EBV Latent Membrane Protein 1 Up-regulates Hypoxia-Inducible Factor 1α through Siah1-Mediated Down-regulation of Prolyl Hydroxylases 1 and 3 in Nasopharyngeal Epithelial Cells

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Abstract

Hypoxia-inducible factor 1 (HIF1) is up-regulated in most malignant tumors usually via interruption of ubiquitination and proteasomal degradation of its subunit α. Recently, we have shown that the principal EBV oncoprotein, latent membrane protein 1 (LMP1), activates HIF1α and subsequently expression of HIF1-responsive genes in epithelial cells. Here, we explore the mechanism for HIF1α activation by LMP1 in nasopharyngeal epithelial cells: LMP1 up-regulates the level of Siah1 E3 ubiquitin ligase by enhancing its stability, which subsequently induces proteasomal degradation of prolyl HIF-hydroxylases 1 and 3 that normally mark HIF1α for degradation. As a result, LMP1 prevents formation of von Hippel-Lindau/HIF1α complex, as shown by coimmunoprecipitation analyses. Thus, Siah1 is implicated in the regulation of HIF1α and is involved in a recently appreciated aspect of EBV-mediated tumorigenesis, namely, the angiogenesis process triggered by LMP1. (Cancer Res 2006; 66(20): 9870-7)

Introduction

Invasion into surrounding tissues is a characteristic property of malignant tumors, including virus-associated cancers such as EBV-associated nasopharyngeal carcinoma and B-cell lymphoproliferative disease. EBV, a ubiquitous human herpesvirus, is associated with other invasive malignancies, including Hodgkin's disease and a subset of gastric cancers; it has also been implicated in some invasive breast cancers (1). In most of these tumors, EBV infection is predominantly latent (1, 2). Both invasion and metastasis involve sequential multistep processes, and the principal EBV oncoprotein, latent membrane protein 1 (LMP1), induces the expression of a series of factors in nasopharyngeal cells that are considered indispensable for these processes, including matrix metalloproteinase 9 (MMP9), which plays a critical role in invasion of basement membrane (3). Expression of LMP1 also induces and causes release of fibroblast growth factor 2 (FGF-2) into extracellular fluid (4), and LMP1 promotes cell migration and invasive growth via Ets-1 expression (5, 6) in human epithelial cells. In addition, LMP1 also induces angiogenic factors such as vascular endothelial factor (VEGF) through induction of cyclooxygenase 2 (COX-2; refs. 3, 7). More recently, we have reported that LMP1 increases the level of the α subunit of hypoxia-inducible factor 1 (HIF1α) in human nasopharyngeal cells, which induces VEGF via activation of reactive oxygen species and p42/44 pathways (8).

HIF1, the principal oxygen-sensing transcription factor, is composed of HIF1α and HIF1β subunits (9). The level of HIF1α increases exponentially in response to decreases in cellular O_2 concentration, whereas HIF1β is not regulated by oxygen tension (9, 10). The specific binding of the heterodimer of HIF1α and HIF1β to the hypoxic response element activates the transcription of genes whose products are required for metabolism, erythropoiesis, vascularization, and tumor progression (9, 11). Under normoxic conditions, HIF1α is degraded via a proteasome-degradation pathway (12). The oxygen-dependent turnover of HIF1α protein is regulated by four isoforms of prolyl HIF-hydroxylases (PHD), termed PHD1-4, that modify HIF1α by hydroxylation at two conserved proline residues located in the oxygen-dependent degradation domain of the protein (13, 14). During normoxia, modification of HIF1α by PHDs permits its binding to von Hippel-Lindau protein (VHL), a recognition component of the E3 ubiquitin ligase complex (15–17). This binding promotes the ubiquitination, which is mediated by a complex of VHL, elongin B, C, Cullin 2, and Rbx1, and subsequently degradation of HIF1α.

The Siah-family proteins are homologues of *Drosophila* seven-in-absentia (*Sina*) protein, which is essential for the formation of R7 photoreceptor cells during development (18). Mice and rats have three unlinked *Siah* genes: *Siah1a, Siah1b*, and *Siah2*, whereas human beings have single *Siah1* and *Siah2* genes (19, 20). The mammalian Siah proteins are highly homologous to one another. Siah1a and Siah1b are 98% identical, whereas Siah1 proteins and Siah2 protein diverge significantly only at their NH_2 terminus. Siah proteins possess E3 ubiquitin ligase activity and regulate the ubiquitination and proteasome-dependent degradation of a number of proteins, such as deleted in colorectal cancer (DCC), β-catenin, c-Myb, and APC, as well as Siah itself (21–23).

Recently, a new pathway for controlling levels of HIF1α has been proposed; murine Siah proteins degrade PHD1 and PHD3, so that HIF1α is stabilized in mouse embryonic fibroblasts (24). In addition, we have recently reported that LMP1 modulates the expression of Siah1 in B lymphoma cells, which results in up-regulation of β-catenin (25). These reports prompted us to investigate whether Siah family proteins are involved in the increased levels of HIF1α detected in cell lines that express LMP1 (8) and to explore the possible mechanisms for such effects. We report here that LMP1 can stabilize Siah1 protein in human

Note: S. Kondo and S.Y. Seo contributed equally to this work. K.L. Jang and J.S. Pagano have equal senior authorship.

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doi:10.1158/0008-5472.CAN-06-1679

epithelial cells, which induces degradation of PHDs and prevents the interaction of VHL and HIF1α, resulting in an increase in levels of HIF1α in EBV-infected human cancer cell lines.

Materials and Methods

Cell culture and transfection. KH-1 and KH-2 lines (gifts of Dr. Maria Masucci, Karolinska Institute, Stockholm, Sweden) are EBV-positive type II cell lines derived from fusion of KR-4 (an EBV-positive type III lymphoblastoid cell line) and HeLa cells (human cervical carcinoma; ref. 26). Ad-AH, kindly provided by Dr. Erik K. Flemington (Tulane University, New Orleans, LA), is an EBV-negative human nasopharyngeal cell line (27). MDA-MB-231 (a human breast cancer cell line) and EBV-infected MDA-MB-231 clones (C4A3, C1D12, C2G6, and C3B4) were described previously (8). MDA-MB-231 cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS), 4 μmol/L L-glutamine, penicillin, and streptomycin. EBV-infected MDA-MB-231 clones were maintained in the same medium, but with 700 μg/mL G418 (Life Technologies, Grand Island, NY). The other cells were maintained in DMEM with 10% FBS and penicillin and streptomycin.

