US3 protein kinase of herpes simplex virus type 2 is required for the stability of the UL46-encoded tegument protein and its association with virus particles

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The herpes simplex virus (HSV) US3 gene encodes a serine/threonine protein kinase (PK). Although US3 PK is not essential for virus replication in cell culture, it plays an important role in the regulation of apoptosis in infected cells. However, the role of US3 PK in virus replication and pathogenicity is not well understood. The UL46 gene encodes virion tegument phosphoproteins, the properties and functions of which are poorly understood. In this study, it is shown that the UL46 protein of HSV type 2 (HSV-2) is affected strikingly by the presence of US3 PK. In the absence of US3 PK, UL46 protein was quite unstable, being much more susceptible to degradation. UL46 protein was undetectable in the extracellular virions of US3-deficient virus. Moreover, in vitro kinase assays using recombinant US3 PK show that UL46 protein is phosphorylated by the US3 PK, suggesting that UL46 can be a direct substrate for US3 PK in infected cells. Together, these findings shed new light on the physiological functions of US3 PK.

INTRODUCTION

Herpes simplex virus (HSV) is a large, enveloped virus, the genome of which comprises at least 74 different genes (Dolan et al., 1998; Roizman & Knipe, 2001). Recent studies have shown that approximately half of these genes are not essential for virus replication in cell culture (Roizman & Knipe, 2001). However, these dispensable gene products are thought to be important for viral growth and spread in the natural host (Nishiyama, 2004). Upon infection, HSV induces the production of many of its own enzymes in infected cells. At least three HSV genes (US3, UL13 and the N-terminal domain of UL39) encode a protein kinase (PK). Of these, US3 was the first to be identified as an HSV-specific serine/threonine protein kinase (Daikoku et al., 1993; Frame et al., 1987; Purves et al., 1987). The US3 PK of HSV-2 has an apparent molecular mass of 66 kDa and has autophosphorylation activity. The enzyme shows optimal activity between pH 9.0 and 9.5 and preferentially phosphorylates substrates that are rich in basic amino acid residues (Daikoku et al., 1993). Some viral proteins, UL34, UL12 and US9, have been implicated as substrates of US3 PK (Daikoku et al., 1994, 1995; Purves et al., 1987, 1991; Ryckman & Roller, 2004). Although the US3 gene is non-essential for virus replication in cell culture and for the establishment of latency, it is important for viral pathogenesis in mice (Kurachi et al., 1993; Mori et al., 2003; Nishiyama et al., 1992). Additionally, US3 was reported to play a role in protecting cells from virus-induced apoptosis (Asano et al., 1999; Galvan & Roizman, 1998; Leopardi et al., 1997; Nishiyama & Murata, 2002). In spite of these observations, the role of US3 PK in virus replication and pathogenicity is not fully understood.

According to its nucleotide sequence, the HSV type 2 (HSV-2) UL46 gene is predicted to encode a protein of 722 aa (Dolan et al., 1998). We have shown that the HSV-2 UL46 gene products are phosphoproteins with apparent molecular masses ranging from 82 to 86 kDa, produced during the late phase of infection. It has also been reported that the UL46 gene of HSV type 1 (HSV-1) is dispensable for replication in cell culture (McKnight et al., 1987) and that an HSV-1 DNA fragment containing the UL46 gene enhances the efficiency of VP16 (α-trans-inducing factor or α-TIF)-mediated α gene expression in transient-expression assays (McKnight et al., 1987). However, deletion of the UL46 gene has no apparent effect on the ability of VP16 to induce the α-regulated thymidine kinase (TK) reporter gene that is resident in 143TK− cells (Zhang et al., 1991). Furthermore, analysis of purified virions obtained from the UL46 deletion mutant showed that UL46 encodes the virion tegument phosphoproteins VP11 and VP12.

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(VP11/VP12) (Zhang & McKnight, 1993). In this paper, we demonstrate that the properties of the HSV-2 UL46 gene products are affected markedly by the presence of US3 and that the UL46 protein is phosphorylated by recombinant US3 PK in vitro.

