Human Herpesvirus 6 Immediate-Early 1 Protein Is a Sumoylated Nuclear Phosphoprotein Colocalizing with Promyelocytic Leukemia Protein-associated Nuclear Bodies*

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Immediate-early (IE) proteins are the first proteins expressed following viral entry and play a crucial role in the initiation of infection. We report the cloning and characterization of a full-length IE1 transcript and protein (IE1B) from human herpesvirus 6 (HHV-6) variant B. The IE1B transcript consists of five exons (3720 nucleotides), three of which are coding for the IE1 protein. The 1078-amino acid-long IE1B protein is 62% identical and 75% similar to the 941-amino acid IE1 from HHV-6 variant A. IE1B protein can be detected at 4 h post-infection (P.I.), and it is distributed as small intranuclear structures. The maximal number of IE1 bodies (~10–12/nucleus) is detected at 12 h P.I. after which the IE1 bodies condense into 1–3 larger entities by 24–48 h P.I. During infection the IE1B protein is phosphorylated on serine and threonine residues. IE1B undergoes further post-translational modification with its conjugation to the small ubiquitin-like modifier (SUMO-1) peptide. IE1B colocalizes with SUMO-1 and promyelocytic leukemia nuclear bodies during infection as well as in transfection experiments. Finally, IE1 from variant B is a weaker transactivator than IE1 from variant A, when assayed using heterologous promoters. Overall, the characterization of the HHV-6 IE1B protein presented highlights the similarity and divergence between IE1 from both variants and provides useful information pertaining to the early phase of infection.

Human herpesvirus 6 (HHV-6) is a betaherpesvirus initially isolated in 1986 from individuals afflicted with lymphoproliferative disorders (1). HHV-6 isolations were subsequently made from immunosuppressed (AIDS) patients from Uganda (2) and Zaire (3). Molecular, biological, and immunological analyses between various isolates led to the sub-division into HHV-6 variant A (U1102 and GS strains) and variant B (Z29 and HST strains) (4). HHV-6 primary infection, occurring within 24 months from birth, is characterized by acute febrile illnesses with intense fever and occasional seizures (5). HHV-6 is the causative agent of the sixth childhood disease exanthem subitum (6). In industrialized countries, primary HHV-6 infections are almost always caused by the B variant (7) although primary infections with variant A have been reported in Africa (8). Other complications of primary HHV-6 infection include hepatitis (9), meningitis (10), fatal hemophagocytic syndrome (11), and fatal disseminated infection (12). More recently, a potential association between HHV-6 infection and the neurological disease multiple sclerosis has been made. Although several papers have reported that HHV-6 could be involved in such pathology (13–15), others have found no such association (16, 17). One undisputed fact, however, is the presence of HHV-6-infected cells within the brain suggesting that HHV-6 is neurotropic and therefore can possibly play a role in neurological diseases. More work is therefore needed to determine the potential link between HHV-6 and multiple sclerosis.

A recent report (18) indicates that HHV-6 infects cells through the widely distributed cell surface CD46 receptor. A second receptor is likely to be necessary for infection given the fact that HHV-6 variants A and B infect different cell lines in vitro and that CD46 appears necessary but not sufficient for infection (18). Infection of susceptible host cells by human herpesviruses, such as herpes simplex virus (HSV) or human cytomegalovirus (HCMV), is often associated with cellular alterations allowing for a successful infection to be initiated. Such cellular modifications include, among others, the shut-off of cellular protein synthesis and destruction of punctate nuclear bodies known as ND10 (nucleolus dot domain) or PODs (promyelocytic leukemia protein (PML) oncogenic domain) (19–23). The first genes expressed following viral entry and associated/responsible for such cellular disturbance are the immediate-early (IE) genes. Transcription of IE genes occurs within minutes to hours post-entry, is independent of de novo protein synthesis, and often relies on virion-associated proteins such as the tegument proteins for proper expression (24). HHV-6 genome contains several putative IE genes encoding proteins having a US22 amino acid sequence motif. Two major loci of IE genes have been identified as follows: locus B encompasses open reading frames 16–19, whereas locus A contains two large open reading frames, U89 and U86 (25–30). Several years ago, the mapping of HHV-6 variant A IE1 transcripts was...
reported (29). By using a probe spanning from the middle of U89 up to the KpnI repeats, the authors have identified four transcripts of 1.0, 1.5, 3.5, and 4.7 kb. However, only the 3.5-kb transcript was expressed under IE conditions, in absence of de novo protein synthesis. This transcript is composed of five (four small and one large (U89)) exons with translation initiating in the middle of the third exon, potentially encoding a 104-kDa protein (29). By using genomic DNA clones, Martin et al. (27) have shown that U89 of HHV-6 variant A was capable of transactivating heterologous promoters suggesting a putative role for IE1 in transcriptional regulation. Finally, using sera of mice immunized with β-galactosidase-IE1 fusion proteins, Takeda et al. (31) have shown that HHV-6 variant B IE1 protein can be detected by 4 h post-infection and that two forms (155 and 170 kDa) of the proteins can be detected in lysates of infected cells.

