Identification of a Physiological Phosphorylation Site of the Herpes Simplex Virus 1-Encoded Protein Kinase Us3 Which Regulates Its Optimal Catalytic Activity In Vitro and Influences Its Function in Infected Cells

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Received 7 January 2008/Accepted 7 April 2008

Us3 is a serine/threonine protein kinase encoded by herpes simplex virus 1 (HSV-1). Here, we report the identification of a physiological Us3 phosphorylation site on serine at position 147 (Ser-147) which regulates its protein kinase activity in vitro. Moreover, mutation of this site influences Us3 function, including correct localization of the enzyme and induction of the usual morphological changes in HSV-1-infected cells. These conclusions are based on the following observations: (i) in vitro kinase assays, a domain of Us3 containing Ser-147 was specifically phosphorylated by Us3 and protein kinase A, while a mutant domain in which Ser-147 was replaced with alanine was not; (ii) in vitro, alanine replacement of Ser-147 (S147A) in Us3 resulted in significant impairment of the kinase activity of the purified molecule expressed in a baculovirus system; (iii) phosphorylation of Ser-147 in Us3 tagged with the monomeric fluorescent protein (FP) VenusA206K (VenusA206K-Us3) from Vero cells infected with a recombinant HSV-1 encoding VenusA206K-Us3 was specifically detected using an antibody that recognizes phosphorylated serine or threonine residues with arginine at the +1 and +2 positions; and (iv) the S147A mutation influenced some but not all Us3 functions, including the ability of the protein to localize itself properly and to induce wild-type cytopathic effects in infected cells. Our results suggest that some of the regulatory activities of Us3 in infected cells are controlled by phosphorylation at Ser-147.

The herpes simplex virus 1 (HSV-1) Us3 gene encodes a serine/threonine protein kinase with an amino acid sequence conserved in the subfamily Alphaherpesvirinae (12, 31, 48). In vitro biochemical studies characterized the consensus target sequence of an HSV-1 Us3 homologue encoded by pseudorabies virus (PRV) as RxnX(S/T)YY, where n is greater than or equal to 2; X can be Arg, Ala, Val, Pro, or Ser; and Y can be any amino acid except an acidic residue (26, 27, 47). The target site specificity of HSV-1 and HSV-2 Us3 and varicella-zoster virus open reading frame 66 (ORF66) has been reported to be broadly similar to that of the PRV homologue (7, 9, 47). Based on studies showing that recombinant Us3 mutant viruses have impaired growth properties in cell cultures and virulence in mouse models (32, 52), it is concluded that HSV-1 Us3 is a positive regulator of viral replication and viral pathogenicity. At present the mechanisms by which this viral protein kinase acts in viral replication and pathogenicity remain largely undetermined, but several lines of evidence suggest possible functions of Us3, as discussed below.

(i) The Us3 protein kinase blocks apoptosis induced by replication-incompetent viruses, osmotic shock, or overexpression of proapoptotic cellular proteins (15, 18, 28, 35, 36, 42). Although the critical Us3 substrate(s) which mediates the antiapoptotic activity of the viral protein kinase has not yet been identified, evidence suggesting mechanisms by which Us3 prevents apoptosis is gradually accumulating. Recently, it has been reported that Us3 activates protein kinase A (PKA), a cellular cyclic AMP-dependent protein kinase with phosphorylation target sequences resembling those of Us3. It has also been reported that both Us3 and PKA phosphorylate the same target protein residues (1), suggesting that the former mediates antiapoptotic activity through phosphorylation of PKA substrates, by activating PKA, and/or by mimicking this cellular protein kinase. In addition, cellular apoptosis-regulating proteins, including Bad, Bid, and procaspase 3, have been implicated as being phosphorylated by Us3 (2–4, 17, 36). However, the biological significance of Us3-mediated phosphorylation of these cellular proteins in infected cells remains to be elucidated.

(ii) The Us3 protein kinase plays a role in nuclear egress of progeny nucleocapsids. In cells infected with Us3 mutant viruses, virions aggregate aberrantly within the perinuclear space in large invaginations (52). Us3 phosphorylates UL31 and UL34, both of which are crucial regulators of primary envelopment of nucleocapsids at the nuclear membrane; the catalytic activity of Us3 protein kinase is required for proper localization of UL31 and UL34 at the nuclear membrane (17, 50, 51, 54, 56). Furthermore, Us3 phosphorylates and alters the
localization of lamin A/C, a fibrous meshwork lining the nucleoplasmic face of the inner nuclear membrane (34). Us3 has also been implicated in phosphorylating the inner nuclear membrane protein Emerin, which interacts with a number of nuclear components, including lamin and the DNA-binding protein BAF, and is suggested to be involved in maintaining nuclear integrity (25, 33). These observations suggest that Us3 regulates nucleolar expression of nucleocapsids by mediating phosphorylation of these viral and cellular proteins. It has been reported that lack of phosphorylation of UL34 is not likely to be responsible for the aberrant nuclear membrane morphology observed in cells infected with Us3 mutant viruses, whereas the role of phosphorylation of other proteins, including UL31, lamin A/C, and Emerin, has not yet been elucidated.

(iii) The HSV-1 Us3 homologues encoded by PRV and Marek’s disease virus mediate actin stress fiber breakdown in infected cells (57, 63). The cytoskeletal rearrangement mediated by the PRV Us3 homologue has been implicated as being associated with the changed morphology of infected cells and intracellular viral spread (11). Consistent with this, it has been reported that the cytopathic effects (CPEs) on cells infected with Us3 mutants of HSV-1 and HSV-2 are different from those with wild-type viruses (36, 38, 48). In addition, overexpression of HSV-2 Us3 in the absence of other viral proteins has been shown to induce actin stress fiber breakdown and to affect the cdc42/Rac pathway, which controls actin cytoskeletal organization (37). Although it remains uncertain whether HSV Us3 is in fact involved in rearrangement of actin stress fibers in infected cells as observed in PRV and Marek’s disease virus infection, these observations indicate that Us3 influences the morphology of infected cells, probably by modifying the cytoskeleton.

(iv) Other than the Us3 substrates described above, ICP22, US9, UL12, UL46, cytokeratin 17, histone deacetylase 1 (HDAC1), and HDAC2 (6, 8, 17, 30, 38, 45, 49) have been reported to be putative substrates for Us3. Although the biological significance of the phosphorylation of the putative substrates by Us3 during the viral life cycle remains to be elucidated, these observations imply an additional novel function(s) of Us3.

