225 cells, a leukemic cell line that is stimulated by the cytokine, IL-2. In these cells, IL-2 utilizes Jak3 and activates STAT5 and STAT3, which have been previously shown to activate the GRR reporter (34). The Kit225 cells were transfected with the LMP1AAAG expression plasmid, and the ability of IL-2 to activate the GRR reporter was tested. Fig. 6B shows that IL-2 was fully functional in the presence of similar levels of LMP1AAAG that were shown to inhibit wt LMP1 signaling. This provides evidence for the specificity of LMP1AAAG and suggests that it acts proximal to the LMP1 molecule itself rather than binding or sequestering signaling machinery that may be utilized by other signaling pathways.

**LMP1AAAG Cellular Localization Is Indistinguishable from that of Wild Type LMP1**—All the mutants of LMP1 utilized in this study were expressed in DG75 B-cells and their cellular localization was investigated by staining with the LMP1 antibody, LMPO25. Cells were transfected with 5 μg each of expression vector and left to express overnight. The cells were fixed with 2% paraformaldehyde and acetone and then stained as described previously. The staining was visualized with a Leica confocal microscope and a Z series was acquired. Fig. 7A shows the staining of wild type LMP1, LMP1AAAG, and tr.LMP1. The staining of wild type LMP1 produced a characteristic pattern, with an accumulation of LMP1 into a “cap” at one end of the cell. The pattern of staining produced by LMP1AAAG was indistinguishable from wild type LMP1. In...

---

**Fig. 5. LMP1AAAG inhibits ICAM-1 protein up-regulation by LMP1.** Jurkat cells were co-transfected with 2 μg of pEGFP-C1 marker plasmid together with variable amounts (0–2.0 μg) of wt LMP1 expression plasmid with or without a constant 20 μg of LMP1AAAG expression plasmid. At 48 h post-transfection, the cells were stained with phycoerythrin (PE)-conjugated CD54 antibodies to ICAM-1 and analyzed by two-color flow cytometry. A, FACS profiles of ICAM-1 staining in the EGFP-positive population of four cultures transfected, respectively, with empty vector, LMP1AAAG, wt LMP1, and wt LMP1 + LMP1AAAG. B, dose-response curves showing the mean fluorescence intensity of ICAM-1 staining of cultures transfected with increasing amounts of wt LMP1 vector with (solid squares) or without (open circles) a constant 20 μg LMP1AAAG vector.
LMP1AAAG could colocalize, constructs that could be distinguished had to be utilized. We used a fusion protein comprising the extracellular and transmembrane domains of rat CD2 tagged to the N terminus of wild type LMP1, and a GFP-tagged LMP1AAAG. Fig. 7A shows the image of an equatorial region of a DG75 cell which had been co-transfected with expression vectors for the CD2-WT-LMP1 and LMP1AAAG-GFP. The cells were fixed and stained for rat CD2 using the OX34 antibody and a Texas Red secondary antibody. GFP was visualized in the fluorescein isothiocyanate channel and a specific channel for Texas Red was used to visualize CD2-tagged LMP1. A comparison of the two pictures shows an overlapping distribution of the two proteins. Taken together, the results suggest that both wild type LMP1 and LMP1AAAG normally localize to the same subcellular region and that, if coexpressed in the same cell, may physically interact.

LMP1AAAG Binds Wild Type LMP1—The images of wild type LMP1 and LMP1AAAG together with the specificity of LMP1AAAG suggested an hypothesis that LMP1AAAG may be forming a complex with wild type LMP1. This complex may inhibit the signaling of wild type LMP1. This hypothesis was tested by investigating whether wild type LMP1 could bind LMP1AAAG by performing co-precipitation experiments. The CD2-tagged wtLMP1 expression vector was transfected alone or together with either LMP1AAAG or tr.LMP1 vectors into DG75 B cells, and cell lysates were immunoprecipitated with a specific CD2 antibody (OX34). The presence of CD2-wtLMP1, LMP1AAAG, and tr.LMP1 in the immunoprecipitates was assayed by Western blotting with a rabbit polyclonal antibody to TRAF2, to bind LMP1. TRAF2 is postulated to be a major component of the LMP1 signaling machinery. DG75 cells were transfected with vectors for wild type LMP1, LMP1AAAG, and the truncated LMP1 (tr.LMP1), in the presence of TRAF2. Cells were left overnight to allow expression. The cells were then lysed and the LMP1 was immunoprecipitated with antibodies
LMP1 Dominant Negative