Antibodies and plasmids. Mouse LMP1 monoclonal antibody was purchased from DAKO (Glostrup, Denmark). Mouse monoclonal antibodies against HIF1α, DCC, and VHL were from Transduction Laboratories (San Diego, CA). Mouse monoclonal antibody to α-tubulin was from Sigma (St. Louis, MO). Mouse monoclonal Myc antibody, rabbit polyclonal histone antibody (H2B), and rabbit polyclonal GRP78 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody against Siah1 and Siah2 were from Transgenic, Inc. (Kumamoto, Japan). Rabbit polyclonal antibody against PHD1, PHD2, PHD3, and PHD4 were from Novus Biologicals (Littleton, CO). pcDNA3-based LMP1 and pcDNA3-LMP1-DM have been described (28). Myc-tagged Siah1 expression plasmid and a mutant form of Siah1 expression plasmid, Myc-tagged Siah1-DN, were kindly provided by Drs. J.C. Reed and S. Matsuzawa (Burnham Institute, La Jolla, CA; ref. 22). Siah1 probe for Northern blots was from Drs. E. Fearon and T. Sakai (Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI; ref. 20).

Transient and stable transfection. For transient expression, cells were transfected for 48 hours with 2 μg appropriate plasmid(s) with the use of Effectene Transfection Reagent (Qiagen, Valencia, CA) following the instructions of the manufacturer. Stable cell lines were established by selecting the transfected Ad-AH cells in the presence of 800 μg/mL G418 as described before (8).

Small interfering RNA transfection. Small interfering RNA (siRNA) duplex oligonucleotides were chemically synthesized from Samchully Pham. (Seoul, South Korea). The PHD1 siRNA (5′-CAUGCAGGGCACGAUAUA-GUCTT-3′) targets the coding region 538 to 558 relative to the start codon. The siRNA for PHD3 (5′-CCACGUGGCGAACAUAACCUGTT-3′) were from positions 389 to 409. A nonspecific duplex oligonucleotide (5′-CUCGCCG-GACACGCUGAACTT-3′) was used as a negative control. Cells were seeded at 30% confluence in antibiotic-free medium 24 hours before transfection. WelFect-Ex PLUS Transfection Reagent (WelGENE, Daegu, South Korea) was used to transfect cells with 100 nmol/L siRNA duplex. The efficacy of the siRNA transfection in each experiment was ascertained by Western blots.

Western blot analysis. Whole-cell lysates were prepared in 500 μL cell lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.50% sodium deoxycholate, 0.10% SDS, 0.2 mmol/L sodium orthovanadate, 0.1 mol/L NaF, and 5 μg fluoride/mL]. Nuclear or cytoplasmic extracts were prepared with the use of NE-Per cell fractionation kit (Pierce, Rockford, IL) in accordance with the protocol of the manufacturer. Protein was electrophoresed on polyacrylamide gels, and transferred onto a nitrocellulose membrane (Osmonics, Minnetonka, MN). The membrane was incubated overnight at 4°C with an appropriate primary antibody, followed by reaction with a horseradish peroxidase–conjugated mouse or rabbit secondary antibody (Amersham, Little Chalfont, United Kingdom) at room temperature for 1 hour. Peroxidase activity was detected by chemiluminescence with the SuperSignal kit (Pierce) as recommended by the manufacturer.

Northern blot analysis. Total RNA was prepared from cells with the RNeasy Mini kit (Qiagen). A 10-μg aliquot of total RNA was denatured and electrophoresed in 1% formaldehyde-agarose gels and blotted onto a nitrocellulose membrane (Osmonics). The cDNA for human Siah1 was labeled with [^{32}P]dCTP using the Strip-EZ DNA, a random-primed probe synthesis kit (Ambion, Austin, TX). Northern blot analysis was done according to the Strip-EZ DNA protocol (Ambion). Equal loading was assessed by analysis of the rRNA bands on ethidium bromide-stained gels.

Coimmunoprecipitation assays. After transient transfection into Ad-AH cells with or without LMP1, the cells were lysed as described before (29). Whole-cell extracts (1 mg) were incubated with HIF1α Siah1, or pVHL antibodies at 4°C for 1 hour; immunocomplexes were incubated with protein A/G-Sepharose beads (Santa Cruz Biotechnology) at 4°C overnight, washed thrice with protein lysis buffer, and then eluted from the buffer with 2× Laemmli buffer by boiling for 5 minutes. Denatured immune complexes were separated by electrophoresis and analyzed by immunoblotting.

Results

The level of Siah1 but not Siah2 protein is increased in latently EBV-infected cells. Nakayama et al. (24) showed that Siah1a (corresponds to Siah1 in human cells) and Siah2 stabilize HIF1α in mouse embryonic cells. To investigate the possibility that Siah proteins also contribute to the increased levels of HIF1α produced by LMP1, we first determined the endogenous levels of Siah1 and Siah2 as well as HIF1α in EBV-infected cell lines. EBV type II latently infected cells express LMPs as well as EBNA1 (1, 2, 30). The KH-1 and KH-2 are type II adherent cell lines that were derived by somatic fusion of cells from an EBV-infected lymphoblastoid suspension cell line, KR4, and adherent HeLa cells (26). The expression level of Siah1 protein is clearly higher in both KH-1 and KH-2 cells, which express EBV latency proteins, including LMP1, than in HeLa cells (Fig. 1*A*). Also, the level of HIF1α seems to correlate with the level of Siah1 as well as the level of LMP1 as reported before (8). However, no significant differences were observed in the level of Siah2, probably due to its intrinsic high expression level in the uninfected cells.

