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Epstein-Barr Virus Latent Membrane Protein Transactivates the Human Immunodeficiency Virus Type 1 Long Terminal Repeat through Induction of NF-κB Activity

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The Epstein-Barr virus latent membrane protein (LMP) is an integral membrane protein that is expressed in cells latently infected with the virus. LMP is believed to play an important role in Epstein-Barr virus transformation and has been shown to induce expression of several cellular proteins. We performed a series of experiments that demonstrated that LMP is an efficient transactivator of expression from the human immunodeficiency virus type 1 long terminal repeat (HIV-1 LTR). Mutation or deletion of the NF-κB elements in the LTR abolished the transactivation, indicating that the LMP effect on HIV expression was due to induction of NF-κB activity. Experiments in which the HIV-1 Tat protein was coexpressed in cells together with LMP showed that Tat was able to potentiate the transactivation. Surprisingly, a synergistic effect of the two proteins was observed even in the absence of the recognized target region for Tat (TAR) in the HIV-1 LTR.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus. EBV infects B lymphocytes and certain epithelial cells in vivo and is the causative agent of infectious mononucleosis. In addition, the virus is strongly associated with both the endemic form of Burkitt's lymphoma and lymphoma in immunocompromised individuals (12). It also shows a strong link with undifferentiated nasopharyngeal carcinoma.

EBV is an efficient agent for immortalization of B cells in vitro. The virus is maintained in a latent state in these cells, and only a small subset of viral genes is expressed. One of these genes gives rise to a 386-amino-acid protein known as the latent membrane protein (LMP) (27, 29, 49). LMP is an integral membrane protein predicted to have six membrane-spanning domains and a large cytoplasmic domain (27). The protein forms patches in the membrane of cells and has also been found to associated with the detergent-insoluble fraction of the cytoskeleton (26).

LMP is believed to play a crucial role in EBV transformation. Rodent fibroblasts expressing the LMP gene product are tumorigenic in nude mice (49), and introduction of the LMP gene can also alter the phenotype of EBV-negative human lymphoblastoid cell lines (50). At the molecular level, LMP has been shown to induce expression of the B-cell activation markers CD23 and CD40 as well as several cellular adhesion molecules (LFA1, LFA3, and ICAM1) (51).

Because of the lack of suitable tissue culture systems, relatively little is known about the interaction of EBV with epithelial cells. However, LMP is regularly expressed in nasopharyngeal carcinoma cells, and it was recently shown that introduction of the LMP gene into human epithelial cells can inhibit the differentiation of these cells (9). Furthermore, transgenic mice that express LMP in epithelial skin cells develop a dermatological disease, indicating a disturbance of the differentiation process (52).

Despite the many studies dealing with phenotypic effects of LMP, the actual molecular mechanisms by which the protein exerts these effects remain to be elucidated. However, on the basis of its localization to the cytoplasmic membrane, its rapid turnover, and its association with the cytoskeleton, it has been hypothesized that LMP may be involved in growth regulation by affecting signal transduction pathways (2, 3, 30).

Here we show that LMP efficiently transactivates expression from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR). The effect maps specifically to the NF-κB elements upstream of the HIV-1 promoter, indicating that LMP is able to induce κB-specific activity. The transactivation is significantly potentiated by the HIV-1 Tat protein even in the absence of the identified target region for this protein (TAR) (42).

MATERIALS AND METHODS

Cell lines and DNA transfections. Raji is an EBV-positive Burkitt's lymphoma cell line, and DG 75 is an EBV-negative Burkitt's lymphoma cell line. These B-lymphoid cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. Monkey CV-1 cells were maintained as monolayers in Iscove's medium supplemented with 10% bovine calf serum. All cells were transfected by modifications of the DEAE-dextran procedure as previously described (19).

Plasmids and plasmid constructions. pSVLMP was constructed by inserting the *Bam*HI-N_{het} fragment of EBV (strain B95-8) into the unique *Xho*I site of the simian virus 40 (SV40) based vector pSVEpR4 after treatment of both vector and insert with T4 DNA polymerase. The construction of pSVEpR4 was described previously (19). The vector pBABY is a derivative of pSVEpR4 (39). pSVSX1 contains the *Sal*I-*Xho*I fragment of HIV-1 (BH10 isolate) inserted into the unique *Xho*I site of this vector (39). pUR-III CAT was originally obtained from W. Haseltine (42). Chloramphenicol

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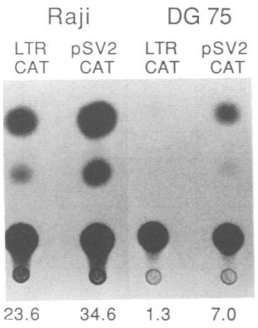

FIG. 1. CAT assays of extract from transfected lymphoid cells. DG75 (EBV-negative) or Raji (EBV-positive) B-lymphoid cells were transfected with plasmids containing the CAT gene under the control of either the HIV-1 LTR (pUR-III CAT) or the SV40 early promoter (pSV2CAT) as indicated. The cells were harvested 48 h posttransfection, and extracts from 10^5 cells were analyzed for CAT activity. The numerical values indicate percent acetylation.

