Characterization of human herpesvirus 6 variant B immediate-early 1 protein modifications by small ubiquitin-related modifiers

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The human herpesvirus 6 (HHV-6) immediate-early (IE) 1 protein undergoes SUMOylation events during the infectious process. In the present work, we report that Lys-802 (K-802) of IE1 from HHV-6 variant B is the only target residue capable of conjugation to SUMO-1/SMT3C/Sentrin-1, SUMO-2/SMT3A/Sentrin-3 or SUMO-3/SMT3B/Sentrin-2 as determined by transfection and in vitro SUMOylation experiments. PolySUMOylated forms of IE1 were also observed, suggesting that SUMO branching occurs at the K-802 residue. Overexpression of SUMO-1, -2 and -3 led to an overall increase in IE1 levels, irrespective of K-802. The SUMO residues could be efficiently removed by incubating SUMOylated IE1 with SENP1, a recently identified SUMO peptidase. SUMOylation-deficient mutants of IE1 co-localized with nuclear promyelocytic leukaemia protein (PML) oncogenic domains (PODs) as efficiently as WT IE1, indicating that POD targeting is independent of IE1 SUMOylation status. However, in contrast to infection, PODs did not aggregate in IE1B-transfected cells, suggesting that other viral proteins are involved in the process. Transactivation studies indicated that IE1, in combination with IE2, could efficiently transactivate diverse promoters, independent of its SUMOylation status. Overall, the results presented provide a detailed biochemical characterization of post-translational modifications of the HHV-6 IE1 protein by SUMO peptides, contributing to our understanding of the complex interactions between herpesviruses and the SUMO-conjugation pathway.

INTRODUCTION

Immediate-early (ie) genes of herpesviruses are the focus of intense research as they play important roles in establishing productive infections, regulating reactivation from latency and creating an appropriate cellular environment for virus replication. Human herpesvirus 6 (HHV-6) is a betaherpesvirus whose genome contains two major IE loci. Major locus A contains the coding sequence for the two major IE proteins, IE1 and IE2. We recently characterized the full-length transcript and IE1 protein of HHV-6 variant B (IE1B) (Gravel et al., 2002). IE1 from HHV-6 variants A and B differ by as much as 38% at the amino acid level. One common feature of both IE1 proteins is the presence of at least one ψKXE motif (where ψ represents a hydrophobic amino acid), a motif that is often predicted for post-translational conjugation by the small ubiquitin-like modifier (SUMO-1) (Desterro et al., 1998; Johnson & Blobel, 1999; Rodriguez et al., 2001; Sternsdorf et al., 1999). The SUMO-1 moiety (11 kDa) is covalently attached to target lysine residues through the successive actions of SUMO hydrolase, SUMO-activating enzyme (E1) (Johnson et al., 1997), SUMO-conjugating enzyme (E2) (Desterro et al., 1997; Johnson & Blobel, 1997) and possibly the participation of a newly identified SUMO ligase (E3) (Sachdev et al., 2001; Takahashi et al., 2001). SUMOylation of herpesvirus IE proteins during the course of infection is also observed for human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) and is thus likely to play an important role in the functions of IE proteins (Adamson & Kenney, 2001; Ahn et al., 2001; Hofmann et al., 2000; Muller & Dejean, 1999; Spengler et al., 2002; Xu et al., 2001). In fact, SUMO-conjugation-defective mutants of HCMV IE2 can affect the ability of IE2 to activate heterologous promoters (Ahn et al., 2001; Hofmann et al., 2000). The only reported function of HHV-6 IE1 is its ability to transactivate heterologous promoters (Flamand et al., 1998; Martin et al., 1991). Our recent work indicates that these proteins differ considerably among HHV-6 variants, with IE1B being much less efficient at promoting transcription than IE1A (Gravel et al., 2002). The reasons for this difference remain elusive. In the present work, we have extended our knowledge by reporting the identification of a single lysine residue (K-802) of IE1B that is a target for SUMO-1, -2 and -3 conjugation and provide a characterization of SUMOylation-deficient mutants of IE1B.
METHODS

Cell culture and virus preparation. The Molt-3 and 293T cell lines were cultured as described previously (Gravel et al., 2002). The Z29 strain of HHV-6 was propagated in Molt-3 cells as described previously (Gravel et al., 2002).

