Identification and Characterization of CCAAT Enhancer-Binding Protein (C/EBP) as a Transcriptional Activator for Epstein-Barr virus Oncogene Latent Membrane Protein 1

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Running head: C/EBP activates LMP1 transcription

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Background: Expression mechanism of EBV oncogene LMP1 is not fully understood.

Results: C/EBP was newly isolated to enhance LMP1 promoter in our transient assay system.

Conclusion: C/EBP transactivate LMP1 promoter at physiological levels.

Significance: This is the first report that showed the significance of C/EBP on LMP1 expression.

SUMMARY

Epstein-Barr virus LMP1, a major oncoprotein expressed in latent infection, is critical for primary B cell transformation, functioning as a TNFR family member by aggregation in the plasma membrane resulting in constitutive activation of cellular signals, such as NF-κB, MAPK, JAK/STAT and AKT. While transcription of LMP1 in latent type III cells is generally under the control of the viral coactivator EBNA2, little is known about EBNA2-independent LMP1 expression in type II latency. We thus screened a cDNA library for factors that can activate the LMP1 promoter in an EBNA2-independent manner, using a reporter assay system. So far, we have screened >20,000 clones, and here newly identified C/EBPε as a transcriptional activator. Exogenous expression of C/EBPα, β or ε efficiently augmented LMP1 mRNA and protein levels in EBV-positive cell lines, while other members of the C/EBP family exhibited modest or little activity. It has been demonstrated that LMP1 gene transcription depends on two promoter regions: proximal (ED-L1) and distal (TR-L1). Interestingly, although we first used the proximal promoter for screening, we found that C/EBP increased transcription from both promoters in latent EBV-positive cells. Mutagenesis in reporter assays and EMSA identified only one functional C/EBP binding site, through which activation of both proximal and distal promoters is mediated. Introduction of point mutations into the identified C/EBP site in EBV-BAC caused reduced
**LMP1 transcription from both LMP1 promoters in epithelial cells. In conclusion, C/EBP is a newly identified transcriptional activator of the LMP1 gene, independent of the EBNA2 coactivator.**

The Epstein-Barr virus (EBV) is a human gamma-herpesvirus that mainly infects and establishes latent infection in B lymphocytes, but it also can infect other types of cells, including NK, T and epithelial cells. Infection of EBV has been implicated in a variety of malignancies, and the expression pattern of viral latent genes varies depending on the tissue of origin and the state of the tumors. Neoplasms such as Burkitt’s lymphoma or gastric carcinoma express only the EBER and EBNA1 (type I latency), whereas some Hodgkin lymphomas, nasopharyngeal carcinomas (NPC) and NK/T lymphomas produce EBER EBNA1, LMP1 and LMP2 genes (type II latency). In addition to the type II genes, EBNA2, EBNA3 and EBNA-LP are also expressed in immunosuppression-related lymphomas or lymphoblastoid cell lines (LCLs) (type III latency).

EBV latent infection integral membrane protein 1 (LMP1) is frequently expressed in latent EBV infections associated with B cell proliferation and with NPC. It is uniformly expressed in latency III EBV infection with human B lymphocyte proliferation in vitro, in resultant LCLs, in primary human infection in vivo, and in lymphoproliferative disorders (LPD) in transplant recipients. LMP1 is also expressed in latent II EBV infection in Hodgkin’s disease B lymphocytes and in NPC epithelial cells.

Since it functions as a constitutive TNFR family member by aggregation in the plasma membrane, resulting in constitutive activation of cellular signaling, through NF-κB, MAPK, JAK/STAT and AKT (1-4), LMP1 is assumed to be the most major oncogene encoded by EBV.

Two promoters regulate LMP1 gene transcription, with mechanisms which differ between type II and type III infection. In latency III lymphocyte infection, LMP1 transcription is turned on by EBNA2 and EBNA-LP from the ED-L1 promoter (5-7). Although EBNA2 does not feature DNA binding activity, it enhances LMP1 promoter activity by acting as a cofactor. It associates with cellular transcriptional factors, including RBP-Jκ and PU.1, which are then recruited onto the LMP1 promoter for transactivation. EBNA-LP also associates with the complex and further helps the activation process (8).

On the other hand, LMP1 is expressed in an EBNA2-independent manner in type II latency, since neither EBNA2 nor EBNA-LP are available in such type II cells. It has been frequently reported that cytokines, such as IL-4, IL-6, IL-10, IL-13 and IL-21, activate the JAK/STAT pathway, thereby inducing LMP1 gene expression through STAT (9-14). In certain latency II infected cells including NPC cells, LMP1 transcription originates from a STAT regulated upstream promoter, termed TR-L1, located within the terminal repeats (TR), in addition to the proximal ED-L1 promoter (10,13,15,16). Involvement of transcriptional factors, such as ATF/CREB (17), Sp1/3 (18) and IRF7 (19), has also been indicated. Despite the presence of these well-targeted, focused reports, there is still a possibility of other yet-unknown factor(s) that play(s) essential roles in EBNA2-independent LMP1 expression, because exhaustive investigations have hitherto not been performed.