While the functional manifestations of Us3 have been gradually unveiled as described above, little is known regarding the mechanisms by which the protein kinase activity of Us3 protein is regulated. In cells, the activity of cellular protein kinases is tightly regulated; they are turned on or off by phosphorylation, binding of regulatory subunits, the presence of small molecules, or by virtue of their subcellular localization (55). Therefore, knowledge of the mechanisms by which protein kinases are activated or inhibited is also crucial for understanding the overall features of the enzymes as well as the functional consequences of their manipulation. In the studies presented here, we focus on phosphorylation of Us3 and its effect on regulatory sequences of their manipulation. This protein kinase has been reported to be autophosphorylated in vitro (17, 47), as well as by another HSV-1-encoded protein kinase, UL13, in infected cells (18). However, a direct link between Us3 autophosphorylation and phosphorylation by UL13, and the regulatory activity of Us3, has not been demonstrated. Here, we report the identification of a physiological Us3 phosphorylation site at Ser-147, which regulates its protein kinase activity in vitro, as well as certain of its functions in infected cells.

MATERIALS AND METHODS

Cells and viruses. Vero, rabbit skin, and Srodoptera frugiperda S09 cells were described previously (21, 59), as was HSV-1 wild-type strain HSV-1(F) (10, 59). Human embryonic lung fibroblast (HEL) cells were kindly provided by Shinya Watanabe and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. A Us3 deletion mutant virus, R7301, and a recombinant virus, R7306, in which the Us3 gene has been replaced (48) were kindly provided by B. Roizman. The recombinant virus YK304 was reconstituted from pYEbac102, containing a complete HSV-1(F) sequence with the bacterial artificial chromosome (BAC) sequence inserted into the HSV intergenic region between UL3 and UL4 (59). YK304 has been shown to have a phenotype identical to that of wild-type HSV-1(F) in cell cultures and in mouse models (59).

Plasmids. (i) A plasmid, pGEUX-Us3-P1, for generating a fusion protein of glutathione S-transferase (GST) and a part of Us3, was constructed by amplifying the domains carrying Us3 codons 98 to 364 by PCR from PBS-Us3 (17) and cloning the DNA fragments into pGEX4T-1 (GE Healthcare) in frame with GST. PBS-Us3/S147A, in which the serine codon at position 147 (Ser-147) of Us3 was replaced with an alanine, was generated by PCR amplifying the DNA fragments into pGEX4T-1 (GV Healthcare) in frame with GST (Stratagene). (ii) Plasmids pMAL-Us3-P3 and pMAL-Us3-P4-S147A, for generating a fusion protein of maltose-binding protein (MBP) and a part of Us3, were constructed by amplifying the domains carrying Us3 codons 1 to 172 by PCR from PBS-Us3 or PBS-Us3/S147A, respectively, cloning the DNA fragments into pMAL-c (New England Biolabs) in frame with MBP. (iii) The transfer plasmid pAcGHLT-Us3S147A, for generating a recombinant baculovirus expressing GST fused to a mutated version of Us3 in which Ser-147 was replaced with an alanine (Us3S147A), was constructed by cloning the EcoRI/NotI fragment of PBS-Us3/S147A into the EcoRI and NotI sites of pAcGHLT-A (Pharmingen) in frame with GST. (iv) The transfer plasmids pBS-Venus/A206K-Us3 and pBS-Venus/A206K-Us3S147A for generating a recombinant HSV-1 expressing a fusion protein of the Us3 protein and UV or Us3S147A protein, respectively, were constructed as follows. Plasmid pBS-Venus was constructed by amplifying the Venus ORF from pCS2 (a generous gift from A. Miyawaki) and cloning it into the EcoRI and SpeI sites of pbBlueScript KSI (+) (Stratagene). Using pBS-Venus, pBS-Venus/A206K, in which an alanine at Venus residue 206 was replaced with lysine, was generated using the QuickChange site-directed mutagenesis XL kit. Because green FP (GFP) and its variants form dimers at high concentrations, the A206K mutation was used to prevent dimerization without significant alteration of FP spectral properties (66). The 1.5-kb sequence upstream of the HSV-1 Us3 start codon was amplified by PCR from pBC1013 (a generous gift from B. Roizman) (22) using the primers 5’-GGCAAGTCTAGGGTCTCCCCACCTG-3’ and 5’-GGGACTTTCC GTGCGACCGTAGTGGCAGC-3’ and cloned into the pBS-Venus/A206K EcoRI and HindIII sites to produce pBS-Venus/A206K-1.5kb. The entire Us3 of the Us3S147A ORF without the Us3 start codon, was amplified by PCR from PBS-Us3 or PBS-Us3/S147A and cloned into pBS-Venus/A206K-1.5kb SpeI and NotI sites in frame with Venus/A206K to produce pBS-Venus/A206K-Us3 and pBS-Venus/A206K-Us3S147A, respectively. The resultant plasmid consisted of Venus/A206K-Us3 or Venus/A206K-Us3S147A bounded by Us3 flanking sequences.

Mutagenesis of viral genomes in E. coli and generation of recombinant HSV-1. To generate a Us3 deletion mutant virus (YK502) in which a gene encoding kanamycin resistance was substituted for the domain of Us3 carrying codons 1 to 220 (Fig. 1), the one-step mutagenesis method known as ET cloning was performed as described previously (60, 67). Briefly, linear fragments containing a gene encoding kanamycin resistance and 50 bp of flanking Us3 sequences on each side were generated by PCR using the primers 5’-GGGAGCCTGTCGCTGCG GGTGCTCGTGGTGCGACACTACCCGCGGCAACGGCTAT-3’ and 5’-AGTGCCCTACAAGCTGCGCTGCTGCGCTGCGCTGCGCTTATCA TACCCGCGGACGAGCTGCGACCAACGGCTAT-3’. These fragments were electroporated into YEBa202, an E. coli DH10B strain containing an HSV-1-F) BAC plasmid (pYEbac102) (59) and pGErotc, encoding recombinases E and T (a generous gift from P. A. Ioannou) (40). These primers were designed to replace codons 1 to 220 of Us3 with a kanamycin resistance gene. Kanamycin-resistant colonies were then screened by PCR with appropriate primers, which led to the identification of E.coli YEBa502 harboring the mutant HSV-1(BAC) plasmid pYEBa502. The Us3 deletion mutant virus YK502 was generated by transfection of pYEBa502 into rabbit skin cells as described previously (59) and verified by Southern blotting.
To construct the recombinant virus YK503 encoding VenusA206K fused to a Us3 mutant protein in which alanine was substituted for Ser-147 (VenusA206K-Us3S147A) (Fig. 1), rabbit skin cells were cotransfected with the transfer plasmid pBS-VenusA206K-Us3S147A and intact YK502 viral DNA, using the calcium phosphate precipitation technique, as described previously (22). Viral DNAs were extracted from infected cells and purified on 5 to 20% potassium acetate gradients as described previously (22). At 3 days posttransfection, the transfected cells were harvested and subjected to freeze-thawing and sonication. The cell lysates were diluted and inoculated onto Vero cells, and plaques were screened for fluorescence with an inverted fluorescence microscope (Olympus IX71). Recombinants were plaque purified three times on Vero cells and verified by Southern blotting.

