SPANDIDOS PUBLICATIONS

Phosphorylation and nuclear translocation of STAT3 regulated by the Epstein-Barr virus latent membrane protein 1 in nasopharyngeal carcinoma

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Abstract. The Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) oncoprotein has been shown to mediate activation of the signal transducer and activator of transcription 3 (STAT3). In the present study, we delineated the mechanism by which LMP1 stimulates STAT3 in a human nasopharyngeal carcinoma (NPC) cell line. LMP1 stimulated STAT3 Tyr 705-dependent nuclear accumulation, as well as the phosphorylation of STAT3 at both Tyr 705 and Ser 727. Treatment of cells with interleukin-6 neutralizing antibody inhibited the phosphorylation of STAT3 Tyr 705 and Ser 727. The differential phosphorylation of STAT3 was found to be a result of activation of Janus kinase 3 (JAK3) and extracellular signal-regulated kinase (ERK). The biological significance of JAK3-mediated activation of STAT3 Tyr 705 phosphorylation was further assessed by treating the cells with an inhibitor (WHI-P131) of JAK3. Inhibition of ERK activity by an inhibitor (PD98059) of MAPK/extracellular signal-regulated kinase kinase (MEK1) decreased the LMP1-induced activation of STAT3 Ser 727. Furthermore, immunohistochemical analysis showed an increased nuclear STAT3 Tyr 705 staining in LMP1-positive cells and STAT3 Tyr 705 phosphorylation related to NPC stages III and IV. Demonstration of the involvement of different kinases in LMP1-induced STAT3 activation supports the involvement of the JAK/STAT and mitogen-activated protein kinase (MAPK)/ERK signaling pathways in the regulation of STAT3 activation by LMP1.

Introduction

The Epstein-Barr virus (EBV), a γ herpes virus, is associated with many malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), and Hodgkin's disease (1). A subset of genes, including latent membrane protein 1 (LMP1), is expressed when EBV transforms resting B lymphocytes into lymphoblastoid cell lines (LCLs). Among the products expressed by EBV during latent infection, LMP1 is regarded as the principal oncoprotein of EBV (2,3).

LMP1 is a 63-kDa integral membrane protein consisting of a short highly charged cytoplasmic amino terminus of 23 amino acids; 6 hydrophobic transmembrane domains required for protein aggregation, and a long acidic cytoplasmic carboxyl terminus of 200 amino acids (4,5). The carboxyl terminus comprises two functional domains: the membrane-proximal C-terminal activation region-1 (CTAR1) and the membrane-distal CTAR2 (6,7). LMP1 can mediate the activation of NF-κB through CTAR1 as well as the activation of both NF-κB and AP-1 through CTAR2. It has been reported that there exists a new domain between CTAR1 and CTAR2 named CTAR3 that is associated with the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway (8).

Mitogen-activated protein (MAP) kinases represent a family of serine/threonine protein kinases comprising extracellular signal-regulated kinase (ERK)1/ERK2, p38/HoG1 (p38), and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) (9-11). Our previous work shows that LMP1 can activate the AP-1 family by activating JNK and regulating the transcription of downstream genes. JNK plays a vital role in the LMP1 signaling pathways. LMP1 promotes JNK phosphorylation by CTAR2 mainly through TRAF/TRADD compounds and hence activates AP-1 (12). In addition, LMP1 can activate the Ras signaling pathway via the Ras/MAPKKK/MAPKK/ERK/Ets-1 signal (13).

STAT3 is a key molecule through which receptors of multiple cytokines and growth factors perform their functions. It plays a significant role in the promotion of cell proliferation (14,15), the inhibition of cell apoptosis (16), immune escape (17,18), angiogenesis (19), and the invasion and metastasis of tumor cells (20-22). STAT3 is thought to play a role in carcinogenesis through its abnormal signal transduction. Phosphorylation of tyrosine 705 (Tyr 705) and serine 727 (Ser 727) are the important functional forms of STAT3. After its activation with the phosphorylation of Tyr 705, STAT3 forms dimers with itself or with STAT1, translocates to nuclei, and then binds to specific DNA sequences and starts

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the transcription of multiple related downstream genes. It has been previously reported that LMP1 can promote STAT3 Tyr 705 phosphorylation and enhance its DNA binding activity in B lymphocytes (8,23). STAT3 Ser 727 phosphorylation is required for the maximal activation of STAT3 signaling, and Tyr 705 phosphorylation alone is not sufficient for the obligatory role of STAT3 signaling in oncogenesis (24-26).

LMP1 expression can instigate an interleukin (IL)-6-mediated signaling loop that renders the cell independent of the initial stimulus and provides continuous STAT3 Tyr 705 activation (27). In B cells and 293 cells, it has been reported that LMP1 can activate the JAK/STAT signaling pathway via CTAR3 (8). Our previous research showed that, in NPC cells, LMP1 can activate the JAK/STAT signaling pathway with the cooperation of the CTAR2 and CTAR3 domains, and that vascular endothelial growth factor (VEGF) can be upregulated by LMP1 through the activation of STAT3 (28,29).

In the present study, we investigated the molecular mechanisms underlying STAT3 phosphorylation induced by LMP1, specifically addressing the role of ERK1/2 and the JAK3 signaling pathway in mediating LMP1-induced translocation of STAT3 to the nucleus. Our finding indicates that LMP1 protein expression has a significant correlation with the prognosis of NPC which may be associated with the activation of STAT3 phosphorylation.

Materials and methods

Cell lines and culture. CNE1 is an LMP1-negative, highly differentiated NPC cell line. CNE1-LMP1 is a stably transfected cell line, established by introducing LMP1 cDNA into the CNE1 cell, in which LMP1 is highly expressed. These cells were maintained in RPMI-1640 (Gibco BRL) medium supplemented with 10% heat-inactivated fetal calf serum. Tet-on-LMP1-HNE2 is an established cell line using a newly developed Tet-on gene expression system in which LMP1 is highly expressed in a dose-dependent manner. Tet-on-LMP1-HNE2 cells were cultured in RPMI-1640 medium with 100 mg/l of G418 and 50 mg/l of hygromycin supplemented with 10% fetal calf serum. To induce LMP1 expression, Tet-on-LMP1-HNE2 cells were treated with doxycycline at a dosage of 0.6 μg/ml (30). All cells were grown at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Reagents. The MAPK/extracellular signal-regulated kinase kinase (MEK1) inhibitor, PD98059, purchased from Cell Signaling Technology, was dissolved in dimethyl sulfoxide and stored at -20˚C. WHI-P131, a JAK3 inhibitor, was kindly provided by Dr Jiming Wang (National Institutes of Health, Bethesda, MD, USA). The BCA Protein Assay Reagent Kit, Supersignal Chemiluminescence System (ECL), and NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit were from Pierce Chemical (Rockford, IL, USA). The p44/p42 MAP Kinase Assay Kit was purchased from Cell Signaling Technology.