**METHODS**

**Cells and viruses.** Vero cells, a stable line of African green monkey kidney cells, were propagated in Eagle's minimal essential medium (MEM) supplemented with 5% calf serum, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Human HEP-2 cells and the monkey kidney cell line COS-1 were propagated in Eagle's modified MEM supplemented with 5% fetal calf serum, 100 U penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Wild-type (186), US3-deficient (L1BR1) and US3- rescued (L1B 11) HSV-2 were propagated and titered on Vero cells as described previously (Nishiyama et al., 1992). Vero cells were infected at an m.o.i. of 3 p.f.u. per cell.

**Construction of plasmids.** For expression of UL46 and US3, expression plasmids were constructed as described previously (Goshima et al., 1998; Kato et al., 2000). To construct pMAL-UL46, cleavage of pcDNA-UL46 with EcoRI and XhoI released the UL46 open reading frame and this DNA fragment was ligated into the multicloning site of pMAL-c (New England Biolabs) in frame with maltose-binding protein (MBP).

**Western blotting.** Proteins were transferred electrophoretically from SDS-PAGE gels to PVDF membranes as described by Towbin et al. (1979). Respective bound primary antibodies were detected by using peroxidase-labelled goat anti-rabbit IgG (BioSource) and ECL Western blotting detection reagents (Amersham Biosciences).

**Metabolic labelling, chase, immunoprecipitation and electrophoresis.** Metabolic labelling was performed as follows. After two washes with methionine-free MEM, cells were preincubated for 30 min at 37°C in methionine-free MEM containing 5% calf serum and labelled for 30 min with 0.2 mCi (7.4 MBq) [³⁵S]methionine ml⁻¹. Labelled cells were chased for various periods of time in complete medium. The chase was terminated by placing the cells on ice. The cells were washed with ice-cold PBS and solubilized in 2 ml ice-cold RIPA buffer [10 mM Tris/HCl (pH 7.4), 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA]. After 1 mM PMSF and 10 µg aprotinin ml⁻¹ were added as protease inhibitors, the lysates were centrifuged at 5000 r.p.m. to remove any cell debris. After preclearing of the supernatants with 20 µl protein A–agarose (Roche Molecular Biochemicals) (1:1 PBS slurry) for 1 h at 4°C, an appropriate amount of polyclonal antibodies was added and immune complexes were precipitated by incubation with 15 µl protein A–agarose. Immunoprecipitates were washed in lysis buffer five times to remove non-specifically adsorbed proteins. Bound antibodies were eluted from the protein A–agarose beads by boiling in 20 µl 2x SDS sample buffer [125 mM Tris/HCl (pH 6.5), 20% β-mercaptoethanol, 4% SDS] containing 10% 2-mercaptoethanol. Purified proteins were separated by SDS-PAGE on a 10% gel. After the gel was fixed with 10% methanol and 10% acetic acid and dried, protein bands were visualized by using a Fujix Bio-Imaging analyser BAS3000 system (Fuji Photo Film Co.).

**Virion purification.** Virions were harvested from the extracellular medium at 36 h post-infection (p.i.). After removal of cell debris by low-speed centrifugation, virions were pelleted from the supernatant by centrifugation at 25 000 r.p.m. for 1 h at 4°C. After washing the virions twice with PBS and centrifugation at 15 000 r.p.m. for 30 min at 4°C, the pellet was resuspended in 1 ml PBS. The viral suspension was layered onto 9 ml of a continuous 10–50% sucrose gradient, followed by centrifugation at 20 000 r.p.m. for 1 h at 4°C. Aliquots (1 ml) of peak virion-containing fractions were added to 100 µl trichloroacetic acid and incubated for 30 min on ice. The lysates were pelleted by centrifugation at 15 000 r.p.m. and were resuspended in 50 µl PBS.

