Matrix Metalloproteinase 9 Expression Is Induced by Epstein-Barr Virus Latent Membrane Protein 1 C-Terminal Activation Regions 1 and 2

HAJIME TAKESHITA,1,2 TOMOKAZU YOSHIZAKI,1,2 WILLIAM E. MILLER,1,3 HIROSHI SATO,4 MITSURU FURUKAWA,2 JOSEPH S. PAGANO,1,3,5 AND NANCY RAAB-TRAUB1,3*

Lineberger Comprehensive Cancer Center,1 Department of Microbiology and Immunology,2 and Department of Medicine,3 University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, and Department of Otolaryngology, School of Medicine,2 and Department of Molecular Virology and Oncology, Cancer Research Institute,4 Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

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Nasopharyngeal carcinoma (NPC), which is closely associated with the Epstein-Barr virus (EBV), is a highly metastatic malignant tumor. An important activity in tumor invasion and metastasis is that of the 92-kDa type IV collagenase or gelatinase, matrix metalloproteinase 9 (MMP-9), which mediates the degradation of the basement membrane and extracellular matrix. The expression of MMP-9 has been shown to be enhanced by the EBV oncoprotein, latent membrane protein 1 (LMP-1). LMP-1, which is expressed in NPC, has two essential signaling domains within the carboxy terminus, termed C-terminal activation regions 1 (CTAR-1) and CTAR-2. This study reveals that either signaling domain can activate the MMP-9 promoter and induce MMP-9 activity; however, LMP-1 deletion mutants lacking either CTAR-1 or CTAR-2 had a decreased ability to induce MMP-9 expression. The deletion of both activation regions completely abolished the induction of MMP-9 activity, while the cotransfection of both the CTAR-1 and CTAR-2 deletion mutants restored MMP-9 activity to levels produced by wild-type LMP-1. The NF-κB and activator protein 1 (AP-1) binding sites in the MMP-9 promoter were essential for the activation of MMP-9 gene expression by both CTAR-1 and CTAR-2. The induction of MMP-9 expression by LMP-1 and both CTAR-1 and CTAR-2 mutants was blocked by the overexpression of IκB. The tumor necrosis factor receptor-associated factor (TRAF) pathway also contributed to the activation of the MMP-9 promoter as shown by the use of TRAF-2 and TRAF-3 dominant-negative constructs. These data indicate that the activation of both the NF-κB and AP-1 pathways by LMP-1, CTAR-1, and CTAR-2 is necessary for the activation of MMP-9 expression. In NPC, LMP-1 may contribute to invasiveness and metastasis through the induction of MMP-9 transcription and enzymatic activity.

Epstein-Barr virus (EBV), a ubiquitous human gamma herpesvirus, is associated with several malignant tumors such as endemic Burkitt’s lymphoma, Hodgkin’s disease, and nasopharyngeal carcinoma (NPC) (23, 46, 49, 60). EBV establishes a latent infection in human B lymphocytes, and infection in vitro results in immortalization (25). Latent membrane protein 1 (LMP-1) is considered the principal oncoprotein of EBV and is essential for lymphocyte immortalization (21). LMP-1 expression has also been detected in rare examples of preinvasive NPC lesions, suggesting that LMP-1 expression is an important contributor to the development of NPC (44). LMP-1 is an integral membrane protein consisting of 386 amino acids (aa). Six transmembrane spanning regions (162 aa) connect a short N-terminal cytoplasmic domain (24 aa) with a long C-terminal cytoplasmic domain (200 aa) (10). Mutational analysis has identified two activation domains in the C terminus of LMP-1: C-terminal activation region 1 (CTAR-1) (residues 187 to 231) and CTAR-2 (residues 351 to 386) (14, 37). LMP-1 associates with the tumor necrosis factor receptor family-associated factors (TRAFs) through a TRAF interaction domain within CTAR-1 (8, 30, 34, 35). TRAF-2 is of particular interest as it mediates the activation of the transcriptional factor NF-κB, following interaction with LMP-1 (8, 22, 34, 48). TRAF-1 and TRAF-3 strongly associate with CTAR-1 and modulate the activation of NF-κB (6, 34, 36, 50). CTAR-2 is a stronger activator of NF-κB than CTAR-1 in reporter assays (11, 14, 37) and has recently been shown to interact with the tumor necrosis factor receptor adaptor protein TRADD (19). Several studies have indicated that LMP-1 activates the c-Jun N-terminal kinase (JNK) pathway through CTAR-2 but not CTAR-1 (9, 12, 24).