In the present study, we therefore adopted a comprehensive approach and screened a cDNA library for cellular factors that can activate LMP1 transcription. We newly cloned the CCAAT Enhancer-Binding Protein (C/EBP) family transcription factor that augments both proximal and distal promoter activation of LMP1 by binding to a motif in the proximal promoter. A functional C/EBP binding site for the LMP1 promoter was identified by reporter mutagenesis and EMSA
assays. We also constructed a mutant EBV with a point mutation in the C/EBP binding site, and confirmed importance of binding for LMP1 expression in latent cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents- HEK293T, Hela-CR2/GFP-EBV, 293EBV-BAC and AGS cells were maintained in Dulbecco’s modified Eagle medium (SIGMA) supplemented with 10% fetal bovine serum. C666-1 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. To prepare Hela-CR2/GFP-EBV cells, an EBV-negative Hela cells were stably transformed with CR2 (CD21, the receptor for the EBV expression vector, and infected with GFP-EBV (20). AGS-CR2 was prepared by retroviral transduction of the viral receptor CR2 (CD21) into AGS cells. Akata(-) cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum. Anti-Flag, -C/EBPα and -tubulin antibodies were purchased from Sigma, Cell Signaling and Santa Cruz, respectively. The anti-LMP1 antibody has been described previously (21). Horseradish peroxidase-linked goat antibodies to mouse/rabbit IgG were from Amersham Biosciences.

Library and Plasmids- A SuperScript Pre-made cDNA library (from Human Bone Marrow) was purchased from Invitrogen and used for screening after exclusion of clones with junk inserts. Control reporter pCMV-Rluc was reported previously (22). For pLMP1/ED-L1-Fluc, the ED-L1 promoter sequence of LMP1 was amplified from B95-8 genome using ED-L1pFor and ED-L1pRev primers (Table S1). The amplified DNA was digested with Xhol and Ncol, and then inserted into the Xhol/Ncol sites of pGL4.10 (Promega). Likewise, luciferase reporter constructs containing various TR sequences were prepared using the following primers: for pLMP1/ED-L1+TR-L1-Fluc, ED-L1p+TR-L1pFor and ED-L1p+TR-L1pRev, for pLMP1/ED-L1+TR-L1pFor and TR-L1pRev, for pLMP1/ED-L1+BS-Fluc, TR-L1p+BSFor and TR-L1p+BSRev (Table S1). Truncated or point-mutated derivatives of the reporter, pLMP1/ED-L1-Fluc, were made by the inverse PCR method using primers shown in Table S1 (From 417For to 268mtRev). The C/EBP expression vectors were made by inserting cDNA fragments of the proteins into EcoRI/XhoI sites of pcDNA3-Flag (23). RNA was obtained from Akata or HEK293T cells, and subjected to RT-PCR using the SuperScript III First-strand System (Invitrogen) and KOD DNA polymerase (TOYOBO). Primers used for the PCR were listed in the Table S1.

Transfection, Luciferase assay and Immunoblotting – Transfections were carried out by lipofection using lipofectamine 2000 reagent (Invitrogen) or by electroporation using a Microoporator (Digital Bio). The total amounts of plasmid DNAs were standardized by addition of an empty vector. Proteins were extracted from cells with the lysis buffer supplied in a Dual-Luciferase Reporter Assay System (Promega) kit and luciferase activities were measured using the kit. Immunoblotting was carried out as described previously (23).

Short hairpin RNA (shRNA) vector – Knockdown of C/EBP was carried out by retrovirus shRNA system (Tsuzuki S. et al, unpublished; (24)). Target sequences for the shRNAs are shown in the Table S2.

Electromobility shift assay (EMSA) and Chromatin immunoprecipitation (ChIP) – EMSA was carried out as described previously (25). Flag-tagged C/EBPα and ε proteins were produced using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer’s instructions. The probe was prepared by 3’-end labeling using the Klenow fragment (TOYOBO) and [32P]-dATP (Institute of Isotopes Co., Hungary). Unincorporated deoxynucleotide triphosphates were removed with Chromaspin-10 columns (Clontech). The in vitro translated protein and
labeled DNA sequences were incubated in the EMSA binding buffer (20 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, 30 mM KCl, 3mM MgCl₂, 0.5 mg/ml poly (dl-dC)) at room temperature for 30 min. The samples were then separated in a 4% non-denaturing polyacrylamide gel in 0.5x TBE buffer and radioactivity was visualized using the BAS2500 system (Fuji Film). The sequences of oligonucleotide probes were listed in Table S3. ChIP assays and real time PCR were carried out as described previously by using anti-C/EBPα antibody (Santa Cruz) (22,25). Primers used for the real time PCR were indicated in the Table S4.

RTPCR- Total cell RNA was purified using TriPure Isolation Reagent (Roche) and subjected to reverse transcription and PCR reactions using the Super Script III First Strand Synthesis System (Invitrogen) and GoTaq Green Master Mix (Promega). Primers used for the RT-PCR were listed in Table S5. The PCR products were then subjected to agarose gel electrophoresis for detection.

Genetic manipulation of EBV-BAC DNA and cloning of HEK293 cells with EBV-BAC - EBV-BAC DNA was provided by W. Hammerschmidt (26). Homologous recombination was carried out in E. coli as described previously (27).

To prepare a C/EBP binding site mutant of EBV-BAC, a transfer DNA fragment for the first recombination was generated by PCR using PpsL-neo (Gene Bridges) as the template, with Neo/stFor and Neo/stRev primrs (Table S6). After the recombination, kanamycin-resistant colonies were selected and checked to make intermediate DNA. The Neo/st cassette in the intermediate DNA was then replaced using the next transfer vector DNA, containing a mutation in the C/EBP binding site of LMP1 promoter. The transfer vector was made by PCR using pLMP1/-268mt-Fluc as the template with the primers in Table S6. Streptomycin-resistant colonies were cloned and checked to make EBV-BAC C/EBP BSmt.

Electroporation of E. Coli was performed using a Gene Pulser III (Bio-Rad) and purification of EBV-BAC DNA was achieved with NucleoBond Bac100 (Macherey-Nagel).

Recombination was confirmed with PCR products of the promoter region, by electrophoresis of the BamHI-digested viral genome, and sequencing analysis.