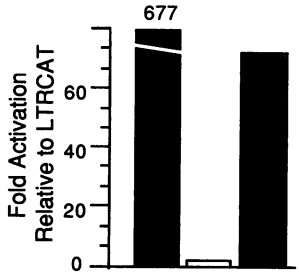

FIG. 2. CAT levels in CV-1 cells cotransfected with pUR-III CAT and plasmids expressing either the EBV LMP protein or the HIV-1 Tat protein or the control plasmid pBABY. Cells were harvested 48 h posttransfection and analyzed for CAT activity as described in Materials and Methods. The graphs represent the average of three independent transfection experiments and show the CAT activity relative to the levels in cells transfected with pUR-III CAT alone. ■, pSVSX1 (Tat); □, pBABY; ▨, pSVLMP.

acetyltransferase (CAT) plasmids containing deleted and mutated HIV-1 LTRs were kind gifts from D. Capon and G. Nabel, respectively (33–35).

CAT assays. The CAT assays were performed essentially as described previously (16). Briefly, the cells were harvested 48 h posttransfection and were sonicated in 0.25 M Tris (pH 7.8). The reactions were done in 0.25 M Tris (pH 7.8) containing 0.53 mM acetyl coenzyme A. The amount of ^{14}C-labeled chloramphenicol added was 0.025 μCi per assay. The reaction mixtures were incubated at 37°C for 30 min. Products of the reactions were extracted with ethyl acetate and analyzed by thin-layer chromatography in chloroform-methanol (95:5). The chromatogram was then sprayed with En^{3}Hance (NEN Research Products) and exposed to X-ray film at −70°C. For quantitation, the spots containing the nonacetylated and different acetylated forms were cut out, and their radioactivities were counted in a liquid scintillation counter.

RESULTS

HIV-1 LTR works efficiently in lymphoblastoid cells immortalized by EBV. Several studies have implicated EBV as a cofactor in the development of AIDS and AIDS-related lymphomas (5, 7, 17, 18, 23, 31, 38, 40). Furthermore, HIV-1 has been shown to replicate efficiently in several EBV-containing lymphoblastoid cell lines (32, 43, 48). To investigate whether this was reflected in high levels of basal expression from the viral LTR in such cells, we transfected two Burkitt's lymphoma cell lines, the EBV-positive cell line Raji and the EBV-negative cell line DG75, with a CAT reporter plasmid containing the HIV-1 LTR (pUR-III CAT) (42). As a control, both cell lines were also transfected with the plasmid pSV2CAT in which expression of the CAT gene is directed by the SV40 early promoter (16). The cells were harvested 48 h posttransfection and assayed for CAT activity (see Materials and Methods). As shown in Fig. 1, significantly higher levels of CAT activity were obtained after transfection of pUR-III CAT into Raji cells compared with DG75 cells. As can be seen in Fig. 1, pSV2CAT also gave higher levels in Raji cells, which might be an indication that the transfection efficiency was higher in these cells than in the DG75 cells. However, when the values were normalized assuming that the levels of the expression from the early SV40 promoter were the same in both cell lines, pUR-III CAT still gave 3.4 times higher activity in the Raji cells. Similar results were obtained with other EBV-positive and -negative cell lines (data not shown).

These results suggested that one or more of the latent EBV proteins was able to directly or indirectly transactivate expression from the HIV-1 LTR. Since LMP has been previously shown to induce expression of some cellular proteins, this protein was an obvious candidate. To test whether LMP was indeed able to transactivate HIV-1 expression, we introduced the LMP gene into the SV40 late replacement vector pSVEpR4 to create the plasmid pSV-LMP (see Materials and Methods). This vector system was previously used to express large amounts of the EBV EBNA1 protein as well as several HIV proteins in CV-1 and COS cells (19, 39, 45). When CV-1 cells were transiently transfected with pSVLMP, they expressed large amounts of the LMP protein (apparent molecular mass, 60 kDa), as shown by Western blot (immunoblot) and immunoprecipitation experiments with LMP-specific antibodies (data not shown). In addition, immunofluorescence analysis of transfected cells showed a specific staining of the cytoplasmic membrane in accordance with previous results (27).