NPC is a highly metastatic and invasive malignant tumor in which the EBV genes encoding LMP-1, LMP-2A and -2B, and EBNA-1 are expressed. Essential steps in the process of tumor invasion and metastasis include the degradation of the extracellular matrix (ECM) and basement membrane (BM). The invasion of the BM by tumor cells is thought to be one of the critical steps in metastasis, which includes sequential multistep processes (26, 40). Many proteolytic enzymes degrade components of the ECM and BM (39, 45). Among these, the matrix metalloproteinases (MMPs) are attractive candidates for enzymes required for tumor metastasis. The MMPs contain a zinc ion at their active sites and can degrade native collagens and other ECM components (27, 31). The MMP family includes four types of collagenase (MMP-1, -8, -13, and -16), three types of stromelysin (MMP-3, -10, and -11), and the 72- and 92-kDa type IV gelatinases or collagenases (MMP-2 and MMP-9) (18). Several membrane-type MMPs that activate pro-MMP-2 to activate MMP2 have also been identified recently (51, 58).
MMP activity is tightly regulated by the following steps: (i) control of gene transcription, (ii) activation of the latent form of the enzyme to its active form by eliminating an N-terminal peptide, and (iii) regulation by endogenous proteins known as tissue inhibitors of metalloproteinases (7, 32). As type IV collagen is one of the integral components of BM, the uncontrolled expression of type IV collagenases, MMP-2 and MMP-9, is believed to play a critical role in the invasion of BM by tumor cells (28). MMP-2 and MMP-9 are often expressed by tumor cells, but their expression is not always coordinated with that of MMP-1 and MMP-3 (52). The release of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57). The expression of MMP-2 and/or MMP-9 has been associated with metastasis in a variety of model systems (2, 13, 55–57).

We have recently shown that the expression of MMP-9, but not that of MMP-2, was induced by the EBV oncoprotein LMP-1 (59). This study reveals that both LMP-1 activation domains, CTAR-1 and CTAR-2, contribute to the full activation of the MMP-9 promoter and the induction of MMP-9 enzymatic activity through the activation of the NF-κB and AP-1 transcription factors.

MATERIALS AND METHODS

Cell lines. C33A epithelial cells, derived from a human cervical carcinoma, were grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Sigma) and antibiotics.

Plasmids. The LMP-1 open reading frame was subcloned downstream of the cytomegalovirus (CMV) immediate-early promoter into the EcoRI site of pcDNA3. A series of 5′-flanking sequences of MMP-9 were inserted upstream of the chloramphenicol acetyltransferase (CAT) reporter gene as described previously (52). The CMV immediate-early promoter-driven IκB expression plasmid was obtained from Albert Baldwin (54). Constructs TRAF-2 dominant negative (DN), containing aa 98 to 501, and TRAF-3-DN, containing aa 345 to 568, were cloned into the pSG5 vector, which contains the simian virus 40 early promoter and intron sequences from the rabbit β-globin gene (Stratagene). All the LMP-1 mutants were cloned into the EcoRI site of the pcDNA3 expression vector and have been previously described (34, 36).

Transient transfection and conditioned media. The transfection of C33A cells was carried out with 5 × 10⁴ cells per 60-mm-diameter dish with the use of Lipofectamine (GIBCO/BRL) following the manufacturer’s protocol. Five micrograms of appropriate reporter and effector plasmids were transfected. Transfected cells were cultured in DMEM with 10% fetal bovine serum overnight and then in a serum-free medium (OPTI-MEM I; GIBCO/BRL) without antibiotics for 5 h at 37°C. Transfection efficiency was monitored by cotransfection with a β-galactosidase reporter construct.