The recombinant virus YK505, in which the alanine replacement of Ser-147 in Us3 of YK503 had been repaired (VenusA206K-Us3-repair) (Fig. 1), was generated as follows. First, recombinant virus YK504, in which a gene encoding kanamycin resistance was substituted for VenusA206K and the domain of YK503 Us3 carrying codons 1 to 220 was constructed. To this end, circular viral DNA of YK503 isolated from infected Vero cells by the Hirt method was electroporated into E. coli DH5a to generate the plasmid pYEbac512. The linear viral DNA of YK503 was then electroporated into E. coli DH5a to generate the plasmid pYEbac514. The plasmids pYEbac512 and pYEbac514 were used to transform E. coli DH5a, and the cells were plated on medium containing kanamycin. The colonies were screened for the presence of the desired recombinant virus, and the recombinant virus YK505 was isolated by plaque purification on Vero cells.
into *E. coli* DH10B (Invitrogen), and *E. coli* YEBac503 harboring the YK503 genome was isolated as described previously (59). ET cloning was performed in *E. coli* YEBac503. The Us3 deletion mutant virus YK503 (Fig. 1) was reconstituted in the same way to generate YK502 as described above. Second, rabbit skin cells were cotransfected with the transfer plasmid pBS-Venus/A206-U3s and intact YK504 viral DNA, and the recombinant virus YK505 was generated in the same way to produce YK503, as described above.

To generate a recombinant virus (YK511) carrying a mycinaminocoline replacing the lysine codon at position 220 (Lys-220) (Fig. 1), the two-step Red-mediated mutagenesis procedure (61) was performed. Briefly, *E. coli* GS1783 (14) containing pYEbac102 (a kind gift from G. A. Smith and N. Osterrieder) was grown in LB medium containing 20 μg/ml of chloramphenicol at 32°C to an optical density at 600 nm of 0.5 to 0.7. The *E. coli* culture was then transferred into a 42°C water bath and left for 15 min to induce the expression of the Red recombination system. Finally, bacteria were cooled on ice for 20 min and harvested, and electrocompetent cells were prepared as described elsewhere (59). Linear fragments containing a gene encoding the I-SceI site, kanamycin resistance, and 60 bp of Us3 sequences carrying the methionine substitution of Lys-220 into the Lys-220 locus of the Us3 gene. The linear PCR-generated fragments were electroporated into the electrocompetent cells. The recombinant virus was reconstituted at 32°C and plated at 1:1000 to 1:5000 to obtain Mb expressing intact YK510 viral DNA, and the recombinant virus YK511 was generated as described previously (17). To generate the recombinant baculovirus Bac-GST-YK510, Bac-GST-YK511, or Bac-GST-UL34 was purified from infected *Sf9* cells using Lipofectin (Invitrogen) as described previously (21). Recombinant baculoviruses were propagated in S9 cells.

**Production and purification of GST and MBP fusion proteins expressed in E. coli.** GST fusion protein (GST-Us3) was expressed in *E. coli* transformed with pGEX-Us3-P1, purified as described previously (19), and used for generation of rabbit polyclonal desorbcty to Us3 as described below. MBP fusion proteins (MBP-Us3-P4, MBP-Us3-P4-S147A, or MBP-UL34) were expressed in *E. coli* transformed with pMAL-Us3-P4, pMAL-Us3-P4-S147A, or pMAL-UL34 (17, respectively), and purified as described previously (21).

**Generation of recombinant baculovirus.** Recombinant baculoviruses Bac-GST-U3, Bac-GST-KS220M, and Bac-GST-BGLF4 have been described previously (17, 21). To generate Bac-GST-U3S147A, pAcHIL-Us3S147A was cotransfected with linearized baculovirus DNA BacOpoGold (Pharmingen) into S9 cells using Lipofectin (Invitrogen) as described previously (21). The recombinant baculoviruses were propagated in S9 cells.

**Purification of GST fusion proteins from baculovirus-infected cells.** GST-U3, GST-U3S147A, and GST-BGLF4 proteins were purified from S9 cells infected with Bac-GST-U3, Bac-GST-KS220M, Bac-GST-U3S147A, and Bac-GST-BGLF4, respectively, as described previously (21). 

**Antibodies.** To generate polyclonal antibody to Us3, two rabbits were immunized with purified GST-U3-P1, following a standard protocol at MBL (Nagoya, Japan). Chicken polyclonal antibody to UL34 was kindly provided by R. Roller (11). Rabbit polyclonal antibodies to UL31 (68) and VP22 (41) were described previously. Rabbit polyclonal antibody to HDAC2 and rat monoclonal antibody to GFP were purchased from Sigma and MBL, respectively. Rabbit phospho-PKA substrate (100G) monoclonal antibody was purchased from Cell Signaling Technology (Danvers, Mass.).

**In vitro kinase assays.** MBP fusion proteins were captured on amyllose beads (New England Biolabs) and used as substrates in in vitro kinase assays with 0.34 μg (3.7 pmol) of purified GST-U3 or GST-U3S147A, as described previously (17). MBP fusion proteins were also used as substrates in in vitro kinase assays with 0.14 μg (3.7 pmol) (Fig. 2C and D) or 0.45 μg (11.9 pmol) (Fig. 2A, B, E, and F) of purified PKA, purchased from Invitrogen. In vitro kinase assays with PKA were performed as described previously (22). Next, (22) PKA reaction buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂) was used. The relative amount of radioactivity in substrates phosphorylated by GST-U3 or GST-U3S147A was quantified with the aid of Dolphi Doc and the software Dolphin-1D (Weltec).

**Immunoblotting and immunofluorescence.** Immunoblotting was performed as described previously (22) except that polyvinylidene difluoride membranes (Millipore) were used with the phospho-PKA substrate antibody. Indirect immunofluorescence assays were performed as described previously (16), except that anti-chicken immunoglobulin G (IgG) conjugated to Alexa Fluor 546 was used as the secondary antibody, and samples were examined with the Zeiss LSM5 laser scanning microscope.

**Immunoprecipitation.** Vero cells were infected with either YK503 or YK505 at a multiplicity of infection (MOI) of 3 PFU. Infected cells were harvested at 18 h postinfection and lysed in NP-40 buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1.0% Nonidet P-40 [NP-40]) containing a protease inhibitor cocktail (Sigma). Supernatants obtained after centrifugation of the cell lysates were preclotted by incubation with protein A-Sepharose beads (GE Healthcare) at 4°C for 30 min. After a brief centrifugation, supernatants were passed through 0.22-μm-pore-size filters and then reacted at 4°C for 1.5 h with 5 μl of agarose-conjugated anti-GFP (MBL), i.e., anti-GFP monoclonal antibody RQ2 coupled to agarose beads. To visualize the agarose bead pellet more easily, additional protein A-Sepharose beads were added to the supernatants. Immunoprecipitates were collected by a brief centrifugation, washed extensively with NP-40 buffer, and analyzed by immunoblotting with anti-phospho-PKA substrate (RRXX/T) antibody or anti-Us3 antibody.