SUMOylation assays in infected cells. Molt-3 cells were infected with the Z29 strain of HHV-6 (m.o.i. of 0-1) for 72 h. Infected cells (1 × 10⁶) were pelleted, lysed and sonicated in a 1:3 dilution of buffer I and II containing 5 mM N-ethylmaleimide (NEM), as described by Desterro et al. (1998) and Wu et al. (1993). Clarified supernatants were incubated overnight with anti-IE1 antibodies (Gravel et al., 2002) and protein A-Sepharose beads, followed by three washes with lysis buffer. Beads were resuspended in Laemmli buffer and boiled for 5 min. Immunoprecipitated proteins were electrophoresed and Western blotting was carried out using anti-IE1 or anti-SUMO-1 antibodies (Zymed Laboratories).

Cloning procedures. A GST–SUMO-1–GG (processed proteins terminating at the diglycine and conjugation-ready) construct was generated by in-frame ligation of the SUMO-1 cDNA from pcDNA3/6 × His-SUMO-1 vector (Desterro et al., 1997) (obtained from R. T. Hay, Centre for Biomolecular Sciences, University of St Andrews, Fife, UK) into the pGEX-2T vector (Amersham Biosciences). The GST–SUMO-2–GG and GST–SUMO-3–GG fusion proteins were generated by in-frame ligation of RT-PCR products amplified from HeLa cell RNA into the pGEX-5X3 vector, using primers as described by Desterro et al. (2001). The same SUMO-2 and SUMO-3 PCR products were ligated in frame with the pCMV2N3T eukaryotic vector (obtained from Didier Trouche, Laboratoire de Biologie Moléculaire Eucaryote, UMR 5099 Centre National de la Recherche Scientifique, Toulouse, France). SENP1 cDNA was obtained following RT-PCR amplification of HeLa cell RNA using the primers 5′-ATGGATGATATTGCTGATAGGATG-3′ and 5′-GGAAATTCATAAGAGATTCTCGTGAGG-3′. The 1.9 kb ampiclon was treated with kinase, digested with EcoRI (underlined in the primer sequence) and cloned in frame in the Smal/EcoRI site of the pCMV2N3T vector. The GST–SENP1 fusion protein was generated by subcloning a blunt-ended SalI/EcoRI fragment from the pCMV2N3T-SENP1 vector into the Smal site of the pGEX-2T vector. A GST–hUBC9 protein was constructed by in-frame subcloning of the hUBC9 cDNA into the pGEX-2T vector. A GST–hUBC9 protein was constructed by in-frame subcloning of the hUBC9 cDNA from pcDNA3/6 × His-SUMO-1 vector (Desterro et al., 1997) (obtained from R. T. Hay, Centre for Biomolecular Sciences, University of St Andrews, Fife, UK) into the pGEX-2T vector (Amersham Biosciences). The GST–hUBC9 fusion protein was constructed by in-frame subcloning of the hUBC9 cDNA into the pGEX-2T vector. The GST, GST–hUBC9 and GST–SENP1 and various GST–SUMO frame subcloning of the hUBC9 cDNA into the pGEX-2T vector. A GST–hUBC9 protein was constructed by in-frame subcloning of the hUBC9 cDNA from pcDNA3/6 × His-SUMO-1 vector (Desterro et al., 1997) (obtained from R. T. Hay, Centre for Biomolecular Sciences, University of St Andrews, Fife, UK) into the pGEX-2T vector (Amersham Biosciences). The GST–SENP1 fusion protein was generated by in-frame ligation of RT-PCR products amplified from HeLa cell RNA into the pGEX-5X3 vector, using primers as described by Desterro et al. (2001). The same SUMO-2 and SUMO-3 PCR products were ligated in frame with the pCMV2N3T eukaryotic vector (obtained from Didier Trouche, Laboratoire de Biologie Moléculaire Eucaryote, UMR 5099 Centre National de la Recherche Scientifique, Toulouse, France). SENP1 cDNA was obtained following RT-PCR amplification of HeLa cell RNA using the primers 5′-ATGGATGATATTGCTGATAGGATG-3′ and 5′-GGAAATTCATAAGAGATTCTCGTGAGG-3′. The 1.9 kb ampiclon was treated with kinase, digested with EcoRI (underlined in the primer sequence) and cloned in frame in the Smal/EcoRI site of the pCMV2N3T vector. The GST–SENP1 fusion protein was generated by subcloning a blunt-ended SalI/EcoRI fragment from the pCMV2N3T-SENP1 vector into the Smal site of the pGEX-2T vector. A GST–hUBC9 protein was constructed by in-frame subcloning of the hUBC9 cDNA into the pGEX-2T vector. The GST, GST–hUBC9 and GST–SENP1 and various GST–SUMO fusion proteins were produced and purified according to standard procedures. Recombinant protein purity was estimated to be > 95% as determined by gel electrophoresis and Coomassie blue staining. Protein concentration was determined using the Bradford reagent (Bio-Rad).

