JOURNAL OF VIROLOGY, Aug. 1993, p. 4842–4847
0022-538X/93/084842-06$02.00/0

Vol. 67, No. 8

The Herpes Simplex Virus UL37 Protein Is Phosphorylated in Infected Cells

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Received 11 March 1993/Accepted 30 April 1993

The herpes simplex virus type 1 (HSV-1) UL37 open reading frame encodes a 120-kDa late (γ1), nonstructural protein in infected cells. Recent studies in our laboratory have demonstrated that the UL37 protein interacts in the cytoplasm of infected cells with ICP8, the major HSV-1 DNA-binding protein. As a result of this interaction, the UL37 protein is transported to the nucleus and can be coeluted with ICP8 from single-stranded DNA columns. Pulse-labeling and pulse-chase studies of HSV-1-infected cells with [^{35}S]methionine and $^{32}P_i$ demonstrated that UL37 was a phosphoprotein which did not have a detectable rate of turnover. The protein was phosphorylated soon after translation and remained phosphorylated throughout the viral replicative cycle. UL37 protein expressed from a vaccinia virus recombinant was also phosphorylated during infection, suggesting that the UL37 protein was phosphorylated by a cellular kinase and that interaction with the ICP8 protein was not a prerequisite for UL37 phosphorylation.

Phosphorylation of proteins is an important posttranslational modification that has been shown to modulate a variety of macromolecular events, including transcription, translation, and viral transformation (14). Most transcription factors are phosphorylated (14), and the phosphorylation of specific amino acid residues has been shown to (i) prevent nuclear localization, (ii) regulate protein binding to DNA sequences, and (iii) regulate the transcription factors' *trans* activation and *trans* repression activities (12, 19, 23). Phosphorylation can also modulate protein function by inducing allosteric conformational changes and by creating electrostatic repulsive effects on protein domains (15, 32).

The herpes simplex virus type 1 (HSV-1) genome is a linear, double-stranded DNA molecule of 160 kbp which encodes at least 75 separate proteins (16, 22). The HSV proteins can be divided into three kinetic classes, termed α, β, and γ, whose expression is coordinately regulated and sequentially ordered in a cascade fashion during lytic infection (13). Analysis of HSV-induced phosphoproteins in infected cells has detected at least 11 separate species (21, 26). HSV phosphoproteins that have been identified to date include transcriptional regulatory proteins, proteins involved in DNA replication and nucleotide metabolism, and structural proteins. The phosphorylated regulatory proteins include the α proteins ICP0, ICP4, ICP22, and ICP27 and the alpha *trans*-inducing factor (1, 20, 21, 26). Papavassiliou and coworkers demonstrated that the phosphorylation state of ICP4 modulates the protein's interaction with different viral promoters (25). The phosphorylated HSV structural proteins include the gB and gE glycoproteins and the tegument proteins encoded by the open reading frames US9 and UL41 (3, 5, 31). The UL41 protein is responsible for an HSV virion-induced shutoff of host cellular gene expression (18). HSV-encoded phosphoproteins with enzymatic functions include the large subunit of ribonucleotide reductase, alkaline exonuclease, and two protein kinases encoded by the open reading frames UL13 and US3 (2, 6, 24, 27, 34). In addition, UL42, the 65,000-M_r double-stranded DNA-binding protein which serves as an accessory protein for the HSV DNA polymerase, is also heavily phosphorylated (20). Pulse-chase studies have shown that the phosphates of several of these proteins cycle on and off during viral replication (34). The functional role of phosphorylation for most of these proteins remains unknown.

Workers in our laboratory have previously reported on the identification and characterization of the HSV-1 UL37 protein. The UL37 open reading frame, which is located in the unique long sequences of the viral DNA genome, encodes a nonstructural protein with an apparent molecular mass of 120 kDa (Fig. 1). Analysis of the kinetics of production of UL37 place it in the γ1 class of HSV genes. In addition, the UL37 protein coelutes from single-stranded and double-stranded DNA columns with ICP8, the major HSV-1 DNA-binding protein (29). We recently discovered that the UL37 and ICP8 proteins interact in HSV-1-infected cells (28). These studies were done with a vaccinia virus recombinant that expresses the UL37 protein (V37) and an HSV-1 ICP8 mutant (*d*21) that encodes a truncated ICP8 protein that does not bind single-stranded DNA and remains cytoplasmic in infected cells. Comparative studies with HSV-1-, V37-, and *d*21-infected-cell proteins demonstrated that (i) the ability of the UL37 protein to bind DNA columns is dependent upon, at least, the presence of a DNA-binding-competent ICP8 protein and (ii) the transport of UL37 to the nucleus of infected cells is mediated by ICP8.