**Immunofluorescence assay.** Vero cells were infected with either YK504, YK511, or YK515 at an MOI of 3. Infected cells were harvested at 18 h postinfection and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium deoxycholate) containing a protease inhibitor cocktail (Sigma). Supernatants obtained after centrifugation of the lysates were preclotted by incubation with protein A-Sepharose beads at 4°C for 30 min and then reacted with the anti-Us3 antibody at 4°C for 1.5 h. Protein A-Sepharose beads were added to the supernatants and incubation continued for another 2 h. Immunoprecipitates were collected by a brief centrifugation and washed twice with high-salt buffer (1 M NaCl, 10 mM Tris-HCl [pH 8.0], 0.2% NP-40), once with low-salt buffer (0.1 M NaCl, 10 mM Tris-HCl [pH 8.0], 0.2% NP-40), six times with radioimmunoprecipitation assay buffer, and finally four times with Us3 kinase buffer (50 mM Tris-HCl [pH 7.5].)
9.0], 20 mM MgCl₂, 0.1% NP-40, and 1 mM dithiothreitol). Thereafter, they were divided into two aliquots. Immunoprecipitates in one aliquot were analyzed by immunoblotting with anti-Us3 beads containing immunoprecipitated Us3 protein kinase and amylose beads and used as substrates in in vitro kinase assays with purified wild-type GST-Us3 or the kinase-negative mutant GST-Us3K220M (Fig. 4A). As shown in Fig. 3C, MBP-Us3-P4 was labeled with [γ-³²P]ATP in kinase assays using GST-Us3 (Fig. 3C, lane 1), while MBP-Us3-P4-S147A was not (Fig. 3C, lane 2). When the kinase-negative mutant GST-Us3K220M was used, none of the MBP fusion proteins were labeled (Fig. 3C, lanes 3 and 4). To confirm that MBP-Us3-P4 labeling by GST-Us3 was due to phosphorylation, the labeled MBP-Us3-P4 was treated with λ-PPase. As shown in Fig. 3E, MBP-Us3-P4 labeling by GST-Us3 was eliminated by phosphatase treatment, indicating that MBP-Us3-P4 was labeled by phosphorylation. The presence of each MBP fusion protein and the radiolabeled MBP-Us3-P4 band was verified by Coomassie brilliant blue (CBB) staining (Fig. 3B and D). These results indicate that Ser-147 of Us3 is one of the in vitro autophosphorylation sites.

**RESULTS**

Identification of the in vitro autophosphorylation site of Us3 as Ser-147. As a first step toward investigation of the regulation of Us3 by phosphorylation, we attempted to identify the in vitro autophosphorylation site(s) of Us3. Based on the consensus Us3 phosphorylation site sequence, we identified a putative autophosphorylation site at Us3 codons 144 to 149 (RRR SRD) (Fig. 3A). To confirm that Ser-147 of Us3 is in fact the autophosphorylation site in vitro, we generated and purified chimeric proteins consisting of MBP fused to peptides encoded by Us3 codons 1 to 172 (MBP-Us3-P4) and its mutant MBP-Us3-P4-S147A in which alanine was substituted for Ser-147 (Fig. 3A). The MBP fusion proteins were captured on amylose beads and used as substrates in in vitro kinase assays with purified wild-type GST-Us3 or the kinase-negative mutant GST-Us3K220M (Fig. 4A). As shown in Fig. 3C, MBP-Us3-P4 was labeled with [γ-³²P]ATP in kinase assays using GST-Us3 (Fig. 3C, lane 1), while MBP-Us3-P4-S147A was not (Fig. 3C, lane 2). When the kinase-negative mutant GST-Us3K220M was used, none of the MBP fusion proteins were labeled (Fig. 3C, lanes 3 and 4). To confirm that MBP-Us3-P4 labeling by GST-Us3 was due to phosphorylation, the labeled MBP-Us3-P4 was treated with λ-PPase. As shown in Fig. 3E, MBP-Us3-P4 labeling by GST-Us3 was eliminated by phosphatase treatment, indicating that MBP-Us3-P4 was labeled by phosphorylation. The presence of each MBP fusion protein and the radiolabeled MBP-Us3-P4 band was verified by Coomassie brilliant blue (CBB) staining (Fig. 3B and D). These results indicate that Ser-147 of Us3 is one of the in vitro autophosphorylation sites.

PKA also phosphorylates Ser-147 of Us3 in vitro. An earlier report indicated that Us3 and PKA phosphorylate the same site of their target protein in vitro (1). These observations led us to test whether PKA also phosphorylates Ser-147 of Us3 in vitro. As shown in Fig. 2B, MBP-Us3-P4 was labeled with [γ-³²P]ATP in kinase assays using purified PKA (Fig. 2B, lane 1), while MBP-Us3-P4-S147A was not (Fig. 2B, lane 2). That this labeling was due to PKA was implied by the observation that MBP-Us3-P4 was not labeled in the presence of a specific inhibitor of PKA, 6-22 amide (Fig. 2D). Thus, MBP-Us3-P4 labeling by PKA was eliminated by phosphatase treatment (Fig. 2F), indicating that MBP-Us3-P4 was labeled by phosphorylation. The presence of each MBP fusion protein and the radiolabeled MBP-Us3-P4 band was verified by CBB staining (Fig. 2A, C, and E). These results indicate that PKA phosphorylates Ser-147 of Us3 in vitro.

The observation that Us3 and PKA phosphorylate the same site of the target protein in our in vitro kinase assays raised the slim possibility that contamination with insect PKA during the purification of GST-Us3 proteins might have been responsible for the protein kinase activity detected using purified GST-Us3. To exclude this possibility, purified GST-Us3 was treated with the specific PKA inhibitor 6-22 amide, followed by addition of the substrate (MBP-Us3-P4). The results (Fig. 2H) indicate that 6-22 amide at a concentration sufficient to completely inhibit PKA...
FIG. 3. (A) Schematic diagram of the genome structures of wild-type virus HSV-1(F) and the location of the Us3 gene. Line 1, linear representation of the HSV-1(F) genome. The unique sequences are represented as unique long (UL) and short (US) domains, and the terminal repeats flanking them are shown as open rectangles with the designation above each repeat. Line 2, structure of the genome domain containing the US2, US3, and US4 ORFs. Line 3, structure of the US3 ORF. The shaded areas represent subdomains I to VI, which are conserved in eukaryotic protein kinases. Line 4, the domains of the US3 gene encoding US3 residues 98 to 364, used in these studies to generate GST-US3-P1 fusion proteins which were used for generation of the rabbit polyclonal antibody to US3. Lane 5, the domains of the US3 gene encoding US3 residues 1 to 172, used in these studies to generate MBP-US3-P4 fusion protein. Line 6, amino acid sequence of US3 residues 135 to 160. Sites with the consensus sequence for phosphorylation by US3 itself are underlined. Line 7, domains of the US3 gene encoding US3 residues 1 to 172 carrying the S147A mutation used in these studies to generate MBP-US3-P4-S147A fusion proteins. (B) Purified MBP-US3-P4 (lanes 1 and 3) and MBP-US3-P4-S147A (lanes 2 and 4) incubated in kinase buffer containing [γ-32P]ATP and purified GST-US3 (lanes 1 and 2) or GST-US3K220M (lanes 2 and 4), separated on a denaturing gel, and stained with CBB. Molecular masses are shown on the left. (C) Autoradiograph of the gel in panel B. (D) Purified MBP-US3-P4 incubated in kinase buffer containing [γ-32P]ATP and purified GST-US3 and then either mock treated (lane 1) or treated with λ-PPase (lane 2), separated on a denaturing gel, and stained with CBB. (E) Autoradiograph of the gel in panel D.
FIG. 4. (A) Schematic diagram of the predicted amino acid sequences of GST-Us3 and its mutants GST-Us3K220M and GST-Us3S147A. The K220M and S147A mutations are also indicated. (B) A silver-stained gel (left panel) and an immunoblot (right panel) of purified GST-Us3S147A, GST-Us3, and GST-BGLF4 from Sf9 cells infected with recombinant baculovirus Bac-GST-Us3S147A (lanes 1), Bac-GST-Us3 (lanes 2), and