Mutagenesis. Conservative mutations from K to R in the SUMO consensus sites were introduced using the Quick-change Site-directed Mutagenesis kit (Stratagene). Point mutations were confirmed by sequencing.

Transfection of 293T cells with pBK-HHV-6 IE1 expression vectors. 293T cells (3 × 10⁵ cells per well) were seeded the day prior to transfection into a six-well plate. Cells were transfected with 2 μg pBK-HHV-6 IE1B expression vectors or pBK control vector (Stratagene) using the ExGen transfection reagent (MBI Fermentas). In some experiments, His–SUMO-1, HA–SUMO-2 and HA–SUMO-3 expression vectors were included in the transfection mixture. Forty-eight hours after transfection, cells were processed for immunoprecipitation (IP)/Western blot analysis as described previously (Gravel et al., 2002). For co-localization studies, acetone-fixed cells were first reacted with an anti-promyelocytic leukaemia protein (PML) monoclonal antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Slides were washed three times 5 min in PBS and then incubated with Alexa 568-labelled goat anti-mouse IgG antibodies for 1 h at room temperature. After three PBS washes, samples were incubated with Alexa 488-labelled rabbit anti-IE1 IgG for 1 h. Slides were washed, mounted and examined as described previously (Gravel et al., 2002). For SUMO-1 and IE1 co-localization studies, cells were first reacted with Alexa 568-labelled rabbit anti-SUMO-1 IgG antibodies, followed by the Alexa 488-labelled rabbit anti-IE1 IgG antibodies.

In vitro SUMOylation/deSUMOylation assay. WT and K mutants of IE1B were in vitro transcribed/translated in the presence of [35S]methionine using rabbit reticulocyte lysates (Promega). Five μl [35S]methionine-labelled in vitro-translated IE1B proteins were combined with 1 μg GST–SUMO-1, -2 or -3, 1 μg GST–hUBC9 or 1 μg GST in a 20 μl reaction containing 50 mM Tris/HCl pH 7-6, 2 mM ATP, 0-5 mM DTT, 5 mM MgCl₂, 19-4 μg creatine phosphokinase ml⁻¹, 15 mM phosphocreatine and 1 U inorganic pyrophosphatase ml⁻¹ and incubated at 37°C for 2 h. In some instances, the reaction mixtures were supplemented with 1μg GST (control) or GST–SENP1 for an extra hour before the reactions were stopped by the addition of SDS sample buffer containing mercaptoethanol. The proteins were fractionated by electrophoresis on 6% SDS-polyacrylamide gels. The gels were dried and exposed to imaging plates (Fuji Medical Systems).

Reporter gene assay. Molt-3 cells (1 × 10⁵) were electroporated (250 V, 960 μF) with 4 μg HHV-1 LTR–luc reporter construct along with 4 μg of each effector plasmid. DNA levels were kept constant at 12 μg by the addition of control pBK vector. Forty-eight hours post-transfection, cells were pelleted and resuspended in 0-25 ml cell lysis buffer (Promega). Twenty μl aliquots were tested for luciferase activity according to the manufacturer’s technical guidelines (Promega) using an MLX luminometer (Dynex Technologies).