Western blot analysis. C33A cells were harvested 48 h after transfection with FLAG-LMP mutants. Whole cell extracts were prepared by washing cells once in cold phosphate-buffered saline solution and then lysing them in 50 mL of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 5 mM dithiothreitol, 0.2 mM Na orthovanadate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin per ml, 5 μg of leupeptin per ml) with repeated freezing and thawing. The supernatant fluid was clarified by centrifugation and was stored at −80°C until use. After sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the proteins were transferred to NitroPlus membranes (Micron Separations Inc.) with the Hoefer semidyli transfer apparatus. Nonproactive reactivity was blocked by incubation overnight in Tris-buffered saline solution containing 0.1% Tween 20 and 5% nonfat dried milk. The membrane was then incubated with a primary antibody to FLAG protein (Santa Cruz Biotechnology, Inc.; 1:200 dilution [34]). A secondary antibody (1:2,000 dilution) was used to detect the bound primary antibody. The reactive protein was detected by enhanced chemiluminescence (Amersham).CAT reporter assay. CAT assays were performed with extracts of C33A cells after transfection. The construction of the MMP-9 promoter reporter series has been described previously (52). Cells were incubated 48 h after transfection in DMEM with 10% fetal bovine serum and antibiotics and then harvested; acetylated [14C]chloramphenicol was quantitated with a PhosphorImager (Molecular Dynamics). The data were evaluated by comparison with the transfection efficiency of β-galactosidase.

Gelatin zymography. MMP-2 and MMP-9 were assayed for gelatinolytic activity by means of gelatin zymography as reported previously (59). The conditioned medium was mixed with an SDS sample buffer (50 mM Tris-HCl [pH 6.8], 1% SDS, 10% glycerol, 10 mM EDTA, 0.1% bromophenol blue) and boiled for 5 min. The sample was loaded onto an 8% polyacrylamide gel with 1% gelatin. After electrophoresis, the gel was washed with 2.5 M NaCl for 2 h and then stained with Coomassie blue.
RESULTS

LMP-1 deletion mutants and polypeptides. The panel of mutants used in this study is shown schematically in Fig. 1A (14, 37). These constructs were cloned into the pcDNA3 vector (Invitrogen), and a FLAG epitope was inserted at the amino terminus to facilitate the detection of protein expression. The expression of proteins of expected size from LMP-1 and the mutated constructs was verified by transfecting each plasmid into EBV-negative C33A cells and assaying by Western blotting with the FLAG antibody. As illustrated in Fig. 1B, all mutant LMP-1 polypeptides were expressed.

MMP-9 activity in cells transfected with LMP-1 deletion mutants. Analysis of MMP gelatinolytic activity revealed an increase in MMP-9 activity in C33A cells transfected with wild-type LMP-1 (Fig. 2A). The ratio of MMP-9/MMP-2 activity, as measured by reverse imaging and densitometric analysis (5), indicated that LMP-1 enhanced activity approximately sixfold above that of the control (Fig. 2B). The LMP-1 mutant lacking the entire carboxy terminus (LMP 1-187) was completely unable to induce MMP-9 activity. The mutants that retained either CTAR-1 (LMP 1-231) or CTAR-2 (LMP del 187-351) had reduced levels of MMP-9 gelatinolytic activity with 66 and 84% of the activity of LMP1, respectively. Interestingly, the coexpression of the deletion mutants LMP 1-231 and LMP del 187-351 restored full activity, suggesting that both domains contribute additively to MMP-9 activation. The levels of the 72-kDa MMP-2 activity were unchanged by LMP-1 or any of the mutants (Fig. 2A).

Analyses of the MMP-9 promoter revealed that LMP-1 activated the transcription of MMP-9 expression construct with a 2.6-fold increase over the vector control. LMP 1-187 did not transactivate the MMP-9 promoter. The deletion mutants LMP 1-231 and LMP del 187-351 had reduced transactivation, with LMP 1-231 and LMP del 187-351 retaining 50 and 72% of LMP1 transactivation, respectively (Fig. 2C). These data indicated a close correlation between the direct measurement of MMP-9 enzyme activity and the transactivation of the MMP-9 promoter. These experiments also show that either activation region of LMP-1, CTAR-1 or CTAR-2, can activate the MMP-9 promoter and induce MMP-9 activity; however, both domains are required for maximal activity.