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Generation of a recombinant virus expressing VenusA206K-U3S147A and the repaired virus. Next, we investigated whether Ser-147 of Us3 is phosphorylated in HSV-1-infected cells. To this end, we attempted to detect Ser-147 phosphorylation of Us3 immunoprecipitated by specific antibody from wild-type HSV-1(F)-infected cells, using the rabbit anti-phospho-PKA substrate monoclonal antibody 100G7. The latter antibody detects proteins containing a phosphorylated serine or threonine residue with arginine at positions −3 and −2 (RRXS or RRXT). In the Us3 polypeptide, the antibody theoretically recognizes only phosphorylated Ser-147. In preliminary experiments, however, we determined that phosphorylation of Us3 immunoprecipitated by rabbit polyclonal antibody from lysates of HSV-1(F)-infected cells was difficult to detect with the anti-phospho-PKA substrate antibody (data not shown). Because the anti-Us3 and the anti-phospho-PKA substrate antibodies were both from rabbits, the secondary antibody against the anti-phospho-PKA antibody in immunoblotting also detected the heavy and light chains of the anti-Us3 antibodies. This resulted in such a high background in the immunoblots that we were unable to discern phosphorylated Us3 protein with the anti-phospho-PKA substrate antibody. To resolve this problem, we generated a recombinant virus, YK503, expressing VenusA206K-tagged Us3 proteins carrying the S147A mutation, and YK505, expressing VenusA206K-tagged Us3, in which the alanine replacement of Ser-147 in YK503 had been repaired. Us3 proteins fused to VenusA206K could then be immunoprecipitated with commercial anti-GFP rat monoclonal antibody. This antibody reacts with the secondary antibody for the detection of the anti-phospho-PKA monoclonal rabbit antibody to a much lesser extent than does the anti-Us3 polyclonal rabbit antibody. Furthermore, VenusA206K-U3s (92 kDa) migrated much more slowly than nontagged Us3 (65 kDa) in denaturing gels. Accordingly, detection of VenusA206K-U3s with the anti-phospho-PKA substrate antibody was much less affected by cross-reaction of the secondary antibody with the heavy chains of the anti-GFP antibody (50 kDa) in immunoblotting. With this system, we could now easily detect Ser-147 phosphorylation of immunoprecipitated VenusA206K-U3s using the anti-phospho-PKA antibody. The strategy for construction of YK503 and YK505 is summarized in Fig. 1. The genotypes of YK502, YK503, YK504, and YK505 were confirmed by Southern blotting and sequencing (data not shown). Expression of the predicted fusion proteins was confirmed by infecting Vero cells with each of the recombinant viruses at an MOI of 3 for 18 h and then assaying the infected
cells for the appropriate fusion proteins by immunoblotting with the anti-Us3 antibody. As shown in Fig. 5A, the anti-Us3 antibody reacted with wild-type Us3 (lane 2), VenusA206K-Us3 (lane 4), and VenusA206K-Us3-repair (lane 5) but not with lysates from cells infected with the Us3 deletion mutant virus (lane 3). The expression levels of VenusA206K-Us3 3S147A mutant protein in Vero cells were similar to those of the proteins from wild-type HSV-1(F) and the recombinant viruses YK304 and YK503, encoding nontagged and tagged wild-type Us3, respectively. These results indicate that neither tagging VenusA206K with Us3 nor the S147A mutation in Us3 has any influence on the accumulation of Us3 proteins in infected cells.

FIG. 5. (A) Immunoblots of electrophoretically separated lysates of Vero cells mock infected (lane 1) or infected with wild-type HSV-1(F) (lane 2), R7041 (ΔUs3) (lane 3), YK503 (VenusA206K-Us3S147A) (lane 4), or YK505 (VenusA206K-Us3-repair) (lane 5) at an MOI of 3. The infected Vero cells were harvested at 18 h postinfection and subjected to immunoblotting with polyclonal antibody to Us3 (upper panel) or VP22 (lower panel). (B) Immunoblots of electrophoretically separated lysates of Vero cells mock infected (lane 1) or infected with YK304 (wild type) (lane 2), YK510 (ΔUs3) (lane 3), YK511 (Us3K220M) (lane 4), or YK513 (repair) (lane 5) at an MOI of 3. The infected Vero cells were harvested at 18 h postinfection and immunoblotted with polyclonal antibody to Us3 (upper panel) or VP22 (lower panel). (C) Immunoblots of electrophoretically separated lysates of Vero cells mock infected (lane 1) or infected with HSV-1(F) (wild type) (lane 1), YK304 (wild type) (lane 2), or YK515 (Us3S147A) (lane 3). The infected Vero cells were harvested at 18 h postinfection and immunoblotted with polyclonal antibody to Us3.

Next, Vero cells were infected with wild-type YK304, YK503 (VenusA206K-Us3S147A), or YK505 (VenusA206K-Us3-repair) at an MOI of 3, and the total virus yield from the cell culture supernatants and the infected cells was harvested at the indicated time points (Fig. 6A). The titers of each sample were determined by standard plaque assays on Vero cells. As shown in Fig. 6A, the growth curve of YK503 was essentially identical to that of YK505, indicating that Ser-147 and its phosphorylation are not critical for optimal viral replication in Vero cells. The FP-tagged recombinant viruses (YK303 and YK505) displayed growth characteristics similar to those of wild-type YK304 but produced a slightly (two- to threefold) lower progeny yield than YK304 (Fig. 6A). Because the growth of the
FP-tagged recombinant virus (YK505) was not appreciably affected by fusing the Us3 protein with VenusA206K and because the other phenotypes of YK505 (VenusA206K-Us3-repair) tested in the present studies were identical to those of wild-type YK304 (Fig. 5; see Fig. 9 and 10), it is likely that tagging VenusA206K with Us3 has little effect on the functions of Us3 in infected cells.

