Characterization of Intercellular Adhesion Molecule-1 Regulation by Epstein-Barr Virus-encoded Latent Membrane Protein-1 Identifies Pathways That Cooperate with Nuclear Factor κB to Activate Transcription

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The latent membrane protein-1 (LMP1) of Epstein-Barr virus induces gene transcription, phenotypic changes, and oncogenic transformation. One cellular gene induced by LMP1 is that for intercellular adhesion molecule-1 (ICAM-1), which participates in a wide range of inflammatory and immune responses. ICAM-1 may enhance the immune recognition of cells transformed by Epstein-Barr virus, and thus combat development of malignancy. Despite growing understanding of the various signaling functions of LMP1, the molecular mechanisms by which LMP1 induces ICAM-1 are not understood. Here, we demonstrate that transcriptional activation by LMP1 is absolutely dependent upon a variant NF-κB motif within the tumor necrosis factor α (TNFα) response element of the ICAM-1 promoter. Although the TNFα response element is sufficient for TNFα induction of the ICAM-1 promoter, LMP1 also required the cooperation of additional upstream sequences for optimal induction. Inhibitor studies of known LMP1-induced signaling pathways ruled out the involvement of c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase, and the Janus-activating tyrosine kinase 3 (JAK3), and confirmed NF-κB as a critical factor for induction of ICAM-1. However, although constitutive activation of NF-κB efficiently induced promoter activity, it was not sufficient to induce either ICAM-1 mRNA or ICAM-1 protein. Using signaling defective LMP1 mutants and deacetylation inhibitors, we showed that the C-terminal activator region 1 of LMP1 delivers a new cooperating signal to induce ICAM-1 mRNA.

The latent membrane protein-1 (LMP1) is considered to be the major oncogene of Epstein-Barr virus (EBV), a persistent herpesvirus that is associated with various malignant diseases (1). LMP1 transforms rodent fibroblasts (2), induces lymphomas in transgenic mice (3), and is essential for EBV-induced immortalization of human primary B-lymphocytes (4). The oncogenic properties of LMP1 are at least in part due to the up-regulation of anti-apoptotic proteins such as Bcl-2, A20, Mcl-1, and Bfl-1 (5–8). In addition, LMP1 up-regulates components of the endogenous antigen processing pathway and some intercellular adhesion molecules, such as ICAM-1 and LFA-3 (9, 10). These latter functions ensure that EBV-transformed lymphocytes can be recognized and regulated by cellular immune responses, which is an important feature of EBV persistence in healthy individuals that normally prevents the development of EBV-positive lymphomas (11).

Consistent with its diverse biological functions, LMP1 has been reported to trigger a number of different signaling pathways, including activation of NF-κB (5, 12), the mitogen-activated protein kinase, JNK and p38, leading to activation of AP-1 and ATF-2 transcription factors (13–15), and the JAK3/STAT1 pathway (16). LMP1 mimics a ligand-independent, constitutive active receptor of the tumor necrosis factor receptor (TNFR) superfamily by binding TNFR-associated factor and TNFR-associated death domain (TRADD) to effect its signaling functions (17, 18). The important regions of LMP1 are the so-called C-terminal activator regions (CTAR-1, -2, and -3), which initiate the signaling function of LMP1 by binding signaling molecules and which need to cooperate together for optimal function (19). CTAR1 induces NF-κB and is probably involved in the p38 pathway; CTAR2 signals through NF-κB, the p38 pathway, and the JNK pathway; and CTAR3 binds JAK3 (13, 16, 19).

The intercellular adhesion molecule, ICAM-1 (CD54), is an inducible cell surface glycoprotein and member of the immunoglobulin supergene family (20, 21). ICAM-1 serves as a counter-receptor for a number of cell surface molecules such as LFA-1 (CD11a) and MAC-1 (CD11b), and it plays a central role in a wide range of inflammatory and immune responses (22). ICAM-1 is constitutively expressed at low levels on vascular endothelium and lymphocytes and at moderate levels on monocytes. Induction of high levels of ICAM-1 occurs in response to various inflammatory mediators, including bacterial lipopolysaccharide, phorbol esters, oxidant stress and pro-inflammatory cytokines, such as TNFα, interleukin-1β (IL-1β), and γ-interferon kinase; MEKK, mitogen-activated protein/extracellular signal-regulated kinase-activating kinase beta; bp, base pair(s); GFP, green fluorescent protein; GST, glutathione S-transferase; C/EBP, CAAT/enhancer-binding protein.