EBV-BAC DNA was transfected into HEK293 cells using lipofectamine 2000 reagent (Invitrogen), followed by culture on 10 cm dishes with 100-150 µg/ml of hygromycin B for 10-15 days for cloning of GFP-positive cell colonies as described previously (27). Briefly, for each recombinant virus, we picked up more than 10 hygromycin-resistant, GFP-positive cell colonies to obtain at least 3 typical clones exhibiting minimal spontaneous expression of viral lytic proteins and significant induction of these upon BZLF1 transfection.

RESULTS

Screening of cellular factors transactivating the LMP1 promoter. In order to exhaustively search for cellular factor(s) that enhance(s) LMP1 transcription, we screened a human bone marrow cDNA expression library for ability to enhance the promoter activity, using reporter assay systems. To this end, we cloned proximal LMP1 promoter (ED-L1) into the promoter-less firefly luciferase vector (pGL4.10) to make pLMP1/ED-L1-Fluc. As a control, pCMV-Rluc, featuring the CMV IE promoter upstream of the Renilla luciferase gene, was used to normalize for transfection efficiency. An example of our screen is shown in Fig. S1. To maximize the number of the cDNAs that could be assayed while assuring that any positive clone would not be missed, we generated cDNA pools with 10 cDNAs per pool. Each cDNA pool was transfected into HEK293T cells together with pLMP1/ED-L1-Fluc and
pCMV-RL. A pool was considered positive when the pLMP1/ED-L1-Fluc reporter was activated 2-fold or more, as compared with the control pCMV-Rluc. Then, the positive pool was re-cloned and assayed again to single out the positive clone, followed by sequencing. So far, we have screened more than 2,000 pools, which means 20,000 clones, and after pseudo positives were excluded, we identified at least 9 clones as possible positive regulators of the LMP1 promoter. All of the hits cloned in the screen turned out to be transcription factors. Among them, we found Ets domain family transcription factors were frequently isolated: two clones of the hits encode Friend Leukemia virus Integration 1 (Fli1), and four clones encode PU.1, also known as Spleen Forming virus (SFFV) Proviral Integration 1(Sp1). Exogenous expression of Fli1 or PU.1 elicited LMP1 promoter activity about 40-50 or 3-5 fold, respectively in the reporter assays. Likewise, one clone of Sp3 was isolated which activated the promoter about 4-8 fold. Because Ets family transcription factor PU.1 (5,6) and Sp1/3 (18) have been reported to bind and activate the proximal LMP1 promoter, we assume credibility of our screen system was proven. Lastly, we newly identified one clone of a b-Zip type transcriptional factor C/EBPε as a LMP1 transcriptional activator. Although CREB/ATF, members of the b-Zip transcriptional factors, are reported to activate the ED-L1 proximal LMP1 promoter (17), we assumed C/EBP to act in a different mode from, because the DNA binding consensus sequence of C/EBP (28,29) is quite distinct form that of CREB/ATF. Therefore, we decided to further analyze molecular mechanisms underlying the activation.

**C/EBPα, β and ε efficiently transactivate the LMP1 promoter.** After newly identifying the transcriptional factor C/EBPε as an activator, we tested if other members of the C/EBP family could also function as transcriptional activators, using a reporter assay system (Fig. 1A). C/EBPα or ε transactivated the promoter relatively efficiently, C/EBPβ had moderate effect, while others had little effects. Only one negative regulator of the family, C/EBPγ, which lacks an activation domain and therefore represses gene transcription by forming inactive heterodimers with other members (30), actually reduced the transcription as expected.

We then transfected the C/EBP expression vectors into an EBV-positive cell line, Hela-CR2/GFP-EBV, to check the effects (Fig. 1B). This cell line was prepared by infecting Hela cells stably expressing CR2 (CD21), the cell surface receptor of the virus, with EBV. We here used the cells for two reasons: first, the EBV in this cell line features type II latency, in which LMP1 is produced in the EBNA2-independent manner, and second, transfection efficiency is very high and thus easy to handle when compared to other cells featuring type II EBV latency. When C/EBPα or ε were exogenously expressed, increased levels of LMP1 protein were readily detected by immunoblotting (Fig. 1B) while other members did not appreciably increase the LMP1 levels. We also tested C666-1, a nasopharyngeal carcinoma cell line naturally infected with EBV (Fig. 1C). At least expression of C/EBPα increased LMP1 mRNA level. In AGS-CR2/GFP-EBV-Bac cells, LMP1 is increased by the exogenous supply of not only C/EBPα and ε, but also β. (Fig. S2)

**Identification of a C/EBP binding site in the LMP1 promoter.** Since we confirmed C/EBP’s potentiating effect of LMP1 transcription, we then carried out truncation and mutagenesis analysis of the promoter region in order to identify any cis-element that might be responsible for the activation. We first prepared reporter vectors in which the promoter sequences were gradually deleted as shown in Fig. 2A. While truncation of the sequence to -320 relative to the transcription start site (+1) did not impair the promoter response to C/EBPα, severing the sequence between nucleotides -320 and -229 markedly diminished the response (Fig. 2B), implying
the presence of the responsible motif(s) between -320 and -229. We thus searched this region for sequences conforming to the consensus C/EBP binding motif, RTTGCGCYAAY, where R indicates A or G, and Y indicates C or T (28), and found three of such possible motifs as shown in Fig. 2C. We named the possible binding motifs as -320, -284 and -268, according to their positions, and introduced point mutations into each as shown in Fig. 2C in order to determine which might be functional. Luciferase assays revealed that C/EBPα-mediated transactivation was severely attenuated when the putative motif at -268 was mutated, whereas replacement of the other two possible motifs did not cause any defect (Fig. 2D). These results suggest that the ATTGCCGCAC motif at the -268 of ED-L1 promoter is the cis-element responsible for the response to C/EBP.