To confirm these results in other cell lines, we tested a set of EBV-infected breast cancer cell lines in which different levels of LMP1 are expressed. MDA-MB-231 is an EBV-negative breast cancer cell line, and C4A3, C1D12, C2G6, and C3B4 are EBV-infected clones derived from it (31). As shown in Fig. 1*B*, the Siah1 level is increased in all EBV-positive cell lines except C4A3 compared with that in the parental line, MDA-MB-231. Although C4A3 does not express a detectable level of LMP1, it is definitely an EBV-positive cell line, as shown by the expression of other EBV latent products such as EBNA1 (31). The levels of HIF1α and Siah1 but not Siah2 seemed to correlate with each other as well as with levels of LMP1. Therefore, direct correlations among endogenous levels of LMP1, Siah1, and HIF1α are evident in the two quite different sets of cell lines.

LMP1 induces expression of Siah1 protein. To clarify whether LMP1 is responsible for induction of Siah1 in the type II epithelial cell lines, we transfected a DN form of LMP1 into C3B4 cells, the EBV-infected breast cancer line that expresses the highest levels of endogenous LMP1 and Siah1. LMP1-DM has point mutations in the COOH-terminal activation regions 1 and 2 and well-established DN properties (28). Although LMP1 and LMP1-DM proteins cannot be distinguished in the Western blots the amount of protein detected reflects the concentration of DM applied. Expression of LMP1-DM reduced levels of Siah1 and HIF1α proteins in a dose-dependent manner (Fig. 1*C*). These results prompted more direct

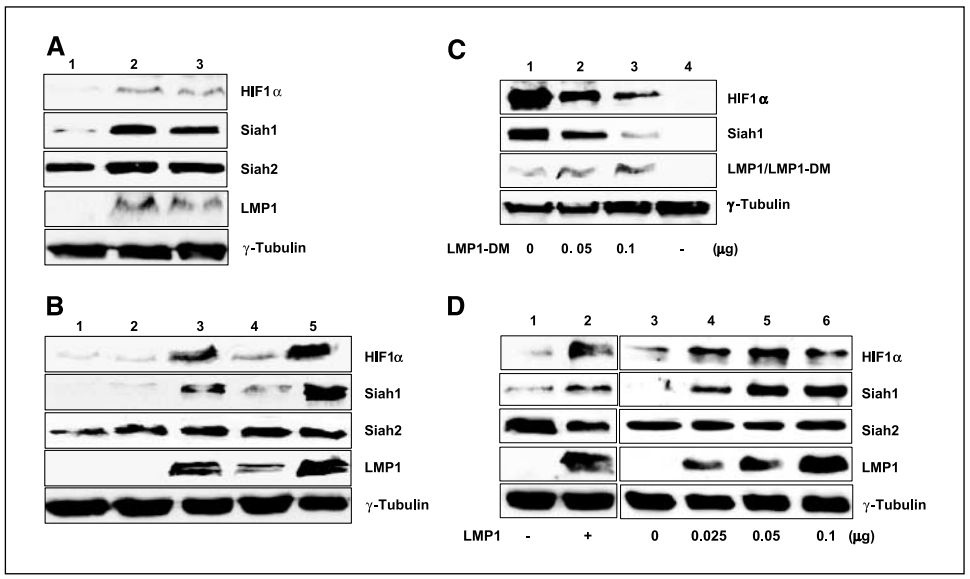

Figure 1. LMP1 up-regulates Siah1 in human epithelial cell lines. *A,* the KH-1 (*lane 2*) and KH-2 (*lane 3*) cell lines are derived from somatic cell fusions of an EBV-infected lymphoblastoid cell line (KR4) with HeLa cells (*lane 1*; ref. 26). Western blots were done to detect HIF1α, Siah1, Siah2, and LMP1 protein. γ-Tubulin was used as loading control. *B,* C4A3 (*lane 2*), C1D12 (*lane 3*), C2G6 (*lane 4*), and C3B4 (*lane 5*) were derived from a human breast cancer cell line, MDA-MB-231 (*lane 1*), after infection with EBV and express different levels of LMP1. *C,* C3B4 cells that express endogenous LMP1 were transiently transfected with increasing amounts of LMP1-DM, a DN mutant of LMP1 (*lanes 1-3*). MDA-MB-231 served as negative control (*lane 4*). *D,* Ad-AH nasopharyngeal epithelial cells were either stably transfected with LMP1 (*lane 2*) or transiently transfected with increasing amounts of LMP1 for 48 hours (*lanes 4-6*) and analyzed by Western blots for HIF1α, Siah1, Siah2, and LMP1.

investigation of whether LMP1 induces expression of Siah1 and its relation to HIF1α.

To explore whether LMP1 induces Siah1, we transfected LMP1 expression vector into Ad-AH cells, an EBV-negative nasopharyngeal epithelial cell line. Ad-AH-LMP1 cells that stably express LMP1 had a significantly higher level of Siah1 than control cells (Fig. 1*D, lanes 1* and *2*), and the HIF1α level was increased as before (8). Moreover, Siah1 induction by LMP1 seemed to be dose dependent in the transient transfection experiment (Fig. 1*D, lanes 3-6*). However, at the highest dose (0.1 μg), the effect of LMP1 was obscured perhaps because overexpression of LMP1 may be toxic to cells (32). Consistent with the results shown in Fig. 1*A* and *B*, no clear differences were observed in the expression level of Siah2 when LMP1 was expressed. Therefore, it seems that LMP1 is responsible for the increased level of Siah1 detected in EBV-infected cells.

Siah1 is responsible for the activation of HIF1α by LMP1. To investigate the functional role(s) of Siah1 in the activation of HIF1α, we first examined whether LMP1-stabilized Siah1 is a functional ubiquitin ligase. For this purpose, we measured levels of DCC protein, a known target of Siah1, as a control for Siah1 activity (21) in LMP1-expressing cells. Consistent with the high Siah1 level, LMP1-transfected cells expressed a lower level of DCC compared with nontransfected control cells (Fig. 2*A* and *B*). Moreover, addition of wild type (WT) Siah1 almost completely abolished expression of DCC protein (Fig. 2*A, lanes 3* and *4*). On the other hand, a dominant-negative (DN) mutant form of Siah1 (21, 22) restored expression of DCC in the presence of LMP1 (Fig. 2*B, lanes 3* and *4*). Therefore, up-regulated Siah1 is functionally active as an E3 ubiquitin ligase for DCC.