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teral induction of ICAM-1 by TNF-α dimers to the C/EBP site was reported to be necessary for upstream of a minimal conalbumin promoter driving the expression of ICAM-1 induction. The 5′-flanking region of the ICAM-1 gene contains numerous potential regulatory elements that could be involved in the activation of the promoter, some of which are tissue-specific and cytokine-dependent (23). For example, γ-IFN and TNFα have been shown to mediate ICAM-1 induction at the level of transcription, using different signal transduction pathways and specific activator sites (28–30). The interferon response element maps to −76 bp to −66 bp, whereas the TNFα-responsive element (TRE) ranges from −227 bp to −177 bp. The TRE was shown to be both necessary and sufficient to induce ICAM-1 promoter activation by TNFα, and it critically required the binding of the NF-κB family of transcription factors, specifically p65 homodimers, to a variant b-site (30). However, flanking sequences surrounding this κB binding site are also required for transcription factor binding and transactivation in TNFα-mediated induction of ICAM-1 (31). The ICAM-1 TRE also contains Sp-1 and C/EBP binding sites located upstream of the modified NF-κB site, and binding of C/EBP homo- or heterodimers to the C/EBP site was reported to be necessary for maximal induction of ICAM-1 by TNFα (32).

The studies described here were designed to define the molecular mechanisms involved in LMP1-induced ICAM-1 expression in lymphocytes. We carried out promoter deletion analysis and luciferase reporter assays to investigate the role of LMP1 at the transcriptional level and to determine the activator sites within the ICAM-1 promoter. Inhibition of specific signaling pathways induced by LMP1 was achieved using chemical inhibitors for the p38 and JAK3-pathways, and dominant inhibitory molecules for the SEK and NF-κB pathways. The importance of these individual pathways for LMP1-induced ICAM-1 promoter and protein expression at the cell surface was thus determined and revealed a hitherto unrecognized function of LMP1 to activate ICAM-1 surface expression. Finally, we will show that this novel function cooperates with one of the C-terminal activator regions of LMP1 and is essential for optimal ICAM-1 induction.

MATERIALS AND METHODS

Cell Lines—Jurrk is a cell line derived from an EBV-negative T cell lymphoma (33). Eli-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 1 is the only viral protein detected (34). DG75 is an EBV-negative Burkitt's lymphoma B cell line, and the derived DG75-tTA-LMP1 line contains a stable transfected tetracycline-regulated LMP1 expression plasmid; this transfectant, together with the control DG75-tTA transfectant, has been described previously (35). All the lymphoid cell lines were grown in suspension in RPMI, 10% fetal calf serum supplemented with 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. The DG75 transfecants were maintained in 1 μg/ml tetracycline and were drug-selected with 0.8 mg/ml hygromycin B plus 2 mg/ml G418 (DG75-tTA-LMP1) or with 0.8 mg/ml hygromycin B only (DG75-tTA).

The ICAM-1 reporter constructs containing 5′ regions upstream of the ICAM-1 gene or a luciferase gene were obtained from Harry C. Ledebur and have been described elsewhere (30). The 3 Enh-luc reporter plasmid, with three κB elements upstream of a minimal conalbumin promoter driving the expression of the firefly luciferase gene (38), and the pIL-6BκB plasmid, containing three copies of the IL-6 promoter κB site in front of a luciferase gene (37), were used to assay NF-κB activity. Plasmid pSG5-LMP1 expresses wild-type LMP1 cloned from the B95.8 strain of EBV and has been described previously (38). The LMP1 mutant CTAR1 plasmids replace amino acids Pro236, Gin238, and Thr232 with alanines, and the CTAR2 mutant replaces Tyr236 with glycine; these mutants were described previously (14, 39). The green fluorescent protein expression plasmid, pEGFP-C1, was purchased from CLONTECH, and the pMERK1 expressing plasmid was from BioLabs.

A constitutively active IκBα/GFP fusion protein vector was generated by amplifying the IκBα gene from the pCMV IκBαΔN plasmid (kindly provided from Dean W. Ballard, Howard Hughes Medical Institute, Nashville, TN) using a forward 5′-primer, which binds to the base pair 240 of the IκBα cDNA, and a reverse 5′-primer lacking the stop codon of the protein. The purified PCR fragment was then cloned into the BglII site of the pEGFP-C1 plasmid to generate plasmid EGFP-IκBαDN. The hemagglutinin-tagged kinase vectors, HA-p46SAPK/ycDNA3 and HA-p38, and the dominant inhibitor SEKDN vector (40) were provided by Aristides Eliopoulos (CRC Institute, Birmingham, United Kingdom). The pyridyl imidazole SB20380 (Calbiochem), a specific inhibitor of the p38 MAPK pathway, was prepared as a 20 mM stock solution in dimethyl sulfoxide and a JAK3 inhibitor (Calbiochem) was also prepared in dimethyl sulfoxide as a 25 μg/ml stock solution. Both inhibitors were added to the cultures at a dilution of 1/1000. The deacetylation inhibitor sodium butyrate (Sigma) was used in a final concentration of 1 mM.