Identification of LMP-1-induced transcription factors that activate the MMP-9 promoter. MMP activity is regulated by the control of gene transcription and also by posttranslational control. The MMP-9 promoter is primarily regulated by NF-κB, AP-1, and, to a lesser extent, secretory protein 1 (7, 32, 59). To determine the contribution of the CTAR-1 and CTAR-2 domains to the activation of NF-κB and AP-1 in the MMP-9 promoter, MMP-9 promoter constructs containing point mu-
tations in the NF-κB or AP-1 sites were cotransfected with LMP-1 or the LMP-1 mutants. The mutation of the NF-κB site slightly increased the basal activity of the promoter by approximately 5% (Fig. 3A), while the mutation of the AP-1 site reduced basal activity by 16% (Fig. 3B). The mutation of the NF-κB site (Fig. 3A) or the AP-1 site (Fig. 3B) abolished transactivation by LMP-1 and both of the LMP-1 mutants. These data indicate that both the NF-κB and AP-1 binding sites are necessary for the activation of the MMP-9 promoter and that both LMP-1 activation regions mediate transactivation through NF-κB and AP-1.

As reported previously, LMP-1 induces nuclear factors that bind to NF-κB and AP-1 sequences in the MMP-9 promoter region. Through the interaction of the TRAFs with CTAR-1 and that of TRADD with CTAR-2, LMP-1 activates NF-κB inducing kinase and JNK (1, 9, 24). Previous studies have indicated that only CTAR-2 activates JNK, and these data also show that a greater amount of AP-1, detected by EMSA, is induced by CTAR-2 (9, 24). However, both LMP-1 activation regions activated AP-1 binding to the MMP-9 promoter and required this site for transactivation (Fig. 3).

Regulation of the MMP-9 promoter through TRAF signaling. In order to determine the involvement of TRAFs in mediating MMP-9 activation induced by the LMP-1 mutants, C33A cells were transiently cotransfected with LMP-1 mutants and plasmids expressing dominant-negative forms of TRAF-2 (TRAF-2DN) or TRAF-3 (TRAF-3DN). The expression of TRAF-2DN has previously been shown to partially inhibit signaling from both CTAR-1 and CTAR-2 (22). In this study, TRAF-2DN reduced the activation by LMP-1 by 58%. TRAF2-DN reduced LMP 1-231 by approximately 43% and LMP del 187-351 by 20%. TRAF-2DN also reduced MMP-9 promoter transactivation by the combined mutants (LMP 1-231 plus LMP del 187-351) by 50% (Fig. 5A).

Previous studies have shown that the activation of NF-κB by CTAR-1 but not CTAR-2 is inhibited by the expression of TRAF-3DN (6, 22, 34). In this study, TRAF-3DN reduced the transactivation of the MMP-9 promoter induced by LMP-1, LMP 1-231, and the reconstruction plasmids by approximately 60% but did not affect transactivation by LMP del 187-351 (Fig. 5B). These data indicate that signaling from CTAR-1 is mediated through both TRAF-2 and TRAF-3, that TRAF2 contributes to signaling from CTAR-2, and that both TRAF-2...
and TRAF-3 contribute to the activation of the MMP-9 promoter.

**IkB inhibits MMP-9 expression.** The activation of NF-κB and NF-κB binding is necessary for the activation of the MMP-9 promoter (6, 22, 30, 34, 48). Therefore, the inhibitory effect of a constitutively activated form of the NF-κB repressor, IkB (17), on the induction of MMP-9 expression by the LMP mutants was determined. In assays performed with the CAT reporter construct, the expression of IkB abolished the induction of the MMP-9 promoter by LMP-1 and the LMP-1 mutants (Fig. 6A). Analysis of MMP-9 activity detected by gelatin zymography also indicated that the cotransfection of the IkB plasmid with the LMP mutants repressed MMP-9 gelatinolytic activity but did not affect the activity of MMP-2 (Fig. 6B and C). The activation of MMP-9 assessed by gelatin zymography correlated with the activation of the MMP-9 promoter. These

![Graph A](image1)

**FIG. 4.** Induction of NF-κB (A) and AP-1 (B) DNA-binding activity in cells transfected with wild-type (WT) LMP-1 and deletion mutants. Nuclear extracts from C33A cells transfected with LMP-1 mutants were mixed with either NF-κB or AP-1 32P-labeled probes. Excesses of nonlabeled NF-κB and AP-1 probes (×100) were used as competitors (NFκB, 5'-GATCGGGTTGCCCCAGTGGAATTCCCCAGCCTT-3'; AP-1, 5'-GATCTTCTAGACCGGATGAGTCATAGCTG-3'). Underlined letters indicate binding sequences in the promoter of the MMP-9 gene. NS, nonspecific binding.

