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RECK is a target of Epstein–Barr virus latent membrane protein 1

Li-Teh Liu[1], Jyh-Ping Peng[2], Hui-Chiu Chang[3] and Wen-Chun Hung*[,4]

[1]*Graduate Institute of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China;* [2]*Department of Otolaryngology, Chang Gung University, Chang Gung Memorial Hospital, Kaohsiung Medical Center, Kaohsiung, Taiwan, Republic of China;* [3]*Department of Physiology, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China;* [4]*School of Technology for Medical Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China*

Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1) has been suggested to be involved in tumor metastasis. However, the molecular mechanism of LMP1-induced metastasis is largely unknown. In this study, we investigated the effect of LMP1 on the expression of RECK, a metastasis suppressor gene, in an EBV-negative nasopharyngeal carcinoma (NPC) cell line. Our data demonstrated that LMP1 induced downregulation of RECK via transcription repression in TW04 cells. In addition, we found that LMP1 acted via an Sp1 site to inhibit RECK promoter activity. We next studied the signaling pathway that mediated the effect of LMP1 on RECK expression. Our results showed that LMP1 potently stimulated the activity of extracellular signal-regulated kinases (ERKs) and inhibition of ERK activity by PD98059 antagonized LMP1-induced downregulation of RECK. Conversely, the c-Jun N-terminal kinase inhibitor SP600125 and p38^{HOG} kinase inhibitor SB203580 had little effect. We also found that the expression of LMP1 increased the invasive ability of TW04 cells. The importance of RECK in LMP1-induced invasiveness was supported by three observations. First, restoration of RECK expression by PD98059 reduced LMP1-induced release of active MMP-9. Second, suppression of PD98059-induced RECK expression by small interference RNA abolished the inhibitory action of PD98059 on LMP1-induced invasiveness. Third, coexpression of RECK with LMP1 in TW04 cells effectively suppressed cell invasiveness induced by LMP1. Taken together, these results suggest that LMP1 inhibits RECK expression via the ERK/Sp1 signaling pathway and this inhibition is a critical step for LMP1-induced tumor metastasis.
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*Correspondence: W-C Hung, School of Technology for Medical Sciences, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, Republic of China;
E-mail: hungl228@ms10.hinet.net
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Introduction

Epstein–Barr virus (EBV), a ubiquitous human gammaherpesvirus, is implicated in the etiology of human malignancies, such as Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma (NPC) (Kieff and Liebowitz, 1990; Pallesen *et al*., 1991; Weiss *et al*., 1991). The association of EBV with NPC is established by the findings that EBV DNA, RNA and proteins were detected in NPC tissues and antibodies against EBV antigens were increased in the sera of NPC patients (Kieff, 1995).

The mechanisms by which EBV induces oncogenic transformation are incompletely understood. Several EBV latent genes, including EBV nuclear antigen-1, -2, -3A, -3C, -LP and latent membrane protein 1 (LMP1), have been demonstrated to be required for cell transformation (Miller, 1990). Among the EBV latency genes, LMP1 is considered an oncoprotein because it can transform rodent fibroblasts (Wang *et al*., 1985). Moreover, studies of primary NPC tissues indicated that LMP1-positive NPCs are more progressive and show an increased tendency of lymph node metastasis than LMP1-negative NPCs (Hu *et al*., 1995; Horikawa *et al*., 2000). These results suggest that LMP1 may contribute to tumor metastasis. However, the molecular mechanism responsible for LMP1-induced metastasis is largely unknown.

Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that selectively degrade components of the extracellular matrix, are involved not only in normal tissue remodeling but also in tumor angiogenesis and metastasis (Stetler-Stevenson *et al*., 1993). MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage. Thus, MMP activity can be regulated in three steps: (1) gene expression, (2) proenzyme processing and (3) direct inhibition of enzymatic activity. Among MMPs, MMP-2 (gelatinase A) and -9 (gelatinase B) are shown to be markedly associated with tumor invasion and metastasis (D'Errico *et al*., 1991; Himelstein *et al*., 1994). A recent study demonstrated that MMP-9 expression was enhanced by LMP1 (Yoshizaki *et al*., 1998). These data suggest that MMPs may be involved in LMP1-mediated metastasis. However, upregulation of MMPs may not be the only mechanism by which LMP1 induces metastasis.

The RECK gene was isolated as a transformation suppressor gene by using an expression cloning strategy designed to identify human cDNA inducing flat reversion in a v-Ki-ras-transformed NIH3T3 cell line (Kitayama *et al*., 1989). This gene encodes a membrane glycoprotein that can negatively regulate MMP-2 and MMP-9 activity, and inhibit tumor angiogenesis and metastasis (Takahashi *et al*., 1998; Oh *et al*., 2001). While RECK mRNA is highly expressed in most of the human tissues and untransformed cells, it is undetectable in many tumor cell lines or in cells expressing active oncogenes (Takahashi *et al*., 1998). In this study, we investigate whether RECK is a downstream target of LMP1 and test the hypothesis that LMP1 may induce metastasis via the inhibition of metastasis suppressor genes.