Transient Transfection—For transient expression, 0.5 to 1 × 107 cells from a suspension culture were transfected by electroporation using a Bio-Rad GenePulsor II electroporator at 280 V and 950 microfarads at room temperature in 500 μl of growth medium. The cells were reseeded in 5 ml of fresh growth medium and were then incubated under normal conditions. Transfection efficiency ranged from 10% to 20% for Eli-Bl and from 40% to 50% for Jurkast, as assessed by cotransfection with the EGFP-C1 expression vector and flow cytometry analysis. Assay for Reporter Activity—The activity of the different reporter plasmids was measured at 18–24 h after transfection. Cells were washed twice in phosphate-buffered saline and lysed in 150 μl of lysis buffer containing 100 mM HEPES, pH 8.0, 2 mM magnesium chloride, 5 mM dithiothreitol, and 2% Triton X-100. Luciferase activity in 50 μl of clarified lysate was analyzed in a Berthold LD9501 luminometer following injection of 100 μl of 0.5 mM luciferin (Amersham Pharmacia Biotech) dissolved in luciferin assay reagent (30 μM glycyglycine, pH 7.9, 1 mM MgCl2, 0.1 mM EDTA, 30 mM dithiothreitol, 0.3 mM coenzyme A, 0.5 mM ATP). Light release was integrated over 10 s.

Assay for Cell Surface ICAM-1 Protein by Flow Cytometry—The induction of ICAM-1 protein in transfected cells was assayed by immunophenotyping of viable cells, followed by flow cytometry using a Becton Dickinson FACSCalibur analyzer as described previously (19). Briefly, at 48 h after transfection, the cells were washed and stained with a phycocerythrin-conjugated monoclonal antibody to human CD54 (MA675PE; Serotec) at 4 °C for 60 min. The transfected population was marked by the expression of cotransfected EGFP-C1 plasmid, and this population was gated for analysis of ICAM-1 staining.

Detection of Proteins by Immunoblotting—Cells were washed in phosphate-buffered saline and lysed for 30 min on ice in lysis buffer. The lysates were centrifuged for 5 min at 13,000 × g. An aliquot of the clarified lysate was added to an equal volume of 2× gel sample buffer (0.1% Triton buffer, pH 6.8, 0.2% dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol, 0.1% bromphenol blue) and boiled for 2 min. The solubilized proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane for immunoblotting using an alkaline phosphatase chemiluminescent detection protocol (41). LMP1 was detected by first incubating the membranes for 1 h with 1 μg/ml CS.1–4 (42) in 1-Bloc (Tropix Inc.), followed by incubating for 1 h with a 1/10,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Bio-Rad 170-6481). The L-MB and EGGF-1BDN proteins were detected with 1 μg/ml rabbit polyclonal antibodies to L-MB (Santa Cruz sc371) followed by an alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad 170-6518). Specific antibody-protein complexes were detected using CDP-Star (Tropix Inc.) development reagent.

Kinase Assays—JNR and p38 in vitro kinase assays were performed as described previously (43). Cells were harvested at 3 h after transfection with either HA-p46SAPK/ycDNA3 or HA-p38, cells were sonicated in 500 μl of kinase lysis buffer (20 mM Tris, pH 7.6, 0.5% Triton X-100, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 2 mM sodium vanadate, 10 μg/ml apotinin, 10 μg/ml leupeptin, and 1 mM dithiothreitol). An aliquot of 250 μg of protein extract was used for immunoprecipitating HA-p38 or HA-JNK using 1 μg of anti-HA antibody (Roche Molecular Biochemicals; 12CA5) on Sepharose beads. The washes immunoprecipitates were
were then subjected to a kinase reaction, and phosphorylation was determined by Western blot. In the JNK assay, phosphorylation of Jun was determined by immunoblot analysis using phospho-c-Jun (Ser63) antibody (New England Biolabs 9261), and in the p38 assay phosphorylation of ATF2 was determined using phospho-ATF2 (Thr71) antibody (New England Biolabs 9221).

**Results**

**LMP1 Induces ICAM-1 Surface Expression and Promoter Activation**—LMP1 up-regulates ICAM-1 protein expression in various cell lines. This is illustrated in Fig. 1A, showing the results of flow cytometry analysis of ICAM-1 expression on Jurkat cells transfected with a control vector or with the SG5-LMP1 expression vector. The cells were cotransfected with a GFP expression vector so that the transfected population could be identified and gated to allow analysis of ICAM-1 expression following staining of the cells with phycoerythrin-conjugated CD54 antibodies. In this representative experiment, the vector control-transfected cells showed basal ICAM-1 surface expression with a mean fluorescence intensity of 29.4 arbitrary units (Fig. 1A, upper histogram), whereas cells transfected with 2 µg of SG5-LMP1 gave a mean fluorescence intensity of 81.3 (Fig. 1A, lower histogram). The LMP1-mediated induction of ICAM-1 protein in Jurkat T lymphocytes (Fig. 1B) and Eli-BL B lymphocytes (Fig. 1C) is dose-dependent. In the experiment illustrated in Fig. 1B, induction of ICAM-1 in Jurkat cells LMP1 caused a maximal 3-fold increase in ICAM1 protein expression. In separate independent experiments, the maximal induction of ICAM-1 by LMP1 in Jurkat cells typically ranged between 2- and 6-fold at 48 h after transfection. In Eli-BL cells (Fig. 1C), a similar magnitude of ICAM-1 induction by LMP1 was observed, but the dose-response curve differed in that Eli-BL cells were responsive to as little as 0.1 µg of SG5-LMP1 plasmid, whereas Jurkat cells required 5 to 10 times more plasmid to elicit a similar response. Nevertheless, the optimal induction of ICAM-1 surface expression in both cell lines was achieved at a plasmid concentration of between 1 and 5 µg of SG5-LMP1, which was used in subsequent experiments.