Western blot analysis. To induce LMP1 expression, Tet-on-LMP1-HNE2 cells were treated with doxycycline (Dox) 0.6 μg/ml for 24 h before harvesting. To neutralize IL-6, anti-human IL-6 neutralizing antibody (R&D Systems) was added to the medium at 10 or 20 μg/ml for 24 h before harvesting. Cells were treated with different kinase inhibitors for 2 h before harvesting. Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit. Protein concentrations were determined using the BCA Protein Assay Reagent Kit, with bovine serum albumin as a standard.

Whole cell lysates were prepared by sonicating cell pellets in sample buffer and boiling for 5 min before loading onto the gel. Proteins were fractionated on 8% polyacrylamide gels and blotted onto a nitrocellulose membrane (Bio-Rad), following the manufacturer's protocol. Antibodies for detecting phosphorylated STAT were purchased from Cell Signaling Technology and used at a 1:1000 dilution in phosphate-buffered saline (PBS) with 5% skim milk. The LMP1 antibody was obtained from Dako. Horseradish peroxidase-conjugated secondary antibody and the ECL Color Developing Kit were purchased from Pharmacia-Amersham and used according to the manufacturer's instructions. The antibodies used were as follows: LMP1 (csl, Dako), STAT3 (#9132, Cell Signaling Technology), phospho-Stat3 (705) (#9136, Cell Signaling Technology), phospho-Stat3 (Ser 727) (#9134, Cell Signaling Technology), actin (sc-8432), nucleolin (MS-3) (SC-8031), anti-human IL-6 neutralizing antibody (AB-206-NA, R&D), p-ERK (E-4) (sc-7383, Santa Cruz), and ERK (c-16) (93, Santa Cruz).

Immunofluorescence analysis. Cellular localization of proteins was investigated by immunofluorescence. Cells were washed with cold PBS and fixed with cold 3.7% polyformaldehyde for 30 min. The primary antibody was diluted 1:200 with PBS and incubated with the cells at 4˚C overnight. Fluorescein-labeled immunoglobulin G (IgG) was diluted 1:1000 with PBS and incubated with the cells to bind with the primary antibody. Cellular localization of proteins was observed using fluorescence microscopy or laser scanning confocal microscopy.

Flow cytometry. Cells were collected, rinsed with PBS, and suspended in 75% ethanol at -20˚C overnight. Fixed cells were centrifuged and washed with PBS twice, treated with 0.25% Triton X-100 for 5 min, washed with PBS, incubated with the primary antibodies at 4˚C overnight, washed with PBS, and incubated with the diluted fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG specific for pSTAT3 at room temperature for 30 min. To detect the fluorescent intensity of certain proteins, cells were counterstained in the dark with 50 μg/ml phosphatidyl inositol (PI) and 0.1% ribonuclease A (RNase A) in 400 μl PBS at 25˚C for 30 min. Stained cells were assayed and quantified using a FACSort flow cytometer (Becton Dickinson).

Immune complex kinase assay. ERK1/ERK2 activity was measured using a nonradioactive p44/42 MAP Kinase Assay Kit in accordance with the manufacturer's instructions. Briefly, cell lysates (200 μg) were incubated with an anti-monoclonal phospho-p44/42 MAP kinase (Thr 202 and Tyr 204) to selectively immunoprecipitate active ERK1/ERK2. Kinase assays were performed by incubating immunoprecipitates in 50 μl of kinase buffer containing 200 μM adenosine triphosphate and 20 μg of Elk-1 fusion protein for

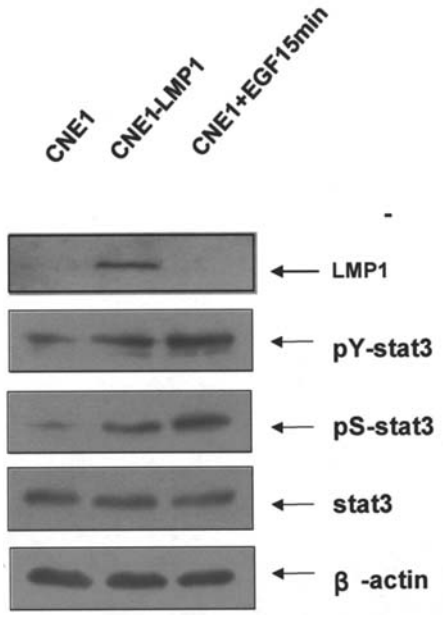

Figure 1. Effect of LMP1 on the phospho-STAT3 level in CNE1 and CNE1-LMP1 cells. Cell lysates were prepared as described. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a nitrocellulose membrane. Quantification of immunoband intensities was determined by densitometry scanning, with ß-actin as an internal control. Lane 1, CNE1; lane 2, CNE1-LMP1; lane 3, CNE1 treated with epidermal growth factor (EGF) (100 ng/ml) for 15 min. LMP1 promoted STAT3 tyrosine 705 and serine 727 phosphorylation.

30 min at room temperature. The reaction was terminated by the addition of 30 μl of 2X Laemmli sample buffer, and samples were then boiled for 5 min. The phosphorylation of Elk-1 was measured by Western blotting with an anti-phospho-Elk-1 antibody (Ser 383).

Clinical samples and staging. A total of 40 NPC tissue samples were collected from the archives of the Department of Pathology at Hunan Province Cancer Hospital. Prior patient consent and approval from the Institute's Research Ethics Committee were obtained for the use of these clinical materials for research purposes. The disease stages of all patients were classified or reclassified according to the 1992 NPC staging system of P.R. China.

Double stain visualization. Immunostaining was performed by the A streptavidin-biotin immunoperoxidase complex method using a commercial kit (Dako).