Results

Expression of LMP1 in TW04 EBV-negative NPC cells induces downregulation of RECK

TW04 EBV-negative NPC cell line was transfected with control or an LMP1-expressing vector. After transfection, cells were incubated in medium containing 10% fetal calf serun (FCS) for 48 h and harvested for several different analyses. The parental cells or mock-transfected cells did not express the LMP1 protein (Lin *et al*., 1990, 1993, and data not shown). In addition, no LMP1 protein was detected in cells transfected with control vector and elevated expression of LMP1 was confirmed by Western blot analysis in cells transfected with the LMP1-expressing vector (Figure 1a). We next investigated the change of expression of LMP1-regulated downstream genes in transfected cells to confirm that the expressed LMP1 was functional. Previous work has shown that LMP1 may stimulate MMP-9, but not MMP-2, expression (Yoshizaki *et al*., 1998). So, we tested whether similar results could be obtained in our LMP1-expressing cells. Our data indeed demonstrated that LMP1 potently upregulated MMP-9 expression in TW04 cells (Figure 1b). Conversely, MMP-2 expression was not affected by LMP1 (Figure 1c). These results indicated that the expressed LMP1 exerted its biological function in transfected TW04 cells. We next investigated the effect of LMP1 on RECK expression. As shown in Figure 2a, the expression of LMP1 induced downregulation of RECK in TW04 cells. In accordance with the result of reverse transcriptase–polymerase chain reaction (RT–PCR) analysis, the RECK protein level was also reduced in LMP1-expressing cells (Figure 2b). We next investigated whether LMP1 suppressed RECK expression at the transcription level. As the human RECK promoter has yet not been cloned, we used mouse RECK promoter–luciferase plasmid to address this question. We found that transfection of LMP1-expressing vector inhibited RECK promoter activity (Figure 2c). These results suggest that LMP1 inhibits RECK expression via transcriptional repression.

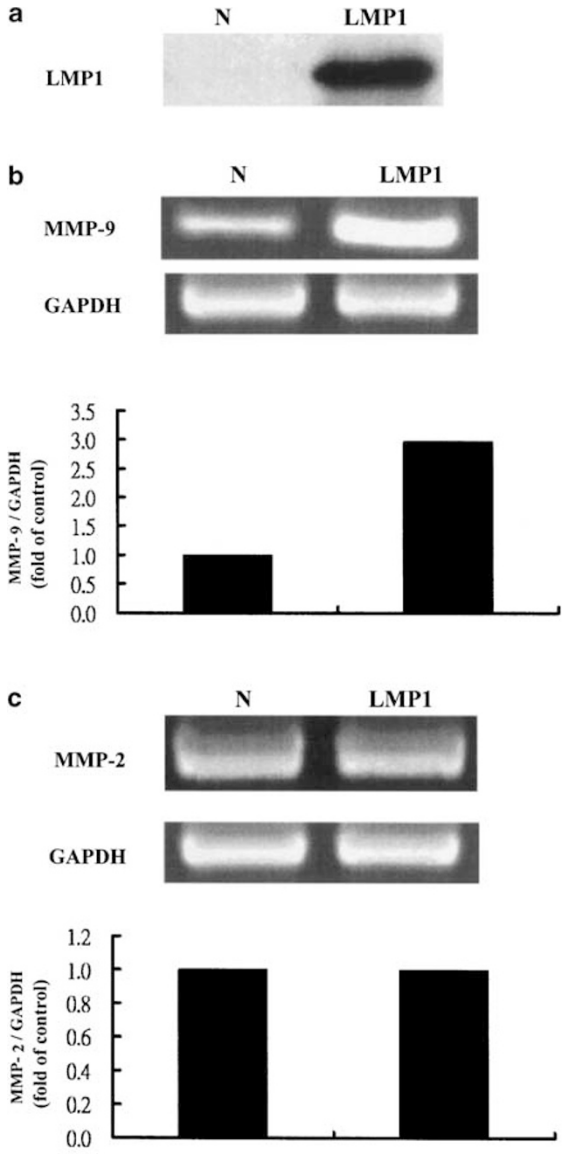
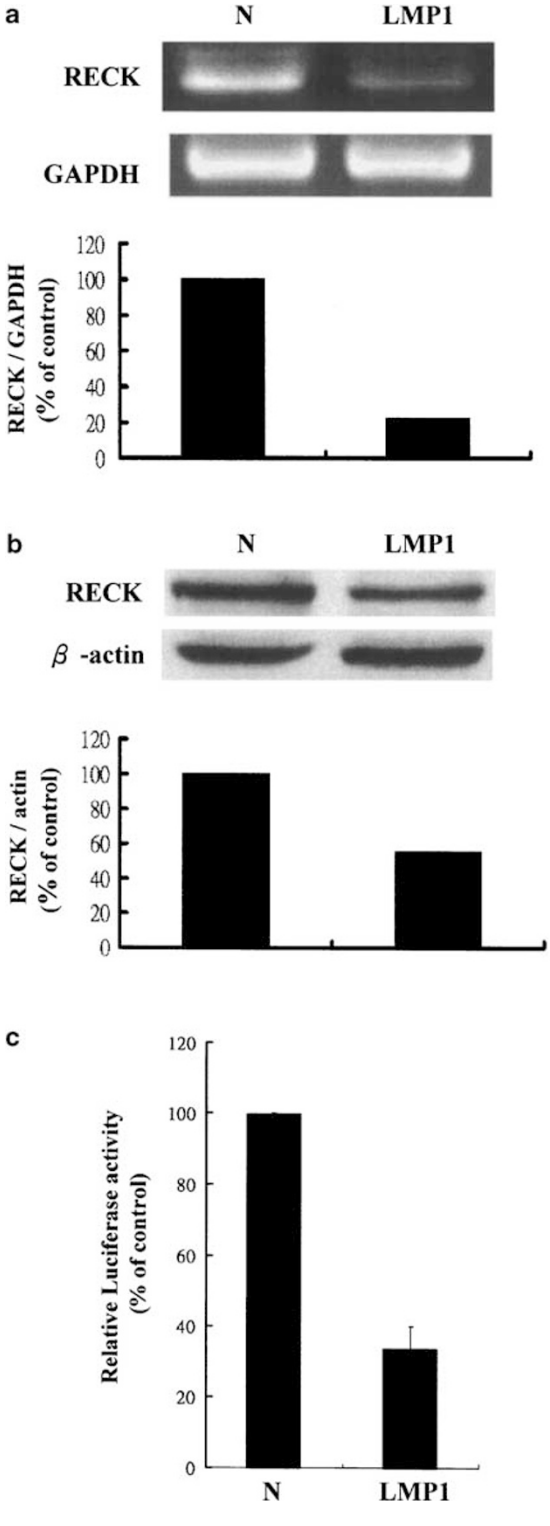