Since LMP1 is known to activate transcription factors, we investigated the effects of LMP1 on the ICAM-1 promoter activation. The 5’ region of the ICAM-1 gene is well described, and the transactivator sites and responsive regions for different members of the TNFα receptor family have been identified (30, 43). Only 1381 base pairs of the 5’-ICAM-1 gene region are required for the ICAM-1 induction by the proinflammatory cytokines TNFα, IL-1β, and γ-IFN. Fig. 2A shows a schematic structure of the promoter region with the potential transcription factor binding sites, and the characterized TNFα response region (TRE). The nuclear transcription factors involved in the regulation of ICAM-1 promoter include: Ap1, NF-κB, C/EBP, 

**Fig. 1. Induction of cell surface ICAM-1 protein expression by LMP1 in lymphocytes.** A, Jurkat cells were transfected with 2 µg of control vector (SG5) or an LMP1-expressing vector (SG5-LMP1), together with pEGFP-C to mark the transfected cell population. At 48 h after transfection, the ICAM-1 surface expression in GFP-positive cells was measured by staining with phycoerythrin-conjugated antibodies to CD54 and analyzing by flow cytometry. The upper histogram shows the basal ICAM-1 cell surface expression of vector control-transfected Jurkat cells, and the lower histogram shows the induced ICAM1 surface expression in Jurkat cells transfected with SG5-LMP1. The mean fluorescence intensity (m.f.i.) is indicated in each case. B, dose response of LMP1-induced up-regulation of ICAM-1 in the Jurkat T cell line. Increasing amounts of SG5-LMP1 plasmid were transfected into Jurkat cells and the ICAM-1 expression measured as in A. Data shown represent the mean values (± S.D.) of at least three independent experiments. C, dose response of LMP1-induced up-regulation of ICAM-1 in the Eli-BL B cell line, analyzed as for Jurkat in B. The results shown are the mean (± S.D.) of at least three independent experiments.

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Ets, STAT, and Sp1. The locations of their binding sites in the ICAM-1 5′-regulatory region are shown, together with the mapped AP2 and AP3 sites, and the translational start sites (CAT/TATA boxes).

As a member of the TNFR superfamily, LMP1 might be predicted to activate ICAM-1 using the TRE region within the ICAM-1 promoter. To test this possibility, we used a series of luciferase reporters regulated by different regions of the ICAM-1 promoter (30). The results of a representative experiment are shown (Fig. 2B) in which the luciferase plasmids were cotransfected with SG5-LMP1 into Eli-BL (black bars) and Jurkat (white columns) cells, and the reporter activities measured at 24 h after transfection. LMP1 induced the ICAM-1 reporters with a similar profile in both cell lines. The maximal luciferase induction of 5–6-fold was only obtained with the full-length 1.3ICAM1-luc construct. Mutants deleted for 779 or 1126 5′-bases (0.1-ICAM1) showed reduced activation levels but were still induced 3–4-fold by LMP1. In contrast, neither the reporter mutant deleted for the TRE (delTRE-ICAM1) nor the mutant deleted for 1248 5′-bases (0.1-ICAM1) was induced by LMP1. It should be noted that the nonresponsive 0.1-ICAM1 reporter contained the intact interferon response element (IRE), and we therefore tested the importance of this site for LMP1-induced ICAM-1 promoter activation. Using the IRE-negative mutant ICAM1 reporter, we always observed an increased background promoter activity of about 2-fold above the basic luciferase reporter activity, which suggests that this functional IRE site regulates basal ICAM-1 levels as well as the inducibility. However, the IRE-negative reporter was not induced further either by LMP1 or by TNFα (Fig. 3). Taken together, the results in Fig. 3 indicate that LMP1 differs from TNFα in utilizing regions upstream of the TRE to maximize the inducibility achieved from the TRE, and that within the TRE the variant IRE site is critical for induction both by LMP1 and by TNFα.

