ABSTRACT

We have shown that the EBV oncoprotein, latent membrane protein 1 (LMP1), induces a constellation of tumor-invasiveness factors. Fibroblast growth factor (FGF)-2 is angiogenic as well mitogenic. Although FGF-2 does not contain a hydrophobic signal sequence for secretion, FGF-2 is released extracellularly. However, the mechanism by which FGF-2 is released is unclear. Here we show first that LMP1 induces in epithelial cells the expression of FGF-2 mRNA and protein through both LMP1 COOH-terminal activation domains, CTAR 1 and CTAR 2, which can activate nuclear factor (NF)-κB signaling and also the p38 mitogen-activated protein kinase pathway. Coexpression of IκBα (S32A/S36A), which cannot be phosphorylated and prevents NF-κB activation, with LMP1 inhibited induction of FGF-2 by LMP1, which suggests that LMP1 induces FGF-2 via NF-κB signaling. Moreover, unlike phorbol 12-myristate 13-acetate 13-acetate LMP1 also induced the release of the Mγ, 18,000 isoform of FGF-2 protein. Transfection of Ad-AH cells with LMP1 deletion mutants lacking either CTAR 1 or CTAR 2 also induced the release of the protein. Secretion was confirmed in 293 cells, which do not contain detectable endogenous FGF-2 protein, cotransfected with FGF-2 and LMP1. Finally, Na+/K+-ATPase participates in FGF-2 release, independently of the classical endoplasmic reticulum/Golgi pathway. In this study, the release of Mγ, 18,000 FGF-2 protein was partially suppressed by ouabain, which inhibits the activity of Na+/K+-ATPase of subunit; but not by Brefeldin A, which inhibits the endoplasmic reticulum/Golgi-dependent secretory pathway. In contrast, the release of Mγ, 18,000 FGF-2 protein was almost completely inhibited by IκBα (S32A/S36A). These results suggest that FGF-2 release is independently mediated by NF-κB signaling, not simply a consequence of induction itself. Thus, NF-κB signaling is involved in induction of expression and release of FGF-2 by LMP1.

INTRODUCTION

EBV is a human herpesvirus associated with several types of malignancies, in particular NPC1 1 (1) and EBV lymphoproliferative disease (2) in which the virus is likely to have a causal role, as well as Burkitt’s lymphoma (3), Hodgkin’s lymphoma (4), and a subset of breast cancers (5) and gastric carcinomas (6). Latent infection is established in all of these tumors. Up to 11 EBV genes are expressed in these latent infections; the number differs in the various malignancies. Two of the genes encode small, nonpolyadenylated EBERs (EBER 1 and EBER 2), 6 encode EBNAs (EBNAs 1, 2, 3A, 3B, 3C, and VP), and 3 encode integral LMPs (LMP 1, 2A, and 2B). Latency is classified into three types (types I, II, and III), based on the pattern of viral gene expression. EBNAs 1 is the only virus-encoded protein constitutively expressed in type I latency and exemplified by Burkitt’s lymphoma. In type II latency, exemplified by NPC, EBNAs 1 is expressed in the absence of the other EBNAs but accompanied by expression of LMP1, LMP2A, and LMP2B. In type III latency, found in EBV B-cell lymphoproliferative diseases, the full set of EBNAs and LMPs is expressed. In all of the latency states, EBERs are expressed (3). Types I and III latency states are captured in B-lymphoblastoid lines, whereas cell lines with a latency II phenotype are not available. Among the nuclear and membrane proteins expressed as a consequence of latent EBV infection, LMP1 is of particular interest because it transforms continuous rodent fibroblast cell lines upon single gene transfer (7). LMP1 protects B lymphocytes from apoptosis through induction of bcl-2 (8). In epithelial cells in transgenic mice, LMP1 induces epidermal hyperplasia and alters keratin gene expression (9), and in epithelial cell culture, LMP1 induces expression of the epidermal growth factor receptor, A20, MFP-9 and COX-2 (10–13). The COOH-terminal portion of LMP1, which is in the cytoplasmic domain of the protein, contains two functional domains. The proximal CTAR 1 interacts with tumor necrosis factor receptor-associated factors and induces NF-κB signaling (14, 15). The distal CTAR 2 associates with the tumor necrosis factor receptor-associated death domain and also mediates NF-κB signaling (16). CTAR 2 also induces the activity of the activator protein-1 (AP-1) transcriptional factor via a signaling pathway that involves the c-Jun NH2-terminal kinase 1 (17). LMP1 can also activate the p38 mitogen-activated protein kinase (MAPK) pathway, which occurs through both CTAR 1 and CTAR 2 domains (18).