We then used EMSA to examine whether the C/EBP protein can actually bind to the ATTGCCGCAC motif at -268 in the ED-L1 promoter (Fig. 3A). Addition of Flag-tagged C/EBPα or ε produced a specific band for the C/EBP-nucleotide complex when the wild-type C/EBP binding site at the -268 of ED-L1 (C/EBP BS) sequence was used, whereas this failed to be produced with mtC/EBP BS, the mutated sequence. Supershift analysis with anti-Flag antibody demonstrated that the band actually contained Flag-tgged C/EBP protein. Therefore, C/EBP binds to the ATTGCCGCAC motif in question.

We also tried to detect binding of endogenous C/EBP to the LMP1 promoter. In AGS-CR2/GFP-EBV-Bac cells, C/EBPε was detected on the promoter sequence (Fig. 3B), although C/EBPα was undetectable (not shown). Because amount of C/EBPα is very low in the cell line, we speculate the ChIP result simply reflected the expression level of the family member.

Previous reports demonstrated that a distal promoter, termed TR-L1, located within the terminal repeats (TR) of the viral genome, is also activated in addition to the proximal ED-L1 promoter in certain cell types with EBNA2-independent LMP1 expression (10,13,15,16), we next examined, by RT-PCR, if C/EBP might affect the TR-L1 promoter, too. An antisense primer was designed to jump the first intron of LMP1 gene (Fig. 4A, primer #3), so that the possibility of genomic contamination could be ignored, and one sense primer was set within the first exon (Fig. 4A, primer #1) and another sense set well upstream of the transcription start site (+1) of ED-L1 promoter (Fig. 4A, primer #2). The result of the RTPCR (Fig. 4B) indicated that C/EBPα markedly enhanced transcription from the TR-L1 promoter. It is not clear, from this result, whether the ED-L1 promoter is also activated or not.

Although we already identified the cis-element responsible for the activation of the proximal ED-L1 promoter (Fig. 2), we then searched to find the cis-element that is crucial for the activation of the distal promoter, since the TR-L1 promoter of LMP1 gene was markedly activated by C/EBPα (Fig. 4). We first prepared a firefly luciferase reporter construct by inserting the TR-L1 promoter (nucleotide -1115 to -544, Fig. 5A, TR). Curiously, this reporter did not respond to exogenous expression of C/EBPα (Fig. 5B, TR), suggesting that a functional cis-element responsible for the activation of the TR-L1 promoter does not exist in the sequence between nucleotides -1115 and -544. Therefore, speculating that the C/EBP binding site located within the ED-L1 promoter might act to influence the TR-L1 promoter activity form downstream, the promoter sequence in the reporter construct was extended to -147, to cover the C/EBP motif (Fig. 5A, TR+BS). Although this reporter contains a part of the ED-L1 promoter, transcription from ED-L1 should not initiate since it does not contain the transcription start site (+1) of the ED-L1 promoter. As shown in Fig. 5B (TR+BS), the vector did respond to C/EBPα, and introduction of a point mutation at the C/EBP BS depressed the response (Fig. 5C,
TR+BSmt). In addition, a reporter containing the TR-L1 and complete ED-L1 promoters (Fig. 5A, TR+ED) acted in a similar manner (Fig. 5D, TR+ED, TR+EDmt). These results suggest that activation of both the TR-L1 and ED-L1 promoters by C/EBP is mediated through the single C/EBP binding site in the ED-L1 promoter.

**Mutation in the C/EBP binding site attenuated activity of both LMP1 promoters in the context of the viral genome.** Experiments so far have indicated there is one functional C/EBP binding site in the ED-L1 promoter through which activation of both ED-L1 and TR-L1 promoters is mediated. To further extend and verify the findings, recombinant EBV with a point mutation at the identified C/EBP binding site was prepared. As shown in Fig. 6A, a part of the LMP1 ED-L1 promoter sequence (-360 to -11), containing the C/EBP binding site (C/EBP BS, ringed in Fig. 6A), was first replaced with the marker cassette (Neo/st), and then this was exchanged with the mutated C/EBP binding site (C/EBP BSmt) sequence, to prepare EBV-BAC C/EBP BSmt. Sequencing analysis confirmed that the EBV-BAC C/EBP BSmt DNA had the same mutation as the pLMP1/-268mt-Fluc vector (Fig. 2C), as intended. Integrity of the BAC DNA was checked by BamHI digestion followed by electrophoresis to confirm that the recombinant viruses did not carry obvious deletions or insertions (Fig. 6B). Recombinant EBV-BAC DNA was introduced into a virus-producing cell line, HEK293, followed by hygromycin selection, to establish cell lines in which multiple copies were maintained as an episome. More than 10 cell colonies from each recombinant virus were obtained and viral protein expression levels in the presence and absence of BZLF1 inductions were examined. The recombinant virus was then infected into AGS-CR2, expressing the cellular receptor for EBV, CR2 (CD21).

Protein levels were examined in the AGS cells, latently infected with wild-type or mutated EBV (Fig. 7A). Production of LMP1 protein in the AGS cells with virus carrying the point mutation at the C/EBP binding site (Fig. 7A, mt) was obviously lower than in the wild-type. The AGS cells expressed little or no EBNA2, in contrast to LCL (Fig. 7A), indicating that the virus established type II latency in the cells (31). Promoter usage patterns were then checked by RT-PCR using the specific primers used for Fig. 4. Transcription from the TR-L1 promoter was remarkably restricted with the mutant (Fig. 7B), although the effect of the mutation on the ED-L1 promoter was not distinguishable from the data. We also checked that EBNA1 levels were comparable (Fig. 7B).