**Activation of NF-κB Is the Major Event in LMP1-induced Up-regulation of ICAM-1**—We wanted to further investigate the importance of the LMP1-induced NF-κB pathway in regulating ICAM-1. Therefore, we examined the effect of the NF-κB

**Comparison of the Effects of LMP1 and TNFα on the ICAM-1 Promoter**—Since the TRE has been shown to be essential and sufficient for TNFα-induced up-regulation of ICAM-1 (30), we tested mutant reporters with or without this region to investigate the similarities between LMP1 and TNFα. We analyzed the luciferase activity of 1.3ICAM1, TRE-ICAM1, and delTRE-ICAM1 (Fig. 2A) together with a full-length reporter in which the variant κB-site in the TRE had been inactivated by point mutation (NF-κBneg-ICAM1). In one representative experiment shown in Fig. 3, cells transfected with each of these reporters were cotransfected either with SG5-LMP1 or with SG5 vector; the SG5 vector transfectants were then treated with TNFα at 12 h after transfection and for 6 h prior to harvesting. The results in Fig. 3 show that LMP1 induced the TRE-ICAM1-luc reporter only to 58% of the full-length promoter activity, whereas TNFα stimulated the TRE-reporter to 140% of the full-length promoter activity. The reduced inducibility of the TRE-ICAM1 reporter by LMP1 suggests that, in contrast to TNFα stimulation, the TRE region is not sufficient for optimal induction of ICAM-1 by LMP1.
inhibitor protein, IκBα, upon LMP1-induced activation of the ICAM-1 promoter reporter and up-regulation of cell surface ICAM-1 protein. To enable endogenous and transfected IκBα to be distinguished, we designed a constitutive active IκBα that was deleted for the first 36 amino acids (thus removing the two regulatory phosphorylation sites) and fused to GFP (EGFP-IκBαDN; see Fig. 4A). Expression of this construct following transfection into Jurkat cells was determined by immunoblotting with a rabbit anti-IκBα antibody (Fig. 4B). In Jurkat cell extracts, this antibody always detected endogenous IκBα as low molecular mass bands, between 39 and 44 kDa, and the transfected EGFP-IκBαDN as a higher molecular mass band, between 68 and 70 kDa, of similar intensity to the endogenous IκBα. The inhibitory function of EGFP-IκBαDN on LMP1-induced NF-κB signaling was analyzed in reporter assays using the b-dependent luciferase reporters 3Enh-luc and IL-6(b)7-luc, which contain triple repeats of b response elements from the Igκ promoter and the IL-6 promoter, respectively. EGFP-IκBαDN was fully functional, being able to inhibit LMP1-induced activation of the 3Enh-luc reporter by 93%, and the IL-6(b)7-luc reporter by 94% (Fig. 4C).

Having confirmed the effectiveness of EGFP-IκBαDN as an inhibitor of the LMP1-induced NF-κB pathway, we investigated its effect on LMP1-induced ICAM-1 up-regulation. Increasing amounts of LMP1 with or without EGFP-IκBαDN were transfected into Jurkat lymphocytes, and the effects upon luciferase activity of the full-length 1.3ICAM-1 promoter reporter (Fig. 5A) as well as ICAM-1 protein expression (Fig. 5B) were measured. The results show that LMP1-induced ICAM-1 promoter activity was completely inhibited by EGFP-IκBαDN at low doses of SG5-LMP1 (≤1 μg), and was inhibited by about 80% at the highest input doses (2 and 4 μg) of SG5-LMP1. It should be noted that the constitutive SV40 promoter of the SG5-LMP1 plasmid itself is not completely unaffected by EGFP-IκBαDN. However, although EGFP-IκBαDN reduced LMP1 expression by up to 50% at the lowest doses of SG5-LMP1, at the higher doses, there was no significant effect on LMP1 expression (data not shown). The flow cytometry analysis of the cell surface ICAM-1 protein expression in the same transfected cell population revealed that LMP1 induction of ICAM-1 protein is completely ablated in the presence of the NF-κB inhibitor. These experiments were also performed with the Eli-BL B cell line and showed similar results (data not shown).

Activation of NF-κB Is Essential but Not Sufficient for ICAM-1 Induction—For confirmation that NF-κB is the major activator of ICAM-1 expression, we tested the effects of a constitutive activated form of MEKK1, a potent inducer of the NF-κB pathway. The active kinase expression plasmid, when transfected into Jurkat cells, was shown to induce the 3Enh-luc NF-κB dependent luciferase reporter by 110-fold (data not shown). Therefore, we transfected MEKK1 with and without SG5-LMP1 into Jurkat cells and measured its effect on the ICAM-1 reporter activation as well as on the expression of cell surface ICAM-1 protein. In the representative experiment shown in Fig. 6, MEKK1 induced the ICAM-1 full-length promoter (1.3ICAM-1) 465-fold over background level, which is 63 times higher than the LMP1-induced reporter luciferase activity (Fig. 6A). In contrast, flow cytometry analysis of cell surface ICAM1 protein expression (Fig. 6B) revealed that, although the cells transfected with LMP1 showed a substantial 10-fold increase in ICAM-1 mean fluorescence intensity, the cells transfected with MEKK1 alone showed no induction of ICAM-1 expression. Cotransfection of LMP1 and MEKK1 showed that the MEKK1 did not interfere with the ability of LMP1 to up-regulate ICAM-1 protein (Fig. 6B). These results show that NF-κB activation alone is sufficient for activation of the ICAM-1 reporter, but is not sufficient to effect up-regulation of ICAM-1 protein. This suggests that other signaling pathways of LMP1, in addition to NF-κB, are required to up-regulate ICAM-1 protein expression.