To test whether LMP was able to transactivate expression from the HIV-1 LTR, we then transfected pSVLMP into CV-1 cells together with pUR-III CAT. Experiments in which LTR-CAT was transfected alone, in conjunction with an SV40 vector lacking an insert (pBABY), or together with a plasmid expressing the HIV-1 Tat protein (pSVSX1) were also performed. The cells were harvested 48 h posttransfection and assayed for CAT activity. A summary of results from three independent transfections is presented in Fig. 2. In these experiments, the CAT levels obtained when pSV-LMP was cotransfected with pUR-III CAT were on the average 70 times higher than those in cells transfected with pUR-III CAT alone, indicating that LMP was indeed able to efficiently transactivate expression from the HIV-1 LTR. As expected, the levels of CAT in cells transfected with pSVSX1 were also very high compared with the levels in cells transfected with the LTR-CAT plasmid alone. No significant transactivation was seen with the vector lacking an insert (pBABY).

The LMP effect maps to the NF-κB elements in the HIV-1 LTR. To investigate whether the effect of LMP could be mapped to a specific region within the HIV-1 LTR, we next performed an analysis using a series of LTR mutants with

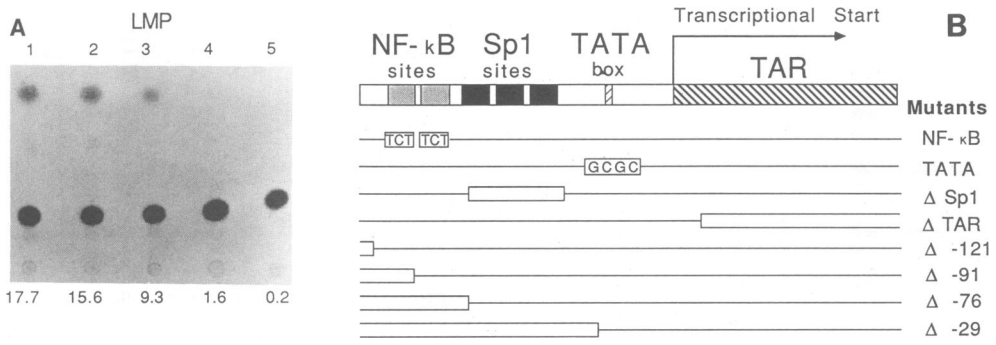

FIG. 3. (A) Thin-layer chromatogram showing CAT activity in cells after cotransfection of pSVLMP with LTR-CAT reporter plasmids containing progressive 5′ deletions in the HIV-1 LTR. The plasmids used contained the following HIV sequences relative to the HIV-1 transcription start site: lane 1, −525 to +232; lane 2, −121 to +232; lane 3, −91 to +232, lane 4, −76 to +232; lane 5, −29 to +232. The values below the chromatogram indicate the percentages of chloramphenicol that were acetylated. (B) Schematic representation of the HIV-1 LTR mutant plasmids used in this study. The 5′ deletion mutants (33) were a gift from D. Capon. The NF-κB, Sp1, TATA, and TAR mutants (34, 35) were a gift from G. Nabel.

progressive deletions from the 5′ end of the LTR (Fig. 3B) (33). The results of experiments in which these mutants were cotransfected into CV-1 cells together with pSVLMP are shown in Fig. 3A. A deletion that removed the sequences between −525 and −121 relative to the transcription start site had no significant effect on the amount of transactivation. In contrast, a further deletion to −91 reduced it to about half, whereas a deletion to −76 virtually obliterated the transactivation. The region between −121 and −91 contains a known binding site for NF-κB transcription factors, and the region between −91 and −76 contains a second NF-κB-binding site (Fig. 3B). Thus, the results of these experiments strongly suggested that LMP mediated its effect on the HIV-1 LTR through the NF-κB elements.