Next, the effects of C/EBP exogenous expression were analyzed in cells carrying recombinant viruses. In AGS cells latently infected with wild-type EBV, intrinsic LMP1 protein was present and ectopic supply of C/EBPα caused prominent increase in LMP1 protein levels (Fig. 7C). On the other hand, in cells with mutant EBV, intrinsic LMP1 protein level was low and C/EBPα expression did not induce increase (Fig. 7C). RT-PCR analysis clearly showed that transcriptional activation of LMP1 gene by C/EBPα in wild-type, at least for the TR-L1 promoter, was diminished in the mutant (Fig. 7D), indicating significance for the motif.

**Knockdown of C/EBP reduced LMP1 levels.** Lastly, we tested the effect of endogenous C/EBP proteins on LMP1 expression levels. To this end, α or ε member of C/EBP family was ablated by shRNA technology. In Hela-CR2/GFP-EBV cells, knockdown of either C/EBPα or ε significantly restricted the amount of LMP1 (Fig. 8A, S3). In AGS-CR2/GFP-EBV-Bac cells, we tested knockdown of C/EBPε. Because levels of endogenous C/EBPα in the cells were very low, knockdown of C/EBPα was not done. Treatment of shC/EBPε caused reduction of LMP1 protein in AGS cells, too (Fig. 8B). These results indicate that C/EBP proteins are involved in LMP1 production, and suggest
that the effect is dependent on cell types.

**DISCUSSION**

The results documented here show clear involvement of C/EBP proteins in up-regulation of the LMP1 gene.

Initially, C/EBPε protein was identified by our screening to increase the proximal LMP1 (ED-L1) promoter activity. We are confident in the screening system, since factors like Sp3 and Ets type transcription factors, both of which have been implicated in the transcriptional regulation of LMP1, were isolated in the screen. Regarding Ets transcription factors, PU.1 has been reported to recruit the viral transcriptional activator EBNA2 and thereby enhance LMP1 ED-L1 promoter activity (5,6), but it is understandable that PU.1 up-regulated transcription even without EBNA2 in the screening experiment, because PU.1 can functionally interact with basic transcriptional regulators, like CBP, TFIID or TBP, or other transcription factors, like GATA or Runx (32,33). Another Ets family transcription factor Fli1 was also identified in our screen. Interestingly, while Fli1 markedly elicited promoter activity (about 40-50 fold) in the reporter assays, exogenous expression of Fli1 did not cause increase in the levels of LMP1 protein in EBV-positive cells (data not shown). Likewise, PU.1 also did not significantly augment the LMP1 protein levels (data not shown). On the other hand, whereas increment of the reporter activity by C/EBPε was not very high (only 2 to 4 fold), exogenous expression of the gene clearly increased the mRNA and protein levels of LMP1 in EBV-positive cells (Fig. 1B,C, 4, S2). Therefore, we must conclude that transient reporter assays do not always reflect the actual promoter activity in the context of viral genome. The reason why overexpression of the Ets family transcription factors fail to increase the LMP1 protein levels in the context of infection is not clear. We speculate either that the promoter might already be occupied with certain Ets family proteins, or that the ability of the Fli1 or PU.1 to enhance the promoter activity might not be sufficiently strong as to counter epigenetic suppression of the gene but high enough for reporter assays.

Subsequent analyses demonstrated that C/EBP enhanced the distal TR-L1 promoter of LMP1, and that the activation was mediated through one C/EBP binding motif in the proximal ED-L1 promoter (Fig. S4). Therefore, the distal TR-L1 promoter is activated by C/EBP binding downstream of transcription start site of the TR-L1 promoter. Because activation of a particular promoter by transcription factor binding downstream of the transcription start site has been demonstrated previously for various promoters (34,35), we assume the activation of TR-L1 promoter by downstream C/EBP binding is reasonable when considered in light of our clear results of reporter assays and point-mutated virus, too. While we obtained substantial amount of evidence that C/EBP enhanced the distal TR-L1 promoter of LMP1, activation of proximal ED-L1 promoter was checked only by the reporter assays. We were not able to confirm this, since PCR primers that detect only the ED-L1 promoter could not be designed.

Previous studies have repeatedly demonstrated that cytokines such as IL-4, IL-6, IL-10 and IL-21 mediate LMP1 gene expression in the absence of the viral coactivator EBNA2, namely in type II latent cells such as B cells (10-12,36), NK/T lymphomas (11), epithelial Hela cells and the NPC-derived cell line CNE2 (14). More amazingly, expression patterns of EBV proteins even in type I or type III cells could be modulated by cytokines to resemble those in type II latency (10-12,36). These reports all showed engagement of the JAK/STAT signaling pathway in the process of induction of LMP1 expression. We here newly suggest involvement of the C/EBP family, which has been implicated in various physiological phenomena, such as differentiation, inflammation and cell growth. In terms of inflammation,
C/EBP proteins have been implicated in induction of a number of cytokines (37,38). To take some examples, C/EBP play a role in transcriptional induction of certain genes by IL-6 (39). IL-10 activates expression of C/EBP and thereby activates transcription of IL-6 in epithelial cells (40). Therefore, it is strongly suggested that cytokines activate the LMP1 promoter through C/EBP, besides JAK/STAT signaling. In addition to cytokine-induced expression of LMP1, it seems likely that C/EBP contributes to produce LMP1 even in the absence of cytokines, because expression of LMP1 in the AGS cell line with recombinant EBV mutated at the C/EBP binding site was notably more subdued than with the wild-type virus (Fig. 7A,B).