Other Signaling Pathways Known to Be Induced by LMP1 Are Not Required to Induce ICAM-1 Protein—In addition to NF-κB, the known LMP1-induced signaling pathways include the JNK pathway, which leads to activation of the c-Jun transcription activator; the p38 pathway, resulting in ATF2 translocation; and a JAK3/STAT pathway. The importance of these individual pathways in LMP1-mediated up-regulation of ICAM-1 was analyzed using specific inhibitors. To inhibit the JNK pathway, we used SEKDN, a dominant negative form of JNK kinase that is deleted for the phosphorylation site and is therefore unable to phosphorylate the Jun-activating kinase, JNK (13). To inhibit the p38 pathway, we used a chemical inhibitor SB203580, which specifically blocks p38 phosphorylation and translocation without affecting other signaling pathways (44). A chemical inhibitor was also used to specifically inhibit JAK3 (45). These inhibitors were all shown to affect their respective targets without affecting the expression of LMP1 from the SG5-LMP1 vector (Fig. 7A and data not shown). The inhibition of LMP1-mediated JNK activation with SEKDN was less efficient than was the inhibition of other pathways (Fig. 7A), but the 60–70% inhibition was similar to that previously reported by other workers (40). Having confirmed the functionality of the inhibitors, we analyzed their effects on LMP1-induced ICAM-1 protein (Fig. 7B). As a positive control for inhibition of ICAM-1 induction, we cotransfected EGFP-IκBαDN together with SG5-LMP1 in Jurkat cells. The results shown demonstrate that, although LMP1-induced up-regulation of ICAM-1 protein was completely inhibited by EGFP-IκBαDN, the other inhibitors tested did not affect the up-regulation of ICAM-1 protein.

MEKK1 Cannot Induce ICAM-1-mRNA—Since LMP1 and MEKK1 induce a similar subset of signaling pathways and MEKK1 very efficiently induces ICAM-1 promoter but was not able to induce ICAM-1 protein, we wanted to investigate those differences in more detail. The inability of MEKK1 to induce ICAM-1 surface expression suggests that LMP1 regulates additional pathways. To further analyze the level at which LMP1 and MEKK1 signal differed, we investigated the effects of MEKK1 on ICAM-1 mRNA levels. DG75 cells were transfected
with MEKK1 or LMP1 together with rat CD2 to identify the transfected cells. Cells were incubated at 37 °C for 24 h to allow expression. The transfected cells were stained with OX34 monoclonal antibody to rat CD2, and were separated by immunomagnetic beads. This resulted in greater than 90% purity of rat CD2-positive cells as assayed by fluorescence-activated cell sorting (data not shown). Total RNA from the purified cells was isolated and mRNA for ICAM-1 was assayed by RNase protection using specific ICAM-1 probes. The results of these experiments are shown in Fig. 8. The upper panel shows a graphic representation of a single experiment and clearly shows that LMP1 can up-regulate ICAM-1 mRNA (compare second lane with first lane). However MEKK1 (third lane) could not induce ICAM-1 mRNA. The lower panel shows the average mRNA levels from the phosphorimager analysis of three different experiments. Although LMP1 induced ICAM-1 mRNA 2–3-fold, MEKK1 had no effect on the ICAM-1 mRNA levels. These data suggest that MEKK1 lacks a key event required for the effective transactivation of the ICAM-1 gene.

CTAR1 and CTAR2 Provide Qualitative Different Signals to ICAM-1—LMP1 regulation of ICAM-1 clearly requires as yet uncharacterized signals in addition to NF-κB. The full nature of these signals remains elusive. In the case of LMP1 signaling, the cooperation of the C-terminal activator regions CTAR1 and CTAR2 has been shown to be essential for LMP1 function (19). Furthermore, histone acetylation of genes and their promoters was identified to be involved in coordinate regulation of transcription (46). We wanted to test if these mechanisms could also be important for LMP1-induced ICAM-1 up-regulation. Therefore, we investigated two point mutants of LMP1, defective for either one (CTAR1−) or the other (CTAR2−) C-terminal activator region and MEKK1 in the presence of a chemical compound that has been shown to inhibit histone deacetylation and thus prevent gene silencing. ELi-BL cells were transfected with control or LMP1−, CTAR1−, CTAR2−, and MEKK1-expressing plasmid together with EGFP-C1 to control for transfection. After 24 h the cells were stimulated with sodium butyrate, and, after an additional 12-h incubation, the ICAM-1 surface expression was analyzed using flow cytometry. The ICAM-1 surface expression of these transfected cells is shown in Fig. 9 as mean values of three independent experiments. LMP1 induces ICAM-1 expression as seen before (column 2 compared with column 1). The CTAR1− mutant (column 3), which has intact CTAR2 and CTAR3 domains, induced only half of the ICAM-1 protein compared with LMP1, and the CTAR2− mutant with intact CTAR1 and CTAR3 induced even less (column 5). However, after incubation with the deacetylation inhibitor sodium butyrate, only the CTAR1−-transfected cells showed ICAM-1 expression comparable to wild type LMP1 levels (column 4 compared with column 2), whereas the ICAM-1 levels of the CTAR2− transfected cells remained low. In the case of the MEKK1-transfected cells, the ICAM-1 expression levels showed no significant difference between cells treated with or without the deacetylation inhibitor. The ICAM-1 surface expression in MEKK1-transfected cells remained at basal levels (columns 7 and 8).