Fig. 8. LMP1AAAG binds wild type LMP1. Western blots, probed with a rabbit antiserum to LMP1, indicating the LMP1 molecules co-immunoprecipitating with wild type LMP1. For each immunoprecipitation, 15 x 10^6 DG75 cells were co-transfected with 10 μg each of the SG5 expression plasmids as indicated, and lysates were prepared 24 h post-transfection. The wild type LMP1 was expressed as a CD2/ LMP1 fusion protein which was precipitated with OX34 monoclonal antibody to rat CD2. Lanes 1–3 show the OX34 immunoprecipitates from cells co-transfected with CD2/LMP1 chimera and SG5 vector (lane 1), LMP1AAAG (lane 2), or tr.LMP1 (lane 3). Lane 4 shows the OX34 immunoprecipitate from cells transfected with LMP1AAAG only, while lane 5 shows the anti-CD2 immunoprecipitate from cells transfected with tr.LMP1 only. The upper blot shows the LMP1 species in the resolved anti-CD2 immunoprecipitates, while the lower blot shows the LMP1 species in the input lysates (equivalent to ~2% of the lysate used for immunoprecipitation).

CS-3 and CS-4. These immunoprecipitates were resolved by SDS-PAGE and the presence of TRAF2 in the immunoprecipitates was detected by Western blotting. The results from a representative experiment are illustrated in Fig. 9A. These show that TRAF2 binds to wild type LMP1 but binds at a substantially reduced level to both LMP1AAAG and tr.LMP1. Experiments were performed to determine whether LMP1AAAG could inhibit the recruitment of TRAF2 to wild type LMP1. This was done by co-transfecting a range of amounts of a vector for GFP-tagged LMP1AAAG (EGFP-LMP1AAAG) with a constant amount of wild type LMP1 (3 μg) and TRAF2 (6 μg) vectors. Cells were lysed and LMP1 was immunoprecipitated as before. Fig. 9B shows that LMP1AAAG prevents the interaction of TRAF2 with wild type LMP1 (top panel). The bottom panel (Fig. 9B) shows the expression of both wild type LMP1 and the GFP-tagged LMP1AAAG.

Clearly, LMP1AAAG interferes with the ability of wild type LMP1 to bind TRAF2 in the cellular context. It is possible that this is a result of LMP1AAAG sequestering wild type LMP1 to a different subcellular location, away from TRAF2. While this appears unlikely from the results in Fig. 7, we conducted additional experiments to examine whether LMP1AAAG interfered with the ability of wild type LMP1 to bind TRAF2 in vitro. In this set of experiments, LMP1 molecules were first precipitated from lysates of transfected DG75 B-cells and were then incubated with TRAF2-containing lysates from separately transfected DG75 cells. Fig. 9C shows that the while immune precipitates of wild type LMP1 can effectively bind exogenously added TRAF2, the complex of wild type LMP1 with LMP1AAAG could not bind TRAF2. Together these immunoprecipitation experiments suggest a mechanism whereby LMP1AAAG binds to wild type LMP1 and prevents the recruitment of TRAF2. This prevents LMP1 signal transduction to the nucleus.

DISCUSSION

This study describes a novel inhibitor of the oncogenic protein LMP1. It characterizes the efficacy, selectivity, and mechanism of the inhibitor. The efficacy was tested by inhibition of reporter constructs and ICAM-1 protein expression changes induced by LMP1. It was shown to be both nonfunctional and to be dominant inhibitory when expressed at similar levels to wild type LMP1. It was shown to be selective as it does not inhibit TNF or IL-2 signaling. Finally an investigation into the mechanism demonstrates that it does not sequester downstream signaling molecules but binds wild type LMP1 and inhibits the binding of at least one signaling molecule, TRAF2.