**Immunofluorescence assay.** Vero cells were grown on coverslips and either mock-infected or infected with HSV-2 186 or L1BR1 at 3 p.f.u. per cell. At various times after infection, the cells were fixed in cold acetone. Indirect immunofluorescence was detected essentially as described previously, using anti-UL46 rabbit polyclonal antibodies (Kato et al., 2000). For secondary antibodies, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Sigma) was used. Fluorescence images were viewed and recorded with a Bio-Rad MRC series confocal imaging system.

**Generation and purification of recombinant glutathione S-transferase (GST)–US3 and its kinase-negative mutant GST–US3K220M.** Construction of recombinant baculoviruses Bac-GST-US3 and Bac-GST-US3K220M and purification of recombinant proteins are described elsewhere in detail (Kato et al., 2005). Bac-GST-US3 and Bac-GST-US3K220M express wild-type (wt) US3-fused GST and a US3 mutant fused to GST, in which lysine at US3 residue 220 (Lys-220) was replaced with methionine by site-directed mutagenesis. Lys-220 was chosen for mutagenesis because there is an invariant lysine at this position in the known protein kinases, and mutation of this lysine in eukaryotic protein kinases results in loss of kinase activity (Hanks et al., 1988). A kinase-negative mutant, GST–US3K220M, was used as a control. GST-fusion proteins were purified from S9 cells infected with the recombinant baculoviruses (Kawaguchi et al., 2003). Purified GST–US3 and GST–US3K220M contained one major band with an apparent molecular mass of approximately 90 kDa, as detected by silver staining, and these proteins reacted with anti-US3 antisera.

**Production and purification of MBP–UL46 protein expressed in Escherichia coli.** MBP–UL46, a chimeric protein consisting of MBP fused to the UL46 gene product, was expressed in E. coli XL-1 Blue transformed with pMAL-UL46 and purified with amyllose resin (New England Biolabs) as described previously (Kawaguchi et al., 2003).

**In vitro kinase assay.** Specific kinase buffer for US3 [50 mM Tris/ HCl (pH 9.0), 20 mM MgCl₂, 0.1% NP-40, 1 mM dithiothreitol] containing 10 µM ATP, 10 µCi (370 kBq) [γ-³²P]ATP and purified GST–US3 or GST–US3K220M was added to the beads (15 µl) that had captured the MBP-fusion protein and the samples were reacted for 30 min at 30°C. After incubation, the samples were washed extensively with TNE buffer [20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA] and subjected to electrophoresis on a denaturing gel that was then stained with Coomassie brilliant blue (CBB) and exposed to X-ray film.

**Transfection and superinfection.** COS-1 cells were transfected with appropriate expression plasmids according to the DEAE–dextran method (Kawaguchi et al., 2001). In the case of superinfection, the transfected cells were mock-infected or infected with 3 p.f.u. 186 or L1BR1 per cell at 24 h post-transfection. The cells were harvested 24 h p.i.

**RESULTS AND DISCUSSION**

**Identification of a novel target protein of US3 PK**

Through extensive investigation, we and other investigators have identified a number of viral and cellular proteins that demonstrate that the properties of the HSV-2 UL46 gene and that the UL46 protein is phosphorylated by recombinant US3 PK in vitro.
are affected by the deletion of US3 PK in HSV-1- and -2-infected cells (Daikoku et al., 1994, 1995; Hagglund et al., 2002; Murata et al., 2002; Purves et al., 1991; Ryckman & Roller, 2004). During the course of our studies, we noticed that the synthesis of a protein of roughly 80 kDa was affected significantly by deletion of US3 PK in HSV-2-infected Vero cells, but it was not investigated further. Fig. 1 shows the result of SDS-PAGE analysis of proteins from HSV-2 186- and L1BR1-infected cells that were labelled with $^{35}$S methionine for 30 min from 5-5 and 11-5 h p.i. and that were harvested at 6 and 12 h p.i., respectively. At 6 h p.i., the electrophoretic pattern of labelled proteins was basically the same for the 186- and L1BR1-infected cells. However, at 12 h p.i., there were some differences in the electrophoretic pattern between 186- and L1BR1-infected cells: a band of approximately 80 kDa was detected only in 186-infected cells. It would be reasonable to expect that the difference in the electrophoretic pattern was the result of a direct or indirect modification of this protein by US3 PK. Our previous study, which used an in vitro phosphorylation assay, showed that HSV-2 US3 PK is associated with phosphorylation of UL12 alkaline nuclease, which has apparent molecular masses ranging from 75 to 80 kDa. However, the UL12 alkaline nuclease is an early gene product and US3 PK does not affect the stability or activity of the alkaline nuclease in HSV-2-infected Vero cells (Daikoku et al., 1995). This 80 kDa band was detected at 12 h, but not at 6 h p.i.; thus, it was likely to be a product of the late phase of infection. From the protein’s molecular mass, the UL46 gene product was considered to be a possible candidate for this protein.