These data suggest that the deacetylation inhibitor, sodium butyrate, can cooperate with CTAR2 and -3 to generate LMP1 wild type ICAM-1 expression. Therefore, we conclude that LMP1 induces a novel additional signal or pathway to induce
ICAM-1 protein, which can be mapped to the CTAR1 region of LMP1.

**DISCUSSION**

Although it has been recognized for some time that the EBV-encoded LMP1 is responsible for the up-regulation of ICAM-1 (10), the mechanism of activation was unclear. The biological and signaling properties of LMP1 share many features with the pro-inflammatory cytokines TNFα and γ-IFN (9, 16–18), both of which transcriptionally regulate ICAM-1 expression through distinct regulatory elements in the ICAM-1 promoter. Our results show that LMP1 acts primarily through the TRE located at −178 bp to −227 bp, but not the interferon response element located at −76 bp to −66 bp. However, LMP1 and TNFα are subtly different in their regulation of ICAM-1 (Fig. 3), which highlights the complex range of signaling pathways and transcription factors involved in ICAM-1 activation. Consistent with this observation, although a similar range of signal transduction factors are known to associate with LMP1 and TNFR1, and an overlapping spectrum of signaling pathways are activated, there are important differences. For example, the death domain at the C terminus of TRADD is involved in TNFRI/TRADD interaction, whereas LMP1 recruits TRADD via its N-terminal domain, and the different topology of TRADD binding affects the mechanisms by which LMP1 and TNFR1 activate NF-κB and JNK (47, 48).

A direct comparison of LMP1 and TNFα signaling (Fig. 3) showed that the TRE is both essential and sufficient for TNFα-induced ICAM-1, as reported previously (30); in contrast, this site was essential but not sufficient for optimal induction by LMP1 (Figs. 2 and 3). Taken together, these data suggest that LMP1 might differ from TNFα by targeting additional transcription activation site(s) located upstream of the TRE between −574 bp and −1381 bp. In this respect, potential sites include an AP1 binding site at position −1284 and a second NF-κB site at position −531. Other upstream regulatory enhancer elements have also been described, which are thought to be more important for constitutive ICAM-1 expression rather than for inducible ICAM-1 (29), although it is possible that they have a role in LMP1 induction of ICAM-1.

Whatever the role of upstream elements, the TRE appears to be critically involved in inducible regulation of ICAM-1 by various stimuli. Within the TRE, a critical feature is the variant NF-κB binding site, which is essential for transcriptional up-regulation of ICAM-1 mediated by LMP1 (Fig. 3), TNFα (30), and other cytokines (24). The critical role for NF-κB in LMP1-mediated up-regulation of ICAM-1, suggested by our analysis with mutant reporters, was supported by complementary experiments showing that a constitutively active IκBα efficiently abolished LMP1-mediated up-regulation both of the full-length 1.3ICAM-1 reporter and expression of ICAM-1 protein (Fig. 5). Our results shed new light on an area of confusion since two previous studies have reported the use of IκBα to inhibit the ability of LMP1 to up-regulate ICAM-1 protein expression, but there was disagreement about the efficiency of this effect (49, 50). Our data, in line with those of Devergne and colleagues (50), show that efficient blocking of NF-κB activation can completely abrogate the ability of LMP1 to up-regulate ICAM-1 protein.

Despite the essential role for NF-κB, it is clear that activation of this transcription factor alone is unable to induce ICAM-1 protein expression. Thus, we have observed previously that transfection and overexpression of p50 and p65 NF-κB species has no effect upon ICAM-1 protein expression in lymphoid cells. We now show that transfection of active MEKK1,
whose effects include activation of NF-κB besides AP-1 and p38 pathway inhibitors, SEKDN and PB203580. Jurkat cells were transfected with control vector (SG5) and SG5-LMP1 in the presence and absence of the inhibitors. At 48 h after transfection, immunocomplex kinase assays were carried out to measure kinase activity using GST-Jun-(1–79) (left panel) or GST-ATF2-(19–96) (right panel) as substrates. Three independent experiments were performed and gave similar results. B, Jurkat cells were transfected with EGFP-C1 and different amounts of SG5-LMP1 DNA with and without dominant negative SEK (SEKDN) or EGFP-IκBαDN plasmids or were treated with 20 μM p38 inhibitor SB203580 or 25 μg/ml JAK3 (Calbiochem) inhibitor as described under “Materials and Methods.” After 48 h, GFP-positive transfected cells were assayed by flow cytometry for ICAM-1 surface expression. The results are shown as ICAM-1 mean fluorescence intensity and are representative of three independent experiments.