Recently, we have shown that LMP1 induces MFP-9 activity (12) and that LMP1 increases production of VEGF through induction of COX-2 (13). These findings suggest that LMP1 is a potent inducer of invasion and angiogenesis in epithelial tumors and prompted us to examine the relation of FGF-2, one of the major angiogenic factors, with LMP1. FGF-2, also known as basic fibroblast growth factor, belongs to a large FGF family of 19 structurally related members. FGF-2 is a multifunctional growth factor in embryogenesis and morphogenesis, but it also functions as an angiogenic factor in wound healing, cardiovascular disease, and neovascularization (19). Overexpression of FGF-2 has been detected in various tumors such as glioblastoma (20), pancreatic cancer (21), non-small cell lung cancer (22), bladder cancer (23), and esophageal cancer (24). The five FGF-2 isoforms result from a process of alternative initiation of translation at five in-frame codons. The translation of the isoform of lowest molecular weight (Mγ, 18,000) is initiated at a canonical AUG codon, whereas the translation of the four high molecular weight isoforms (Mγ, 22,000, 22,500, 24,000, and 34,000) of FGF-2 initiates at four upstream AUG codons (25, 26). The main structural feature of the high molecular weight isoforms is presence of a nuclear localizing sequence. These isoforms of FGF-2 localize in the nucleus, whereas Mγ, 18,000 FGF-2 lacks the nuclear localizing sequence and is mostly cytosolic (27). Although all FGF-2 isoforms do not contain a hydrophobic signal peptide sequence, FGF-2 can be detected extracellularly and is released from FGF-2-producing...
cells in an ATP-dependent, classical ER/Golgi-independent pathway (28, 29). Recently, it has been reported that Na+/K+ -ATPase participates in FGF-2 release (30, 31). In this report, we show that LMP1 induces the expression of FGF-2 in epithelial cells. Moreover, LMP1 promotes the release of FGF-2 into the extracellular fluid. Both of these phenomena appear to be mediated by NF-κB signaling. Promotion of protein secretion is a newly detected function of LMP1.

MATERIALS AND METHODS

Plasmids. pcDNA3-based LMP1 has been described (12). FLAG-tagged LMP1 mutants (LMP1 WT, LMP1 1–231, LMP1 1–187, and LMP1 del 187–351) were kindly provided by Dr. Nancy Raab-Traub (University of North Carolina, Chapel Hill, NC) and have been described (32). The LMP1 1–187 mutant has the entire COOH-terminal domain deleted, whereas LMP1 1–231 retains CTAR 1 only, and LMP1 del 187–351 has CTAR 2 only (Fig. 2A). The FLAG-tagged M, 18,000 FGF-2 expression plasmid, in which FLAG epitope tags are inserted at the COOH terminus of FGF-2 cDNA, was the generous gift of Dr. Robert Levenson (Penn State College of Medicine, Hershey, PA; Ref. 32). The FLAG-tagged IκBα (S32A/S36A) expression plasmid (sIκBα) was kindly provided by Dr. Albert Baldwin (University of North Carolina). IκBα (S32A/S36A) is not phosphorylated because of the S32 and S36 substitutions, which result in prevention of both degradation of IκBα and subsequent translocation of NF-κB into the nucleus.

Cell Culture and Transient Transfection. Ad-AH cells, kindly provided by Dr. Erik K. Flemington (Tulane University, New Orleans, LA), are an EBV-negative human nasopharyngeal carcinoma, and human embryonal kidney 293 cells were obtained from American Type Culture Collection. Cells were maintained in DMEM with 10% FBS and penicillin and streptomycin.