To further test this hypothesis, we performed additional transactivation experiments using mutants with mutations in specific regions of the HIV-1 LTR (Fig. 3B) (34, 35). In these experiments, the effect of LMP was compared with that of the HIV-1 Tat protein, which has been shown to transactivate the HIV-1 LTR specifically through the TAR element downstream of the HIV promoter (46). The results of this analysis are summarized in Fig. 4. As expected, deletion of TAR had a dramatic effect on Tat transactivation. In contrast, the transactivation observed with LMP was only slightly reduced with this mutant compared with the wild-type LTR plasmid. Deletion of the Sp1 sites or mutation of the TATA element also reduced the level of CAT observed in response to the Tat protein rather dramatically. This has been previously described and is likely to reflect the fact that both of these elements are essential components of the basal HIV promoter (13, 42). Consequently, the CAT levels observed with these constructs in conjunction with pSVLMP were also lower than those obtained with the wild-type LTR. However, in both cases the levels with LMP were higher than those obtained with the Tat protein. This indicated that the LMP effect did not directly map to either of these regions.

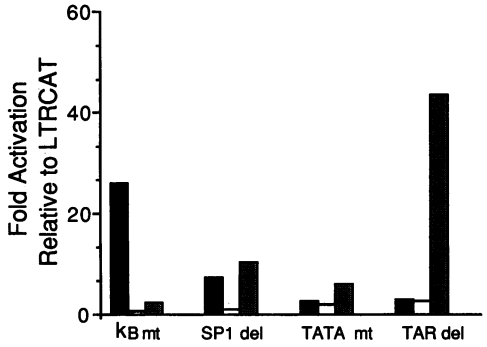

FIG. 4. LMP transactivation of HIV-1 LTR plasmids with mutations in specific regions of the LTR. Cells were transfected with the indicated plasmids (Fig. 3B) and analyzed as described in the legend to Fig. 2. The graphs represent the average of three independent transfection experiments. ■, pSVSX1 (Tat); □, pBABY; ▩, pSVLMP.

The LTR CAT plasmid that contained a specific mutation (GGG to TCT) in both copies of the NF-κB-binding sites was still efficiently transactivated by Tat. However, no significant transactivation was observed when pSVLMP was cotransfected with this plasmid. This NF-κB mutant plasmid was previously shown to be refractory to active NF-κB induced by either mitogen or phorbol ester stimulation (34). The results of these experiments thus confirmed the hypothesis that LMP acts through the NF-κB elements and indicate that LMP is able to specifically induce NF-κB activity.

The LMP effect is potentiated by the HIV-1 Tat protein even in the absence of the TAR element. We next performed experiments in which the Tat-expressing plasmid pSVSX1 and pSVLMP were cotransfected into cells together with the different mutated LTR constructs. A summary of these results is shown in Fig. 5.

Cells transfected with pSVSX1 and pSVLMP together with the NF-κB mutant plasmid showed slightly lower CAT activity than cells transfected with this mutant and pSVSX1 alone. In contrast, severalfold-higher levels of CAT were obtained when pSVSX1 and pSVLMP were transfected with either the Sp1 or TATA mutant than with either of these plasmids alone. This suggests that Tat and LMP show a synergistic effect in their transactivation of the HIV-1 LTR. These results provide further confirmation that the effect of LMP maps specifically to the NF-κB elements. Surprisingly, the levels of CAT expressed from the TAR mutant were also significantly higher in cells expressing both Tat and LMP than in cells expressing LMP alone. Thus, Tat has additional effects in the presence of NF-κB activity induced by

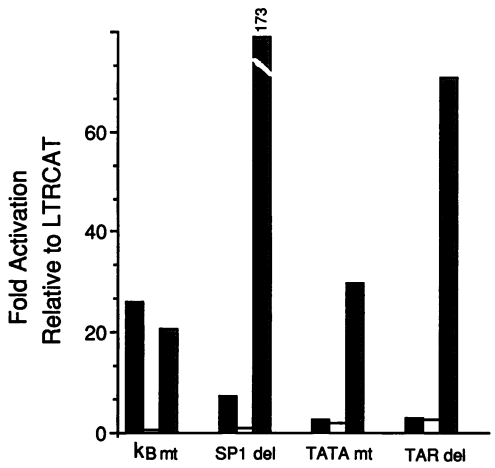

FIG. 5. Transactivation of specific HIV-1 LTR mutants by the LMP protein in conjunction with HIV-1 Tat. Cells were transfected with the indicated plasmids and analyzed as described in the legend to Fig. 2. The graphs represent the average of three independent transfection experiments. ■, pSVSX1 (Tat); □, pBABY; ▨, pSVSX1 plus pSVLMP.

pSVLMP beyond those mediated by binding of Tat to the TAR element.