In the present work we report the cloning, expression, and characterization of HHV-6 variant B (Z29 strain) IE1 gene and protein in infected cells. Kinetics of IE1 mRNA, proteins, and nuclear distribution are reported. Post-translational modifications of HHV-6 IE1 protein are presented. Finally, a comparative functional analysis of IE1 from variants A and B is presented.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Virus Preparation**—The Molt-3 cell line, obtained from the American Type Culture Collection (ATCC), was passaged twice a week and cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Sigma) and M-Phasmonic (InvivoGen, San Diego, CA) (complete medium) to prevent mycoplasma contamination. The 293T cell line was cultured in complete Dulbecco's modified Eagle's medium (Sigma) and passaged twice a week. The Z29 strain of HHV-6 was propagated on Molt-3 cells. Briefly, 5 × 10⁶ uninfected Molt-3 cells were mixed with 5 × 10⁶ HHV-6-infected Molt-3 cells and seeded at a density of 5 × 10⁵ cells/ml in complete RPMI 1640 medium. By day 5–6, when cytolytic effects were evident in most of the cells (ballooning), the cultures were centrifuged at 2,300 × g for 20 min at 4 °C. The supernatant was harvested and kept at 4 °C, and the cell pellet was frozen-thawed three times. The pellet lysates were centrifuged at 2,300 × g for 15 min at 4 °C. The supernatants were harvested and combined with the supernatant obtained following the initial centrifugation. HHV-6 was concentrated by ultracentrifugation at 38,800 × g for 160 min. Virions were resuspended in a minimal volume of complete RPMI 1640 medium, aliquoted, and stored at −150 °C. HHV-6 infectivity titer was determined on Molt-3 cells, 24 h after infection with varying dilutions of HHV-6. Infected cells were fixed in cold acetone for 10 min, air-dried, and reacted with Alexa 488-labeled anti-IE1 antiserum (Amersham Biosciences). After three 5-min washes in PBS, slides were mounted with glycerol and observed under fluorescent microscopy. After calculating the percentage of IE1-positive cells, the HHV-6 titer was determined to be 6 × 10⁶ infectious particles/ml. HHV-6 GS strain was propagated and concentrated as described previously (32).

**RNA Isolation, cDNA Library, and Sequencing**—To obtain RNA species from the majority of HHV-6 (229) genes, RNA was isolated from Molt-3 cells infected under the following conditions: under IE conditions (7-h infection in the presence of 10 μg/ml cycloheximide (CHX), under early conditions (2-day infection in the presence of 100 μg/ml of phosphonoacetic acid), and under late conditions (3-day infection in absence of inhibitors). Total RNA was obtained using the Trizol reagent (Invitrogen). One hundred μg/ml of [³²P]methylene/ [¹⁴C]methionine (PerkinElmer Life Sciences) was added to the medium for 5 h, followed by a 5 min washes in PBS, slides were mounted with glycerol observed under fluorescent microscopy. After calculating the percentage of IE1-positive cells, the HHV-6 titer was determined to be 6 × 10⁶ infectious particles/ml. HHV-6 GS strain was propagated and concentrated as described previously (32).

**Cloning of a Recombinant GST-IE1 Fusion Protein and Anti-IE1 Antibody Generation**—A 1.3-kb BglII-StuI fragment derived from HHV-6 type A U89 was subcloned in-frame with the glutathione S-transferase (GST) of the pGEX-6X-2 prokaryotic expression vector (Amersham Biosciences). The recombinant 75-kDa fusion protein was purified using a glutathione-Sepharose 4B column (Amersham Biosciences) and eluted with glutathione elution buffer (20 mM reduced glutathione in 50 mM Tris-HCl, pH 12). After neutralization of the protein sample, the purified protein (500 μg) was emulsified in complete Freund's adjuvant and subcutaneously injected into New Zealand rabbits. After the third immunization, rabbits were bled by cardiac puncture. Immunoglobulins were purified using a HiTrap Protein G-Sepharose HP affinity column (Amersham Biosciences). For fluorescence analysis, anti-IE1 IgG were labeled with the Alexa-Fluor 488 protein labeling kit (Molecular Probes, Eugene, OR).

**Expression of IE1 mRNA Expression in HHV-6-infected Molt-3 Cells**—Molt-3 cells were infected with HHV-6 (m.o.i. of 0.02) for times ranging from 2 to 96 h. At the indicated times, total RNA was isolated with the Trizol reagent. To determine whether IE1 gene was expressed under IE conditions, cells were first incubated with 10 μg/ml cycloheximide for 30 min prior to and during infection (8 h). Ten micrograms of total RNA was separated through electrophoresis in 1% agarose-formaldehyde gel and transferred onto nylon membranes. Membranes were prehybridized in 5× SSPE, 1% SDS, 50% formamide, 10% dextran sulfate, 1× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA for 3 h and hybridized overnight at 42 °C in the same buffer without salmon sperm DNA but supplemented with 1 × 10⁶ cpm/ml of [³²P]-labeled probe. Membranes were washed once in 2× SSPE for 15 min at 42 °C, twice with 2× SSPE, 2% SDS for an additional 15 min at 65 °C, and once with 0.1× SSPE at room temperature for 15 min. Membranes were exposed to imaging plates for several hours and analyzed using a PhosphorImager system (Fuji Medical Systems, Stamford, CT).