For transfections, cells were grown in 100-mm dishes. Cells were transfected with 1–2 μg of DNA with the use of Effectene reagent (Qiagen) according to the manufacturer’s protocol. Forty-eight h after transfection, Ad-AH and C33A cells were held for 24 h in DMEM with neither FBS nor antibiotics and with or without treatment with PMA (100 ng/ml; Sigma). After transfection, 293 cells were cultured in DMEM containing 10% FBS and antibiotics for an additional 48 h with or without ouabain (100 μM; Calbiochem) or Brefeldin A (5 μg/ml; Sigma), followed by incubation in DMEM without FBS and antibiotics and with or without ouabain or Brefeldin A. Cell viability was assessed by the trypan blue exclusion test (34) and MTT assay (35). The difference in ratio of nonviable cells did not exceed >3% in trypan blue exclusion tests. In MTT assay, absorbance was not reduced, nor was there any visible cytotoxic effect at any of the concentrations of drug used for the experiments.

Western Blot Analysis. Cell lysates were extracted in 500 μl of lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.10% SDS, 0.2 mM sodium orthovanadate, 0.1 M NaF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride], and protein concentration was determined by a Bio-Rad protein assay. Protein (100 μg) was boiled in SDS sample buffer for 3 min, electrophoresed on 10–15% SDS polyacrylamide gels, and transferred onto a nitrocellulose membrane (Osmonics). Nonspecific reactivity was blocked by incubation for 30 min in Tris-buffered saline solution containing 0.1% Tween 20 and 10% nonfat dried milk. The membrane was incubated overnight at 4°C with: (a) mouse anti-LMP1 monoclonal antibody (DAKO); (b) mouse anti-FLAG M2 monoclonal antibody (Sigma); (c) rabbit anti-FGF-2 polyclonal antibody (Santa Cruz); and (d) mouse anti-γ-ubulin antibody (Sigma) or mouse anti-Na+/K+ -ATPase α1 subunit (Upstate Biotechnology). The membrane was washed with Tris-buffered saline solution containing 0.1% Tween 20 (TBST), incubated with horseradish peroxidase-conjugated antimouse (for monoclonal primary) or antirabbit (for polyclonal primary) secondary antibody (Amersham) at room temperature for 1 h, and washed three times for 15 min with TBST. Peroxidase activity was detected by enhanced chemiluminescence.
The medium of cultured cells was collected and cleared by centrifugation at 13,000 rpm for 15 min at 4°C. For detection of FGF-2 in medium, the medium was concentrated by ultrafiltration through Centricon (Amicon) and applied for electrophoresis.

Gelatin Zymography. MMP-2 and MMP-9 were assayed by gelatinolytic activity by means of gelatin zymography as described previously (12). The concentrated medium was fixed with SDS loading buffer [50 mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, and 0.01% bromphenol blue], followed by incubation at 37°C for 20 min. The mixtures were then electrophoresed on 10% SDS-PAGE polyacrylamide gel containing gelatin at a final concentration of 0.1%. After electrophoresis, the gel was rinsed in 2.5% Triton X-100 for 2 h and incubated in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 10 mM CaCl₂, and 0.02% NaN₃ at 37°C overnight. The MMPs were identified after staining of the gel in 0.1% Coomassie blue R250 (Sigma) dissolved in 40% methanol, 10% acetic acid, and destaining in the same solution, but without Coomassie blue. Gelatinolytic activity was visualized as a clear band against a dark background of stained gel.

RNA Extraction and RNase Protection Assay. Total RNA was extracted from cells with the use of RNeasy total RNA isolation kit (Qiagen). Plasmid DNA, to be used as a template in the preparation of riboprobe for detection of human FGF-2, was the generous gift of Selective Genetics, Inc. When linearized with XbaI and transcribed with T7 RNA polymerase, the length of the [α-³²P]UTP-labeled riboprobe was 189 nucleotides, which protects 169 nucleotides of FGF-2 mRNA (36). GAPDH was supplied by United States Biochemicals, Inc. (37). RNase protection assays for the detection of FGF-2 mRNA were performed with total RNA with RNase protection kit II (Ambion) according to the manufacturer’s protocol. Briefly, 10 μg of RNA were dissolved and heated at 95°C for 3 min in hybridization solution containing [α-³²P]UTP-labeled FGF-2 probe (100,000 cpm) and [α-³²P]UTP-labeled GAPDH probe (500,000 cpm). After hybridization overnight at 37°C, the solution was diluted in RNase digestion buffer containing RNases A and T1 and incubated for 30 min. Ethanol-precipitated samples were resuspended in 6 μl of gel loading buffer and heated at 95°C for 5 min. Samples were electrophoresed on a 4% polyacrylamide gel containing 7 M urea, and then the gel was dried under vacuum at 80°C and visualized with Kodak BioMax film. Signal intensities were calculated with a Molecular Dynamics Phosphorimager (Sunnyvale) and ImageQuant software. The intensity of each band for FGF-2 mRNA was normalized to the intensity obtained with the GAPDH probe in the same hybridization reaction.