Nonspecific reaction was blocked with normal mouse serum. The slides on which the cell lines grew were incubated with acetate-formaldehyde fixative for 20 min at room temperature and then washed in PBS, followed by quenching of endogenous peroxidase activity with 0.15% hydrogen peroxide in methanol. Next the slides were incubated with the primary antibodies (diluted 1:100) at 4˚C overnight. After washing with 0.01 M PBS, the slides were incubated with a biotinylated secondary antibody for 30 min and finally incubated with streptavidin peroxidase for 30 min. 3,3'-diaminobenzidine tetrahydrochloride (0.05%) was used as the final chromogen. Counterstaining of the nucleus was achieved with hematoxylin.

Table I. The intensity of fluorescence of p-STAT3 and STAT3 in NPC cells.

	STAT3 Tyr 705	STAT3 Ser 727	STAT3
CNE1	18.16	12.33	34.16
CNE1-LMP1	27.82	21.29	35.46
Tet-on-LMP1-HNE2 Dox 0	18.58	12.72	50.92
Tet-on-LMP1-HNE2 Dox 0.6	29.69	20.62	55.66

The intensity of fluorescence showed the effect of LMP1 on the phospho-STAT3 level and on the total STAT3 level in CNE1, CNE1-LMP1, Tet-on-LMP1-HNE2 Dox 0 μg/ml, and Tet-on-LMP1-HNE2 Dox 0.6 μg/ml cells as detected by flow cytometry. Control, without primary antibody.

Statistical analysis. The significance of differences between experimental conditions was determined using the Spearman analysis and χ^2 test.

Results

EBV-LMP1 upregulation of STAT3 phosphorylation in NPC. To define the regulation of LMP1 on STAT3 phosphorylation, we used CNE1 (LMP1-negative) and CNE1-LMP1 as the cell models for the detection of STAT3 phosphorylation by Western blotting. CNE1 treated with 100 ng/ml epidermal growth factor (EGF) was used as the positive control. CNE1-LMP1 had markedly higher STAT3 Tyr 705 (pY-Stat3) and Ser 727 (pS-stat3) phosphorylation than did CNE1, but STAT3 expression levels showed no distinct difference (Fig. 1).

To confirm this observation, fluorescent intensity analysis was performed using flow cytometry. We found CNE1-LMP1 had higher fluorescent intensity for both STAT3 Tyr 705 phosphorylation and STAT3 Ser 727 phosphorylation than did CNE1. Similarly, STAT3 Tyr 705 and Ser 727 phosphorylation was elevated by 0.6 μg/ml doxycycline in Tet-on-LMP1-HNE2 cells, in which the expression of LMP1 would be stimulated by doxycycline in a dose-dependent manner and LMP1 maximally induced by 0.6 μg/ml doxycycline (30). The data demonstrated that LMP1 had a marked effect in promoting STAT3 Tyr 705 and Ser 727 phosphorylation in the NPC cell lines (Fig. 2, Table I).

Previous studies support a positive feedback loop of IL-6-induced STAT activation by LMP1 (27), and we were able to confirm these results using Tet-on-LMP1-HNE2 cells. Expression of LMP1 led to a striking increase in both Tyr 705 and Ser 727 phosphorylation of STAT3 protein (Fig. 3). By blocking IL-6 activation through the use of an anti-IL-6 neutralizing antibody, we found that activation of STAT3 in response to IL-6 was dependent on phosphorylation of both tyrosine and serine amino acid residues. The suppression of STAT3 Ser 727 phosphorylation was observed at 10 μg/ml during IL-6 inhibition, whereas the suppression of STAT3 Tyr 705 phosphorylation was observed at 20 μg/ml. However, in doxycycline-untreated cells, IL-6 neutralizing antibody did