RESULTS AND DISCUSSION

Lys-802 of IE1 from variant B is a target for SUMO-1 conjugation

HHV-6 IE1B undergoes SUMOylation during the infectious process (Gravel et al., 2002; Stanton et al., 2002). An example of IE1 SUMOylation during infection of Molt-3 cells by the Z29 strain of HHV-6 is presented in Fig. 1(A). Three days post-infection, cells were lysed in buffer supplemented with 5 mM of the deSUMOylating isopeptidase inhibitor NEM. Sonicated and clarified supernatants were immunoprecipitated overnight with anti-IE1 rabbit antibodies and protein A–Sepharose beads, as previously reported (Gravel et al., 2002). Immunoprecipitated proteins were analysed by Western blotting using anti-IE1 or anti-SUMO-1 antibodies. Using the anti-IE1 serum, two major proteins of 170 and 195 kDa were detected by Western blotting (Fig. 1A, left panel), while the anti-SUMO-1 antibodies reacted with several high molecular mass forms of IE1 including two dominant proteins of 195 and 210 kDa (Fig. 1A, right panel). These results indicated that the 170 kDa protein represented unmodified IE1, while the 195, 210 kDa and larger proteins corresponded to SUMOylated IE1. In an effort to increase our knowledge of the importance of SUMOylation on the functionality of HHV-6 IE1B, we conservatively mutated the lysine residues (K281 and K802) from the SUMO consensus site (ψKXE) to arginine residues. Wild-type (WT) (pBK-IE1B),
single mutants (pBK-IE1B K281R and pBK-IE1B K802R) and the double lysine mutant (pBK-IE1B DM) were transfected together with a SUMO-1 expression vector into 293T cells and analysed for their ability to undergo SUMOylation events. Forty-eight hours post-transfection, WT and K mutants of IE1B were immunoprecipitated using an anti-IE1 antiserum. WT and IE1B mutants were expressed equally in 293T cells (Fig. 1B, left panel). In both the WT and the K281R IE1B mutant, a higher molecular mass form of IE1 (195 kDa) was detected, while no such protein was present in cells transfected with the K802R or DM mutant. To determine whether this high molecular mass species of IE1B represented SUMOylated IE1, the immunoprecipitates were reacted with anti-SUMO-1 antibodies (Fig. 1B, right panel). SUMO-1-conjugated IE1 could only be detected in cells transfected with the pBK-IE1B and pBK-IE1B K281R mutant vectors. These results indicated that K-802 of IE1 from variant B is a target for SUMO-1 conjugation, while K-281 is not. IE1 from HHV-6 variant A contains one SUMOylation consensus sequence (IKSE; K-665) located in a position analogous to that of the functional distal SUMO site (K-802) of variant B. In previous work, we reported that IE1 from variant A is SUMOylated during the course of an infection (Gravel et al., 2002), suggesting that K-665 is the residue targeted for SUMO-1 conjugation.

In a recent paper, Stanton et al. reported that HHV-6 IE1 is expressed at higher levels in SUMO-1-overexpressing cells, suggesting that an association with SUMO-1 may enhance the stability of IE1 (Stanton et al., 2002). Using the expression vectors encoding WT and SUMOylation-deficient IE1s, we therefore tested the effects of SUMO-1 overexpression on IE1 levels. 293T cells were co-transfected with SUMO-1 and IE1 expression vectors (WT, K281R, K802R or DM mutant) and the levels of IE1 were determined by IP and Western blotting. As observed in Fig. 1(C), expression levels of WT IE1 were enhanced by over-expressing SUMO-1, confirming the results of Stanton et al. (2002). Interestingly, similar results were obtained with the SUMO-1-conjugation-defective mutants of IE1 (K802R and DM), indicating that the greater accumulation of IE1 in SUMO-1-overexpressing cells was not a direct consequence of SUMO-1 conjugation to IE1.