Ser-147 of Us3 is phosphorylated in infected cells. To investigate whether Ser-147 of Us3 was phosphorylated in infected cells, Vero cells were infected with YK503 expressing VenusA206K-Us3S147A or with YK505 expressing VenusA206K-Us3-repair at an MOI of 3, harvested at 18 h postinfection, solubilized, immunoprecipitated with the anti-GFP antibody, and analyzed by immunoblotting with anti-Us3 antibody (left panel) or anti-phospho-PKA substrate antibody (right panel). As shown in Fig. 7, the anti-phospho-PKA substrate antibody reacted with VenusA206K-Us3-repair purified from YK505-infected Vero cells but not with VenusA206K-Us3S147A purified from YK503-infected Vero cells. The expression levels of total Us3 proteins in Vero cells infected with YK505 were equivalent to those in cells infected with YK503 (VenusA206K-Us3-repair) or with YK505 expressing VenusA206K-Us3-repair at an MOI of 3, harvested at 18 h postinfection, solubilized, immunoprecipitated with the anti-GFP antibody, and finally analyzed by immunoblotting with the anti-phospho-PKA substrate antibody or anti-Us3 antibody. As shown in Fig. 7A, the anti-phospho-PKA substrate antibody reacted with VenusA206K-Us3-repair purified from YK505-infected Vero cells but not with VenusA206K-Us3S147A purified from YK503-infected Vero cells. The expression levels of total Us3 proteins in Vero cells infected with YK505 were equivalent to those in cells infected with YK503. To confirm that the VenusA206K-Us3-repair product detected by the anti-phospho-PKA substrate antibody was due to phosphorylation, material purified by immunoprecipitation was treated with λ-PPase. As shown in Fig. 7B, the reactivity of VenusA206K-Us3-repair with the anti-phospho-PKA substrate antibody was eliminated by phosphatase treatment, indicating that the antibody did
Indeed, these results indicate that the anti-phospho-PKA substrate antibody specifically recognized the phosphorylated Ser-147 of Us3 and that this residue is phosphorylated in infected cells.

The S147A mutation in Us3 affects enzyme localization in infected cells. An earlier publication by Ryckman and Roller reported that in Vero cells infected with a recombinant HSV-1 encoding enzymatically inactive Us3, the mutant Us3 protein was aberrantly localized to large, punctate structures distributed throughout the cells, indicating that the protein kinase activity of Us3 regulates its localization (56). This observation, together with our results that Ser-147 of Us3 is required for optimal kinase activity of Us3 in vitro, led us to examine the localization of the Us3S147A mutant in infected cells more closely. However, Reynolds et al. had noted that the anti-Us3 antibody used in earlier studies had a consistent background fluorescence in the cytoplasm of cells infected with HSV-1 Us3 null mutants in immunofluorescence assays (52), making it difficult to draw conclusions about the exact localization of Us3. Consistent with this, the anti-Us3 antibody generated in the present study also showed such background fluorescence in some cell lines infected with the Us3 deletion mutant virus (R7041) (data not shown). Therefore, we investigated the effect of the S147A mutation in Us3 on localization of this molecule in infected cells using recombinant viruses (YK503 and YK505) encoding FP-tagged Us3. Because Us3 was tagged with FP in YK503 and YK505, the specific subcellular localization of Us3 proteins in live infected cells could easily be investigated without immunofluorescence assays relying on anti-Us3 antibodies. Vero cells were mock infected or infected with YK503 (VenusA206K-Us3S147A) or YK505 (VenusA206K-Us3-repair) at an MOI of 10 or 12 h and then examined by confocal microscopy. As shown in Fig. 8, the VenusA206K-Us3-repair protein was detected mainly in the cytoplasm of YK505-infected Vero cells, and as infection progressed, it accumulated preferentially at the juxtanuclear regions (Fig. 8b, d, f, and h). In contrast, when Vero cells were infected with YK503, Us3 localization differed significantly from that in YK505-infected Vero cells. In Vero cells infected with YK503, the VenusA206K-Us3S147A protein was aberrantly localized to large, punctate structures in the cytoplasm, the appearance of which was strongly reminiscent of those reported earlier by Ryckman and Roller (56). The number of these punctate structures increased as infection progressed (Fig. 8a, c, e, and g). These results indicate that Ser-147 of Us3 is necessary for the correct localization of the enzyme in infected cells.

The S147A mutation in Us3 affects the ability of the protein to induce wild-type CPEs in infected cells. It has been reported that the CPEs of Us3 deletion mutant viruses of HSV-1 and HSV-2 are clearly different from those of wild-type viruses (36, 38, 48), indicating that Us3 influences the morphology of infected cells. To confirm this, Vero cells were infected with wild-type HSV-1(F), R7041 (ΔUs3), and R7306 (repair) at an MOI of 10 for 24 h, and then CPEs of infected cells were observed by confocal microscopy. Consistent with the previous reports, CPEs of R7041 (ΔUs3) were apparently different from those of wild-type HSV-1(F) or R7306 (repair) (Fig. 9A). Infection of Vero cells with wild-type HSV-1(F) or R7306 (repair) efficiently induced cell rounding, while R7041 (ΔUs3) did so only partially. Next, we examined whether the ability of Us3 to induce wild-type CPEs is associated with catalytic activity of Us3, because this issue has not been addressed. To this end, we generated three Us3 mutant viruses, i.e., YK510 (ΔUs3), YK511 (Us3K220M), and their repaired version, YK513 (Fig. 1). YK510 is a Us3 null mutant, whereas YK511 encodes the enzymatically inactive Us3 mutant Us3K220M (17), in which the lysine codon at position 220 is replaced by methionine. The genotypes of YK510, YK511, and YK513 were confirmed by Southern blotting and sequencing (data not shown). Expression of the predicted proteins was confirmed by infecting Vero cells with each of the recombinant viruses and assaying the infected cells for the appropriate proteins by immunoblotting (Fig. 5B). As shown in Fig. 9B, CPEs in Vero cells infected with YK511 (Us3K220M) were similar to those in cells infected with YK510 (ΔUs3), with both viruses only partially inducing cell rounding. In contrast, wild-type HSV-1(F), YK304, and YK513 (repair) efficiently induced cell rounding. These results indicate that the catalytic function of the Us3 protein is necessary for induction of wild-type CPEs in infected cells.