**In Vitro Transcription/Translation of HHV-6 IE1 Protein**—The full-length IE1 cDNA was cloned into the pBK vector (Stratagene) under the control of the strong CMV promoter. The vector also contains a T3 promoter allowing the cDNA to be transcribed and subsequently translated in vitro using the TNT T3-coupled reticulocyte lysate system (Promega). IE1 protein was radio-labeled by incorporating 20 μCi of [³²P]methionine in the reaction mixture (Amersham Biosciences). After 90 min at 30 °C, the samples were mixed with Laemmli buffer, boiled, and electrophoresed through a 6% SDS-PAGE. After migration, the gels were dried and exposed to imaging plates for several hours and analyzed using a PhosphorImager system (Fuji).

**Transfection of 293T Cells with pBK-HHV-6 IE1 Expression Vector**—293T cells were plated (3 × 10⁵ cells/well) the day before transfection in a 6-well plate. Cells were transfected with 2 μg of pBK-HHV-6 IE1B expression vector or pBK control vector (Stratagene) using the ExGen transfection reagent (MBI Fermentas, Flamborough, Ontario, Canada). Transfected cells were post-transfected for 1–2 days. Cells were processed for immunofluorescence or immunoprecipitation-Western blot analysis as described above. In some experiments, metabolic labeling of IE1 protein was performed. Briefly, 48 h post-transfection, cells were rinsed twice with PBS and incubated for 30 min in methionine-cysteine-free complete Dulbecco's modified Eagle's medium containing 10% dialyzed fetal bovine serum (Invitrogen). One hundred μCi/ml of [³⁵S]methionine/ [¹⁴C]methionine (PerkinElmer Life Sciences) was added to the medium for 5 h, after which the cells were washed 3 times in PBS, centrifuged, and lysed in 1 ml of lysis buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% glycerol and 1% Triton) for 30 min at 4 °C. Insoluble material was removed by centrifugation, and the IE1 protein in the supernatant was immunoprecipitated with 34 μg of rabbit anti-IE1 IgG and protein A-Sepharose. After separating the immunoprecipitated proteins through a 6% SDS-PAGE, the gels were dried and exposed to imaging plates for several hours and analyzed using a PhosphorImager system (Fuji).

**Kinetics of IE1 Protein Expression in HHV-6-infected Molt-3 Cells**—Molt-3 cells were infected with HHV-6 (m.o.i. of 0.02) for times ranging from 2 to 96 h. At selected times, cells were either prepared for immunofluorescence (IFA) analysis or immunoprecipitation, followed by Western blot analysis. For IFA, cells were washed once in PBS and deposited onto 10-well microscope glass slides (ICN Pharmaceuticals, Costa Mesa, CA). When dry, slides were immersed in cold acetone (−20 °C) for 10 min. Infected and uninfected cells were stained with HHV-6 Alexa 488-labeled rabbit anti-IE1 IgG and protein A-Sepharose. After separating the immunoprecipitated proteins through a 6% SDS-PAGE, the gels were dried and exposed to imaging plates for several hours and analyzed using a PhosphorImager system (Fuji).
Supernatants were collected and supplemented with 34 μg of anti-IE1 rabbit antisera and protein A-Sepharose. After an overnight incubation at 4 °C on a rotary device, beads were washed 3 times with lysis buffer and resuspended in Laemmli buffer. Samples were boiled for 5 min and electrophoresed through a 6% polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride membranes and blocked for HHV-6 IE1 protein using a rabbit anti-IE1 antisera. After 1 h the blots were washed with TBST and incubated with peroxidase-labeled goat anti-rabbit IgG antibodies followed by chemiluminescent detection (PerkinElmer Life Sciences).

Post-translational Phosphorylation of HHV-6 IE1 Protein—Phosphorylation of HHV-6 IE1 protein was determined in 3-day-old infected Molt-3 cells. In the first series of experiments, cells were metabolically labeled for 5 h with inorganic [32P]orthophosphate. Radiolabeled infected or uninfected cells were washed in PBS and lysed for 30 min at 4 °C in a phosphate lysis buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, and 1% Triton) supplemented with 1 mM sodium orthovanadate and 25 mM calcine as phosphatase inhibitors and a mixture of protease inhibitors (Roche Diagnostics, Lavai, Quebec, Canada). Samples were centrifuged at 18,000 × g for 10 min at 4 °C to remove insoluble materials. Supernatants were subjected to IE1 immunoprecipitation using an anti-IE1 antiserum and protein A-Sepharose beads. After an overnight incubation, beads were washed 3 times in lysis buffer, resuspended in Laemmli buffer, boiled for 5 min, and subjected to electrophoresis through a 6% polyacrylamide gel. After migration, the gels were dried and exposed to imaging plates. In a second series of experiments, uninfected and HHV-6-infected cells were lysed in phosphate lysis buffer, immunoprecipitated with anti-IE1 antisera and protein A-Sepharose, and processed for Western blot using either anti-IE1, anti-phosphoserine (1 μg/ml) (Chemicon, Temecula, CA), anti-phosphothreonine (0.5 μg/ml) (Zymed Laboratories Inc., San Francisco, CA), or anti-phosphorysine (2 μg/ml) (Upstate Biotechnology, Inc., Lake Placid, NY) antibodies. After incubation with specific antibodies, blots were washed and reacted with peroxidase-labeled goat anti-rabbit IgG or anti-mouse IgG antibodies. Reactive proteins were detected by chemiluminescence.