RESULTS

LMP1 Stimulates the Expression of FGF-2 Protein and mRNA in Epithelial Cells. To test whether LMP1 can induce the expression of FGF-2, Ad-AH cells, an EBV-negative nasopharyngeal epithelial cell line, were transiently transfected with an LMP1 expression plasmid. FGF-2 protein was detected by Western blotting of cell extracts.
LMP1 induced the expression of FGF-2 protein, depending on the amount of transfected LMP1 expression plasmid. Treatment of Ad-AH cells with PMA also stimulated the expression of FGF-2. The amount of transfected LMP1 expression plasmid, or both LMP1 and FLAG-tagged IκBα (S32A/S36A) expression plasmids, depends on the level of protein and mRNA in Ad-AH cells (Fig. 3, A and B). At the mRNA level, IκBα (S32A/S36A) suppressed the induction of FGF-2 mRNA by LMP1 by 86% (Fig. 3C). These results indicate that NF-κB signaling is involved in induction of FGF-2 by LMP1. 

Both CTAR 1 and CTAR 2 Domains Induce FGF-2 Expression in Epithelial Cells. The COOH-terminal portion of LMP1 contains two signal transduction-activating domains, CTAR 1 and CTAR 2 (Fig. 2A). To determine whether these domains are implicated in FGF-2 expression, FLAG-tagged LMP1 mutant plasmids were used. As shown in Fig. 2, B–D, LMP1 1–187, which lacks both CTAR 1 and CTAR 2 domains, did not induce FGF-2 expression in Ad-AH cells. On the other hand, LMP1 1–231, which contains only CTAR 1, and LMP1 del 187–351, which contains only CTAR 2, induced the expression of FGF-2 at the level of both protein and mRNA. FGF-2 expression induced by these mutants, which contain either CTAR 1 or CTAR 2, was lower than the level of FGF-2 induced by LMP1 WT expression plasmid. These data suggest that both CTAR 1 and CTAR 2 domains contribute additively to the induction of FGF-2.

Induction of FGF-2 Expression by LMP1 Is Related to NF-κB Signaling. LMP1 activates NF-κB through both CTAR 1 and CTAR 2 (14–16). Therefore, we tested whether NF-κB signaling is involved in induction of FGF-2 by LMP1. The FLAG-tagged IκBα (S32A/S36A) expression plasmid, cotransfected with LMP1 expression plasmid, suppressed the induction of FGF-2 by LMP1 at the level of protein and mRNA in Ad-AH cells (Fig. 3, A and B). At the mRNA level, IκBα (S32A/S36A) suppressed the induction of FGF-2 mRNA by LMP1 by 86% (Fig. 3C). These results indicate that NF-κB signaling is involved in induction of FGF-2 by LMP1. Na+/K+-ATPase α1 subunit protein expression was not affected by LMP1 in Ad-AH cells.

$M_1$, 18,000 FGF-2 Protein Increases in the Culture Medium of LMP1-transfected Epithelial Cells. All FGF-2 isoforms do not contain a hydrophobic signal peptide sequence. However, FGF-2 can be released extracellularly (28, 29). To detect the FGF-2 protein released from Ad-AH cells, Western blot analysis was performed on culture medium. $M_1$, 18,000 FGF-2 protein was detected in concentrated medium from LMP1-transfected Ad-AH cells, and the protein level was dependent on the amount of LMP1 expression plasmid transfected (Fig. 2A). High molecular weight isoforms of FGF-2 ($M_r$ 22,000, 22,500, and 24,000) were not detected in the medium. Interestingly, although treatment of Ad-AH cells with PMA induced a large amount of $M_1$, 18,000 FGF-2 protein detected in cell lysates (Fig. 1A), concentrated medium from Ad-AH cells treated with PMA contained much less $M_1$, 18,000 FGF-2 protein than did medium from LMP1-transfected cells (Fig. 4A). Furthermore, the amount of $M_1$, 18,000 FGF-2 protein in medium from LMP1 1–231 or LMP1 del 187–351 transfected cells was clearly more than that in medium from Ad-AH cells.
These results indicate that LMP1 stimulates the extracellular release of the $M_1$ 18,000 isoform of FGF-2. To analyze the effect of LMP1 mutants on FGF-2 release, 293 cells were cotransfected with FLAG-tagged $M_1$ 18,000 FGF-2 plasmid and the LMP1 mutants. Both LMP1 1–231, which contains only CTAR 1, and LMP1 del 187–351, which contains only CTAR 2, stimulated the extracellular release of FLAG-tagged $M_1$ 18,000 FGF-2 protein in 293 cells, but to a lesser extent than by LMP1 WT, accompanied by a decrease of FLAG-tagged $M_1$ 18,000 FGF-2 protein level in cell lysates. LMP1 1–187, which contains neither CTAR 1 nor CTAR 2, did not affect the release of the protein (Fig. 5C and D). These data indicate that both CTAR 1 and CTAR 2 contribute additively to stimulating the release of $M_1$ 18,000 FGF-2 protein.