FIG. 7. Effects of inhibitors of LMP1-induced signaling. A, kinase assays were carried out to confirm the functionality of the Jun and p38 pathway inhibitors, SEKDN and PB203580. Jurkat cells were transfected with control vector (SG5) and SG5-LMP1 in the presence and absence of the inhibitors. At 48 h after transfection, immunocomplex kinase assays were carried out to measure kinase activity using GST-Jun-(1–79) (left panel) or GST-ATF2-(19–96) (right panel) as substrates. Three independent experiments were performed and gave similar results. B, Jurkat cells were transfected with EGFP-C1 and different amounts of SG5-LMP1 DNA with and without dominant negative SEK (SEKDN) or EGFP-IκBαDN plasmids or were treated with 20 μM p38 inhibitor SB203580 or 25 μg/ml JAK3 (Calbiochem) inhibitor as described under “Materials and Methods.” After 48 h, GFP-positive transfected cells were assayed by flow cytometry for ICAM-1 surface expression. The results are shown as ICAM-1 mean fluorescence intensity and are representative of three independent experiments.

FIG. 8. RPA analysis of ICAM-1 mRNA levels in transfected DG75 cells. DG75 cells were transfected with rCD2GFP and either control vector or LMP1- or MEKK1-expressing plasmids. After 48 h, the cells were immunomagnetically sorted for CD2 expression. The RPA was performed on total RNA samples prepared from those positive sorted cells. The upper panel shows a representative RPA autoradiogram (8-h exposure) of ICAM-1 mRNA levels in the individual samples indicated above each track. Levels of ICAM-1 mRNA were quantitated on a phosphorimager. The data shown in the lower panel represent ICAM-1 mRNA levels relative to the housekeeping gene L32 mRNA levels. Three independent experiments were performed and gave similar results (S.D. for control mRNA levels was less than 10%).

FIG. 9. Effects of deacetylase inhibitor on induced ICAM-1 protein expression. Analysis of ICAM-1 surface expression in Eli-BL cells transfected with EGFP-C1 marker plasmid and either control plasmid, LMP1, LMP1 mutants (CTAR12 mutated at amino acids 204, 206, and 208; CTAR22 construct mutated at amino acid 384), or MEKK1-expressing plasmid. After 24 h cells were treated with sodium butyrate (NaB, 1 mM). ICAM-1 protein expression in GFP-positive cells was assayed by flow cytometry after an additional 12-h incubation. Bars represent the average (± S.D.) of the ICAM-1 mean fluorescence intensity (×1000) in three independent experiments.

Regulation of ICAM-1 Expression by LMP1
ICAM-1 protein expression (Fig. 6) is intriguing. There are a number of possible factors that could be involved, including mRNA stabilization, promoter accessibility regulated by nucleosomes, and various post-transcriptional mechanisms. With regard to mRNA stability, both phosphor esters and γ-IFN have been shown to stabilize the otherwise labile ICAM-1 mRNA in murine fibroblast and monocytic cell lines (54, 55). Furthermore, stabilization of mRNA has been shown to be a feature of LMP1-mediated up-regulation of Bf-1 in lymphocytes as by as yet unknown mechanisms (8). However, using the same human lymphoid cell model (BJAB cells transfected with a tetracycline-regulated LMP1 expression vector) that was used to demonstrate Bf-1 mRNA stabilization, we found that LMP1 does not affect the stability of ICAM-1 mRNA (data not shown).

The major difference regarding MEKK1 and LMP1 lies in the ability to induce mRNA, since LMP1 induces ICAM-1 mRNA 2–3-fold but MEKK1-transfected cells have no increased ICAM-1 mRNA levels (Fig. 8). Thus, the LMP1-specific effect was shown to be critical for induction of ICAM-1 protein by LMP1 (Fig. 7), sodium butyrate was unable to cooperate with the NF-κB activated by the LMP1-CTAR2 mutant or by MEKK1 (Fig. 9). There are several possible explanations for this; one being that there is yet another unknown signaling function of LMP1. The precise analysis of these new features of LMP1 signaling is ongoing.

In summary, the present study demonstrates that NF-κB is a key signaling pathway stimulated by LMP1 in the transcriptional regulation of ICAM-1, and that the other known signaling pathways activated by LMP1 appear not to be critical for up-regulation of ICAM-1. However, NF-κB activation is not sufficient by itself to up-regulate ICAM-1 protein. We described a new cooperating signaling mechanism induced by LMP1, which may act at the level of promoter accessibility and maps to the CTAR1 domain of LMP1. Further characterization of this pathway and the detailed analysis of the signal-regulated acetylation event and gene transcription are the next critical steps toward our understanding of LMP1 function.

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Characterization of Intercellular Adhesion Molecule-1 Regulation by Epstein-Barr Virus-encoded Latent Membrane Protein-1 Identifies Pathways That Cooperate with Nuclear Factor κB to Activate Transcription

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