Figure 1 LMP1 induces MMP-9 expression in TW04 EBV-negative NPC cells. Cells were transfected with control (N) or LMP1-expressing (LMP1) vector and cultured in medium containing 10% FCS for 48 h. (**a**) Cellular proteins were harvested and Western blot analysis was performed to investigate the protein level of LMP1 in transfected cells. (**b**) Total RNA was extracted and MMP-9 mRNA level was examined by RT–PCR. A housekeeping gene, GAPDH, was used as an internal control to verify the efficiency of cDNA synthesis and PCR amplification. Quantitative analysis of MMP-9 and GAPDH mRNA ratio is shown. (**c**) The same RNA sample was subjected to RT–PCR analysis to investigate the MMP-2 mRNA level. Quantitative analysis of MMP-2 and GAPDH mRNA ratio is shown

LMP1 acts via the ERK/Sp1 signaling pathway to inhibit RECK

We next studied the signal transduction pathway by which LMP1 inhibited RECK expression. Previous studies have demonstrated that LMP1 may activate various signaling pathways, including NF- κB, ERK, p38^{HOG}, c-Jun N-terminal kinase (JNK) and Janus

kinase pathways (Hammarskjold and Simurda, 1992; Kieser *et al*., 1997; Roberts and Cooper, 1998; Eliopoulos *et al*., 1999; Gires *et al*., 1999). In this study, we analysed the involvement of various mitogen-activated protein kinases (MAPKs) (including ERK, JNK and p38^{HOG}) in this process. The effects of different kinase inhibitors (PD98059 for MEK, SP600125 for JNK and SB203580 for p38^{HOG}) on LMP1-induced downregulation of RECK were examined. Our RT–PCR results showed that PD98059, but not SP600125 and SB203580, could effectively reverse the inhibition of RECK by LMP1 (Figure 3a). The antagonistic action of PD98059 was not due to a nonspecific effect of this drug on LMP1 expression, because the LMP1 protein level in transfected cells treated without or with kinase inhibitors was similar (Figure 3b). These data imply that LMP1 may act via the ERK signaling pathway to inhibit RECK expression. So, we tested whether LMP1 stimulated ERK activity in TW04 cells. As shown in Figure 4a, transfection of LMP1-expressing vector, but not control vector, significantly activated ERK activity in TW04 cells and this activation was suppressed by PD98059. Moreover, PD98059 reversed the inhibition of RECK promoter