The increase in Siah1 produced by the transfection of Siah1-Myc-WT plasmid seemed to increase the level of HIF1α detected in the presence of LMP1 (Fig. 2*A*). In addition, expression of Siah1 DN almost completely abolished the elevated level of HIF1α produced by LMP1 (Fig. 2*B*). Thus, we conclude that the LMP1-stabilized Siah1 is responsible for the stabilization of HIF1α. In addition, the fact that the Siah1-DN form, which lacks its E3 ligase activity, showed effects opposite to those produced by the WT form suggests that HIF1α activation in the presence of LMP1 is dependent on the ligase activity of Siah1.

LMP1 stabilizes Siah1 protein. To clarify how LMP1 up-regulates expression level of Siah1, we first investigated whether LMP1 induces transcription of Siah1. However, Northern blot analyses revealed no significant difference in amounts of RNA detected in stably LMP1-transfected Ad-AH cells and control cells (Fig. 3*A*). Therefore, LMP1 does not seem to affect transcription of Siah1.

Because Siah1 protein is itself ubiquitinated and proteasomally degraded (21), we explored whether LMP1 affects the half-life of Siah1 in cells in which protein synthesis is blocked. For this purpose, mock or Ad-AH cells stably expressing LMP1 were treated with cycloheximide. In the absence of protein synthesis, Siah1 was

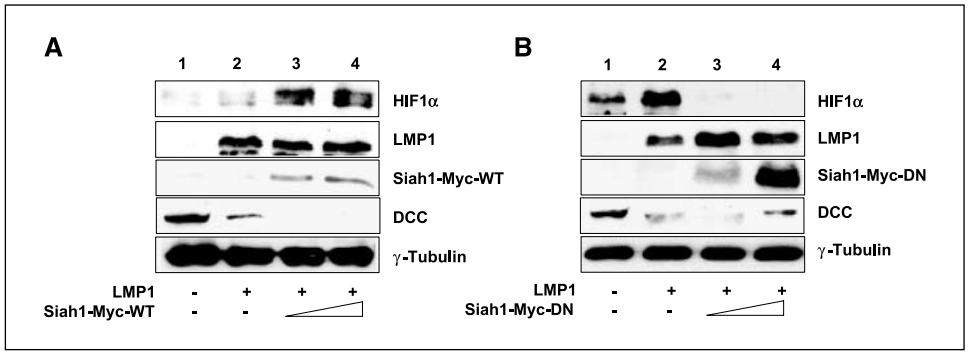

Figure 2. Siah1 is responsible for the activation of HIF1α by LMP1. Graded amounts (0.05 and 0.1 μg) of either WT (Siah1-Myc-WT; *A, lanes 3* and *4*) or a DN mutant form (Siah1-Myc-DN) of Siah1 (*B, lanes 3* and *4*) were transiently cotransfected with LMP1 (0.1 μg) into Ad-AH cells, and Western blots were done. Ad-AH cells transfected with either empty vector (0.1 μg; *lane 1*) or LMP1-expressing plasmid (0.1 μg; *lane 2*) were used as controls. DCC was included as a positive control for Siah1 ubiquitin ligase activity.

still detectable in the LMP1-expressing cells up to 4 hours after addition of the inhibitor, whereas in the control cells not expressing LMP1, Siah1 degrades more rapidly and almost completely disappeared after 2 hours (Fig. 3*B*). Next, to prove that the high level of Siah1 in LMP1-transfected cells is caused by inhibition of proteasome degradation pathways, cells were treated with MG132, a potent proteasome inhibitor, and Western blots were done. In control AdAH cells, MG132 led to significant accumulation of Siah1 after 1 and 3 hours (Fig. 3*C, lanes 1, 3*, and *5*). However, in LMP1-expressing cells, addition of MG132 had little if any additional effect on the Siah1 level (Fig. 3*C, lanes 2, 4*, and *6*). Therefore, the difference in Siah1 levels produced by LMP1 can be attributed not to differential transcription but to differential degradation of the ligase. Thus, we conclude that Siah1 is stabilized by LMP1.

Siah1 induced by LMP1 localizes in both the nucleus and cytoplasm. In previous reports, Siah1 was detected in both cytoplasm and nucleus by immunofluorescence microscopy (33, 34). After separation of nuclear and cytoplasmic fractions from stable transfectants of AdAH cells, we confirmed that Siah1 is found in the cytoplasm and nucleus in LMP1-negative cells (Fig. 3*D*). After induction with LMP1, Siah1 was still distributed in both cell compartments, but the increase in Siah1 protein was predominantly in the cytoplasm.

Degradation of PHD1 and PHD3 by Siah1 is responsible for the activation of HIF1α by LMP1. Next, we investigated the mechanism by which LMP1 stabilizes HIF1α via Siah1. Nakayama et al. (24) showed that Siah1a and 2 bind and degrade PHD1 and PHD3 in mouse embryonic cells, resulting in stabilization of HIF1α. According to the results in Fig. 4*A*, LMP1 down-regulates expression of PHD1 and PHD3 among the four PHD isoforms in both transient and stable transfection experiments. Treatment with a proteasomal inhibitor, MG132, almost completely abolished the deregulation of PHD1 and PHD3 by LMP1 (Fig. 4*B*), suggesting that LMP1 stimulates degradation of PHD1/PHD3 through a proteasomal degradation pathway.

To investigate whether the down-regulation of PHD1 and PHD3 is attributable to the enhanced Siah1 level in the presence of LMP1, either WT or the DN form of Siah1 were introduced into LMP1-expressing cells. The levels of PHD1 and PHD3 decreased further in the presence of exogenous WT Siah1, but recovered to almost the control level when Siah1 DN was introduced (Fig. 4*C*). Neither PHD2 nor PHD4 was affected by transfection of Siah1 WT and DN. These results suggest that LMP1 down-regulates the expression of PHD1 and PHD3 via Siah1 in human nasopharyngeal cells.