**Production of UL46 in the presence or absence of US3 PK**

The UL46 gene products of HSV-2 are phosphoproteins with apparent molecular masses ranging from 82 to 86 kDa in infected Vero cells, and the UL46 protein is produced during late-phase infection in a manner highly dependent on viral DNA synthesis (Kato et al., 2000). The lysates of 186- and L1BR1-infected Vero cells were subjected to Western blotting analysis using anti-UL46 antiserum (Fig. 2). In 186-infected cells, the UL46 gene products were observed as at least five species of 86, 85, 84, 83 and 82 kDa [Fig. 2a(i)]. All five species of UL46 gene product in 186-infected cells were detected by 9 h p.i. and increased gradually in amount until 18 h p.i. In L1BR1-infected cells, four species were detected by 12 h p.i., but only as faint bands, and increased slightly in amount until 18 h p.i. [Fig. 2a(i)]. In L1BR1-infected cells, these were observed as three or four species of 87, 86, 84 and 83 kDa, but the 82 kDa variant was not observed. In L1B'11 (US3 revertant virus)-infected cells, the profile of the UL46 gene products was similar to that of 186-infected cells. In these blotting experiments, expression of the UL48 and UL42 gene products

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**Fig. 1.** Vero cells were mock-infected (M) or infected with L1BR1 (1, 3) or 186 (2, 4). Cells were labelled with $^{35}$S methionine for 30 min and harvested 6 (1, 2) or 12 (3, 4) h p.i. A band of roughly 80 kDa was detected in 186-infected cells at 12 h p.i. (arrowhead).

**Fig. 2.** Vero (a) and HEp-2 (b) cells were mock-infected (M) or infected with 186 [upper row in (i), (ii) and (iii)], L1BR1 [middle row in (i); lower row in (ii) and (iii)] or L1B'11 [lower row in (i)] and harvested at 3 (1), 6 (2), 9 (3), 12 (4), 18 (5) or 24 (6) h p.i. Lysates of 186 (7), L1BR1 (8) or L1B'11 (9)-infected cells harvested at 24 h p.i. were subjected to SDS-PAGE and Western blotting at the same time. Western blotting was performed with anti-UL46 (i), anti-UL48 (ii) and anti-UL42 (iii) antibodies.
products was also examined as a control. The UL48 protein is known as a major tegument protein, expressed in the late phase of infection, and as the trans-inducing factor in immediate-early infection. The UL42 gene encodes a DNA polymerase-accessory protein and is expressed during early infection. The UL48 [Fig. 2a(ii)] and UL42 [Fig. 2a(iii)] proteins were detected by 6 h p.i. and there was no significant difference in quantity between 186- and L1BR1-infected cells. Similar results were obtained in HEp-2 cells (Fig. 2b).