To summarize, we could successfully identify a new factor C/EBP as transcriptional activator of the major EBV LMP1, and made an initial characterization of the molecular mechanisms of how LMP1 expression is reinforced by the transcription factor in an EBNA2-independent manner. Because LMP1 is the major oncogene of EBV, suppression of LMP1 gene expression by inhibiting the C/EBP family may provide potential targets of therapeutic drugs for EBV-positive cancers, especially for type II cancers, such as NK/T lymphomas, NPC and Hodgkin lymphomas. Search for small molecules that inhibit LMP1 expression is already under way.
REFERENCES

1. Soni, V., Cahir-McFarland, E., and Kieff, E. (2007) Adv Exp Med Biol 597, 173-187
2. Lam, N., and Sugden, B. (2003) Cell Signal 15, 9-16
3. Shair, K. H., Bendt, K. M., Edwards, R. H., Bedford, E. C., Nielsen, J. N., and Raab-Traub, N. (2007) PLoS Pathog 3, e166
4. 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
5. Johannsen, E., Koh, E., Mosialos, G., Tong, X., Kieff, E., and Grossman, S. R. (1995) J Virol 69, 253-262
6. Laux, G., Adam, B., Strobl, L. J., and Moreau-Gachelin, F. (1994) EMBO J 13, 5624-5632
7. Grossman, S. R., Johannsen, E., Tong, X., Yalamanchili, R., and Kieff, E. (1994) Proc Natl Acad Sci U S A 91, 7568-7572
8. Harada, S., and Kieff, E. (1997) J Virol 71, 6611-6618
9. Kis, L. L., Gerasimcik, N., Salamon, D., Persson, E. K., Nagy, N., Klein, G., Severinson, E., and Klein, E. (2011) Blood 117, 165-174
10. Kis, L. L., Salamon, D., Persson, E. K., Nagy, N., Scheeren, F. A., Spits, H., Klein, G., and Klein, E. (2010) Proc Natl Acad Sci U S A 107, 872-877
11. Kis, L. L., Takahara, M., Nagy, N., Klein, G., and Klein, E. (2006) Blood 107, 2928-2935
12. Konforte, D., Simard, N., and Paige, C. J. (2008) Virology 374, 100-113
13. Chen, H., Lee, J. M., Zong, Y., Borowitz, M., Ng, M. H., Ambinder, R. F., and Hayward, S. D. (2001) J Virol 75, 2929-2937
14. Chen, H., Hutt-Fletcher, L., Cao, L., and Hayward, S. D. (2003) J Virol 77, 4139-4148
15. Sadler, R. H., and Raab-Traub, N. (1995) J Virol 69, 4577-4581
16. Hsiao, J. R., Chang, K. C., Chen, C. W., Wu, S. Y., Su, I. J., Hsu, M. C., Jin, Y. T., Tsai, S. T., Takada, K., and Chang, Y. (2009) Cancer Res 69, 4461-4467
17. Sjoblom, A., Yang, W., Palmqvist, L., Jansson, A., and Rymo, L. (1998) J Virol 72, 1365-1376
18. Tsai, C. N., Lee, C. M., Chien, C. K., Kuo, S. C., and Chang, Y. S. (1999) Virology 261, 288-294
19. Ning, S., Hahn, A. M., Huye, L. E., and Pagano, J. S. (2003) J Virol 77, 9359-9368
20. Maruo, S., Yang, L., and Takada, K. (2001) J Gen Virol 82, 2373-2383
21. Kanda, T., Yajima, M., Ahsan, N., Tanaka, M., and Takada, K. (2004) J Virol 78, 7004-7015
22. Murata, T., Hotta, N., Toyama, S., Nakayama, S., Chiba, S., Isomura, H., Ohshima, T., Kanda, T., and Tsurumi, T. (2010) J Biol Chem 285, 23925-23935
23. Murata, T., Sato, Y., Nakayama, S., Kudoh, A., Iwashori, S., Isomura, H., Tajima, M., Hishiki, T., Ohshima, T., Hijikata, M., Shimotohno, K., and Tsurumi, T. (2009) J Biol Chem 284, 8033-8041
24. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Cancer Cell 2, 243-247
25. Murata, T., Noda, C., Saito, S., Kawashima, D., Sugimoto, A., Isomura, H., Kanda, T., Yokoyama, K. K., and Tsurumi, T. (2011) J Biol Chem 286, 22007-22016
26. Delecluse, H. J., Hilsendegen, T., Pich, D., Zeidler, R., and Hammerschmidt, W. (1998) Proc Natl Acad Sci U S A 95, 8245-8250
27. Murata, T., Isomura, H., Yamashita, Y., Toyama, S., Sato, Y., Nakayama, S., Kudoh, A., Iwahori, S., Kanda, T., and Tsurumi, T. (2009) Virology 389, 75-81
28. Ramji, D. P., and Foka, P. (2002) Biochem J 365, 561-575
29. Osada, S., Yamamoto, H., Nishihara, T., and Imagawa, M. (1996) J Biol Chem 271, 3891-3896
30. Cooper, C., Henderson, A., Artandi, S., Avitahl, N., and Calame, K. (1995) Nucleic Acids Res 23, 4371-4377
31. Yoshiyama, H., Imai, S., Shimizu, N., and Takada, K. (1997) J Virol 71, 5688-5691
32. Burda, P., Laslo, P., and Stopka, T. (2010) Leukemia 24, 1249-1257
33. Gupta, P., Gurudutta, G. U., Saluja, D., and Tripathi, R. P. (2009) J Cell Mol Med 13, 4349-4363
34. Sugiyama, T., Uchida, C., Oda, T., Kitagawa, M., Hayashi, H., and Ichiyama, A. (2001) FEBS Lett 508, 16-22
35. Kilareski, E. M., Shah, S., Nonnemacher, M. R., and Wigdahl, B. (2009) Retrovirology 6, 118
36. Kis, L. L., Takahara, M., Nagy, N., Klein, G., and Klein, E. (2006) Immunol Lett 104, 83-88
37. Tsukada, J., Yoshida, Y., Kominato, Y., and Auron, P. E. (2011) Cytokine 54, 6-19
38. Kalvakolanu, D. V., and Roy, S. K. (2005) J Interferon Cytokine Res 25, 757-769
39. Kordula, T., and Travis, J. (1996) Biochem J 313 (Pt 3), 1019-1027
40. Robb, B. W., Hershko, D. D., Paxton, J. H., Luo, G. J., and Hasselgren, P. O. (2002) Surgery 132, 226-231