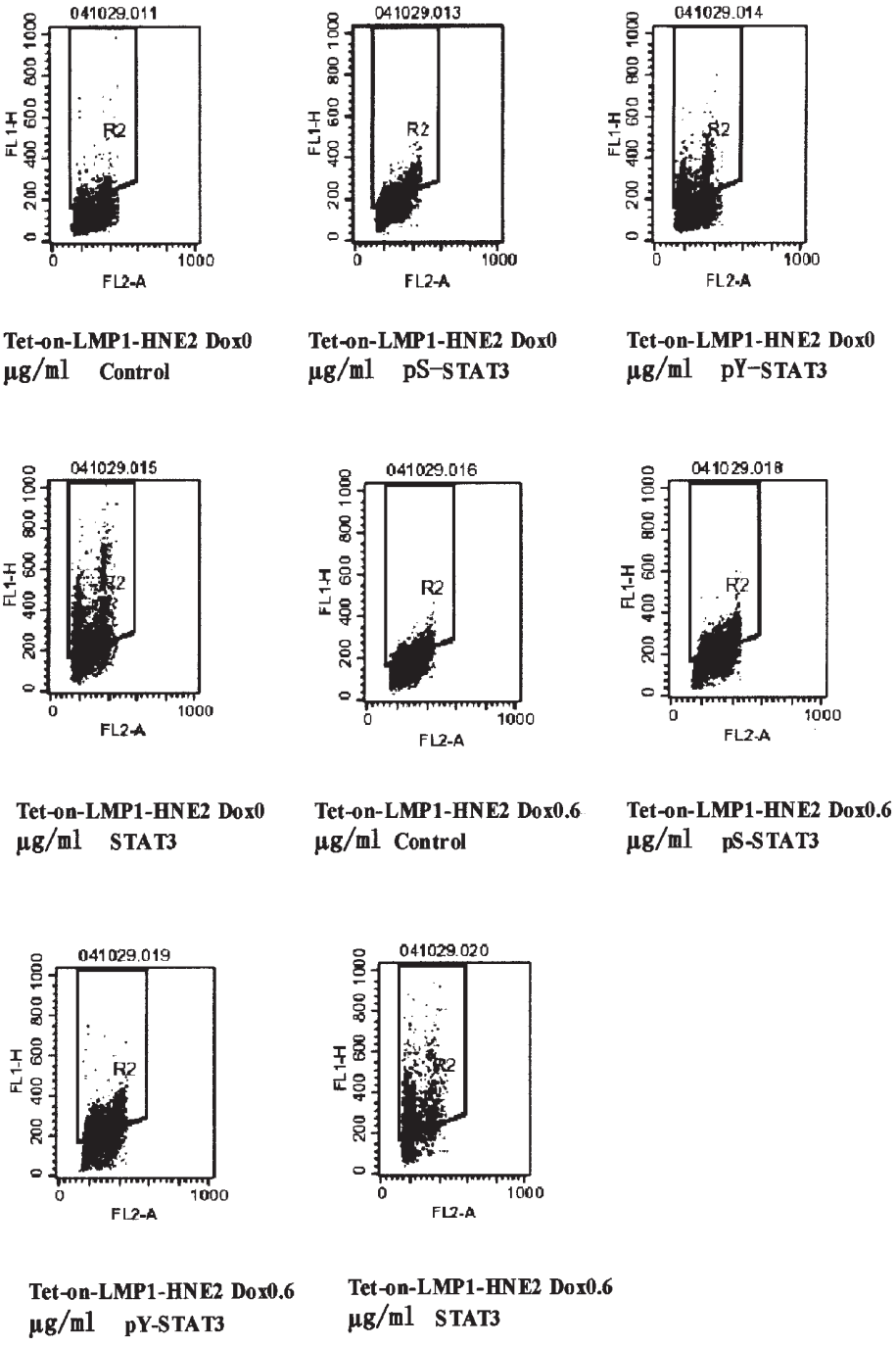

Figure 2. The intensity of fluorescence shows the effect of LMP1 on phospho-STAT3 and the total STAT3 levels in CNE1, CNE1-LMP1, Tet-on-LMP1-HNE2 doxycycline (Dox) 0 μg/ml, and Tet-on-LMP1-HNE2 Dox 0.6 μg/ml cells as detected by flow cytometry. Control, without primary antibody. FITC-labeled anti-mouse IgG is specific for pSTAT3. Cells were counterstained with propidium iodide to visualize the nuclei. LMP1 induced by 0.6 μg/ml Dox had a higher intensity of fluorescence of tyrosine 705-phosphorylated STAT3 and serine 727-phosphorylated STAT3.

not cause an appreciable decrease in the phosphorylation of STAT3 Tyr 705 and STAT3 Ser 727. This finding suggests that the treatment of cells with IL-6 neutralizing antibody partially abrogates the LMP1-induced activation of STAT3 Tyr 705 and STAT3 Ser 727. These results indicate that LMP1 activates STAT3 phosphorylation indirectly.

EBV-LMP1 promotes nuclear translocation of phosphorylated STAT3. Nuclear translocation was essential for STAT3 function; after its activation with the phosphorylation at tyrosine 705, STAT3 formed dimers with itself or STAT1, translocated to nuclei to bind a specific DNA sequence, and started the transcription of multiple related downstream genes. To test the effects of LMP1 on the nuclear translocation of phosphorylated STAT3, we detected phosphorylated STAT3 (Tyr 705, Ser 727) in the nuclei and cytoplasm of CNE1 and CNE1-LMP1 cells, respectively, through Western blotting. The data suggest that CNE1-LMP1 exhibited more nuclear accumulation and less cytoplasmic accumulation of Tyr 705-phosphorylated STAT3 (pY-stat3) and Ser 727-

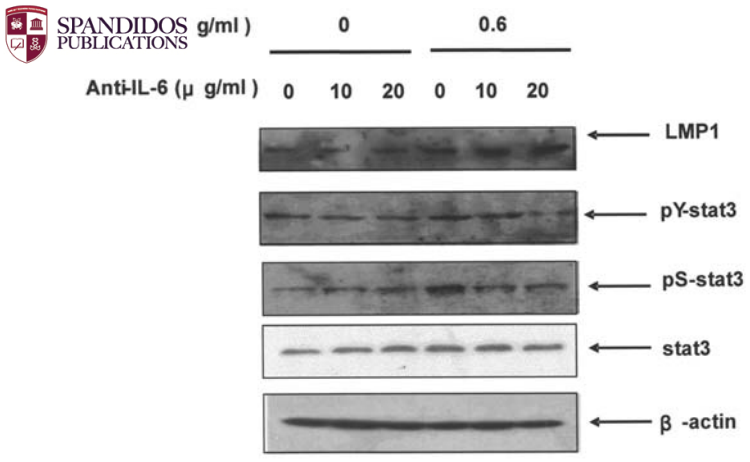

Figure 3. Anti-IL-6 neutralizing antibody prevents the ability of LMP1 to induce phosphorylation of STAT3 Tyr 705 and STAT3 Ser 727 in Tet-on-LMP1-HNE2 cells. Tet-on-LMP1-HNE2 cells were stimulated with Dox 0.6 μg/ml and anti-IL-6 neutralizing antibody for 24 h before harvesting the lysates. Dox 0.6 μg/ml caused an increase in STAT3 Tyr 705 and STAT3 Ser 727 phosphorylation. However, the presence of the anti-IL-6 neutralizing antibody prevented the LMP1-induced phosphorylation of STAT3 Tyr 705 and STAT3 Ser 727 in a dose-dependent manner. Total cellular levels of STAT3 were used as loading controls.

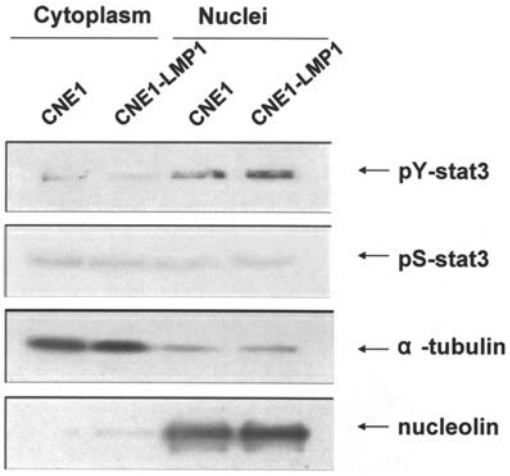

Figure 4. Nuclear translocation of phospho-STAT3 induced by LMP1. Cytoplasmic and nuclear proteins of CNE1 and CNE1-LMP1 cells were prepared, and Western blot analysis was performed. pY-stat3, pS-stat3, α-tubulin, and nucleolin were examined. Nucleolin and α-tubulin were the controls for nuclear protein and cytoplasmic protein, respectively. LMP1 promoted pY-stat3 and pS-stat3 nuclear translocation.

phosphorylated STAT3 (pS-stat3) in comparison to CNE1 cells (Fig. 4). Meanwhile, immunofluorescence analysis was performed to localize phosphorylated STAT3 (Tyr 705, Ser 727) in these cell lines. We found that CNE1-LMP1 had a relatively higher intensity of fluorescence of phosphorylated STAT3 (Tyr 705, Ser 727) in the nuclei compared with CNE1. Stronger signals were observed for pY-stat3 than for pS-stat3 providing important evidence that under LMP1 regulation phosphorylated STAT3 (particularly pY-stat3) had nuclear accumulation in NPC tissues. The data suggest that phosphorylated STAT3 (Tyr 705, Ser 727) nuclear translocation may be Tyr 705-phosphorylation dependent in LMP1-positive cells (Fig. 5).