We next examined the effect of US3 PK on the expression of UL46 protein in transfected cells. COS-1 cells were transfected with various combinations of three plasmids, a control plasmid pcDNA, pcDNA-UL46 and pcDNA-US3, and the expression of UL46 protein in these cells was investigated by Western blotting (Fig. 3). In cells singly expressing UL46, UL46 protein was detected faintly as a single band of 82 kDa. In UL46 and US3 co-expressing cells, the UL46 protein was also detected as a single band of 82 kDa. However, the intensity of this band was enhanced, compared with UL46 singly expressed cells. Next, we performed transfection with the same combinations of these plasmids, followed by superinfection with L1BR1 or 186. In UL46-transfected, L1BR1-superinfected cells, UL46 protein was observed as three or four faint bands. In US3-transfected, L1BR1-superinfected cells, however, UL46 protein bands were easily detectable and showed a similar pattern to empty vector-transfected, 186-superinfected cells. These results indicate that the presence of the UL46 protein was affected greatly by US3 and that the UL46 protein may be modified directly or indirectly by US3 PK. Moreover, our data suggest that some other viral protein is also involved in the modification of the UL46 protein.

**Intracellular localization of the UL46 protein**

The intracellular localization of the UL46 protein was examined by indirect immunofluorescent staining at various times after infection (Fig. 4). In 186-infected cells, specific fluorescence became detectable in the cytoplasm at
6 h p.i. UL46 protein was detected mainly as a mass in the perinuclear region of the cytoplasm. Later in infection, this perinuclear staining increased and fine speckles were observed in other parts of the cells. In L1BR1-infected cells, weak but specific fluorescence became detectable in the cytoplasm at 6 h p.i. Fluorescence intensity did not increase as much as in 186-infected cells. The mass in the perinuclear region tended to be smaller and overall production (or antibody recognition of UL46 protein) seemed lower, highlighting the effect of US3 deficiency.

**US3 PK is important for the association of UL46 protein with viral particles**

The above experiments indicated that the detectability of the UL46 protein decreased in the absence of US3 PK, both in infected and in transfected cells. Therefore, to determine whether deletion of US3 PK alters uptake of UL46 into viral particles, association of the UL46 protein with extracellular virions was examined (Fig. 5). Purified and concentrated virions were subjected to silver staining and Western blotting after SDS-PAGE. The silver-staining patterns were similar for both 186 and L1BR1 virions (Fig. 5a). Although the UL46 protein was detectable as several bands ranging from 82 to 86 kDa in the lysate of 186-infected cells, as shown above, it was detected as a single band of 82 kDa in extracellular virions. In extracellular virions from L1BR1-infected cells, UL46 protein was not detectable (Fig. 5b). Conversely, the major tegument protein VP16 (the UL48 gene product) was clearly detectable as a single band of 62 kDa in extracellular virions from both 186- and L1BR1-infected cells (Fig. 5c). However, several minor bands, which seemed to be degradation products of VP16, were observed in L1BR1 extracellular virions. These results suggest that the 82 kDa UL46 protein is a tegument protein that constitutes the virion and that the UL46 protein requires US3 PK in order to associate with and be incorporated into the viral particle.

**UL46 protein is stabilized by US3 PK**

In UL46-transfected cells and in HSV-2-infected cells and viral particles, UL46 protein was undetectable or only faintly detectable in the absence of US3 PK. We inferred that the UL46 protein may be unstable in the absence of US3 PK. To determine whether the UL46 protein is destabilized in the absence of US3 PK, metabolic pulse–chase studies were performed (Fig. 6). Confluent monolayers of Vero cells were mock-infected or infected with 186 or L1BR1 at an m.o.i. of 3 p.f.u. per cell. At 11.5 h p.i., the cells were labelled with [35S]methionine for 30 min in methionine-free medium, washed and then incubated for 30–120 min in growth medium containing cold methionine. After the chase period, UL46 protein was immunoprecipitated with an anti-UL46 polyclonal antibody. Analysis by SDS-PAGE revealed that the UL46 protein was unstable in cells infected with L1BR1, but not 186 (Fig. 6a). The labelled UL46 protein in 186-infected cells was observed as two species of 82 and 83 kDa during the
chase. UL46 protein in L1BR1-infected cells was observed as the same two species at the initiation of chase, but as a single band of 83 kDa after 30 min. Thereafter, band intensity decreased rapidly to 50% of its initial expression level, whilst the intensity in 186-infected cells increased gradually during the chase period (Fig. 6b). UL48 protein was detected equally in both 186- and L1BR1-infected cells (data not shown).