**SUMO-1, SUMO-2 and SUMO-3 are efficiently conjugated to IE1**

Three different SUMOs (1, 2 and 3) have been described so far (Kamitani et al., 1998; Lapenta et al., 1997; Mahajan et al., 1997; Matunis et al., 1996; Saitoh & Hinchey, 2000). SUMO-2/SMT3A/Sentrin-3 and SUMO-3/SMT3B/Sentrin-2 are highly homologous to one another (95% identity) and differ from SUMO-1 by 50%. Previous studies have indicated that SUMO-2 and SUMO-3 can be conjugated to proteins via mechanisms similar to that of SUMO-1 (Saitoh & Hinchey, 2000). To study the conjugation of the SUMO variants to IE1, we co-transfected 293T cells with IE1 and SUMO-1 (His-tagged) or HA–SUMO-2 or HA–SUMO-3 expression vectors and detected the conjugated forms of IE1 by IE1 IP and Western blotting using anti-SUMO-1 or anti-HA antibodies. The first notable observation was that in the absence of SUMO-1 overexpression, a single IE1 protein of 170 kDa was detected following IP and Western blotting (Fig. 2A, lane 2, top panel), suggesting that SUMOylated IE1s are present at much lower levels than unconjugated IE1 or that SUMO peptidases are very active and only partly inhibited by NEM in our lysis buffer. When SUMO-1 was overexpressed, the overall levels of IE1 were increased (Fig. 2A, lane 3, top panel), as also shown in Fig. 1(C) and reported previously (Stanton et al., 2002). In addition, slower-migrating IE1 species, corresponding to SUMO-1-conjugated IE1s were detected (Fig. 2A, lane 3, middle panel), HA–SUMO-2 (Fig. 2A, lane 4) and HA–SUMO-3 (Fig. 2A, lane 5) overexpression also led to increased levels of IE1, in a manner similar to that of SUMO-1. When the immunoprecipitated IE1s were analysed for SUMO-2 or -3 conjugation by reprobing the blot with anti-HA antibodies, one major immunoreactive IE1 protein of approximately 210 kDa (arrowhead) was detected (Fig. 2A, lanes 4 and 5, bottom panel). Higher and lower molecular mass IE1 species containing SUMO-2 and -3 were also detected following film overexposure (not shown). The results presented in Fig. 2(A) indicated that IE1 was efficiently conjugated by SUMO-1, -2 and -3. Two differences of likely biological significance between SUMO-1 and SUMO-2/3 exist. First, compared with SUMO-1, SUMO-2 and -3 are much more abundantly expressed within cells. Secondly, and possibly directly related to the first difference, there are large quantities of free SUMO-2/3 available for conjugation in contrast to the majority of SUMO-1, which is attached to target proteins (Saitoh & Hinchey, 2000). To determine whether IE1 was preferentially SUMOylated by SUMO-1, -2 or -3, we overexpressed SUMO-1 and SUMO-2 or SUMO-1 and SUMO-3 together with IE1 in 293T cells and analysed the SUMOylation patterns of IE1. Overexpression of SUMO-1 (Fig. 2B, lanes 3–5) led to the detection of a major protein of approximately 195 kDa, identified as a SUMO-1-conjugated IE1 (indicated by an asterisk), as well as several minor larger IE1–SUMO-1 proteins (Fig. 2B, middle panel). When SUMO-2 and SUMO-1 were both overexpressed (Fig. 2B, lane 4), three major IE1 proteins of 170, 195 and 210 kDa were detected (top panel). These respectively corresponded to unconjugated, SUMO-1-conjugated (Fig. 2B, middle panel) and SUMO-2-conjugated IE1 (Fig. 2B, bottom panel). The 195 kDa band observed in Fig. 2(B) (lane 4, bottom panel) corresponds to IE1 linked to a single SUMO-2 residue (asterisk). The 195 kDa band is therefore a mixture of SUMO-1 and SUMO-2 conjugated IE1. Although we could not firmly conclude that both SUMO-1 and SUMO-2 were simultaneously conjugated to IE1, co-migration of a 210 kDa SUMO-1-conjugated (Fig. 2B, middle panel) and SUMO-2-conjugated (Fig. 2B, bottom panel) IE1 (arrowhead) suggested that this was the case. This was not unexpected considering that SUMO-2 contains an internal
SUMOylation site allowing its conjugation to SUMO proteins (Tatham et al., 2001). Simultaneous overexpression of both SUMO-1 and -3 (Fig. 2B, lane 5) had little effect on the SUMOylation patterns of IE1 with detection of multiple SUMO-1-conjugated IE1s (Fig. 2B, middle panel) and a major 210 kDa SUMO-conjugated IE1 (Fig. 2B, bottom panel, arrowhead), most likely linked to a SUMO-3/SUMO-1 dimer. Whether polySUMOylated proteins behave differently