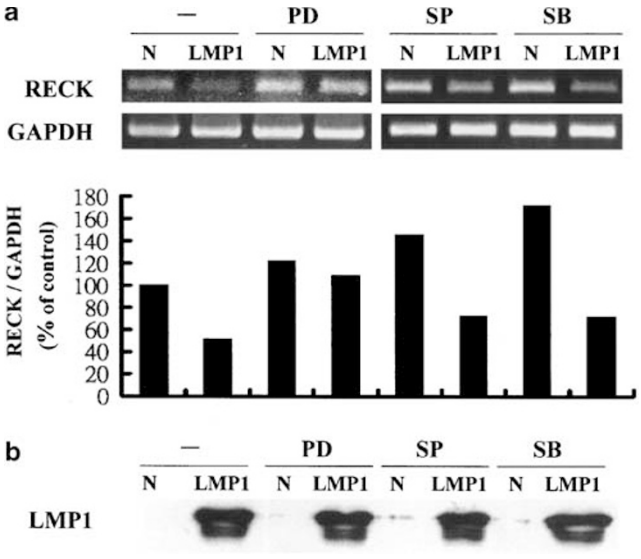


Figure 3 LMP1-induced downregulation of RECK is reversed by ERK inhibitor PD98059. (**a**) Cells were transfected with control (N) or LMP1-expressing (LMP1) vector and cultured in 10% FCS medium containing vehicle (–) or 10 μM of PD98059 (PD), SP600125 (SP) or SB203580 (SB) for 48 h. Total RNA was extracted and RECK mRNA level was examined by RT–PCR. The ratio of RECK and GAPDH mRNA of cells transfected with control vector and incubated with vehicle was defined as 100%. (**b**) Cells treated as described above were harvested and LMP1 protein level was investigated by immunoblotting

Figure 2 LMP1 downregulates RECK via transcription repression in TW04 cells. Cells were transfected with control (N) or LMP1-expressing (LMP1) vector and cultured in medium containing 10% FCS for 48 h. (**a**) Total RNA was extracted and RECK mRNA level was investigated by RT–PCR. Quantitative analysis of RECK and GAPDH mRNA ratio is shown. (**b**) Cellular proteins were harvested and Western blot analysis was performed to investigate the protein level of RECK in transfected cells. Quantitative analysis of RECK and β-actin protein ratio is shown. (**c**) Cells were cotransfected with 2 μg of RECK promoter–luciferase plasmid, 1 μg of renilla luciferase reporter vector and 1 μg of control or LMP1-expressing vector. Luciferase activity was determined at 48 h after transfection by a dual-luciferase reporter assay system

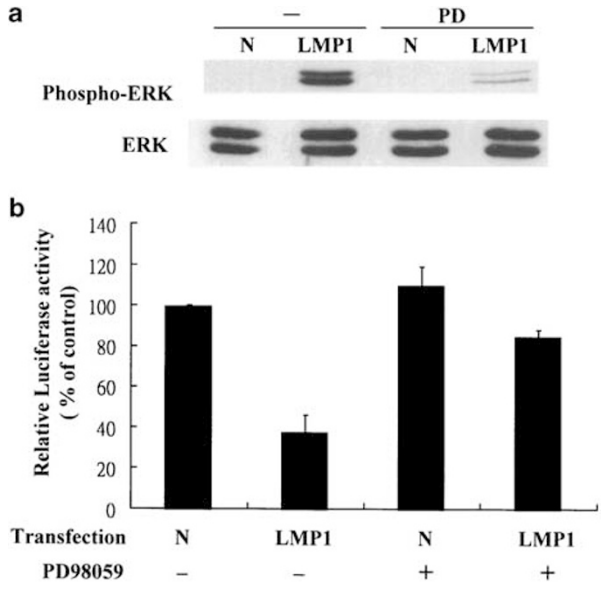


Figure 4 PD98059 inhibits ERK activation and RECK down-regulation induced by LMP1. (**a**) Cells were transfected with control (N) or LMP1-expressing (LMP1) vector and cultured in 10% FCS medium containing vehicle (−) or 10 μM of PD98059 (PD) for 48 h. After treatment, cellular proteins were extracted and subjected to SDS–PAGE. ERK activation was investigated by probing the membranes with anti-phospho-ERK antibody. Equal loading of proteins in each lane was confirmed by probing the membrane with anti-ERK antibody. (**b**) Cells were cotransfected with 2 μg of RECK promoter–luciferase plasmid and 1 μg of control or LMP1-expressing vector. After transfection, cells were incubated with vehicle (−) or 10 μM of PD98059 (+) for 48 h. and luciferase activity was determined. RECK promoter activity of cells transfected with control vector and incubated with vehicle was defined as 100%

activity by LMP1 (Figure 4b). These data strongly support the notion that LMP1 activates the ERK signaling pathway to suppress RECK expression.