Next, we examined whether the S147A mutation in Us3...
FIG. 9. Digital confocal microscope images of CPEs in Vero cells infected at an MOI of 10 with (A) wild-type HSV-1(F) (a), R7041 (ΔUs3) (b), or R7306 (repair) (c); (B) wild-type HSV-1(F) (a), wild-type YK304 (b), YK510 (ΔUs3) (c), YK511 (Us3K220M) (d), or YK513 (repair) (e); (C) YK503 (VenusA206K-Us3S147A) (a) or YK505 (VenusA206K-Us3-repair) (b); and (D) wild-type YK304 (a), YK511 (Us3K220M) (b) YK515 (Us3S147A) (c), or YK517 (repair) (d). The live cells were examined at 24 h postinfection by confocal microscopy. Differential interference contrast (DIC) (A, B, and D) and fluorescence (C) images are shown.
affects the ability of the protein to induce wild-type CPEs. As shown in Fig. 9C, CPEs in Vero cells infected with YK503 (VenusA206K-Us3S147A) resembled those in cells infected with the Us3 mutant viruses YK510 (ΔUs3), R7041 (ΔUs3), and YK511 (Us3K220M) (Fig. 9A and B). In contrast, YK505 (VenusA206K-Us3-repair) induced cell rounding efficiently in Vero cells, as observed with the wild-type viruses HSV-1(1-F) and YK304 (Fig. 9A and B).

We also generated an additional Us3 mutant virus (YK515) in which Ser-147 of nontagged Us3 was replaced with alanine (Fig. 1). The repaired version was also created (YK517) (Fig. 1). The genotypes of YK515 and YK517 were confirmed by Southern blotting and sequencing (data not shown). We then examined the effect of the SI47A mutation in nontagged Us3 on the ability of the protein to induce wild-type CPEs. As shown in Fig. 9D, the results obtained with YK515 (Us3SI47A) and YK517 (repair) were consistent with those obtained with FP-tagged viruses (Fig. 9C). These results indicate that Ser-147 of Us3 is required for induction of wild-type CPEs in infected cells. We also examined growth of YK515 in Vero cells at an MOI of 3 or 0.01 and the level of expression of Us3 in infected cells. Vero cells infected with wild-type YK304, R7041 (ΔUs3), YK511 (Us3K220M), YK503 (VenusA206K-Us3SI47A), YK505 (VenusA206K-Us3-repair), or YK515 (Us3SI47A) at an MOI of 10, fixed at 15 h postinfection, and processed for immunofluorescence with anti-UL34 antibody. As shown in Fig. 10E and F, the UL34 protein localized mainly at the nuclear envelope with a uniform distribution in cells infected with HSV-1(1-F) (wild type), YK304 (wild type), YK503 (VenusA206K-Us3SI47A), YK505 (VenusA206K-Us3-repair), or YK515 (Us3SI47A), while it was mislocalized to punctate structures as reported previously (51, 56) in cells infected with the Us3 mutant virus R7041 (ΔUs3) or YK511 (Us3K220M). These results indicate that Ser-147 and phosphorylation of Ser-147 are not required for proper localization of UL34 in infected cells.

The SI47A mutation in Us3 does not affect the total Us3 protein kinase activity in infected cells. Finally, we investigated protein kinase activity of Us3 carrying the SI47A mutation in infected cells. Vero cells infected with wild-type YK304, YK515 (Us3SI47A), or YK511 (Us3K220M) at an MOI of 3 were harvested at 18 h postinfection, solubilized, and immunoprecipitated with the anti-Us3 antibody. The immunoprecipitates were then used in in vitro kinase assays with purified MBP-UL34 as a substrate. To reduce the possibility that the anti-Us3 antibody might coprecipitate a contaminating kinase(s), the precipitated material containing Us3 protein kinase was washed with a high-salt buffer containing 1 M NaCl prior to the assays. As shown in Fig. 11A, MBP-UL34 was labeled with [γ-32P]ATP in the presence of Us3SI47A mutant protein immunoprecipitated from YK515-infected cells at a level similar to that in the presence of wild-type Us3 protein immunoprecipitated from wild-type YK304-infected cells. In contrast, MBP-UL34 was not labeled in the presence of the kinase-negative mutant Us3K220M protein immunoprecipitated from YK511-infected cells. The labeling of Us3 proteins was due to phosphorylation, as determined by studies showing that it was eliminated by phosphatase treatment (Fig. 11B). The presence of MBP-UL34 protein and the radiolabeled MBP-UL34 band was verified by Ponceau S staining. Similar results were also observed with the FP-tagged recombinant viruses YK503 (VenusA206K-Us3SI47A) and YK505 (VenusA206K-Us3-repair) (data not shown). These results indicate that the total protein kinase activity of the Us3SI47A mutant in infected cells is similar to that of wild-type Us3.

DISCUSSION

HSV-1 possesses at least three protein kinases encoded by the UL13, Us3, and UL39 genes. The amino acid sequence of
Us3 is conserved in the *Alphaherpesvirinae* subfamily, while UL13 is conserved throughout all herpesvirus subfamilies (5, 31, 58). In *Alphaherpesvirinae*, only HSV-1 and HSV-2 have a third viral protein kinase, RR1, encoded by the UL39 gene, in addition to Us3 and UL13 (20). Thus, every herpesvirus encodes at least one viral protein kinase, and these have been reported to play multiple roles in viral replication by regulation of viral gene expression (49), apoptosis (28, 43), encapsidation (64), nuclear egress of nucleocapsid (13, 24, 52), and replication of viral genomes (64). While downstream events of her-
Herpesviral protein kinases have been extensively investigated, there is a dearth of information on any herpesviral protein kinases regarding how their activity is regulated. The question we have posed in the studies reported here is whether the activity of the Us3 protein kinase is regulated by phosphorylation. The salient features of these studies are as follows.

(i) Ser-147 of Us3 is phosphorylated in vitro and in infected cells. In the studies presented here, bioinformatics analysis predicted a putative autophosphorylation site of Us3 at codons 144 to 149 (RRRSDR), and in vitro kinase assays confirmed that Us3 phosphorylates Ser-147 of Us3. Consistent with the previous report that the activity of Us3 kinase functionally overlaps that of PKA in vitro and in infected cells (1), we have also demonstrated that PKA phosphorylated Ser-147 of Us3 in vitro. It is known that some protein kinases are somewhat promiscuous in vitro if allowed sufficient reaction time and provided with enough substrate. Therefore, in vitro phosphorylation, although a necessary characteristic, is not sufficient for defining a new phosphorylation site; thus, the site identified in vitro must be shown to be a physiological phosphorylation site in cells in vivo. To this end, we used an anti-phospho-PKA substrate antibody which recognizes phosphorylated Ser or Thr residues in the context of the sequence RRXS or RRXT. Phospho-substrate antibodies have been extremely useful for identifying and characterizing phosphorylation sites of new substrates over the past several years (29). In the studies presented here, we have demonstrated that the anti-phospho-PKA substrate antibody specifically reacted with phosphorylated Ser-147 of Us3 purified by immunoprecipitation from infected cells. This indicates that Ser-147 of Us3 is phosphorylated in infected cells and therefore fulfills the requirements for a physiological phosphorylation site. At present, the protein kinase(s) responsible for the phosphorylation of Ser-147 of Us3 in infected cells is unknown, although in vitro assays suggest that Us3 itself and PKA are involved. As mentioned above, in vitro phosphorylation often does not reflect in vivo phosphorylation, and the sequence flanking Ser-147 of Us3 also matches the consensus phosphorylation sites for other protein kinases, including a set of protein kinases in the AGC protein kinase family, such as protein kinase C, protein kinase G, and p21 activation kinase 2 (23, 62). Further studies will be needed to clarify which protein kinase(s) is responsible for the phosphorylation of Us3 Ser-147 in infected cells.