**FOOTNOTES**

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The abbreviations used are: EBV, Epstein-Barr virus; C/EBP, CCAAT enhancer-binding protein; LMP1, latent membrane protein 1; NPC, nasopharyngeal carcinomas; TR, terminal repeats.
**FIGURE LEGENDS**

Fig. 1. C/EBP efficiently transactivates the LMP1 promoter. (A) C/EBPα and ε augmented LMP1 promoter function in reporter assays. HEK293T cells were transfected with 10 ng of reporter plasmid pLMP1/ED-L1-Fluc, 1 ng of control pCMV-Rluc and 100 ng of indicated C/EBP family expression vector or the empty vector (pcDNA3). Luciferase assays were carried out after 1 day as described in the Experimental Procedures. The firefly luciferase activity was normalized to Renilla luciferase activity and shown as mean fold-activation of that with the control vector (pcDNA3) and SD. (B) Potentiation of LMP1 levels by ectopic expression of C/EBPα and ε in Hela cells latently infected with EBV. Hela-CR2/GFP-EBV cells were transfected with empty vector (pcDNA3) or the indicated C/EBP family expression vector. After 60 h, cell proteins were harvested and subjected to immunoblotting with anti-LMP1, -tubulin, and -Flag antibodies. (C) C/EBPα transactivated LMP1 levels in a nasopharyngeal carcinoma cell line. C666-1 cells were transfected with empty vector or C/EBPα expression vector. After 48 h, cell RNAs were collected and subjected to RTPCR.

Fig. 2. Identification of the sequence motif responsible for activation of the LMP1 ED-L1 promoter by C/EBPα. (A) Schematic representation of reporter constructs with truncated LMP1 ED-L1 promoter sequences. Possible C/EBP binding sites between -320 and -229 are ringed. (B) The C/EBPα expression plasmid or its empty vector were cotransfected into HEK293T cells with the truncated reporter plasmid in (A) and pCMV-Rluc. Luciferase assays were carried out after 1 day as described in the Experimental Procedures. The firefly luciferase activity was normalized to Renilla luciferase activity. Bars indicate averages of the fold activation on transfection of C/EBPα, compared with those with the empty vector, and SD, for each reporter. (C) Schematic representation of the mutated derivatives of pLMP1/ED-L1-Fluc. Possible C/EBP binding sites between -320 and -229 are ringed. The putative C/EBP binding motifs were replaced with the sequences below. (D) C/EBPα expression plasmid or its empty vector were cotransfected into HEK293T cells with the mutated reporter plasmid in (C) and pCMV-Rluc. Luciferase assays were carried out after 1 day as described in the Experimental Procedures. The firefly luciferase activity was normalized to Renilla luciferase activity. Bars indicate averages of the fold activation by transfection of C/EBPα, compared with those with empty vector, and SD, for each reporter. The numbers in the figure indicate nucleotide positions relative to the transcription start site (+1).

Fig. 3. Binding of C/EBPα and ε to the binding site in the LMP1 ED-L1 promoter. (A) EMSA was carried out as described in the Experimental Procedures. Flag-tagged C/EBPα (left panel) or ε (right panel) were produced in vitro and incubated with 32P-labeled wild-type (C/EBP BS) or point-mutated (mtBS) probe. Supershift analysis was performed using mouse anti-Flag monoclonal antibodies. The samples were then separated in a 4% polyacrylamide gel and analyzed with Fuji Image Analyzer BAS2500. (B) Binding of endogenous C/EBPε to LMP1 promoter. AGS-CR2/GFP-EBV-Bac cells, latently infected with EBV, were subjected to ChIP assays using anti-C/EBPε antibody (Santa Cruz), followed by real time PCR analysis for quantification.

Fig. 4. Activation of the LMP1 TR-L1 promoter by C/EBPα in cells latently infected with EBV. (A) Schematic representation of the regulatory sequence of the LMP1 gene. The 2.8 and 3.5 kb LMP1 mRNAs and the primers used for RTPCR in (B) are depicted. (B) Hela-CR2/GFP-EBV cells were transfected with empty vector (pcDNA3) or the indicated C/EBPα expression vector. After 60 h, cell RNAs were harvested and subjected to RTPCR using the primers indicated above.
Fig. 5. Identification of the sequence motif responsible for the activation of the LMP1 TR-L1 promoter by C/EBPα. (A) Schematic representation of reporter constructs with truncated and/or mutated LMP1 promoter sequences. Identified C/EBP binding sites in the ED-L1 promoter are ringed. (B) The C/EBPα expression plasmid or its empty vector were cotransfected into HEK293T cells with the mutated reporter plasmid in (A) and pCMV-Rluc. Luciferase assays were carried out after 1 day as described in the Experimental Procedures. The firefly luciferase activity was normalized to Renilla luciferase activity. Bars indicate averages of the fold activation by transfection of C/EBPα, compared with those with the empty vector, and SD, for each reporter. The numbers in the figure indicate nucleotide positions relative to the transcription start site (+1).