Ouabain Partially Suppressed the Extracellular Release of FLAG-tagged $M_1$ 18,000 FGF-2 Protein, but Brefeldin A Did Not. To analyze the mechanism of stimulation of release of $M_1$ 18,000 FGF-2 protein by LMP1, we compared the effect of ouabain, which inhibits the activity of the $Na^+\!/K^+\!-ATPase$ α1 subunit isoform, and Brefeldin A, which inhibits the classical ER/Golgi-dependent secretory pathway. It has been reported that ouabain inhibits release of $M_1$ 18,000 FGF-2, but Brefeldin A does not (30, 31). 293 cells are known to express a ouabain-sensitive α1 subunit isoform (39). These cells, transiently transfected with FLAG-tagged $M_1$ 18,000 FGF-2 expression plasmid, with or without LMP1, were maintained in DMEM with 10% FBS and antibiotics, with or without 100 nM ouabain or 5 ng/ml Brefeldin A for 48 h. After brief washing with PBS, the cells were

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**Fig. 5.** LMP1 induces extracellular release of $M_1$ 18,000 FGF-2 protein. A, detection of FLAG-tagged $M_1$ 18,000 FGF-2 protein in concentrated media from 293 cells transfected with control vector (pcDNA3) or two different amounts of LMP1 expression plasmid, or from cells treated with PMA (100 ng/ml). The gelatinolytic activity of MMP-2 was used as loading control. B, Western blotting of FLAG-tagged $M_1$ 18,000 FGF-2 or LMP1 protein in 293 cell lysates of the same samples as A. C, detection of FLAG-tagged $M_1$ 18,000 FGF-2 in the concentrated medium from 293 cells transfected with control vector (pcDNA3) or LMP1 mutant expression plasmids (pcLMP1 WT, pcLMP1 1–231, pcLMP1 1–187, or pcLMP1 del 187–351). The gelatinolytic activity of MMP-2 was used as a loading control. D, expression of FLAG-tagged $M_1$ 18,000 FGF-2 protein in 293 cell lysates from the same samples as in C.
incubated in fresh DMEM containing neither FBS nor antibiotics, but with or without ouabain or Brefeldin A for 24 h. As shown in Fig. 6, Brefeldin A did not affect the release of FLAG-tagged M<sub>r</sub> 18,000 FGF-2 protein. Treatment with ouabain reduced release of FGF-2 from 293 cells transfected with LMP1. Na<sup>+</sup>/K<sup>+</sup>-ATPase α1 subunit protein expression was affected by neither LMP1, ouabain, nor Brefeldin A.

**LMP1 Stimulates Extracellular Release of FLAG-tagged M<sub>r</sub> 18,000 FGF-2 Protein via NF-κB Signaling.** 293 cells, transiently cotransfected with FLAG-tagged M<sub>r</sub> 18,000 FGF-2 expression plasmid and LMP1 and/or IκBα (S32A/S36A), were maintained in DMEM with 10% FBS and antibiotics for 48 h. After brief washing with PBS, the cells were incubated in fresh DMEM containing neither FBS nor antibiotics for 24 h. Cell lysates and concentrated media were analyzed by Western blotting. Interestingly, M<sub>r</sub> 18,000 FGF-2 in medium was decreased by transfection with srIκBα (Fig. 3D). This result prompted examination of the role of NF-κB signaling for the release of M<sub>r</sub> 18,000 FGF-2 protein. In 293 cells cotransfected with LMP1 and IκBα (S32A/S36A) expression plasmid, extracellular release of FLAG-tagged M<sub>r</sub> 18,000 FGF-2 was almost completely suppressed (Fig. 6A) and was accompanied by an increase in the intracellular stock of the protein (Fig. 6B). These data indicate that NF-κB signaling plays an important role in the extracellular release of M<sub>r</sub> 18,000 FGF-2 protein over and above the effect of NF-κB in signaling induction of FGF-2.