Previous study has shown that *ras* oncogene negatively regulates RECK via an Sp1 site (Sasahara *et al*., 1999). As ERK is a major downstream effector of Ras, we studied whether LMP1 also acted via the same Sp1 site to repress RECK. Our data showed that the shortest promoter–luciferase construct containing the upstream 52-bp region of the RECK promoter was fully responsive to LMP1 (Figure 5). However, LMP 1-induced inhibition was totally abolished after mutation of the Sp1 site (originally designated as Sp1 (B) in the study of Sasahara *et al*., 1999) within the promoter. Thus, LMP1 and *ras* act via the same Sp1 site to repress RECK expression.

Inhibition of ERK activity attenuates LMP1-stimulated MMP-9 activity and cell invasion

Since our results demonstrated that PD98058 restores the expression of RECK in TW04 cells, we tested whether PD98059 could suppress LMP1-stimulated MMP-9 activity and cell invasion. Our data showed that the amount of secreted MMP-9 protein detected in the culture medium of parental TW04 cells was

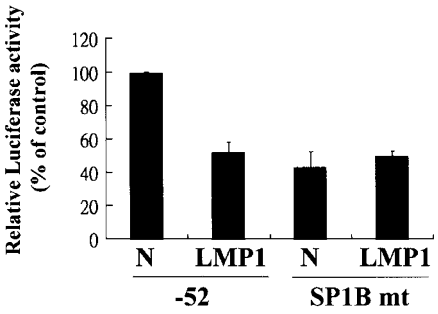


Figure 5 LMP1 inhibits RECK via an Sp1 site. Cells were cotransfected with 2 μg of RECK promoter–luciferase plasmid containing the upstream 52-bp region of RECK promoter (−52) or its mutant (Sp1B mt), in which the Sp1 site (originally designated as Sp1(B) in the study of Sasahara *et al*., 1999) was mutated and 1 μg of control (N) or LMP1-expressing (LMP1) vector. Luciferase activity was determined at 48 h after transfection. Promoter activity of cells transfected with RECK promoter–luciferase plasmid containing the upstream 52-bp region of RECK promoter and control vector was defined as 100%

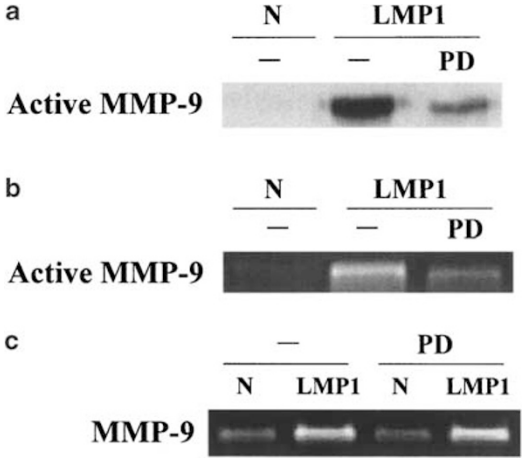


Figure 6 Inhibition of LMP1-induced MMP-9 activity by PD98059. Cells were transfected with control (N) or LMP1-expressing (LMP1) vector and cultured in 10% FCS medium containing vehicle (−) or 10 μM of PD98059 (PD) for 48 h. (**a**) Conditioned medium from equal number of cells was subjected to SDS–PAGE and probed with MMP-9 antibody to detect the amount of active MMP-9. (**b**) Conditioned medium from equal number of cells was also separated by 10% acrylamide gels containing 0.1% gelatin and gelatinolytic activity was visualized as a clear band against a dark background of stained gelatin. (**c**) Cells treated as described above were harvested and MMP-9 mRNA level was investigated by RT–PCR

negligible (Figure 6a). Similarly, gelatin zymography assay also detected less MMP-9 activity in the conditioned medium (Figure 6b). This is not consistent with the RT–PCR results showing that parental TW04 cells expressed significant amounts of MMP-9 mRNA (as shown in Figure 1b). We hypothesized that this might be due to the coexpression of RECK, which was known to block MMP-9 secretion and its enzymatic activity, in TW04 cells (as shown in Figure 2a and b). Transfection of LMP1 significantly increased the amount of active MMP-9 protein in the culture medium (Figure 6a). In

addition, increase of MMP-9 activity in LMP1-transfected cells was also detected by gelatin zymography (Figure 6b). Inhibition of the ERK signaling pathway by PD98059 significantly attenuated LMP1-induced increase of MMP-9 protein and enzymatic activity in the conditioned medium (Figure 6a and b). However, it should be noted that PD98059 did not affect LMP1-induced increase of the MMP-9 mRNA level in TW04 cells (Figure 6c). Therefore, reduction of active MMP-9 protein and enzymatic activity in the culture medium by PD98059 was not due to the inhibition of MMP-9 expression. Instead, PD98059 might restore RECK expression to suppress MMP-9 release and activation in TW04 cells. As LMP1 significantly increased the amount of active MMP-9 in the culture medium, we studied whether LMP1 expression might enhance the invasive ability of TW04 cells. Cells were transiently transfected with control or LMP1-expressing vector for 48 h and subjected to cell invasion assays as described in Materials and methods. Figure 7 showed that a 2.4-fold increase of the number of penetrated cells was found in LMP1-expressing cells. Incubation of PD98059 inhibited cell-invasive ability induced by LMP1. To confirm that RECK indeed plays a role in the inhibition of LMP1-induced cell invasion by PD98059, we used two approaches to clarify this question. First, we used small interference RNA (siRNA) to down-regulate RECK expression and tested its effect on cell invasiveness. The specificity and efficacy of this siRNA on the suppression of RECK expression has been