Colocalization Studies—Uninfected and HHV-6-infected Molt-3 cells were processed for IFA as described above. Fixed cells were first reacted with an anti-PML monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Slides were washed three times for 5 min in PBS and then incubated with Alexa 568-labeled goat anti-mouse IgG antibodies for 1 h at room temperature. After three PBS washes, samples were incubated with Alexa 488-labeled rabbit anti-IE1 IgG. After a 1-h incubation, slides were washed, mounted, and examined as described above. For SUMO-1 and IE1 colocalization studies, first cells were reacted with Alexa 568-labeled rabbit anti-SUMO-1 IgG antibodies followed by the Alexa 488-labeled rabbit anti-IE1 IgG preparation.

In Vivo Sumoylation of HHV-6 IE1 Proteins—Ten million Molt-3 and HS6-2 cells were infected with the Z29 or GS strain of HHV-6 (m.o.i. of 0.1), respectively, for 72 h. Infected cells were pelleted, lysed, and sonicated in a 1:3 dilution of buffer I and II containing 5 mM N-ethylmaleimide, as described previously (35, 36). Clarified supernatants were incubated overnight with anti-IE1 antibodies plus protein A-Sepharose, followed by three washes with lysis buffer. Beads were resuspended in Laemmli buffer and boiled 5 min. Immunoprecipitated proteins were electrophoresed and processed for Western blot using anti-IE1 or anti-SUMO-1 monoclonal antibody (Zymed Laboratories Inc.).

Reporter Gene Assay—Ten million Molt-3 cells were electroporated (250 V, 960 microfarads) with 4 μg of HIV-1 LTR-luc reporter construct along with 4, 8, or 12 μg of pBK-HHV-6 IE1B or pR56 vector, which contains the entire IE1A coding region (27). Forty eight hours post-transfection, cells were pelleted and resuspended in 0.2 ml of Cell Lysis Buffer (Promega). Twenty-μl aliquots were tested for luciferase activity according to the manufacturer’s technical guideline (Promega) using an MLX lumimeter (Dynex Technologies, Chantilly, VA).

Nucleotide Sequence Accession Number—The sequence reported has been deposited with GenBank™ under accession number AF0397932.

RESULTS
Characterization of HHV-6 Variant B (Z29 Strain) IE1 Transcript and Comparison of IE1 Protein from Both Variants—Following the screening of a cDNA library derived from HHV-6 (Z29)-infected Molt-3 cells, we successfully isolated a transcript encoding a full-length IE1 protein (IE1B). The transcript is 3720 nucleotides long, of which 3237 nucleotides are coding for the encoded IE1 protein is 1078 amino acids long, most of which (975) are derived from the fifth exon, and has a calculated molecular mass of 120 kDa. Overall, the IE1 protein is acidic, with an isoelectric point of 5.1. Kyte and Doolittle analysis suggests that the IE1 protein is mostly hydrophilic throughout its entire length (not shown). Computer analyses of the primary amino acid sequence of IE1 have identified several putative casein kinase II and protein kinase C serine-threonine phosphorylation sites, one tyrosine kinase phosphorylation site, one cAMP-, cGMP-dependent protein kinase phosphorylation site, and two putative SUMO consensus conjugation sites (sKXE), where ψ represents a hydrophobic amino acid (Fig. 1B, top drawing). Line diagrams of IE1 protein from variant B (Z29 strain) and variant A (U1102 strain) are presented in Fig. 1B. The IE1 protein from Z29 is larger (1078 aa)
hybridized with 32P-labeled probe to detect IE1 transcripts. Blots were stripped and hybridized with an 18 S ribosomal RNA oligonucleotide probe to monitor the amount of RNA loaded. IE1 and 18 S hybridization signals were quantified, normalized, and compared with the 2-h time point, arbitrarily set at 1. Arrows represent the size (kb) of the various IE1 transcripts detected. B, Molt-3 cells were pretreated with CHX (10 μg/ml) for 30 min before and during infection with HHV-6 (m.o.i. 0.02). At 8 h P.I., total RNA was extracted and probed for IE1 mRNA and 18 S ribosomal subunit.