DISCUSSION

The experiments presented here show that the EBV LMP efficiently transactivates expression from the HIV-1 LTR. This may contribute to the ability of HIV-1 to replicate efficiently in EBV-positive B-cell lines (32, 43, 48). Recently, we have found that a second EBV protein expressed in latently infected cells, EBNA2, is also able to transactivate HIV-1 expression through activation of the HIV-1 LTR. The effect of this protein maps outside of the NF-κB elements (44a). Previously, another EBV protein (the BZLF1 gene product) was reported to efficiently transactivate HIV-1 expression (28). However, since this protein is not expressed in latently infected cell lines, it is not likely to play a significant role for replication of HIV in such lines unless the HIV infection induces its expression.

The fact that the effect of LMP maps specifically to the NF-κB elements suggests that LMP is able to induce active NF-κB in the transfected cells. NF-κB was first detected by its binding to a specific sequence in the immunoglobulin κ-gene enhancer and was shown to be an efficient transactivator of transcription from the κ-gene promoter (44). Identical or related DNA sequences capable of binding NF-κB have since been found in the enhancer regions upstream of several additional promoters including many viral promoters (reviewed in reference 24). Cloning of genes encoding proteins involved in NF-κB activity has shown that NF-κB, rather than being a single protein, is derived from a family of related genes. These genes all show partial homology to the proto-oncogene c-*rel* and the *Drosophila* morphogen *dorsal* (4).

Several different agents are able to induce NF-κB activity that stimulates transcription from the HIV-1 LTR. These include phorbol esters, UV irradiation, and tumor necrosis factor α (10, 21, 34, 47). It also appears that several viruses including at least two of the other human herpesviruses (herpes simplex virus and human herpesvirus 6) can activate HIV-1 through this element (11, 15).

Many of the agents shown to induce active NF-κB have been postulated to mediate their effects through activation of protein kinases such as protein kinase C and calcium-dependent kinases. Protein kinase C is of special interest since it has been specifically shown to inactivate IκB, an inhibitor of NF-κB that retains heterodimers of NF-κB p50 and p65 in the cytoplasm, through phosphorylation of this protein (14). This in turn leads to the dissociation of the NFκB-IκB complex, leaving NFκB free to migrate into the nucleus. We are presently performing transfection experiments using specific inhibitors of protein kinase C. Preliminary results indicate that LMP activates NF-κB through a protein kinase C-independent pathway. Since cells stably transfected with the LMP gene have been reported to show increased levels of intracellular Ca^{2+} (50), it seems possible that LMP activates NF-κB through activation of calcium-dependent kinases.

In most cells, Tat transactivation of the HIV-1 LTR is absolutely dependent on the TAR element downstream of the HIV promoter. It has been shown that Tat binds to this element in the RNA and enhances RNA elongation and probably also further initiation of transcription (for a recent review, see reference 22). In this study, we observed a synergistic effect of Tat and LMP even in the absence of TAR. Such TAR-independent Tat effects have been previously observed after HIV-1 infection of phorbol ester-treated T cells (20), which have been shown to contain large amounts of active NF-κB. Thus, Tat seems to have additional effects on the HIV-1 promoter that are observed only in the presence of active NF-κB.

NF-κB is believed to play an important role in B- and T-cell activation. Active NF-κB is constitutively expressed in both immunoglobulin-producing B cells and mitogen- and antigen-activated T cells. EBV infects small resting B cells, leading to activation and immortalization of these cells. The phenotype of immortalized cells is similar to that of antigen- and mitogen-activated B cells (37). On the basis of the results presented here, it seems conceivable that induction of NF-κB by LMP is an important step in the EBV-mediated activation. In this context, it should be mentioned that the Tax protein of human T-cell leukemia virus type I (HTLV-I), a virus that infects, activates, and immortalizes T cells, also has been shown to be an effective inducer of NF-κB (6, 8, 25). In addition, it was recently reported that HIV-1 is capable of inducing NF-κB in certain cells (1, 41). Thus, NF-κB activation seems to be a common denominator for several lymphotropic viruses.

For HTLV-I, it has been speculated that abnormal induction of NF-κB causes unregulated T-cell growth and that such proliferation might predispose the infected T lymphocytes to a second transforming mutation leading to frank leukemia (24). In addition, a chromosomal translocation involving an NF-κB gene (lyt), with concomitant activation of expression of this gene, was recently reported in three cases of human lymphoma (36). Preliminary experiments in our laboratory indicate that mutants of LMP defective for transformation also fail to transactivate the HIV-1 LTR. It thus seems conceivable that induction of NF-κB activity may play a key role in EBV transformation. However, confirmation of this hypothesis and the exact role that NF-κB plays in the replication of lymphotropic viruses awaits further experimentation.

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