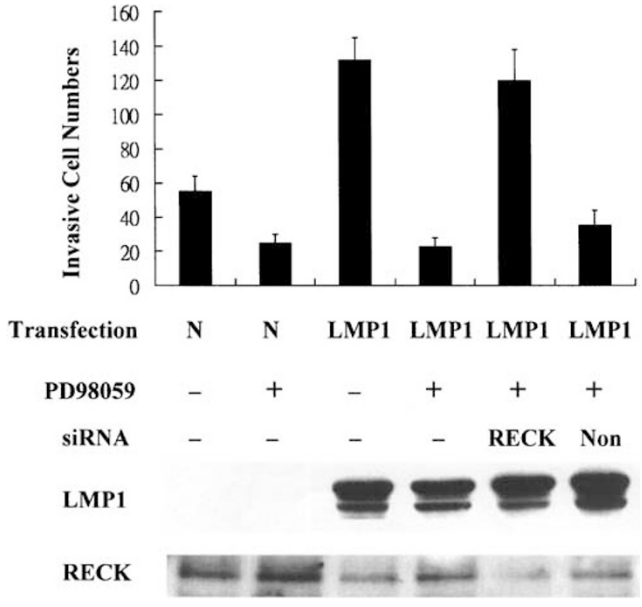


Figure 7 PD98059 inhibits LMP1-induced cell invasion via RECK. Cells were transfected with control (N) or LMP1-expressing (LMP1) vector in combination with nonspecific (Non) or RECK-specific (RECK) siRNA and treated with vehicle (−) or 10 μM of PD98059 (+). *In vitro* invasion assay was performed as described in 'Materials and methods'. Migrated cells on the bottom surface of the membrane were fixed in formaldehyde, stained with Giemsa solution and counted under a microscope. The LMP1 and RECK protein levels in tranfected cells were also shown

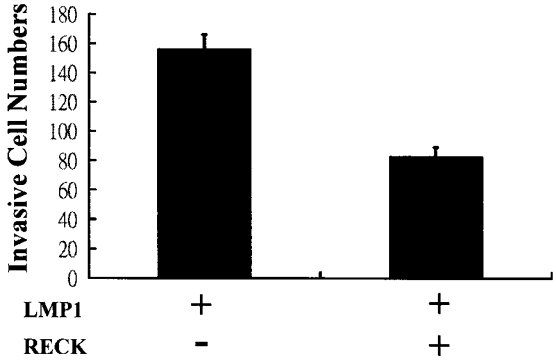


Figure 8 RECK suppresses LMP1-induced cell invasion. Cells were cotransfected with LMP1-expressing vector and control (−) or RECK expression vector (+) and cultured in 10% FCS medium for 48 h. *In vitro* invasion assay was performed as described in 'Materials and methods' to investigate the effect of RECK on LMP1-induced cell invasion

investigated in our recent study (Liu *et al*., 2003). In accordance with our previous results, RECK-specific siRNA inhibited RECK expression in TW04 cells (Figure 7). Conversely, nonspecific double-strand RNA had no effect. We found that RECK-specific siRNA, but not nonspecific double-strand RNA, reversed the inhibition of LMP1-induced cell invasiveness by PD98059. Second, we cotransfected RECK and LMP1 expression vectors into TW04 cells and examined the effect of RECK on LMP1-stimulated cell invasiveness. As shown in Figure 8, transfection of the RECK-expressing vector, but not the control vector, inhibited LMP1-induced cell invasiveness. Collectively, these data indicate that LMP1 stimulates MMP-9 activation and cell invasion in EBV-negative NPC cells, and this stimulatory effect can be counteracted by PD98059 via the restoration of RECK expression.

Discussion

In this study, we provide the first evidence that LMP1 may inhibit the expression of a metastasis suppressor gene RECK via the activation of the ERK/Sp1 pathway. Previous studies have demonstrated that LMP1 is involved in cell transformation and tumor metastasis of NPC. LMP1 may induce cell transformation via the induction of DNA synthesis (Peng and Lundgren, 1992), suppression of apoptosis (Henderson *et al*., 1991) and inhibition of differentiation (Yang *et al*., 2000). The involvement of LMP1 in tumor metastasis was supported by the observations that LMP1 expression in cells resulted in increased cell motility and invasive growth (Kim *et al*., 2000). Moreover, analysis of primary NPC tissues also demonstrates that LMP1-positive NPCs are more progressive and show an increased tendency toward lymph node metastasis than LMP1-negative NPCs (Hu *et al*., 1995; Horikawa *et al*., 2000). However, the mechanism by which LMP1 induces tumor metastasis is largely unknown.