**UL46 protein is phosphorylated directly by US3 PK in vitro**

To test whether the UL46 protein is phosphorylated by US3 PK, we generated and purified chimeric proteins consisting of MBP fused to full-length UL46 protein. This MBP-fusion protein was captured on amylose resin and used as a substrate for in vitro kinase assays in the presence of purified, wt GST-US3 and the kinase-negative mutant GST-US3K220M, both of which were produced in insect Sf9 cells infected with recombinant baculoviruses. GST-US3K220M was used as a control to rule out the possibility that the protein-kinase activity detected in experiments using GST-US3 was due to a kinase contaminant co-purified through the purification procedure. In the autoradiographic image of MBP-UL46 protein incubated in kinase buffer containing GST-US3 wt and \([\gamma-32P]ATP\) (Fig. 7b), two bands with apparent molecular masses of 124 (upper arrowhead) and 114 (lower arrowhead) kDa were labelled. The 124 kDa band was thought to be full-length MBP-UL46, whilst the 114 kDa band probably represents a degradation product. In contrast, MBP-UL46 was labelled scantily in the presence of the kinase-negative mutant GST-US3K220M (Fig. 7b). The amount of MBP-fusion protein and the identities of the radioisotope-labelled bands were verified by CBB staining (Fig. 7a). The MBP-UL46 protein was labelled and was shifted up due to phosphorylation.

Our study demonstrates that the presence of US3 is required for stable expression of UL46 and that the UL46 protein is phosphorylated by US3 PK in vitro. US3 PK seems to phosphorylate UL46 directly in infected cells and, even in transfected cells, UL46 seems to depend on US3 for stable existence. The band patterns differ considerably in wt and US3-deleted virus, both in molecular mass and the number of bands detected. In addition, UL46 was undetectable in L1BR1 virions, despite its abundance in wt virus 186. To our knowledge, such a profound effect on another gene product has not been observed by deleting a single gene. US3 seems to play an indispensable role for UL46 in HSV-2 infection.

Pulse-chase analysis and immunoprecipitation studies of L1BR1 showed that the UL46 protein is degraded, or decreases in antibody recognition, as soon as 60 min after synthesis, whilst a wt virus showed a steady increase in immunoprecipitated UL46. As the anti-UL46 rabbit antiserum used in this study was generated by using UL46 protein produced in E. coli and reacts specifically with HSV-2 UL46 proteins produced in both E. coli and eukaryotic cells, it seems unlikely that the reactivity of the polyclonal antibodies to the UL46 protein was changed markedly by modification with US3 PK. Rather, it is much more likely that US3 PK affects the stability of UL46.

We have previously reported the possibility of a physical interaction between the HSV-2 UL46 and UL48 gene products (Kato et al., 2000). Additionally, McKnight et al. (1986) have suggested that there is a functional interaction between UL46 and UL48 (VP16). In HSV-2-infected cells, UL46, the major tegument protein VP16 and the major capsid protein VP5 accumulate in a juxtanuclear domain, forming aggresome-like structures. There, they colocalize with cellular chaperone proteins including Hsp40 and Hsp70, as well as mitochondria and Golgi-derived vesicles. These structures are thought to play significant roles in viral maturation and egress (Nozawa et al., 2004). US3 PK has also been shown to directly modify viral proteins such as UL34 (Ryckman & Roller, 2004) and UL31 (Kato et al., 2005), which act in the capsid envelopment/de-envelopment process at the nuclear membranes. US3 may indirectly affect the stability and function of VP16 by phosphorylating UL46 and may regulate the process of virion egress or immediate-early expression of viral genes. If this is indeed the case, US3 must then be regarded as a truly important and multifunctional protein of HSV.

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