All described previously, mutants of LMP1 that are unable to activate NFκB have had deletions of either CTAR1 or CTAR2. LMP1AAAG is the first mutant that is unable to activate NFκB and contains only point mutations. This reduces the likelihood of dramatic structural effects that can result from amino acid deletions. It strengthens the hypothesis that all the signals required for NFκB activation lie completely within CTAR1 and CTAR2.

While in terms of NFκB, this is not surprising, it is still an important confirmation of our expectations. However, the fact that proper functioning of CTAR1 and CTAR2 is required for GRR lucerase activity, and thus STAT activation, was unexpected since Gires et al. (14) recently described CTAR3 as a Jak-binding domain. Our result does not necessarily contradict the results from Gires et al. (14) but casts them in a new light. It suggests that while CTAR3 may be a distinct signaling motif of LMP1, it does not function in isolation to activate STATs.

The mechanism of this activation is intriguing and is currently the subject of investigation. Gires et al. (14) measured STAT1 DNA binding whereas this present study has measured STAT transcriptional activity. STAT proteins have been shown to be modulated by both tyrosine and serine phosphorylation (34, 40, 42) and the Jak kinases can only phosphorylate tyrosine residues. The serine phosphorylation of STATs has been shown to be important for transcriptional activity but not DNA binding. Distinct signaling pathways have been shown to be utilized by both IL-2 (34) and interferon signaling (42) for the regulation of STAT transcriptional activity. Thus, while CTAR3 may control STAT tyrosine phosphorylation, CTAR1 or CTAR2 may be required for other signaling pathways necessary for STAT transcriptional activity.

The interplay between the domains of LMP1 has been postulated previously. It has been shown that for optimal functions, CTAR1 and CTAR2 must be in the same signaling complex (21). The inhibitory effects of LMP1AAAG further elucidates the importance of cooperation between LMP1 molecules. It is apparent that this cooperation is not just of a structural nature but also requires the cooperative binding of signaling molecules. We have shown that LMP1AAAG does not prevent normal cellular localization of LMP1 (Fig. 7). Furthermore, a complex of wild type LMP1 and LMP1AAAG is unable to bind TRAF2, a key signaling molecule, both in vitro and in whole cells (Fig. 9). This suggests that the cooperation between the domains of LMP1 is required between molecules for the regulation of gene expression events. Furthermore, this cooperation acts at the level of the recruitment of receptor proximal signaling molecules and not at the level of cooperating nuclear signals, demonstrating the importance of the formation of an intact signaling complex. TRAF2 has been shown to bind the TNF receptor, to which LMP1 has been compared, as a trimeric
FIG. 9. LMP1AAAG has impaired TRAF2 binding ability and it inhibits the binding of TRAF2 to wt LMP1. Western blots indicating the co-precipitation of TRAF2 with LMP1 complexes. For each immunoprecipitation, 15 × 10⁶ DG75 cells were transfected as indicated and lysates were prepared at 24 h post-transfection. Immunoprecipitations were performed using a pool of CS.3 and CS.4 monoclonal antibodies to LMP1. A, to compare the ability of different LMP1 molecules to bind TRAF2, cells were transfected with 6 µg pCMV-TRAF2(8–26) together with 3 µg of either pSG5 vector (first lane), pSG5-LMP1 (second lane), pSG5-LMP1AAAG (third lane), or pSG5-LMP1Δ(1–128) (fourth lane). The upper panel shows a Western blot of material immunoprecipitated with antibodies to LMP1, and probed with rabbit anti-TRAF2 antibodies. The middle and lower panels show Western blots of the input lysates probed with rabbit anti-TRAF2 antibodies (middle panel) or with CS.1–4 antibodies to LMP1 (lower panel). B, the dose responsive dominant-negative effect of LMP1AAAG on wt LMP1 binding to TRAF2 was assayed. LMP1AAAG was expressed as an EGFP-LMP1AAAG-tagged protein so that the expression levels obtained with the indicated doses of pEGFP-LMP1AAAG DNA (1–8 µg) could be compared with the expression of the lower molecular weight wt LMP1 coexpressed from 3 µg of pSG5-LMP1 plasmid. A Western blot of the LMP1 immunoprecipitates was probed with rabbit anti-TRAF2 antibodies (upper blot), and the input lysates were probed with CS.1–4 anti-LMP1 antibodies (lower blot). C, the in vitro binding of TRAF2 to wild type LMP1 or a complex of wild type LMP1 and LMP1AAAG was investigated by adding lysates that contained TRAF2 to immunoprecipitates of LMP1 from DG75 cells that had been transfected with wild type LMP1 or both wild type LMP1 and LMP1AAAG. The immunoprecipitates were then resolved by SDS-PAGE and the presence of newly bound TRAF2 was investigated by Western blotting.
REFERENCES