A possible candidate that participated in LMP1-induced metastasis is MMP-9. Our and others' data clearly demonstrated that transfection of LMP1 strongly enhanced MMP-9 expression. Therefore, upregulation of MMP-9 may be one of the mechanisms by which LMP1 increases the metastatic ability of tumor cells. It should be noted, however, that enhancement of MMP expression does not always implicate increase of MMP activity because most of the MMPs are synthesized as inactive precursors and must be cleaved for activation. In this study, we found that MMP-9 mRNA was expressed in parental TW04 cells (as shown in Figure 1b). However, the amount of active MMP-9 detected in the conditioned medium was very less. We hypothesized that secretion and activation of MMP-9 might be suppressed in TW04 cells. This speculation was supported by the observation that parental TW04 cells expressed significant amount of RECK, which was known to regulate MMP-9 activity negatively via the suppression of enzyme secretion and direct inhibition of enzyme activity. After transfection of the LMP1-expressing vector, RECK expression was significantly downregulated and the enzymatic activity of MMP-9 in the culture medium was significantly increased. These results suggest that in addition to upregulation of MMP-9 expression, LMP1 must inhibit RECK expression to guarantee full activation of MMP-9 and to promote tumor metastasis. This can also explain why LMP1-positive NPCs are more progressive and show an increased tendency of lymph node metastasis than LMP1-negative NPCs. Therefore, we have revealed an important mechanism responsible for LMP1-induced metastasis in this study.

Additionally, we have also explored the signaling pathway by which LMP1 inhibits RECK expression. LMP1 has been shown to induce activation of various signal transduction pathways in transfected cells. Previous study demonstrated that LMP1 might activate the NF- κB signaling pathway to enhance MMP-9 expression (Yoshizaki *et al*., 1998). In this study, we provide strong evidence that LMP1 inhibits RECK via the activation of the ERK/Sp1 signaling pathway. Interestingly, a previous study demonstrated that oncogenic *ras* might inhibit RECK via the same Sp1 site in RECK promoter (Sasahara *et al*., 1999). It will be an important issue to elucidate the molecular action by which ERKs control RECK expression. Taken together, these results suggest that LMP1 may simultaneously modulate multiple signal transduction pathways to promote metastasis, and inhibitors of the ERK signaling pathway may be useful for the prevention of metastasis of EBV-associated tumors such as NPC. Additionally, the combination of inhibitors of the ERK and NF-κB signaling pathway (which can upregulate RECK and inhibit MMP-9 simultaneously) may act synergistically and exert a more significant effect on the inhibition of LMP1-mediated metastasis. These results are of important clinical significance and warrant further *in vivo* study.

Materials and Methods

Cell line

The NPC-TW04 human NPC cell line was kindly provided by Dr Lin CT (National Taiwan University) and was routinely cultured in DMEM/F12 medium supplemented with 10% heat-inactivated FCS and antibiotics. This cell line is an EBV-negative NPC cell line and the characteristics have been reported previously (Lin *et al*., 1990, 1993).

Plasmids and reagents

The control and LMP1-expressing vector were kindly provided by Dr YS Chang (Chang Gung University). Mouse RECK promoter–luciferase plasmid was a generous gift from Dr Noda. PD98059 and SB203580 were purchased from Biomol (Polymouth Meeting, PA, USA). SP600125 was obtained from Tocris (Ellisville, MO, USA). Anti-ERK and anti-phospho-ERK antibodies were obtained from New England Biolabs (Beverly, MA, USA). The anti-LMP1 antibody was obtained from DAKO (Copenhagen, Denmark). The anti-RECK antibody was purchased from MBL (Nagoya, Japan). LipofectAMINE reagent was obtained from Invitrogen (Carlsbad, CA, USA) and luciferase assay system was obtained from Promega (Madison, WI, USA).