JAK mediates LMP1-induced STAT3 Tyr 705 phosphorylation. Upon confirming the promotion of STAT3 (Tyr 705, Ser 727) phosphorylation and nuclear translocation by LMP1, we further investigated the mechanism of LMP1 regulation of STAT3. Our previous study suggested that JAK3 activity was involved in LMP1 activation of STAT3, which required the cooperation of the CTAR2 and CTAR3 domains (28). As shown in Fig. 3, treating CNE1-LMP1 cells with WHI-P131 (31-33), a JAK3 inhibitor, for 24 h effectively inhibited JAK3-induced STAT3 Tyr 705 phosphorylation. In response to the inhibition, STAT3 Tyr 705 phosphorylation markedly decreased, but STAT3 Ser 727 phosphorylation and STAT3 protein levels did not change (Fig. 6). Thus, JAK3 was particularly important to STAT3 Tyr 705 phosphorylation induced by LMP1.

ERK mediates LMP1-induced STAT3 Ser 727 phosphorylation. To determine the role of ERK in LMP1-induced STAT3 Ser 727 phosphorylation, the activity of ERK was analyzed in the presence of the specific inhibitor PD98059 in CNE1-LMP1 cells and compared with control CNE1 cells. ERK was immunoprecipitated, and its activity was determined by kinase assays *in vitro* using Elk as a substrate. In comparison to CNE1, the CNE1-LMP1 cells showed an increase in ERK activity (Fig. 7A). The ERK activation was suppressed by PD98059 in a dose-dependent manner in CNE1-LMP1 cells. To determine a correlation between ERK activation and the activation of STAT3 Ser 727 phosphorylation, we blocked the activation of ERKs using PD98059, the MEK1 inhibitor. As shown in Fig. 7B, treatment with PD98059 caused a decrease in the ERK phosphorylation induced by LMP1. The levels of phosphorylated STAT3 Ser 727 in CNE1-LMP1 cells were reduced in a dose-dependent manner by PD98059. Total cellular levels of STAT3 ERK and STAT3 Tyr 705 phosphorylation, however, remained unchanged (Fig. 7B). These results strongly suggest that ERK is responsible for LMP1-induced STAT3 Ser 727 phosphorylation.

It was previously reported that JAK3 activation occurred upon ligand-mediated receptor multimerization and that the activated JAK3 subsequently phosphorylated STAT3 at a conserved tyrosine residue. This phosphotyrosine permitted the STAT3 to form dimers and enter the nuclei. To determine whether LMP1-mediated nuclear localization of STAT3 is an event dependent on the phosphorylation of STAT3 on tyrosines and/or serines, nuclear translocation of phosphorylated STAT3 (Tyr 705, Ser 727) was determined by laser scanning confocal microscopy with anti-FITC antibodies and rhodamine-conjugated secondary antibody. STAT3 phosphorylation and translocation into the nucleus decreased after being in the presence of WHI-P131 for 24 h in CNE1-LMP1 cells. As shown in Fig. 8, we did not observe the same decline in nuclear accumulation of phosphorylated STAT3 (Tyr 705, Ser 727) when CNE1-LMP1 cells were treated with PD98059, a small-molecule MEK1 inhibitor, for 2 h (34,35). The data suggested that phosphorylated STAT3 (Tyr 705, Ser 727) nuclear translocation was Tyr 705 phosphorylation dependent and Ser 727 phosphorylation independent.

A close association of phosphorylated STAT3 with NPC stage. To evaluate the possible effects of STAT3 phos-

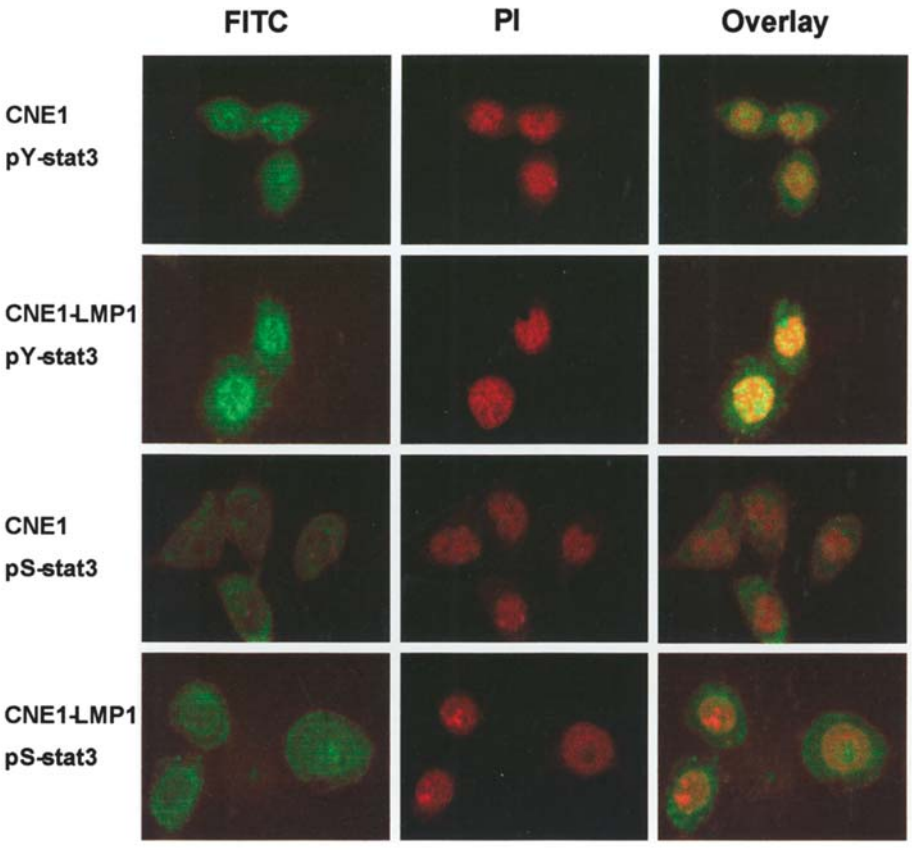

Figure 5. Fluorescence microscopy shows nuclear translocation of pY-stat3 and pS-stat3 induced by LMP1. FITC-labeled anti-rabbit IgG is specific for pSTAT3. Cells were counterstained with propidium iodide to display the nuclei. The fluorescent images were visualized with a laser scanning confocal microscope (x1,000). When the two images were merged, yellow signals were observed indicating the localization of phospho-STAT3 in nuclei. PBS was substituted for antibody as a blank control.