**DISCUSSION**

FGF-2 is a mitogenic and growth-promoting protein (40). In addition, FGF-2 induces endothelial cell proliferation, migration, and angiogenesis in vitro and in vivo (41). These phenomena suggest that FGF-2 is involved in malignant tumor progression. In this study, we have shown that both CTAR 1 and CTAR 2 of LMP1 induce FGF-2 at the protein and mRNA levels. The induction was in part dependent on NF-κB signaling because it was strongly suppressed by the expression of IκBα (S32A/S36A). There are five GC boxes, which may represent stimulatory protein-1 (SP-1) binding sites, and one potential activator protein-1 (AP-1) binding site within the FGF-2 core promoter region (42). Moffett et al. (43) identified a unique −555/−513 bp growth factor-responsive element and a separate region (−624/−556 bp) essential for PKC and cyclic AMP stimulation. There has been no report showing the existence of an NF-κB binding site on the FGF-2 promoter. Although the mechanism of FGF-2 induction by LMP1 is unclear, it might be an indirect effect. One candidate involved in the induction of FGF-2 by LMP1 may be COX-2. COX-2 catalyzes the first two steps in the biosynthesis of the prostaglandins from arachidonic acid. PGH<sub>1</sub> is transformed into the primary prostanoids, PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, PGI<sub>2</sub>, and thromboxane A<sub>2</sub> (44, 45). Recently, we have shown that LMP1 induces COX-2 through both CTAR 1 and CTAR 2 domains, and NF-κB is essential for induction of COX-2 by LMP1. LMP1 increased production of PGE<sub>2</sub> in a COX-2-dependent manner and finally increased VEGF production (13). PGF<sub>2α</sub> induces FGF-2 in vascular smooth muscle cells (46), and there is a possibility that COX-2 induces FGF-2 expression in a manner similar to induction of VEGF by LMP1 through COX-2 (13).

FGF-2 lacks the hydrophobic signal-peptide sequence, so that the mechanism by which FGF-2 is released is an intriguing question. Florkiewicz et al. (28) have shown that only the M<sub>r</sub> 18,000 isoform of FGF-2 was released extracellularly from COS-1 cells transfected with all four FGF-2 isoforms. Albuquerque et al. (47) have also shown that only M<sub>r</sub> 18,000 FGF-2 release was enhanced by matrix proteins, 17β-estradiol, and a PKC-signaling pathway. In contrast, there have been some reports that show the high molecular weight isoforms of FGF-2 can also exit the cell. Azuma et al. (48) examined the release of FGF-2 protein in six human salivary gland cell clones. They identified FGF-2 isoforms in conditioned medium, but the expression patterns of each FGF-2 isoform were different with each clone. Although the mechanism that underlies these discrepancies is unknown, there may be cell specificities in the type of extracellular release of each FGF-2 isoform. Interestingly, although PMA strongly induced expression of M<sub>r</sub> 18,000 FGF-2, very little could be detected in concentrated medium. In contrast, large amounts were detected in the medium of LMP1-transfected cells (Fig. 4A). Furthermore, both CTAR 1 and CTAR 2 domains contributed to the stimulation of the M<sub>r</sub> 18,000 FGF-2 protein release. These results suggest that LMP1 induces the extracellular release of M<sub>r</sub> 18,000 FGF-2 protein through both CTAR 1 and CTAR 2 domains. This observation was confirmed.
that oleandrin, a polyphenolic cardiac glycoside derived from the et al. appeared to be suppressed (50). In addition, Manna et al. that FGF-2 binds directly or indirectly to the Na+/H+ATPase. Although they demonstrated that FGF-2 binds directly or indirectly to the Na+/K+-ATPase α1 subunit (28, 30), the mechanism of inhibition of FGF-2 release is unclear. In our study, ouabain partially inhibited the extracellular release of FLAG-tagged M, 18,000 FGF-2 protein, but Brefeldin A, an inhibitor of the ER/Golgi pathway, did not. This finding is consistent with previous reports. In our data, Na+/K+-ATPase α1 subunit protein expression was not affected by LMP1, ouabain, Brefeldin A, or IκBα (S32A/S36A). This result suggests that Na+/K+-ATPase activity is involved in release of M, 18,000 FGF-2 protein, but the Na+/ K+-ATPase expression level is not.