(ii) The S147A mutation attenuated the activity of Us3 in vitro and affected some but not all Us3 functions in infected cells. It is well established that activity of cellular protein kinases is controlled by phosphorylation of the enzymes (55). Consistent with this, we have demonstrated that the protein kinase activity of the GST-Us3S147A mutant generated in a baculovirus expression system was much lower than that of wild-type GST-Us3 in vitro (Fig. 4), indicating that optimal kinase activity of the GST-Us3S147A mutant generated in a baculovirus expression system was much lower than that of wild-type GST-Us3 in vitro (1), we have shown that Us3 from recombinant viruses carrying the S147A mutation in Us3 failed to localize correctly (Fig. 8) and did not alter the morphology of cells like the wild-type virus (Fig. 9), two activities both requiring the catalytic activity of Us3 (Fig. 9) (56). Together with the observation that Ser-147 is the physiological phosphorylation site of Us3, this makes it reasonable to conclude that phosphorylation of Us3 Ser-147 is the event that regulates the optimal kinase activity of this enzyme and the manifestations of its catalytic activity in infected cells. However, we cannot formally exclude the slim possibility that the phenotypes observed with recombinant viruses carrying the S147A mutation in Us3 are due to steric hindrance of Us3 caused by the amino acid replacement, rather than prevention of phosphorylation.

(iii) HSV-1 Us3 has been reported to encode two transcriptional units directing the synthesis of the Us3 and Us3.5 proteins (44, 53). The Us3.5 protein is a truncated form of Us3.
protein and is expressed in wild-type HSV-1-infected cells at a much lower level than Us3 protein (46). Like Us3 protein kinase, Us3.5 mediates posttranslational modification of HDAC1, HDAC2, the PKA regulatory II subunit, and UL31, suggesting that Us3.5 protein is a protein kinase (44). In contrast, these proteins differ with respect to their functions in blocking apoptosis and in nuclear egress of progeny nucleocapsids (44). Since the physiological phosphorylation site (Ser-147) in Us3 identified in this study is present in Us3.5 protein, it is possible that phosphorylation of Ser-147 also controls regulatory activities of Us3.5. Further studies, such as studies of the effects of Ser-147 mutation in Us3.5 on its protein kinase activity in vitro and its functions in infected cells, are of interest and are under way in this laboratory.

(iv) It appears that Ser-147 in Us3 is conserved among HSV-1 strains, whereas the residue is not among other alphaherpesvirus Us3 homologues and even the HSV-2 Us3 protein lacks a comparable serine residue (Fig. 12). This suggests that the phosphorylation of Us3 at Ser-147 and its relevance in viral replication are specific to HSV-1 and are not applicable to Us3 homologues of other alphaherpesviruses. However, we note that the amino acid residue in Us3 of HSV-2 strain HG52 corresponding to HSV-1 Us3 Ser-147 is aspartic acid (Asp-147) (Fig. 12), and Asp-147 in Us3 appears to be conserved among HSV-2 strains because Asp-147 is also present in Us3 of HSV-2 strain G (data not shown). It is known that acidic amino acids such as Asp or glutamic acid sometimes mimic the negative charges exerted by phosphorylation (65). Therefore, it would be interesting to investigate whether Asp-147 in HSV-2 Us3 plays roles in its proper localization and induction of the wild-type morphological change in HSV-2-infected cells.

(v) The total protein kinase activity of Us3 purified by immunoprecipitation from cells infected with the recombinant virus YK503 or YK515 carrying the SI47A mutation in Us3 was similar to that from cells infected with recombinant viruses expressing wild-type Us3 (YK505 and YK304). These observations appeared to contradict the result that Ser-147 is critical for optimal protein kinase activity of Us3 in vitro. One hypothesis to account for this discrepancy is that only a small fraction of Us3 molecules are phosphorylated at Ser-147 at any one time, and another is that phosphorylation of the majority does take place but is transient in HSV-1-infected cells. Thus, phosphorylation of Us3 on Ser-147 is presumably tightly regulated in HSV-1-infected cells and occurs transiently or only in a limited subcellular domain(s). Therefore, the majority of Us3 in HSV-1-infected cells would be unphosphorylated on Ser-147 at any one time, resulting in little difference in the total protein kinase activity between wild-type Us3 from cells infected with wild-type viruses and Us3S147A mutants from cells infected with YK503 or YK515. This hypothesis is supported by the finding that only some of the Us3 functions examined here, including regulation of localization of Us3 itself and the morphology of infected cells, were affected by the S147A mutation, while others, including regulation of localization of UL34 and modification of UL31 and HDAC2, were not. Probably overexpression of large amounts of GST-Us3 in the baculovirus expression system in insect cells resulted in a different phosphorylation status of Us3 proteins than for the majority of Us3 expressed in mammalian cells infected with HSV-1. Consistent with this possibility, the phosphorylation status of GST-Us3 expressed in the baculovirus expression system was clearly different from that of GST-Us3S147A (Fig. 4C), while the phosphorylation status of Us3 from Vero cells infected with the wild-type viruses, detected in a denaturing gel, could not be differentiated from that of Us3S147A in Vero cells infected with YK515 (Fig. 5D). Thus, GST-Us3 may be efficiently phosphorylated at Ser-147 in insect cells infected with the recombinant baculovirus, resulting in significant differences in protein kinase activity between GST-Us3 and GST-Us3S147A. Further studies, such as time-space tracking of phosphorylation of Us3 on Ser-147 in HSV-1-infected cells by using antibodies that specifically recognize phosphorylated Ser-147 of Us3 and identification of the protein kinase(s) responsible for this phosphorylation, will be needed and are in progress in this laboratory. Such information will provide further insights into the important issue of how Us3 functions are regulated.

ACKNOWLEDGMENTS

We thank B. Roizman for R7041, R7306, and pBC1013; R. Roller for chicken polyclonal antibody to UL34; A. Miyawaki for Venus/ pRS2-lo-LoaG, A. Ioannou for pGE128/C; G. A. Smith for E. coli GS1783; N. Osterrieder for E. coli GS1783 containing pYEBac102 and pEPkan-S; and S. Watanabe for HELs. We thank S. Koyama for excellent technical assistance.

This study was supported in part by Grants for Scientific Research and Grants for Scientific Research in Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan. A.K. and R.A. were supported by a Research Fellowship of the Japanese Society for the Promotion of Science for Young Scientists.

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