![Graph B](image2)

**FIG. 5.** The effect of transient expression of TRAF-2DN or TRAF-3DN on MMP-9 transcriptional activity induced by wild-type (WT) LMP-1 or deletion mutants. Solid bars represent the CAT activity of LMP-1 and deletion mutants, while open bars represent the CAT activity when cells were cotransfected with TRAF2-DN (A) and TRAF-3DN (B). The data were compared with the transfection efficiency as determined by β-galactosidase assay, and activities were given relative to the activity of pcDNA3 without TRAF-DN, which was defined as 1. The mean values and standard deviations (error bars) are the results of five experiments.
DISCUSSION

LMP-1 is essential for the transformation of B lymphocytes and has profound effects on cellular gene expression (4, 38). In addition LMP-1 can activate the type IV collagenase MMP-9, which is implicated in tumor invasion of BM (59). Whether this effect is unique among the oncogenic viruses is unknown. The two activation domains of LMP-1, CTAR-1 and CTAR-2, both activate NF-kB yet also have distinct properties (14, 34, 37). CTAR-1, which interacts with TRAFs, induces the expression of the epidermal growth factor receptor through a pathway distinct from NF-kB activation, as epidermal growth factor receptor expression is not induced by CTAR-2 (34). In contrast, CTAR-2 has a greater ability to activate NF-kB in reporter gene assays (14, 34, 37) and is thought to be responsible for JNK activation by LMP-1 (9, 24). As LMP-1 also induces the expression of MMP-9, which is known to be regulated by NF-kB and AP-1 (59), it was of interest to determine the contribution of CTAR-1 and CTAR-2 to this transactivation. The data presented here reveal that both CTAR-1 and CTAR-2 of LMP-1 can activate the MMP-9 promoter and induce MMP-9 activity and that the domains have an additive effect for transactivation. These results suggest that the complete activation of the MMP-9 promoter by LMP-1 requires both CTAR-1 and CTAR-2, which can be present on separate molecules. It is likely that the oligomerization of LMP-1, mediated by the transmembrane domain, results in complexes that contain both signaling domains, albeit on separate molecules.

Although previous studies have suggested that JNK activation is mediated through CTAR-2 (9, 24), in this study both CTAR-1 and CTAR-2 induced AP-1, as detected by EMSA, with CTAR-2 inducing a greater amount. CTAR-1 interacts with TRAF-2 (8, 30, 34, 35), CTAR-2 interacts with TRADD, which binds TRAF-2 (19), and both domains are partially inhibited by TRAF-2DN. These results suggest that TRAF-2 signaling is a common pathway arising from these two domains. TRAF-2 has previously been shown to activate both NF-kB and JNK through distinct pathways (1, 20, 29, 41, 47). Thus, it is not surprising that both CTAR-1 and CTAR-2 would activate both NF-kB and AP-1, as revealed by these studies of the MMP-9 promoter. The previous studies of JNK activation have analyzed JNK activity on a glutathione S-transferase–Jun substrate in the presence of overexpressed JNK-1 (9, 24, 29, 38). The data presented here detect activated AP-1 on an authentic AP-1 site in the MMP-9 promoter. This activity may reflect the activation of distinct JNK kinases in vivo or indicate that CTAR-1 activates AP-1 through some other indirect mechanism.

The data also indicate that both NF-kB and AP-1 are essential for the activation of the MMP-9 promoter. The effects are not additive; thus, the mutation of either the NF-kB or AP-1 site eliminates the transactivation of the promoter by LMP-1. The complete inhibition of promoter activity by the constitutive active form of IκB also indicates that NF-κB binding is essential for MMP-9 promoter activity. These data suggest that both the NF-κB and AP-1 sites must be occupied to initiate transcription.
As NPC is a highly metastatic tumor with frequent expression of LMP-1 (3), the activation of MMP-9 may be an important contributing factor to pathogenesis. The data presented here reveal that NF-κB and AP-1 are both essential for this activation. Thus, agents that specifically inhibit NF-κB activation or JNK activation may be effective in preventing the metastasis of NPC (33). These findings suggest that the biologic phenotype of tumors associated with EBV latency types in which LMP-1 is expressed may include a potential for metastasis.

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