RNA extraction and RT–PCR

Cells were transiently transfected with control or LMP1-expressing vector by the LipofectAMINE method. Total RNA was isolated from cells by an RNeasy Mini Kit from Qiagen (Santa Clarita, CA, USA). A measure of 1 μg of total RNA was reverse-transcribed to cDNA by the OneStep RT–PCR kit (Qiagen) according to the manufacturer's protocol. Each polymerase chain reaction (PCR) was performed in 50 μl of a reaction mix, containing 10 mM Tris-HCl (pH 8.3), 50 mM KC1, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 600 nM primers and 3 U of HotStar *Taq* DNA polymerase. Volumes of 5 μl of the reverse-transcribed cDNA samples were added to the reaction mixture and amplified. A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was used as an internal control to verify the efficiency of cDNA synthesis and PCR amplification. The condition for PCR was 30 cycles of denaturation (94°C/min), annealing (60°C/min), extension (72°C/min) and one cycle of final extension (72°C/10 min). The predicted sizes for PCR products for RECK, MMP-2, MMP-9 and GAPDH were 477, 605, 263 and 346 bp, respectively. The primers used were: RECK-forward: 5′-CCT CAGTG AGCACAGTTCAGA-3′, RECK-reverse: 5′-GCA GCACACACACTGCTGTA-3′, MMP-2-forward: 5′-GTGC TGAAGGACACACTAAAGAAGA-3′, MMP-2-reverse: 5′-TTGCCATCCTTCTCAAAGTTGTAGG-3′, MMP-9-forward: 5′-CACTGT CCACCCCTCAGAGC-3′, MMP-9-reverse: 5′-GCCACTTGTCGGCGA TAAGG-3′, GAPDH-forward: 5′-CCCATCACCATCTTCCAG-3′, and GAPDH-reverse: 5′-CAGTCTTCTGGGTGGCAGT-3′. After reaction, PCR products were analysed on a 2% 0.5 × TBE agarose gel and visualized under UV light.

Promoter activity assays

Promoter activity of RECK gene was analysed as described previously (Lee *et al*., 2000). In brief, cells were plated onto six-well plates at a density of 100 000 cells/well and grown overnight. Cells were then cotransfected with 2 μg of RECK promoter–luciferase plasmid and 1 μg of control or LMP1-expressing vector. Luciferase activity was determined at 48 h

after transfection by using an assay system according to the procedures of the manufacturer. Luciferase activity was normalized for protein concentration in cell lysate and expressed as an average of three independent experiments. In some experiments, the renilla luciferase reporter vector was cotransfected with the RECK promoter-luciferase plasmid and luciferase activity was determined by a dual-luciferase reporter assay system (Promega).

Western blot analysis

Cells were transfected with control or LMP1-expressing vector and cultured in 10% FCS medium for 48 h. Cells were harvested in a lysis buffer and equal amounts of cellular proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously (Pan and Hung, 2002). Proteins were transferred on to nitrocellulose membranes and blots were probed with anti-LMP1 or anti-RECK antibody. Enhanced chemiluminescence reagents were used to depict the protein bands on the membranes.

Assessment of ERK activation

Cells were transfected with 2 μg of control or LMP1-expressing vectors and cultured in 10% FCS medium containing 0.1% DMSO (vehicle) or 10 μM of PD98059 for 48 h. After treatment, cellular proteins were extracted and subjected to SDS–PAGE. ERK activation was investigated by probing the membranes with anti-phospho-ERK antibody. Equal loading of proteins in each lane was confirmed by probing the membranes with anti-ERK antibody.

Zymography

Cells were transfected with control or LMP1-expressing vector and cultured in serum-free medium containing 0.1%. DMSO or 10 μM of PD98059 for 48 h. The conditioned medium was collected and concentrated by using Centricon YM-50 columns (Amicon, Bedford, MA, USA). Cell numbers were determined by a hemocytometer. The conditioned medium from equal number of cells was separated by 10% acrylamide gels containing 0.1% gelatin (Invitrogen, Carlsbad, CA, USA). The gels were incubated in 2.5% Triton X-100 solution at room temperature with gentle agitation to remove SDS and then soaked in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.5 mM $ZnCl_2$) at 37°C overnight. After the reaction, the gels were stained for 1 h with staining solution (0.1% Coomassie brilliant blue, 30% methanol and 10% acetic acid) and destained in the same solution, but without Coomassie brilliant blue. Gelatinolytic activity was visualized as a clear band against a dark background of stained gelatin.

In vitro *invasion assays*

In vitro invasion assay was performed as described previously (Kim *et al*., 2001) using a 24-well transwell unit with polycarbonate filters coated on the upper side with Matrigel (Becton Dickinson Labware, Bedford, MA, USA). Cells were transfected with control or LMP1-expressing vector for 24 h and then incubated with vehicle or 10 μM of PD98059 for another 24 h. Cells were harvested and resuspended in 100 μl of 1% FCS DMEM/F12 medium and placed in the upper part of the transwell unit and allowed to migrate for 6 h. The lower part of the transwell unit was filled with 10% FCS medium. After incubation, nonmigrated cells on the upper part of the membrane were removed with a cotton swab. Migrated cells on the bottom surface of the membrane were fixed in formaldehyde, stained with Giemsa solution and counted under a microscope.

siRNA experiments

siRNA designed to target RECK 5′-AAGACCCAGCC-CUUGCCUCAA-3′ (sense strand) and a nonspecific RNA 5′-AACGUUGCGAUAGCGUAGUAC-3′ were synthesized and transfected by LipofectAMINE reagent as previously described (Liu *et al*., 2003). Cell invasion assays were performed as mentioned above.

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