from monoSUMOylated ones requires a clearer understanding of the biological differences between SUMO-1 and SUMO-2/3. To confirm and compare the SUMOylation of IE1 by SUMOs, we generated matured GST–SUMO-1, -2 and -3 fusion proteins, as described previously (Tatham et al., 2001). A GST–hUBC9 recombinant protein was used as a source of SUMO-conjugating enzyme. WT and K mutants (K281R, K802R and DM) of IE1B were in vitro transcribed/translated and incubated with 1 μg GST–SUMO-1, -2 or -3, 1 μg GST–hUBC9 or 1 μg GST in a 20 μl reaction volume. In the presence of GST or GST plus GST–hUBC9, only the unmodified form of IE1 was detected (Fig. 2C, lanes 1 and 2). Transcription/translation of an empty pBK control vector under similar conditions yielded background noise only (data not shown). The K mutants of IE1 were as efficiently transcribed/translated as WT IE1 with a single major band of 170 kDa detected. When GST–SUMO-1 was added to the reaction mixture (Fig. 2C, lane 3), SUMOylation of WT IE1 and the K281R mutant was observed, while the K802R and DM mutants failed to undergo SUMO-1 conjugation. These results were in accordance with those presented in Fig. 1(B) indicating that K-802 represents the target residue for SUMO-1 conjugation in transfection experiments. Similar experiments were carried out using GST–SUMO-2 and -3 proteins (Fig. 2C, lanes 4 and 5). The results obtained indicated that WT IE1 and the K281R mutant could be efficiently modified by both SUMO-2 and SUMO-3. The K802R and DM mutants failed to undergo SUMOylation in the presence of SUMO-2 or SUMO-3 suggesting that K-802 is the only residue capable of conjugating all forms of SUMO. Interestingly, both SUMO-2 and SUMO-3 have a ψKXE motif within their primary amino acid sequence, raising the possibility that multimers of SUMO can form. In fact, a recent paper has indicated that this is indeed the case (Tatham et al., 2001). Considering that only K-802 of IE1 is a target for SUMOylation, we presumed that the high molecular mass IE1 proteins observed (> 195 kDa) in Fig. 1(A) represented IE1 proteins carrying multimers of SUMO-1, -2 and/or -3 on K-802. Post-translational protein modifications by SUMO residues are often technically difficult to study. This is partly attributable to deSUMOylating peptidase activity in cellular extracts. One recently characterized deSUMOylating enzyme is SENP1 (Gong et al., 2000). We cloned and purified a GST–SENP1 protein to near homogeneity and its deSUMOylating activity was tested using the RanGAP1 protein. RanGAP1 was the first SUMO-modified protein to be reported (Matunis et al., 1996) and is one of the easiest to study. In vitro-transcribed/translated RanGAP1 undergoes spontaneous SUMOylation in the presence of rabbit reticulocyte lysate. In vitro-generated RanGAP1 protein was incubated with purified GST or GST–SENP1 for 1 h at 37 °C, then electrophoresed and exposed to imaging plates. The results obtained (Fig. 2D, upper panel) confirmed that GST–SENP1 is capable of removing SUMO residues from RanGAP1 as revealed by the disappearance of the slower-migrating RanGAP1 species (Gong et al., 2000). We next proceeded to determine whether SENP1 could remove SUMO-1, -2 and/or -3 from IE1. In vitro SUMO-conjugated IE1 was incubated with GST or GST–SENP1 and analysed by gel electrophoresis. Our results (Fig. 2D, lower panel) indicated that SUMO-1-conjugated IE1 (Fig. 2D, lane 3) could be efficiently deconjugated by SENP1 (Fig. 2D, lane 4). These results also indicated that SENP1 could cleave IE1–SUMO-2 (Fig. 2D, lanes 5 and 6) and IE1–SUMO-3 (Fig. 2D, lanes 7 and 8) residues, highlighting a potential role for SENP1 in controlling the levels of SUMO-modified proteins within cells. So far, three mammalian (SMT3IP1, SENP1 and SUSP1) and one yeast (Ulp1) SUMO peptidases have been identified (Gong et al., 2000; Kim et al., 2000; Li & Hochstrasser, 1999; Nishida et al., 2000; Suzuki et al., 1999). Although these enzymes share some homologies within the C-terminal region, their distribution within cells differs considerably. For example, SENP1 has a diffuse nuclear distribution (Gong et al., 2000) and SUSP1 is exclusively cytoplasmic (Kim et al., 2000), while SMT3IP1 associates with the nucleoli (Nishida et al., 2000). Although sharing a common property in cleaving SUMO residues from conjugated proteins, the differing cellular localization of the SUMO peptidases suggests that they act on different SUMOylated target proteins. How SUMO peptidases can modulate the course of a virus infection remains unclear, but considering that the functionality of some viral proteins, such as the HCMV IE86 (Ahn et al., 2001; Hofmann et al., 2000), are influenced by their SUMOylation status, these enzymes are expected to play an integral part in the infectious process.