Fig. 6. Construction of a recombinant EBV featuring point mutation in the C/EBP binding site of the LMP1 promoter. (A) Schematic arrangement of the recombination of the EBV genome using the tandemly arranged neomycin-resistance and streptomycin-sensitivity genes (Neo/st). The sequences around the C/EBP binding site (C/EBP BS, ringed) of the B95-8 LMP1 promoter (-360 to -11) were first replaced with the Neo/st cassette, which was then replaced with a point mutated C/EBP BS sequence (ringed X) to construct EBV-BAC C/EBP BS mt. (B) Electrophoresis of the recombinant viruses. The recombinant EBV genomes were digested with BamHI and separated in an agarose gel.

Fig. 7. Decrease in LMP1 protein and mRNA levels with point mutation of the C/EBP binding site of the EBV-BAC LMP1 promoter. (A) LMP1 protein levels in AGS cells latently infected with wild-type or point-mutated EBV-BAC. Immunoblotting was performed using anti-LMP1, -tubulin and -EBNA2 antibodies. Proteins from LCL were also included as a positive control for EBNA2. (B) Both TR-L1 and ED-L1 promoters were attenuated by point mutation of the C/EBP binding site of EBV-BAC LMP1 promoter. RNA was collected from AGS cells latently infected with wild-type or point-mutated EBV-BAC, and subjected to RTPCR using the primers shown in Fig. 5A. EBNA1 and GAPDH levels were also checked. (C) Response to ectopic expression of C/EBPα was diminished by point mutation of the C/EBP binding site of EBV-BAC LMP1 promoter. AGS cells latently infected with wild-type or point-mutated EBV-BAC were transfected with the C/EBPα expression vector or its empty vector (pcDNA3). After 60 h, cell proteins were harvested and subjected to immunoblotting with anti-LMP1, -tubulin, and -Flag antibodies. (D) Responses of both TR-L1 and ED-L1 promoters to ectopic expression of C/EBPα were diminished by point mutation of the C/EBP binding site of EBV-BAC LMP1 promoter. AGS cells latently infected with wild-type or point-mutated EBV-BAC were transfected with C/EBPα expression vector or its empty vector (pcDNA3). After 60 h, RNA was collected and subjected to RTPCR using the primers shown in Fig. 4A.

Fig. 8. Knockdown of C/EBPε decreased levels of LMP1. Hela-CR2/GFP-EBV (A) and AGS-CR2/GFP-EBV-Bac (B) cells were treated with control shRNA or shRNA for the C/EBPε. Cell proteins were harvested and subjected to immunoblotting with anti-LMP1, -C/EBPε and –tubulin antibodies.
Fig. 1

(A) 

Fold activation

- pDNA3
- C/EBPα
- C/EBPβ
- C/EBPγ
- C/EBPδ
- C/EBPε

(B) 

Hela-CR2/GFP-EBV cell

- anti-LMP1
- anti-tubulin
- anti-flag

(C) 

C666-1 cell

- LMP1
- GAPDH
Fig. 3

(A)

| Probe | C/EBP BS | C/EBP BS | mt BS | C/EBP BS | C/EBP BS | mt BS |
|-------|---------|---------|-------|---------|---------|-------|
| Ab    | -       | -       | -     | -       | -       | -     |
| Protein | Control Flag C/EBP\(\alpha\) Flag C/EBP\(\gamma\) Flag C/EBP\(\epsilon\) Control Flag C/EBP\(\gamma\) Flag C/EBP\(\epsilon\) |

C/EBP\(\alpha\)-C/EBP BS complex

Free probe

(B)

| 1/1000 input | normal IgG ppt | C/EBP\(\epsilon\) ppt |
|--------------|----------------|---------------------|
| % input      |                |                     |
| 0.12         | 0.08           | 0.04                |
| 0.1          | 0.06           | 0.04                |
| 0.08         | 0.04           | 0.04                |
| 0.06         | 0.04           | 0.04                |
| 0.04         | 0.04           | 0.04                |
Fig. 5

(A) Diagram showing regulatory elements and luciferase activity:
- TR-L1p
- C/EBP BS
- ED-L1p
- -268 to +1
- luc TR+ED
- luc TR
- luc ED
- luc TR+BS
- luc TR+ED mt
- luc TR+BS mt
- luc pGL4.10 (vector)

(B) Bar graph showing fold activation:
- pGL4.10
- ED
- TR
- TR+BS

(C) Bar graph showing fold activation:
- pGL4.10
- ED+BS
- TR+BS mt

(D) Bar graph showing fold activation:
- pGL4.10
- ED+ED mt
- TR+ED
- TR+ED mt
Fig. 6

(A) 

EBV-BAC WT 

↓ Homologous recombination 

EBV-BAC LMP1 Neo/st 

↓ Homologous recombination 

EBV-BAC C/EBP BS mt 

TR-L1p 3.5kb 

TR1 

-360 C/EBP BS 

-268 

+1 

ED-L1p 2.8kb 

Neo/st 

exon a 

exon b 

exon c 

exon a 

exon b 

exon c 

(B) 

WT 

mt 

9416 

6557 

4361 

2322 

2027
Fig. 8

(A) sh control sh/C/EBPε-1 sh/C/EBPε-2
anti-LMP1
anti-C/EBPε
anti-tubulin
Hela-CR2/GFP-EBV cell

(B) sh control sh/C/EBPε
anti-LMP1
anti-C/EBPε
anti-tubulin
AGS-CR2/GFP-EBV-Bac cell
Identification and characterization of CCAAT enhancer-binding protein (C/EBP) as a transcriptional activator for Epstein-Barr virus oncogene latent membrane protein 1

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