Characterization of HHV-6 IE1 Protein

We first characterized the IE1 protein using an expression vector encoding the full-length variant B IE1 (pBK-IE1B) by in vitro transcription/translation experiments. As shown in Fig. 3A, a protein with an estimated size of 150 kDa was synthesized using the pBK-IE1B, whereas no such protein was generated with the empty pBK vector. To detect the HHV-6 IE1B protein, we generated antibodies following immunization of rabbits with purified recombinant GST-IE1 fusion protein. The IE1 regions fused to the GST correspond to amino acids 480–920 of the mature protein of variant A. To validate our anti-IE1 antisera, we first tested it on 293T cells transfected with the pBK-IE1B expression vector. Immunofluorescence analysis indicates that the anti-IE1 antibodies detect several discrete nuclear substructures in pBK-IE1B-transfected cells (Fig. 3B). The anti-IE1 antisera did not show any reactivity against cells transfected with the pBK control vector. We next performed an immunoprecipitation on pBK or pBK-IE1B-transfected 293T cells that were metabolically labeled with radioactive amino acids. The anti-IE1 antisera immunoprecipitated a 150-kDa radioactive protein from IE1B-transfected cells but not from control transfected cells (Fig. 3C). Finally, using a combination of immunoprecipitation and Western blot analysis on pBK and pBK-IE1B transfected cells, we could detect a reactive protein with an approximate molecular mass of 150 kDa (Fig. 3D), in accordance with results obtained in Fig. 3, A and C. No such bands were detected in cells transfected with a control vector.

By having obtained a serum that reacted specifically with the IE1 protein, we performed a kinetic of IE1B expression in
Characterization of HHV-6 IE1 Protein

HHV-6-infected Molt-3 cells. By using immunofluorescence assays, the IE1B protein was detected at 4 h HHV-6 post-infection (Fig. 4A). At such time point, the IE1B protein was detected as small granules in the nuclei of infected cells. Over the next 8–12 h P.I., IE1B became easily detectable; the cells were getting larger, and the number of IE1B bodies increased to ~8–10/nucleus. By 24–48 h P.I., the number of IE1B bodies diminished (~1–3/nucleus), whereas the size of each body became enlarged (arrows). By 96 h P.I., up to three large IE1B bodies per nucleus could be detected in many infected cells. Some cells also displayed numerous small IE1B bodies, most likely reflecting second round infection initiated by the release of newly synthesized progeny virions (arrowhead). These results suggest that during the early phase of HHV-6 infection (4–8 h), IE1 starts to be expressed and is distributed in small and numerous bodies, whereas at later time points (24–96 h), the IE1B bodies fuse together into a unique or few large bodies.

We next studied the IE1B electrophoretic mobility profile during the course of an infection. At early time points (~24 h), detection of IE1B by Western blot was difficult, and we therefore studied its expression at later time points (48–96 h P.I.). Infected cells were lysed and analyzed for IE1B expression by immunoprecipitation and Western blot analysis. The results obtained indicate that IE1B expression continues to increase up to 96 h P.I. (Fig. 4B). No modification in IE1B electrophoretic mobility could be detected at these time points under these conditions suggesting the existence of a single form of IE1B.

Post-translational Modifications of the HHV-6 IE1B Protein—By using computer analysis to identify motifs within the IE1B protein of HHV-6, we have identified numerous putative phosphorylation sites. A single tyrosine consensus sequence, 21 serine-threonine protein kinase C sites, 37 casein kinase II sites, and one cAMP-, cGMP-dependent kinase sites were identified (Fig. 1B). Our first experiment was designed to determine whether the IE1B protein undergoes phosphorylation events during the course of an HHV-6 infection. HHV-6-infected (72 h) and uninfected Molt-3 cells were labeled with inorganic radioactive phosphorus for several hours and lysed in buffer supplemented with phosphatase inhibitors. The anti-IE1 antibodies immunoprecipitated a radioactive 150-kDa IE1B protein from infected cells (Fig. 5A) indicating that under in vivo conditions this protein undergoes phosphorylation events. No such protein was immunoprecipitated from uninfected cells.

Because tyrosine and serine-threonine phosphorylation sites were recognized from the IE1B primary amino acids sequence, we sought to determine the nature of the phosphorylation events. Infected cells were immunoprecipitated with the anti-IE1B antibodies followed by Western blot analysis for anti-phosphoserine, anti-phosphothreonine, or anti-phosphotyrosine. The results obtained indicate the IE1B is phosphorylated on both serine and threonine residues (Fig. 5B). However, no tyrosine phosphorylation of IE1 could be detected under the same conditions. The 4G10 anti-phosphotyrosine antibody has been used extensively by numerous investigators and has demonstrated excellent reactivity against several proteins having a single phosphorylated tyrosine residue. We are therefore confident that the HHV-6 IE1B is not tyrosine-phosphorylated on residue 920 during infection. This result is further supported by the observation that the entire radioactivity from phosphorylated-IE1 is lost following incubation in 1 M KOH, a treat-
Characterization of HHV-6 IE1 Protein

Post-translational phosphorylation of HHV-6 IE1B protein. A, Molt-3 cells were infected with HHV-6 (m.o.i. 0.02) for 3 days followed by metabolic labeling with radioactive inorganic phosphate. Cells were processed for immunoprecipitation (IP) using anti-IE1 antibodies and protein A-Sepharose beads. After protein separation by electrophoresis, gels were dried and exposed to imaging plates for several hours and analyzed using a PhosphorImager system. B, IE1 proteins from mock (M) or HHV-6 infected (I) Molt-3 cells were immunoprecipitated using anti-IE1 antibodies and protein A-Sepharose beads. Immunoprecipitated proteins were analyzed by Western blot using anti-phosphoarginine (PY), anti-phosphoserine (PS), anti-phosphothreonine (PT), or anti-IE1 antibodies.