**SUMO-1 modification is not required for POD targeting by IE1**

During infection, IE1 is expressed as a nuclear protein that co-localizes with the nuclear PML oncogenic domains (PODs) and SUMO (Gravel et al., 2002; Stanton et al.,...
To determine whether the IE1 SUMOylation-deficient mutants had a similar nuclear distribution to that of WT IE1B, 293T cells were transfected with vectors encoding WT or K mutants of IE1 and processed for dual colour immunofluorescence 48 h later. Cells were labelled with anti-IE1 (green) and anti-PML (red) or with anti-IE1 and anti-SUMO-1 (red) antibodies as described previously (Gravel et al., 2002). Most of the WT IE1B was expressed as intranuclear granules (5–10 per nucleus) (Fig. 3A). The majority of IE1B co-localized with PML antigens found in nuclear bodies, as well as with the SUMO-1 protein. Given the fact that both IE1 and PML are SUMOylated proteins, their co-localization with SUMO-1 was expected. However, in contrast to HHV-6-infected T cells, transfection of 293T cells with IE1 did not lead to PML body condensation into one to three entities per cell. However, due to our inability to detect IE1 in transfected T cells, we could not firmly conclude that this was actually the case in T cells. The K mutants of IE1B behaved as WT IE1B with regard to their punctate nuclear distribution and co-localization with PML (Fig. 3A). All mutants, even the SUMO-conjugation defective K802R and DM mutants, co-localized with SUMO-1, as a result of their close association with PML. Although expression of WT and K mutants of IE1B did not affect PML body morphology, as is observed during infection (Gravel et al., 2002), changes in SUMO-1 distribution were noticeable. In control cells (pBK vector), the SUMO-1 antigen was barely detectable (Fig. 3A). Expression of WT and K mutants of IE1B led to easily detectable nuclear SUMO-1 body formation, suggesting that IE1 caused SUMO-1 to aggregate in sufficient quantities to allow its detection. SUMO conjugation-defective mutants of IE1B caused the same phenotypic changes as WT IE1B, suggesting that IE1B expression, irrespective of its SUMOylation status,
SUMOylation of HHV-6 IE1B protein

is associated with nuclear redistribution of SUMOylated cellular proteins.

Transfection experiments indicated that IE1B is unable to cause PML body aggregation as it is observed during the course of an infection, suggesting that other viral proteins are essential for this event to occur. To determine the nature of HHV-6 proteins associated with POD coalescence, we carried out an infection in the presence or absence of phosphonoacetic acid (PAA; 100 μg ml⁻¹), a viral DNA polymerase inhibitor restricting virus expression to IE and early genes only. As observed in Fig. 3(B), mock-infected cells did not express IE1 and had a normal PML distribution with minimal SUMO-1 reactivity. Three days post-infection, cells express IE1 as a few large intranuclear granules that co-localized with enlarged POD and SUMO aggregates. In the presence of PAA, HHV-6 retained the ability to cause POD and SUMO-1 aggregation (Fig. 3B), indicating that IE and early gene expression was sufficient for these events to occur.