To provide more direct evidence for the role of PHD1 and PHD3 in the stabilization of HIF1α in the Ad-AH cells, we attempted to knockdown PHD1 and PHD3 expression by introducing specific siRNA for either PHD1 or PHD3 into the cells. As expected, expression of PHD1 and PHD3 was specifically decreased by the corresponding siRNA (Fig. 4*D*). Accordingly, HIF1α expression was increased, proving that PHD1 and PHD3 play a direct role in the repression of HIF1α in these cells. Therefore, LMP1 appears to stabilize HIF1α by modulation of PHD activity through Siah1.

LMP1 disrupts interaction between HIF1α and VHL. Next, we investigated whether the down-regulation of PHD expression is connected to the decrease in the interaction of HIF1α and VHL, which subsequently results in stabilization of HIF1α. Because VHL E3 ligase is an important component involved in the proteasomal degradation of HIF1α (15, 16), we first examined whether LMP1 affects the expression level of VHL. The level of VHL was not altered by the introduction of either LMP1 (Fig. 5, *lanes 5* and *6*) or Siah1 (data not shown).

Finally, we examined whether LMP1 affects the binding of VHL to HIF1α. For this purpose, cell lysates were prepared from either control or the LMP1-expressing nasopharyngeal cell line and immunoprecipitated with VHL antibody. When the resulting immunocomplexes were immunoblotted with an antibody to HIF1α, a strong interaction between VHL and HIF1α was detected in empty vector-transfected cell lysates (Fig. 5, *lanes 1* and *2*). In the presence of LMP1, however, the interaction between VHL and HIF1α decreased dramatically. A similar result was obtained with the reverse sequence of immunoprecipitation experiments. In this case, as expected, more HIF1α was detected from the precipitates of the LMP1-expressing cells (Fig. 5, *lanes 3* and *4*). However, the

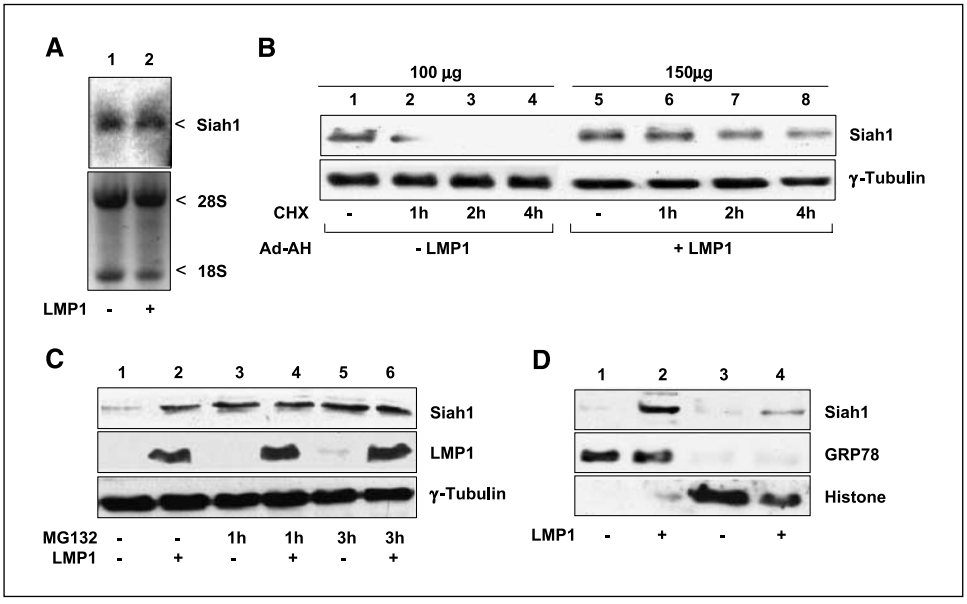

Figure 3. LMP1 stabilizes Siah1 protein but does not affect its transcription. *A,* total RNA (10 μg) from Ad-AH cell lines stably transfected with either empty vector (*lane 1*) or LMP1-expressing plasmid (*lane 2*) were analyzed with Northern blots to detect levels of Siah1 transcripts. Levels of 28S and 18S rRNA are shown as loading controls. *B,* the stably transfected Ad-AH cell lines were treated with cycloheximide (10 μmol/L) for the indicated times to block protein synthesis. To compensate for the different levels of Siah1 in the presence or absence of LMP1, different amounts of protein (100 or 150 μg) were used. *C,* Ad-AH cell lines were treated with a proteasomal inhibitor, MG132 (100 μmol/L), for the indicated time, followed by Western blots for Siah1. *D,* cytoplasmic (*lanes 1* and *2*) and nuclear (*lanes 3* and *4*) fractions of Ad-AH cells stably expressing LMP1 were analyzed by Western blots. Histone (H2B) and GRP78 were used as nuclear and cytoplasmic markers, respectively.