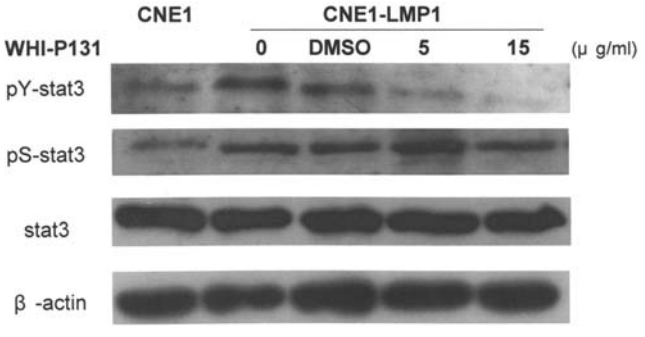

Figure 6. JAK3 mediates LMP1-induced STAT3 Tyr 705 phosphorylation. WHI-P131 (10 μg/ml) inhibited JAK3-induced tyrosine phosphorylation of STAT3 in CNE1-LMP1 cells. CNE1-LMP1 cells were treated with WHI-P131, a JAK3 inhibitor, for 24 h. Cell lysates were prepared, and equal amounts of protein were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Quantification of immunoband intensities was determined by densitometry scanning, with ß-actin as an internal control. LMP1 increased STAT3 tyrosine 705 phosphorylation through JAK3.

Table II. Relativity of LMP1 and pY-stat3, pS-stat3 in 40 NPC tissue samples.

Group	n	pY-stat3	pS-stat3
LMP1 (-)	15	(7) 46.7%	(6) 40.0%
LMP1 (+)	25	(20) 80.0%	(18) 72.0%
r_s		0.345	0.316
p		0.029	0.047

Statistical analysis of the correlation of LMP1 and pY-stat3 or pS-stat3 in 40 tissue samples. from NPC patients. The EnVision Doublestain method was applied as described. Data show that LMP1 correlates with both pY-stat3 (r_s=0.345, p=0.029) and pS-stat3 (r_s=0.316, p=0.047).

phorylation and LMP1 expression on the progression of NPC, immunohistochemisty (IHC) was performed on formalin-fixed, paraffin-embedded tissue sections of human NPC using the EnVision Doublestain system to determine the expression of phosphorylated STAT3 and LMP1. Coexpression of phosphorylated STAT3 and LMP1 signals were distinctly observed, and pY-stat3 and pS-stat3 were mainly located in the nuclei (Fig. 9). The IHC Doublestain technique was also applied to 40 NPC patient tissue samples to evaluate the relationship between LMP1 and phosphorylated STAT3 (Tyr 705, Ser 727), as well as the correlation of phosphorylated STAT3 (Tyr 705, Ser 727) with NPC clinical stages. Statistical analysis indicated that the positive percentage of LMP1 in NPC patient tissues was ~62.5% (25/40), the positive percentage of pY-stat3 was ~67.5% (27/40), and the positive percentage of pS-stat3 was ~60.0% (24/40).

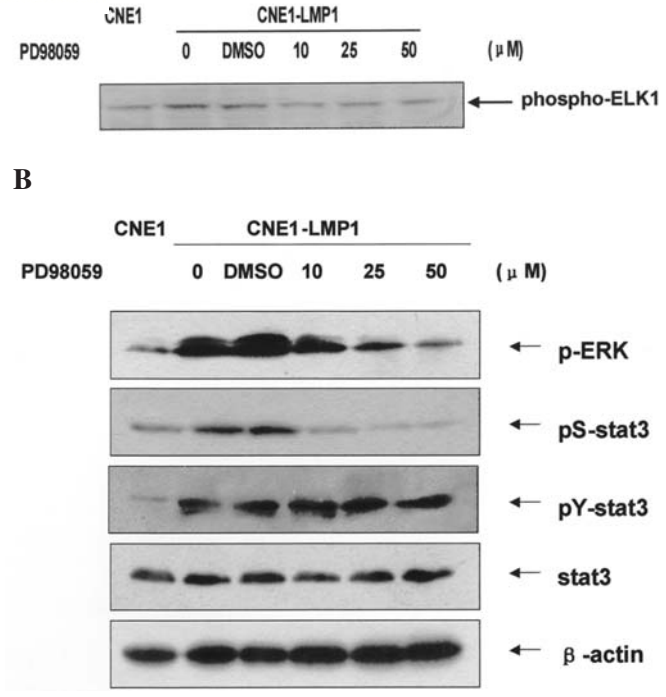

Figure 7. (A) LMP1 induces the activity of ERK. Phosphorylation inhibitor PD98059 inhibited p44/42 MAPK activity in CNE1-LMP1 cells. CNE1 cells were used as a negative control. (B) ERK mediates LMP1-induced STAT3 Ser 727 phosphorylation. ERK phosphorylation inhibitor PD98059 inhibited serine phosphorylation of STAT3 in CNE1-LMP1 cells. CNE1-LMP1 cells were treated with PD98059 for 2 h. Cell lysates were prepared, and equal amounts of protein were separated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Quantification of immunoband intensities was determined by densitometry scanning, with ß-actin as an internal control.

Moreover, the positive percentage of pY-stat3 was ~80.0% (20/25) in LMP1-positive tissue and 46.7% (7/25) in LMP1-negative tissue (r_s=0.345, p=0.029). The positive percentage of pS-stat3 was ~72.0% (18/25) in LMP1-positive tissue and 40.0% (6/15) in LMP1-negative tissue (r_s=0.316, p=0.047) (Table II). Thus, there existed a marked statistical difference. Of the 40 NPC patient tissue samples, 8 were CIN I or II, and 32 were CIN III or IV. Of the 8 that were CIN I or II, 25.0% (2/8) had a positive proportion of pY-stat3, and 62.5% (5/8) had a positive proportion of pS-stat3. Of the 32 that were CIN III or IV, 78.1% (25/32) had a positive proportion of pY-stat3, and 59.4% (19/32) had a positive proportion of pS-stat3. These results revealed the relevancy of LMP1 with phospho-STAT3 after statistic analysis (P<0.05) and a close association of phosphorylated STAT3 (Tyr 705, Ser 727) with the development of NPC (Table III).