In Vivo Sumoylation of HHV-6 IE1 Protein—Primary sequence analysis of the IE1 protein indicates two putative SUMO conjugation sites (Lys-281 and Lys-802) for the variant B and one site (Lys-665) for the variant A. The consensus site is defined as a stretch of 4 amino acids consisting of a hydrophobic amino acid followed by a lysine, an irrelevant residue, and a glutamic acid (ϕKXE) (35, 38–40). The SUMO moiety (11 kDa) is attached to the target lysine residue through the successive action of SUMO-activating enzyme (41), SUMO-conjugating enzyme (42, 43), and possibly with the participation of a newly identified SUMO ligase (44, 45). To test for the presence of SUMO-IE1, Molt-3 and HS6-2 cells were infected for 72 h with HHV-6 Z29 and GS strains, respectively. Using conventional RIPA lysis buffer, IE1B proteins with reduced electrophoretic migration profiles, suggestive of modified IE1B, were never detected (Figs. 3D and 4B). However, using a lysis buffer containing 5 mM N-ethylmaleimide, which inhibits desumoylating isopeptidases (46, 47), SUMO-conjugated IE1B proteins could be detected. By using a combination of immunoprecipitation-Western blot for IE1, two species of IE1B were detected as follows: the major band migrates at 150 kDa representing the SUMO-unconjugated IE1B, and a protein migrates at 170 kDa possibly representing a SUMO-modified IE1B (Fig. 6, left panel). The IE1 immunoprecipitates were therefore analyzed by Western blot using an anti-SUMO-1 monoclonal antibody (Fig. 6, right panel). Several species of SUMO-1-modified IE1B were detected with the 170-kDa SUMO-IE1 representing the major species. Several low abundance SUMO-IE1B proteins with larger molecular weights were also detected. Similar results were obtained with HHV-6 variant A (data not shown). These results suggest that under in vivo conditions the IE1 protein from variant B can be sumoylated on more than one target lysine residue, that SUMO multimerization can occur at a single site or that both events are occurring.

Colocalization of HHV-6 IE1 with SUMO and PML during Infection—The IE proteins of several herpesviruses, including HCMV, HSV, and Epstein-Barr virus were reported to transiently colocalize and disrupt PML bodies shortly after infection (19, 20, 22, 47, 48). We therefore performed kinetic studies of HHV-6 IE1B nuclear localization and its effect on PML bodies integrity. By using dual color immunofluorescence assay with antibodies against IE1 and PML or against IE1 and SUMO-1, we could detect a perfect colocalization pattern between IE1B and PML at 8 h post-infection (Fig. 7A). Similar results were obtained at earlier time points (data not shown). IE1B also colocalized perfectly with SUMO-1. This was somewhat expected given the fact that IE1B undergoes sumoylation during the infection process and that PML, within PODs, is sumoylated (Fig. 6). Contrary to HCMV and HSV, however, HHV-6 variant B infection does not lead to the dispersal of PML bodies. During the early phase of infection (0–12 h), PML bodies have a normal distribution with 8–10 bodies per nucleus. As infection progresses (24 h), PML bodies tend to fuse into a few larger entities in which IE1B and SUMO remain associated. By 96 h post-infection, a mixture of normal and fused PML bodies is observed, the result of secondary infection events. Similar results were obtained with HHV-6 variant A (data not shown). To determine whether IE1B by itself can cause PML bodies to coalesce, we transfected 293T cells with an IE1B expression vector and performed double IFA for IE1 and PML. The results obtained indicate that IE1B expression, in the absence of other viral proteins, is present in small nuclear dots that perfectly colocalize with PML (Fig. 7B). IE1B and PML did not coalesce into one or a few large bodies, suggesting that other HHV-6 proteins are necessary for this event to occur.

Heterologous Promoter Transactivation by IE1 from HHV-6 Variants A and B—In a previous report Martin et al. (27) presented data to the effect that a genomic segment encoding HHV-6 variant A IE1 was capable of transactivating several heterologous promoters, a finding that we were able to confirm (49). To compare the transactivating potential of the IE1 protein from both variants, we transfected Molt-3 cells with a HIV-LTR luciferase reporter construct along with increasing quantities of IE1 expression vectors. The HHV-6 variant A IE1, encoded by the pRS56 vector, was capable of efficiently transactivating, in a dose-dependent manner, the HIV-LTR promoter, as reported previously (Fig. 8) (27). By contrast, IE1 from
variant B was unable to activate efficiently the HIV-LTR promoter, with a 2-fold induction obtained under the best experimental conditions. IE1 from variant B also failed to significantly transactivate promoters driven by NF-κB or CRE elements (data not shown). These results suggest that IE1 proteins from both variants differ significantly with regard to their ability to activate heterologous promoters.