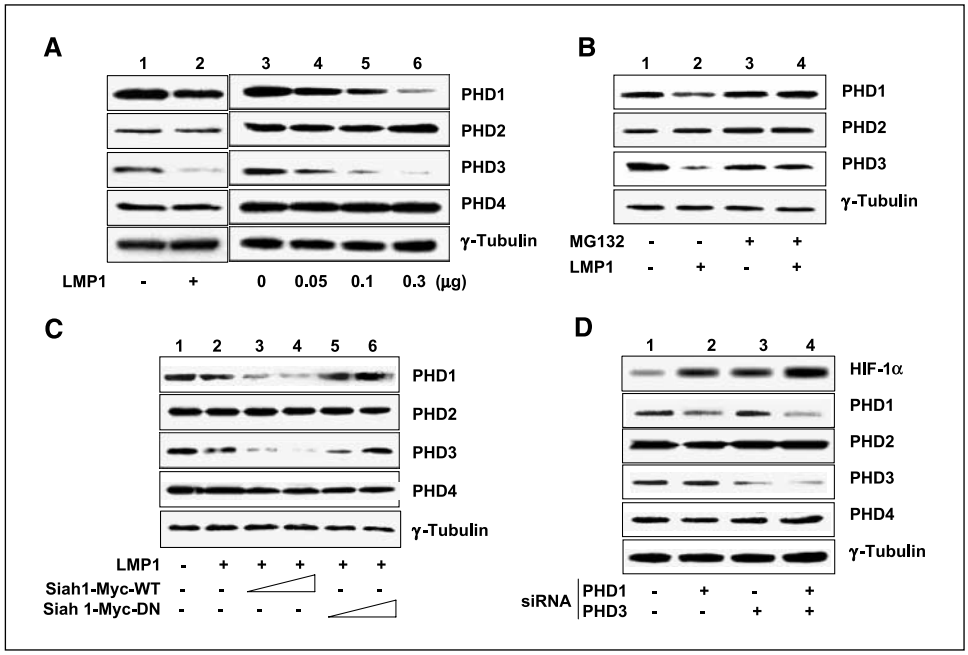

Figure 4. Degradation of PHD1 and PHD3 by Siah1 is responsible for the activation of HIF1α by LMP1. *A,* expression of LMP1 decreases levels of PHD1 and PHD3. Ad-AH cells were either stably transfected with LMP1 (*lane 2*) or transiently transfected with increasing amounts of LMP1 for 48 hours (*lanes 4-6*) and were analyzed for four isoforms of PHD. *B,* LMP-1 increases proteasomal degradation of PHD1 and PHD3. Ad-AH cell lines were treated with MG132 (100 μmol/L) for 3 hours, followed by Western blots for HIF1α and PHDs (*C*). Decrease of PHD1 and PHD3 levels induced by LMP1 depends on Siah1 ubiquitinating activity. Increasing amounts of either WT (*lanes 3* and *4*) or a DN form (*lanes 5* and *6*) of Siah1 were transiently transfected into LMP1-expressing Ad-AH cells. *D,* expression of PHD1 and PHD3 causes stabilization of HIF1α. Ad-AH cells were transfected with 100 nmol/L siRNAs: a control (*lane 1*), PHD1 (*lane 2*), PHD2 (*lane 3*), or both PHD1 and PHD2 (*lane 4*).

amount of VHL coprecipitated with HIF1α was much lower with the LMP1-expressing cells than from the control extracts, which indicates a weak interaction of VHL and HIF1α in the presence of LMP1.

Discussion

LMP1 is essential for human B-cell immortalization by EBV and is the only EBV protein that transforms rodent fibroblasts (35), human epithelial cells (36), and human keratinocytes (37), and also induces B-cell lymphoma in transgenic mice (38). Consequently, the oncogenic properties of LMP1 have been the focus of many investigations. In addition, it is well known that nasopharyngeal carcinoma and EBV-associated lymphoproliferative diseases are invasive and metastatic: LMP1 is characteristically expressed in most of these malignancies (30). More recently, we have addressed this aspect of EBV oncogenesis by putting forth varied and increasing evidence that LMP1 induces the expression of comprehensive set of invasion, metastasis, and angiogenic factors, including MMP9, COX2, VEGF, and FGF2 (3, 4, 7, 30).

We have also reported that expression of HIF1α is elevated in EBV type II and III latently infected cells, which correlates with the induction of VEGF. LMP1 can modulate protein levels of HIF1α via activation of reactive oxygen species and p42/p44 mitogen-activated protein kinase (MAPK) pathways (8). Previous results, including our own with LMP1, indicated that some stimuli can induce synthesis of HIF1α (8, 39). However, rescue of the protein from destruction remains an additional possibility and is reexamined here.

Recently, Nakayama et al. (24) have reported that Siah1a (which corresponds to Siah1 in human cells) and Siah2 stabilize HIF1α under hypoxic conditions by targeting PHD1 and PHD3 for proteasome-mediated degradation. In this study, we observed a similar phenomenon, but under normoxic conditions. LMP1 induces stabilization of Siah1, which, in turn, destabilizes PHD1 and PHD3 and eventually stabilizes HIF1α. Therefore, up-regulation of Siah family proteins might be a general mechanism for the stabilization of HIF1α. However, unlike the induction of Siah1a and Siah2 under hypoxic conditions (24), LMP1-mediated Siah1 activation does not occur at the transcriptional level. Instead, it involves stabilization of the protein through inhibition of a proteasomal degradation pathway. Another difference is that LMP1 seems not to cause stabilization of HIF1α via Siah2, because LMP1 does not affect Siah2 levels (Fig. 1).

The stability of HIF1α protein in human cells is regulated by three isoforms of PHDs, termed PHD1 to PHD3, that modify HIF1α by hydroxylation at two conserved proline residues (13, 14). Initial analysis has established that all have the ability to hydroxylate HIF1α polypeptides *in vitro* (13) and that all can suppress HIF1α activity when overexpressed in cells (40). Each PHD isoform seems to contributes to the regulation of HIF1α in different cell types under particular culture conditions (41). The precise function of each PHD isoform *in vivo*, and indeed whether HIF1α is the physiologic substrate for all PHD isoforms, are unanswered questions.

Recently, Berra et al. (42) suggested that PHD2 is the key oxygen sensor setting low steady-state levels of HIF1α in normoxia. According to the present study, although the three isoforms are expressed with some variations in the nasopharyngeal epithelial cell lines, only PHD1 and PHD3 are affected by LMP1. In addition, knockdown of either PHD1 or PHD3 levels was enough to stabilize

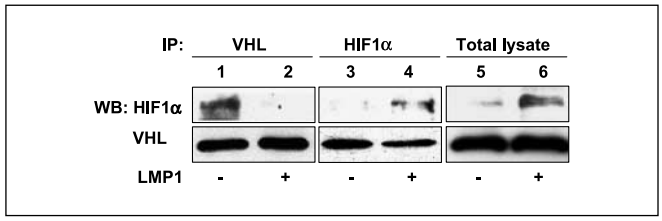

Figure 5. LMP1 disrupts interaction between HIF1α and VHL but does not affect VHL levels. Cell extracts from Ad-AH-pcDNA3 (*lanes 1, 3*, and *5*) and Ad-AH-LMP1 (*lanes 2, 4* and *6*) were immunoprecipitated (*IP*) with either VHL (*lanes 1* and *2*) or HIF1α (*lanes 3* and *4*) antibody, followed by western blots for HIF1α and VHL. Total lysates were included as control (*lanes 5* and *6*).