Discussion

EBV-LMP1 is one of several latently expressed genes essential for the growth transformation of B lymphocytes *in vitro* (3). The frequent expression of LMP1 in undifferentiated NPC points to a role for this viral oncoprotein as a key effector molecule in NPC pathogenesis. LMP1 affects the control of cellular growth and differentiation of epithelial cells *in vitro*. The underlying molecular events behind this are still unknown.

Table III. Relativity of pY-stat3, pS-stat3 and clinical stages in 40 NPC tissue samples.

Clinical stages	n	pY-stat3	pS-stat3
I, II	8	(2) 25.0%	(5) 62.5%
III, IV	32	(25) 78.1%	(19) 59.4%
χ^2		4.167	0.000
p		0.041	1.000

Statistical analysis of the correlation of pSTAT3 with NPC clinical stages in 40 tissue samples from NPC patients. The EnVision Doublestain method was applied as described. Data show that pY-stat3, but not pS-stat3, correlates with clinical stages III and IV.

The goals of this study were to demonstrate that LMP1 is capable of activating STAT3 through a complex mechanism involving the MAPK/ERK and JAK/STAT pathways that directly phosphorylate STAT3 at Ser 727 and Tyr 705, respectively. LMP1 resulted in increased phosphorylation of STAT3 at Tyr 705 and Ser 727 in CNE1-LMP1 cells as compared with CNE1 cells. Blocking of extracellular IL-6 with anti-IL-6 neutralizing antibody abolished the LMP1-mediated increase in STAT3 tyrosine and serine phosphorylation in Tet-on-LMP1-HNE2 cells. The tyrosine phosphorylation of STAT3 induced by LMP1 led to functional activation, as demonstrated by the ability of LMP1 to induce nuclear translocation of STAT3 in CNE1-LMP1 cells.

LMP1 is known to engage signaling on the JAK/STAT pathway, and inhibition of this pathway by coexpression of a constitutively active CTAR2 mutant abolished STAT3 pTKs3 luciferase reporter activity (28). Mutational analysis of the LMP1 cytoplasmic COOH terminus identified both the CTAR2 and CTAR3 domains as contributing to JAK/STAT signaling. The effects of JAK on LMP1-mediated STAT3 activation were found to occur as treatment of CNE1-LMP1 cells with WHI-P131 partially inhibited the upregulation of STAT3 Tyr 705 phosphorylation. This suggests that LMP1 modulates the IL-6 and JAK pathways that phosphorylate STAT3 Tyr 705 indirectly or directly, respectively.

In addition to tyrosine phosphorylation, we found that STAT3 is serine phosphorylated at a single residue (Ser 727) in response to IL-6. At present, the signaling events downstream of the gp130 receptor have not been fully explored, and the kinase responsible for IL-6-induced STAT3 Ser 727 phosphorylation has not been identified. STAT3 Ser 727 is phosphorylated via different pathways under different stimulations. Receptor tyrosine kinases, IL-2, the B cell receptor (BCR), and the T cell receptor (TCR) phosphorylate STAT3 Ser 727 via the MAPK/ERK pathway (36,37); ultraviolet (UV) radiation, high saturation stress, tumor necrosis factor (TNF)-α, and lipopolysaccharide (LPS) induce STAT3 Ser 727 phosphorylation via the MAPK/Jun N-terminal kinase (JNK) pathway; v-src stimulates STAT3 Ser 727 phosphorylation via MAPK/p38 (38); and IL-6 activates STAT3 Ser 727 phosphorylation through the protein kinase C (PKC) pathway (39). The binding of IL-6 to its cellular receptor simultaneously activates both ERK1/2 and STAT3 (40,41).

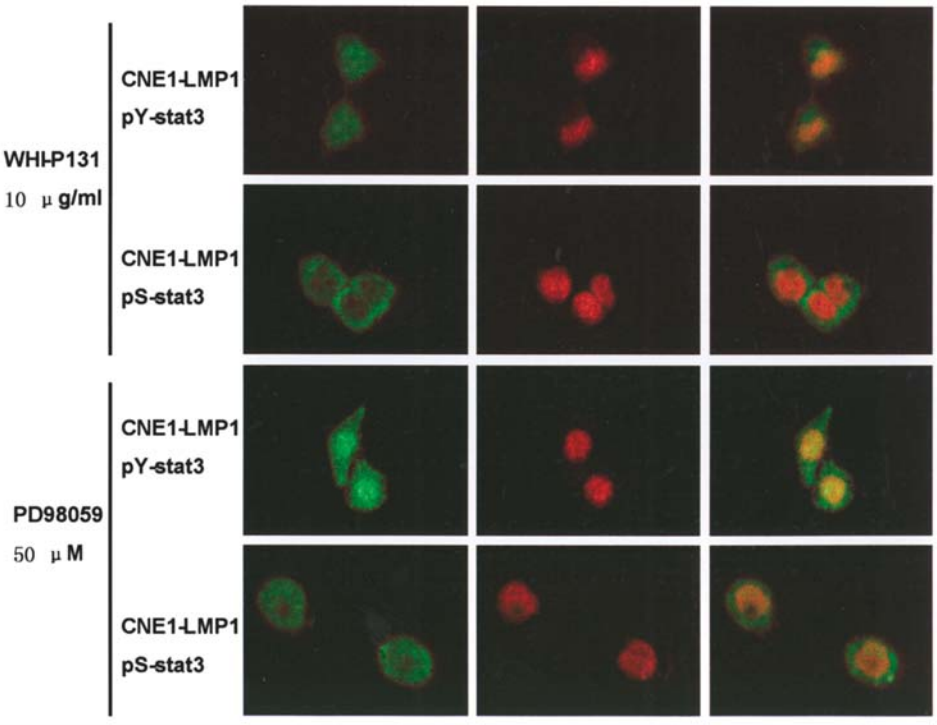

Figure 8. Fluorescence microscopy shows nuclear translocation of pY-STAT3 and pS-STAT3 induced by LMP1 in a tyrosine 705 phosphorylated STAT3-dependent manner. FITC-labeled anti-rabbit IgG is specific for pSTAT3. Cells were counterstained with propidium iodide to display the nuclei. The fluorescent images were visualized with a laser scanning confocal microscope (x1,000). When the two images were merged, yellow signals were observed indicating the localization of phospho-STAT3 in nuclei. PBS was substituted for antibody as a blank control. CNE1-LMP1 cells were treated with WHI-P131 (10 μg/ml) for 24 h and with PD98059 (50 μM) for 2 h. Data showed that LMP1 induced the nuclear localization of phospho-STAT3 in a tyrosine 705-phosphorylated STAT3-dependent manner.