1. Rickinson, A. B., and Kieff, E. (1996) in Fields Virology (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) Vol. 2, pp. 2397–2446, Lippincott-Raven, Philadelphia.
2. Kaye, K. M., Izumi, K. M., and Kieff, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1150–1154.
3. Wang, D., Liebowitz, D., and Kieff, E. (1985) Cell 43, 831–840.
4. Baichwal, V. R., and Sugden, B. (1988) Oncogene 2, 461–467.
5. Kulwichit, W., Edwards, R. H., Davenport, E. M., Baskar, J. F., Godfrey, V., and Raab-Traub, N. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11963–11968.
6. Kieff, E., and Rickinson, A. B. (1995) Eur. J. Immunol. 25, 1575–1586.
7. Rowe, M., and Jones, M. (2001) in Virus Protocols (Wilson, J. B., ed) Humana Press Inc., Totowa, NJ, in press.
8. Kaye, K. M., Izumi, K. M., and Kieff, E. (1993) Cell 73, 381–393.
9. O ’ Hare, P., Krummel, M. F., and Allison, J. D. (1995) Science 269, 1442–1446.
10. de la Rosette, J., and Traut, W. (1994) J. Urol. 152, 317–320.
11. deKruif, E. J., and Kieff, E. (1999) in Cell Surface Receptors: From Structure to Function (Fields, B. N., ed) Academic Press, New York, in press.
12. Bunnell, S., and Allison, J. D. (1999) J. Immunol. 163, 1429–1433.
13. Krammer, P. H., and Kieff, E. (1993) J. Exp. Med. 177, 1374–1384.
14. Siegal, G., and Kieff, E. (1997) Cell 87, 565–576.
15. Krammer, P. H., and Kieff, E. (1997) J. Virol. 71, 679–689.
16. Longnecker, R., Kieff, E., and Rickinson, A. (1991) Cell 65, 1107–1115.
17. Kaye, K. M., Izumi, K. M., and Kieff, E. (1993) Cell 73, 381–393.
18. Nakamura, M., and Kieff, E. (1999) J. Biol. Chem. 274, 599–606.
19. Honjo, T., and Kieff, E. (1994) J. Exp. Med. 180, 1429–1437.
20. Krammer, P. H., and Kieff, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12592–12597.
21. Liu, Z. G., Heu, H., Goeddel, D. V., and Karin, M. (1996) Cell 87, 565–576.
22. Bunnell, S., and Allison, J. D. (1999) J. Immunol. 163, 1429–1433.
23. Decker, T., and Kovarik, P. (1999) Science 286, 296–299.
24. Weber-Nordt, R. M., Egen, C., Wehinger, J., Ludwig, W., Gouilleux-Gruart, V., Mertelsmann, R., and Finke, J. (1996) Blood 88, 809–816.
25. Busson, P., Ganem, G., Flores, P., Mugneret, F., Clavel, B., Caillou, B., Braham, K., Wakasugi, H., Lipinski, M., and Turz, T. (1988) Int. J. Cancer 42, 599–606.
26. Kim, S. H., Shin, Y. K., Lee, J. S., Bae, Y. M., Sohn, H. W., Suh, Y. H., Ree, H. J., Rowe, M., and Park, S. H. (2000) Blood 95, 294–300.