HIF1α in such cells. A similar role for PHD1 and PHD3 was observed in hypoxia-induced HIF1α stabilization, which is also mediated by Siah proteins (24). Two explanations for the discrepancy with the finding that PHD2 sets levels of HIF1α in normoxic conditions (42) can be made. First, PHD isoforms may act dominantly in different cell types under specific conditions, as suggested by Appelhoff et al. (41). Therefore, PHD1 and PHD3 might play a dominant role in the regulation of HIF1α stability mediated by Siah family proteins in nasopharyngeal carcinoma cells under our experimental conditions. Second, LMP1 is a multifunctional protein, regulating several signal transduction pathways and altering biochemical activities in both cytoplasm and nucleus. Therefore, LMP1 may be able to mimic hypoxic conditions in which PHD1 and PHD3 act dominantly to regulate HIFα.

Why Siah proteins specifically target PHD1 and PHD3 but not others is unknown. Protein abundance does not seem to be an important determinant: relatively low levels of PHD3 in the nasopharyngeal cells correlate with a strong induction of HIF1α when PHD3 expression was diminished by the introduction of either LMP1 or siRNA (Fig. 4). Instead, the specific interactions between Siah proteins and PHD1/3, as shown by Nakayama et al. (24), might be important for the specific action of Siah proteins. Subcellular localization of the PHD isoforms could be another determinant. At least under conditions of overexpression, PHD1 is exclusively present in the nucleus, PHD2 is mainly located in the cytoplasm, and PHD3 was homogeneously distributed in cytoplasm and nucleus (40). According to the present study, the levels of Siah1 in the presence of LMP1 increase in both the nucleus and cytoplasm (Fig. 3*D*). More detailed studies are required to correlate the abundance of Siah proteins in the nucleus and PHD1/3 degradation.

Recently, we showed that in B-lymphoma cells, LMP1 inhibits Siah1 ubiquitin ligase expression, which results in up-regulation of β-catenin (25). Our observations in the present study show that in contrast to B-lymphomas, the effect of LMP1 on Siah1 in epithelial cells is quite the reverse: Instead of reducing, LMP1 increases Siah-1 levels. Furthermore, although LMP1-dependent regulation of Siah1 in B cells occurs on the transcriptional level (25), in the epithelial cells LMP1 affects Siah1 protein levels by preventing its proteasomal degradation (Fig. 3*C*). More detailed studies are needed to clarify the distinct effects of LMP1 on the expression of Siah1 in different cell types.

The mechanism by which LMP1 blocks proteasomal degradation of Siah1 remains unknown. Recently, Xu et al. (43) have reported that apoptotic stimuli lead to stabilization of Siah1 protein via a mechanism dependent on c-Jun-NH_2-kinase (JNK) pathway activation and on Siah tyrosine phosphorylation. Therefore, it is possible that activation of the JNK pathway by LMP1 (44) results in stabilization of Siah1. Another route for rescue of ubiquitinated Siah1 from proteasomal degradation by LMP1 is activation of Siah1 deubiquitination. Considering that EBV-induced cell transformation regulates activity of deubiquitinating enzymes (45), it is intriguing to speculate that LMP1 might participate in the stabilization of Siah1 through activation of cellular deubiquitinating enzymes in nasopharyngeal carcinomas.

Another question is how Siah1 escapes self-ubiquitination while maintaining E3 ubiquitin ligase activity. One possibility is that LMP1 may specifically alter a component(s) or a step(s) required for the self-ubiquitination of Siah1 without affecting other activities of Siah1 as an E3 ubiquitin ligase. Activation of the JNK pathway induces Siah1 phosphorylation of tyrosines 100 and 126, which permits its interaction with POSH (plenty of SH3s) to result in Siah1 stabilization (43). Therefore, it is possible that the interaction with POSH may stabilize Siah1 without affecting other E3 ubiquitin ligase activities. However, it remains to be determined whether Siah1 is phosphorylated and thus interacts with POSH in the presence of LMP1. In addition, at this point, whether it is at least possible to stabilize Siah1 without loss of its ubiquitin ligase activity, as shown by treatment with apoptotic stimuli (43), is unknown.

Modification of HIF1α by PHDs permits its binding to VHL, which promotes the ubiquitination and subsequently degradation of HIF1α (16, 17). Therefore, the reduced binding of VHL and HIF1α in the presence of LMP1 is obviously due to the down-regulation of PHDs by Siah1. Moreover, binding between the two proteins was at least in part restored when Siah1-DN was introduced into cells expressing them (data not shown). Based on all these observations, we propose a model for the stabilization of HIF1α by LMP1 through enhancing Siah1 levels, as depicted in Fig. 6. Under normal conditions, HIF1α is easily modified by the action of PHD1, PHD3, or both. The modified HIF1α then recruits VHL and other components of the ubiquitin ligase system to facilitate ubiquitination of HIF1α, which finally leads to proteasomal degradation. Therefore, under normoxic conditions, the expression level of HIF1α is quite low. However, as Siah1 is stabilized by either LMP1 or hypoxia, it induces degradation of both PHD1 and PHD3. As long as the levels of PHD1 and PHD3 are maintained below a certain level, HIF1α is spared from ubiquitin-mediated protein degradation and accumulates in the cell.