**DISCUSSION**

In the present paper we report the cloning of a transcript encoding the full coding sequence for HHV-6 variant B (Z29) IE1 protein. IE1 transcripts for HHV-6 variant A (U1102 strain) have been described previously (29). The overall structures of HHV-6 IE1 transcripts are similar in both A and B variants and are composed of five exons with translation initiating in the middle of the third exon. The 3.7-kb IE1 transcript from variant B is slightly larger than the 3.5-kb of variant A. Two deletions of 123 and 215 nucleotides within the coding region of IE1 from the A strain account for most of the differences, which translates into a 941-amino acid protein for variant A compared with 1078 amino acids for variant B. IE1 proteins from both variants share 62% overall amino acid identity and 72% similarity (Ref. 25 and this work), whereas the amino acid identity between strains of variant B IE1 (HST and Z29 strains) is 92% (26). In addition to the 3.5-kb IE1A transcript, Schiewe et al. (29) reported the presence of a lately expressed 4.7-kb transcript encoding for the same IE1A protein. Similarly, a 5.6-kb mRNA and two additional larger transcripts of 7.0 and 8.9 kb were observed during our Northern blot analysis of HHV-6 variant B infected cells. Characterization of these transcripts has yet to be performed, but these clearly suggest that, depending on the stage of infection, transcription of IE1-related genes can be initiated from multiple promoters. None of these large transcripts were detected under IE conditions, suggesting different kinetics of expression than the 3.7-kb IE1 mRNA. Whether these larger transcripts encode variants of the IE1B protein remains to be determined.

Although the HHV-6 IE1 gene is a positional homologue of HCMV IE1 gene (UL123), no significant identity between the encoded proteins exists. Functionally, a genomic segment coding for HHV-6 IE1 from variant A and the HCMV IE1 protein was shown to activate heterologous promoters (27, 49–52). Because the only known measurable activity of HHV-6 IE1 pertains to its effect on heterologous promoter transactivation, we compared the ability of variants A and B to activate the HIV LTR promoter. IE1 from variant B has marginal activity compared with the IE1 from variant A in promoting transcription.

**FIG. 7.** HHV-6 IE1B protein colocalizes with PML and SUMO in infected cells. A, Molt-3 cells were infected with HHV-6 (Z29 strain) for the indicated times, fixed in acetone, and processed for immunofluorescence as described under “Experimental Procedures.” Cells were first reacted with anti-PML or anti-SUMO antibodies followed by incubation with Alexa 488–conjugated secondary antibodies (red). Finally, cells were incubated with Alexa 488–labeled anti-IE1 (green) antibodies. Colocalized proteins are represented in the merge pictures by a yellow color. B, 293T cells were transfected with the control pBK or pBK-IE1B vectors and processed, after 48 h, for dual color immunofluorescence using anti-IE1 and anti-PML antibodies as described under “Experimental Procedures.” Same field images were captured for IE1 and PML.

**FIG. 8.** HIV-LTR promoter activation by HHV-6 IE1. Molt-3 cells were transfected by electroporation with the pcDNA control vector (12 μg) or varying amounts of pBK-IE1B or pR56 (coding for variant A IE1) plasmids along with the HIV-LTR luciferase reporter (4 μg). DNA levels were kept constant (16 μg) by the addition of the control pcDNA vector. Data are presented as the mean fold activation ± S.D. and is representative of three independent experiments.
from the HIV LTR. This difference in the behavior of IE1 may account for some of the divergence in biological properties between both variants. Conflicting reports exist regarding the ability of HHV-6 infection to activate or repress HIV (53–57). The reasons for this remain unknown but are likely attributable to the varying experimental procedures used and do not appear related to the ability of IE1 from both variants to differentially activate the HIV LTR.

HHV-6 IE1B protein, like that of HCMV, is phosphorylated and is expressed in the nuclei of infected cells. In terms of kinetics of expression, we first could detect HHV-6 IE1B at 4 h P.I. Attempts to detect the protein at earlier time points (2 h) proved unsuccessful. Efficient cell-free HHV-6 infections are sometimes difficult to achieve, a feature that could have explained our inability to detect IE1B at such early time points. However, given the fact that HHV-6 IE1B mRNA can easily be detected by 2 h of infection, the protein may not be synthesized in sufficient quantity to be detected. Thus, in terms of kinetics, HCMV IE1 is possibly expressed more rapidly (i.e., at 2 h) upon infection than IE1B of HHV-6. By 4–6 h post-HCMV infection, the punctate pattern is lost, and IE1 is diffusely distributed throughout the nuclei of infected cells (19). In sharp contrast, HHV-6 IE1B is first observed as small granules whose intensity increases with time. By 12 h P.I., the number of IE1B punctate bodies is maximal, reaching 8–10 such entities per nucleus. By 24–48 h P.I., the IE1B bodies condense together into 1 or few large bodies per nucleus, while at later time points (72–96 h P.I.), a mixture of large and small IE1B granules is observed, the latter presumably resulting from secondary infection.