IE1 and IE2 transcriptional modulating activities

The function of IE1 from HHV-6 variant B remains largely elusive, with the ability to weakly transactivate heterologous promoters the only known activity of IE1 (Gravel et al., 1995).
Fig. 3. POD targeting by SUMOylation-deficient IE1s. (A) 293T cells were transfected with a control (pBK) or expression vectors encoding WT IE1 or K mutants of IE1. Two days after transfection, cells were fixed in cold acetone and reacted with an anti-PML antibody followed by incubation with Alexa 568-conjugated secondary antibodies (red) or Alexa 568-labelled rabbit anti-SUMO-1 antibodies (red). Cells were then incubated with Alexa 488-labelled anti-IE1 (green) antibodies. Co-localized proteins are represented in the merged pictures by a yellow colour. (B) Molt-3 cells were mock infected or HHV-6 infected in the presence or absence of 100 μg PAA ml⁻¹ for 3 days. Cells were processed for immunofluorescence as described above.
2002). We recently cloned a full-length transcript encoding IE2, the second major protein from the IE-A locus of HHV-6 (Gravel et al., 2003). In contrast to IE1, IE2 is a strong promiscuous transcriptional activator. Given the fact that both genes are expressed with similar kinetics (Gravel et al., 2003), we tested the effects of IE1 on the ability of IE2 to activate several promoters, including the HIV LTR, as well as more simple promoters containing NF-κB-binding consensus sequences, nuclear factor of activated T cells or cyclic-AMP-responsive elements. Similar results were obtained using all these promoters; Fig. 4 shows the results using the HIV LTR promoter. Molt-3 T cells transfected with an IE1B expression vector promoted marginal activation (<twofold) of the HIV LTR, as previously reported (Gravel et al., 2002). In contrast, transfection with an expression vector for HHV-6 IE2 led to an 18-fold activation in promoter activity. By combining IE1 and IE2 expression vectors, the activity increased up to almost 30-fold, suggesting that both proteins may act additively in promoting transcription. We next tested the impact of IE1 SUMOylation-deficient mutants of IE1 on transactivation capability. First, the mutants were individually tested and shown to have poor transactivation potential, similar to that of WT IE1B. Next, in combination with IE2, all IE1 mutants demonstrated levels of activation almost identical to that of WT IE1. These results indicated that IE1, together with IE2, can efficiently transactivate heterologous promoters and that this effect is independent of the SUMOylation status of IE1.

Post-translational SUMOylation of proteins is now recognized as an important modification system that can influence the function of a given protein. For herpesviruses, the importance of SUMOylation on IE protein functions is not clear. Here, we have provided evidence that the weak transactivating potential of IE1 from variant B is independent of its SUMOylation status. A recent report has also indicated that a SUMOylation-deficient IE72 from HCMV, a positional homologue to HHV-6 IE1, behaves as WT in promoter transactivation assays (Spengler et al., 2002). IE proteins of HCMV, HHV-6 and EBV target PODs shortly after infection. The PML protein represents the major POD structural constituent and depends on SUMO-1 for POD targeting (Muller et al., 1998). It was thus conceivable to hypothesize that SUMO modification of herpesvirus IE proteins might play a role in directing IE proteins to PODs. However, it is now apparent that IE proteins from HCMV and HHV-6 target PODs, irrespective of their SUMOylation status. Unlike HCMV, HHV-6 variant B infection does not lead to POD dispersal but rather to POD fusion, with PML-containing PODs becoming enlarged and their number reduced to one to three per cell. At present, the functional consequences of SUMOylation of the HHV-6 IE1 protein remain undetermined and further work is needed to understand the role of SUMO in the herpesvirus infectious process. Considering that most herpesviruses studied so far are known to interact, at least in some way, with the SUMOylation process, it is conceivable that this biochemical post-translational pathway is of importance for the efficient propagation of these viruses.

**ACKNOWLEDGEMENTS**

A Canadian Institutes of Health Research (CIHR) grant (MOP-14437) to L.F. supported this work. We thank R. T. Hay and Didier Trouche for providing plasmids. Jean Gosselin is a senior scholar from the FRQS and Louis Flamand is the recipient of a CIHR New Investigator Award.

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