Thus, the evidence from these findings is that EBV LMP1 increases the HIF1α level by preventing its proteasomal degradation. For this outcome, LMP1 up-regulates the level of Siah1 E3 ubiquitin ligase, which subsequently induces proteasomal degradation of PHD1 and PHD3 that are involved in the regulation of HIF1α stability. Another gammaherpesvirus, Kaposi's sarcoma–associated herpes virus, also up-regulates HIF1α, which results in increased VEGF expression and secretion (46). Stimulation of HIF1α by the Kaposi's sarcoma–associated herpes virus G protein-coupled receptor involves the phosphorylation of its regulatory/inhibitory domain by the p38 and MAPK signaling pathways, thereby enhancing its transcriptional activity. In addition, hepatitis B virus X protein, which is closely involved in the development of hepatocellular carcinoma, increases the transcriptional activity and protein level of HIF1α (47). Hepatitis B virus X protein directly interacts with the bHLH/PAS domain of HIF1α and decreases the binding of pVHL to HIF1α to prevent its ubiquitin-dependent degradation. Therefore, it seems likely that up-regulation of HIF1α is a common strategy by which viral oncoproteins promote angiogenesis in human tumors although the mechanisms are slightly different in detail.

Although it has been suggested that the Siah1 protein is a potential candidate for key functions in biological processes such as cell cycle, programmed cell death, and oncogenesis (48), there are few revelations of its functional roles. Human cancer-derived cells selected for suppression of their tumorigenic phenotype exhibit constitutively elevated levels of Siah1 mRNA (49). Moreover, this gene is activated during the physiologic program of cell death in the intestinal epithelium (49). Therefore, most studies have focused on Siah1 as a candidate tumor suppressor gene that may be inactivated during tumorigenesis. Interestingly, however, it has been shown recently that activation of the transforming growth

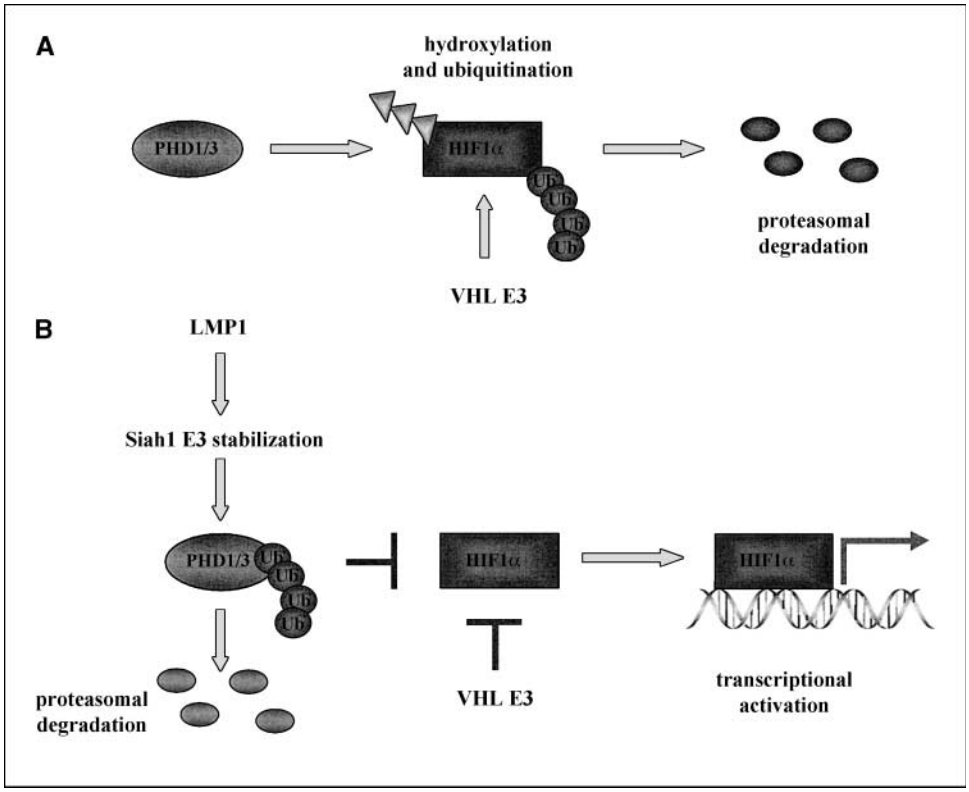

Figure 6. A proposed model for the stabilization of HIF1α by LMP1 through Siah1. *A*, in the absence of LMP1, HIF1α is extremely unstable because PHD1, PHD3, or both effectively hydroxylate it at specific Pro residues. The hydroxylated Pro residues of HIF1α then recruit VHL and other components of the ubiquitin ligase system to facilitate ubiquitination of HIF1α, which finally leads to proteasomal degradation. *B*, the presence of LMP1, however, stabilizes Siah1 through an unknown mechanism. Siah1 can then cause degradation of PHD1 and PHD3. As long as the levels of PHD1 and PHD3 are maintained below a certain level, HIF1α is spared from ubiquitin-mediated degradation and accumulates in the cell. HIF1α can then translocate into the nucleus to activate transcripion of its target genes.

factor β (TGF-β)/Smad pathway is regulated through the ubiquitin-proteasome pathway by the regulation of the stability of the TGF-β–inducible early protein through Siah1 (50). Therefore, the activation of Siah1 by LMP1 may provide a mechanism that cancer cells use to evade cell cycle regulation by the growth-inhibitory effects of TGF-β. Moreover, we have found that LMP1-stabilized Siah1 also degrades one of the tumor suppressor proteins, DCC, which is important in the pathobiology of another carcinoma, colorectal cancer (51). Therefore, the activation of Siah1 by LMP1 may play important roles during EBV-mediated tumorigenesis and tumor progression, at least through elevation of HIF1α at the angiogenesis step. Extensive studies will be needed to fully evaluate this novel effect of LMP1 on Siah1 activation and its contribution and overall significance for EBV pathogenesis.

Acknowledgments

Received 5/9/2006; revised 7/20/2006; accepted 7/26/2006.

Grant support: National Cancer Institute grant P01CA19014 (J. Pagano).

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We thank Drs. Shu-ichi Matsuzawa, John C. Reed, Eric R. Fearon, and Toshiyuki Sakai for their generous gifts of materials; Drs. Erik K. Flemington and Maria Masucci for providing cell lines; and Dr. Julia Shackelford for her insights and suggestions in the course of this work.

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