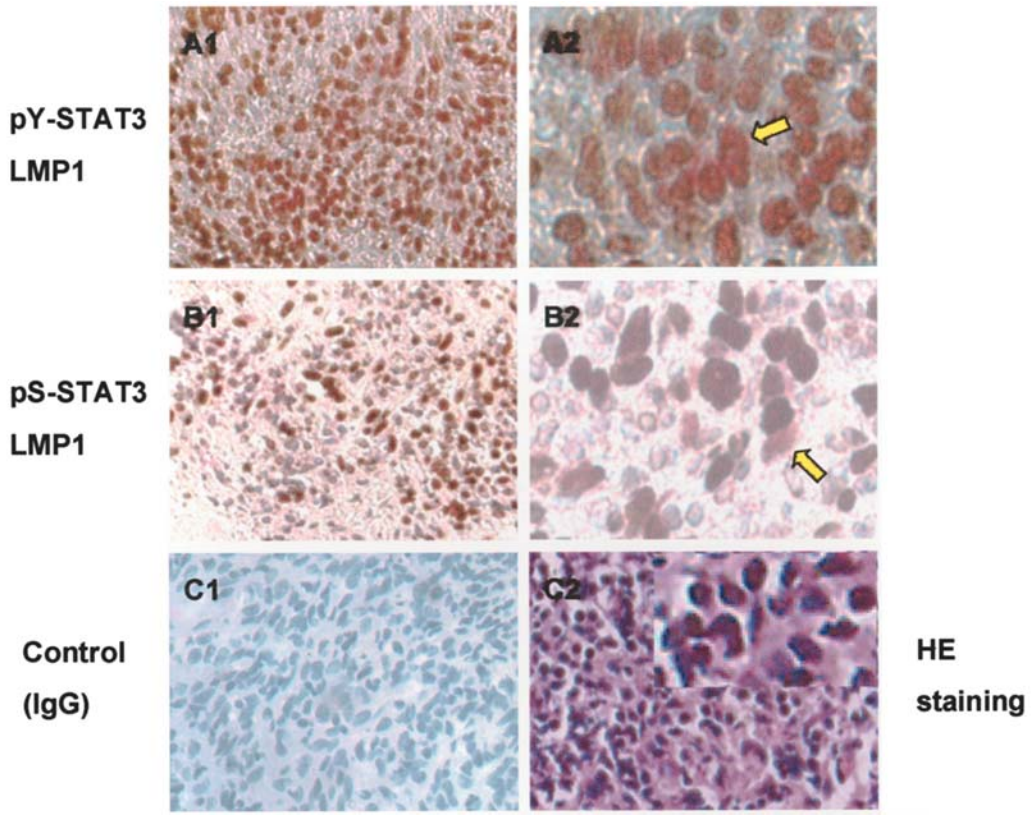

Figure 9. Coexpression of pY-STAT3 or pS-STAT3 with LMP1 in NPC tissue with the IHC EnVision Doublestain method. (A1) pY-stat3 (brown) and LMP1 (red), x150; (A2) pY-stst3 (brown) and LMP1 (red), x300; (B1) pS-stat3 (brown) and LMP1 (red), x150; (B2) pS-stat3 (brown) and LMP1 (red), x300; (C1) control, x300; (C2) hematoxylin and eosin (HE) staining, x150 (top right corner, x300). Control, mouse and rabbit IgG. Cells were counterstained with hematoxylin to display the nuclei. Mouse and rabbit IgG was substituted for the antibody as a blank control. Data show that LMP1 induced the nuclear localization of phospho-STAT3 in NPC tissues.

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study, we provide evidence of another signaling activated by LMP1 that induces STAT3 Ser 727 phosphorylation. We showed that LMP1 expression led to the activation of ERK, as evidenced by the ability of LMP1 to induce ERK phosphorylation. Furthermore, ERK immunoprecipitated from LMP1-positive cells induced the phosphorylation of Elk, one of the downstream targets of ERK. Since LMP1-induced IL-6 signaling engages STAT3 Ser 727 phosphorylation, the question of whether these two pathways run on the same axis or in parallel has not been clarified. We also demonstrated that PD98059 inhibited ERK activity and phosphorylation in CNE1-LMP1 cells. Thus, the functional implications of ERK-mediated STAT3 Ser 727 phosphorylation were demonstrated by the ability of PD98059 to downregulate LMP1-activated STAT3 Ser 727 phosphorylation. The JNK pathway did not influence STAT3 phosphorylation, which eliminates the possibility of JNK involvement in the process of LMP1-induced STAT3 Ser 727 phosphorylation (data not shown). Furthermore, LMP1 promotion of phosphorylated STAT3 nuclear translocation has been observed. This process is STAT3 Tyr 705-phosphorylation dependent and Ser 727-phosphorylation independent.

pY-stat3 has a high correlation with NPC stages III and IV, suggesting its role in NPC invasion and metastasis by IHC Doublestain. We conclude that the overexpression of p-STAT3 may have an important role in the development of NPC tumors. Abnormal activation of STAT3 may be related to the metastasis potential in NPC, and the simultaneous detection of p-stat3 and LMP1 may aid in predicting the progression of NPC. STAT3 promotes metastasis and angiogenesis by inducing expression of the metastasis gene matrix metalloproteinase-2 (MMP-2) and the potent angiogenic gene VEGF (20,22). Our present findings contribute toward expanding the knowledge of the mechanism of NPC metastasis and in identifying new predictive biomarkers and therapeutic targets.

Overall, this study demonstrates the ability of a viral protein, EBV-encoded LMP1, to activate the MAPK/ERK and JAK/STAT pathways responsible for LMP1-induced STAT3 activation. The continued discovery of unique signaling pathways and the identification of crucial signaling molecules greatly enhances the current knowledge of intracellular mechanisms involved in LMP1 signaling.

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