We have shown that IκBα (S32A/S36A) almost completely suppressed the extracellular release of endogenous M, 18,000 FGF-2 protein. In addition, this finding was confirmed in 293 cells, which do not contain a detectable level of endogenous FGF-2 protein, transfected with M, 18,000 FGF-2 expression plasmid. These results suggest that M, 18,000 FGF-2 release is independently mediated by NF-κB signaling, not simply a consequence of induction itself. Although the relation between Na+/K+-ATPase and NF-κB signaling is unclear, there are several reports about inhibition of Na+/K+-ATPase and NF-κB signaling (49, 50). Aizman et al. (49) showed that ouabain acts as a biological inducer of intracellular calcium oscillation that elicits activation of NF-κB. This result seems to conflict with the present findings. However, experimental procedures in that study were completely different. They maintained cells in much higher concentrations of ouabain (250 μM) for 40 min compared with our concentration (100 nm) for 3 days. Actually, in another report, ouabain, at <100 μM, did not stimulate NF-κB signaling; rather, it appeared to be suppressed (50). In addition, Mann et al. (50) showed that oleandrin, a polyphenolic cardiac glycoside derived from the leaves of Nerium oleander, blocked tumor necrosis factor-induced activation of NF-κB in a concentration- and time-dependent manner. Smith et al. (51) exposed prostate cancer cell lines to a nontoxic concentration of ouabain (170 ng/ml) for up to 72 h and produced 48.4% inhibition of FGF-2 release from the cells. These results suggest that a low concentration of ouabain is effective enough to inhibit the release of FGF-2 protein. Furthermore, Mann et al. (50) have shown that oleandrin also produced an inhibition of FGF-2 release. It is known that PMA, a PKC activator, activates at least two pathways: NF-κB and mitogen-activated protein kinase pathways (52). However, PMA induced the release of a much lesser amount of M, 18,000 FGF-2 protein compared with LMP1 in our system. Also noteworthy is the inhibition of Na+/K+-ATPase activity by PKC (53). This information suggests that the relation between the Na+/K+-ATPase and NF-κB signaling pathways should be reevaluated to elucidate the mechanism of FGF-2 release stimulation by LMP1. Incidentally, LMP1 has pleiotropic effects, but this is the first description of extracellular release of protein produced by this viral oncoprotein and fits well with the proposal that it not only has transforming properties but can also promote tumor invasiveness by inducing specific cellular proteins and signaling pathways (12, 13, 35, 38).

Recently, a significant correlation between expression of LMP1 and microvesicle counts in NPC tissue was reported (54). Microvesicle counts also correlated with the progression of lymph node metastasis (54). In addition to VEGF and FGF-2, LMP1 induces the expression of interleukin 8 at the transcriptional level (55). Interestingly, these angiogenic molecules are regulated by the NF-κB signaling pathway in LMP1-expressing cells (13, 55). Taken together, there is the possibility that LMP1 contributes to metastasis by inducing angiogenesis through NF-κB signaling. Furthermore, suppression of the NF-κB pathway might provide a basis for LMP1-expressing epithelial tumors.

ACKNOWLEDGMENTS

We thank Drs. David C. Lee and Luwen Zhang for critical comments and suggestions. We are also grateful to Drs. Nancy Raab-Traub, Robert Levenson, and Andrew Baldwin for the excellent technical assistance. We are grateful to Drs. David Lee and Luwen Zhang for critical comments and suggestions. We are also grateful to Drs. Nancy Raab-Traub, Robert Levenson, and Andrew Baldwin for the excellent technical assistance.

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Epstein-Barr Virus Latent Membrane Protein 1 Induces and Causes Release of Fibroblast Growth Factor-2

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