Targeting/disruption of PML bodies by herpesviruses IE proteins appears to be a generalized phenomenon as it is observed following infection by HCMV, HSV, and Epstein-Barr virus (19–21, 23, 48, 58, 59). Likewise, HHV-6 IE1 from both variants target PML bodies soon after infection. A major difference with other human herpesviruses, however, is that HHV-6 infection does not lead to the dispersal of PML bodies but rather leads to the condensation of PML bodies into few large entities into which IE1 and SUMO-1 colocalize. HHV-6 IE1 remains associated with PML bodies throughout the infection process. In transfection experiments and in the absence of other viral proteins, IE1 is present in numerous nuclear entities that colocalize with PML bodies. The PML bodies patterns remain unaffected suggesting that additional viral proteins are necessary for IE1 and PML bodies condensation. Modification (dispersal or condensation) of PODs following herpesviruses infections likely represents an important step for efficient transcription and replication of the viral genome. In fact, treatment of cells with arsenic trioxide, which increases the stability of PODs, effectively inhibits the generation of infectious HSV particles in vitro (60). Input of viral genomes and transcription of IE genes were reported to occur at or in the very close proximity of PODs (61–63). It is tempting to speculate that the targeting of PODs by herpesviruses represents a way to free cellular factors implicated later during the infectious process such as those involved in replication. In the case of HHV-6, however, where PODs condensation rather than dispersal is observed, this event may bring together factors involved in viral DNA replication. In support of this hypothesis, our preliminary results suggest that maximal PODs aggregation correlates with the initiation of viral DNA replication (24 h). Current work is in progress to address the precise localization of the viral DNA replication compartments and their position relative to the aggregated PODs. The condensation of PODs into one or two larger nuclear entities is somewhat reminiscent of the phenotype of triple mutant of PML (3M-PML), which can no longer be sumoylated (64). In PML–/–-transfected cells, the 3M-PML protein is distributed into a few large entities, in striking contrast to the wild type PML speckled pattern (64). It is possible that HHV-6 IE1, by sequestering the majority of free SUMO residues, present in limited amounts within cells, prevents the SUMO conjugation and hence the improper nuclear distribution of the newly synthesized PML proteins. Thus, in a manner similar to HSV-1, HHV-6 infectious process may be associated with the loss of SUMO-conjugated PML isoforms causing the aberrant distribution of PML. Finally, the possibility that PODs may play a role in the establishment of an antiviral state following treatment with interferons has also been proposed. Following interferon treatment, twice as many PODs can be observed within the nucleus. Expression of the main proteins composed within PODs, the PML and Sp100 proteins, are strongly up-regulated by interferon stimulation (65). Overexpression of the PML protein has been reported to affect the replication of RNA viruses such as the human foamy virus, the vesicular stomatitis virus, and the influenza virus by interfering with viral mRNA and protein synthesis (66, 67). Thus, by modifying the overall PODs architecture, viruses may render the mounting of an antiviral state initiated following interferon stimulation.

Two putative SUMO conjugation sites are present within variant B IE1 protein. At least one of the sites is efficiently conjugated by SUMO-1 during the infectious process. Our results also suggest that more than one SUMO-modified form of IE1 is present in infected cells. Whether lysine 281, lysine 802, or both are covalently modified remains to be determined. IE1 from variant A has only one SUMO site (Lys-665) that gets sumoylated during infection. This suggests that Lys-802 of variant B, a positional homologue of Lys-665 from variant A, is likely a target for SUMO conjugation. Higher molecular weight species of SUMO-IE1 is also suggestive of multiple SUMO conjugation events occurring. At least three forms of SUMO have been described so far (46, 68–71). Although multimerization of SUMO-1 has never been reported, branching of SUMO-2 and SUMO-3 has been described (72). A SUMO conjugation site within SUMO-2 and –3 allows these proteins to be sumoylated themselves in a manner analogous to the polyubiquitination reaction. Thus, the high molecular weight forms of SUMO-IE1 may represent polysumoylated IE1. Work is in progress to address this issue. Sumoylation of herpesviruses IE proteins have also been reported for HCMV and Epstein-Barr virus (36, 47, 48, 73, 74). The effects of SUMO conjugation on the functionality of HHV-6 IE1 remain unknown. SUMO conjugation is reported to affect proteins function in a variety of different ways. For example, the ability of the p53 and HCMV IE2 proteins in promoting transcriptional activation is positively influenced by SUMO conjugation (73–76). Whether a similar activity can be expected for IE1 of HHV-6 remains to be determined. Alternatively, SUMO conjugation could affect the half-life through competition with the ubiquitination process of IE1, as in the case of iBeo (35). Finally, in a manner analogous to the RanGAP1 protein (68, 77), SUMO conjugation of IE1 may facilitate its transport to the nuclear pore complex. Further work is needed to address these possibilities.

The IE1 protein encoded by the IE-A locus of HHV-6 is the second most divergent protein (after U91) between A and B strains with an identity of sequence of 62%. HHV-6 variants A and B possess divergent biological properties such as their ability to infect the HSB-2 and the Molt-3 cell lines, respectively. Another example of segregation between HHV-6 variants A and B is the association with roseola infantum, where the vast majority of cases is caused by B variant infections (6). Until the true functions of IE proteins are fully understood, an
association between the divergence of IE1 proteins and biological and pathogenic differences between A and B variants of HHV-6 remains speculative. Precise comparative studies on IE1 proteins from both HHV-6 variants are therefore essential for a complete understanding of the importance of IE-A locus divergence between A and B variants and their respective biological differences.

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