In this article, we report that the UL37 protein was stably phosphorylated in HSV-1-infected cells. Phosphorylation of the UL37 protein did not require interaction with the ICP8 protein and was most likely the result of the action of a cellular kinase. The phosphorylation of the UL37 protein occurred soon after translation, and the phosphate did not appear to cycle on and off during viral replication.

MATERIALS AND METHODS

Cells and viruses. Vero cells (American Type Culture Collection) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% (vol/vol) Serum-Plus (JRH, Rockville, Md.) and 50 μg of gentamicin (USB, Inc.,

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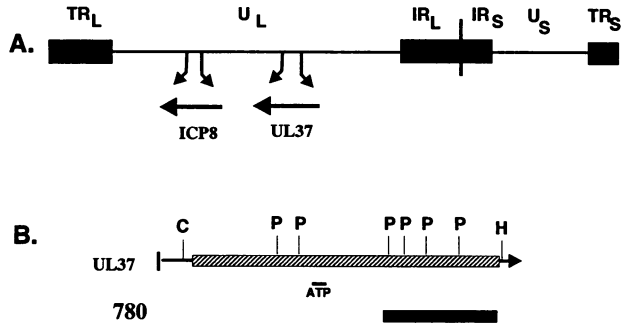

FIG. 1. (A) Arrangement of the HSV-1 DNA genome, showing the locations of the unique sequences of the L and S components (U_L and U_S) and of the terminal (TR_L and TR_S) and inverted (IR_L and IR_S) repeats and the ICP8 and UL37 genes. (B) Schematic of the UL37 gene. The hatched box represents the UL37 coding region. The solid box represents the domain of the UL37 protein in the MalE–1.1-kbp UL37 fusion protein used to generate the 780 antiserum described in Materials and Methods. C, *Cla*I; P, *Pst*I; H, *Hin*dIII. The location of a potential ATP-binding domain is indicated by the small narrow bar.

Cleveland, Ohio) per ml. The properties of HSV-1(F) and the vaccinia virus recombinants V37 and V8 have been described previously (4, 28, 29). HSV-1(F) and vaccinia virus stocks were prepared and titered on Vero cells as described previously (4, 29).

Antisera. The production of ICP8-specific and UL37-specific rabbit polyclonal antisera has been described previously (28). The ICP8-specific antiserum is directed against wild-type ICP8 protein, and the UL37-specific antiserum 780 is directed against a MalE-UL37 fusion protein consisting of the *Escherichia coli* maltose-binding protein (product of *malE*) fused to the terminal one-third amino acid residues of UL37 (Fig. 1B). The ICP6-specific monoclonal antibody 38S was obtained from Martin Zweig, National Cancer Institute, Frederick, Md. (30).

Preparation of ^{32}P-labeled infected-cell proteins. Confluent monolayers of Vero cells were incubated in phosphate-free medium for 2 h before and after infection with 5 PFU of either HSV-1(F), V37, or V8 per cell. ^{32}P labeling was performed by the addition of 50 μCi of $^{32}P_i$ (carrier free; New England Nuclear, Boston, Mass.) per ml. Cells were labeled from 1 to 6, 7 to 12, or 12 to 24 h postinfection (hpi) and harvested at the end of each labeling period by rinsing the monolayers with phosphate-buffered saline and scraping the cells into 1 ml of RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris-HCl [pH 7.5]) supplemented with 0.01 mM TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone), 0.01 mM TLCK (*Nα-p*-tosyl-L-lysine chloromethyl ketone), and aprotinin (1:100). Lysates were frozen at −70°C.

Pulse-chase radiolabeling of virus-infected-cell proteins. (i) ^{35}S labeling. Confluent monolayers of Vero cells were incubated in EMEM containing 1/10 the normal concentration of methionine for 1 h prior to and following infection with 5 PFU of HSV-1(F) per cell. ^{35}S pulse labeling was performed by incubating the infected cells in EMEM containing reduced methionine plus 37.5 μCi of [^{35}S]methionine per ml for 30 min. Following the pulse, a cold chase was performed by incubating the cells in EMEM containing the normal concentration of methionine. Mock-infected cells were labeled in the same way.

(ii) ^{32}P labeling. Confluent monolayers of Vero cells were incubated in phosphate-free EMEM for 1 h prior to and following infection with 5 PFU of the appropriate virus per cell. ^{32}P labeling was performed by incubating the cells in phosphate-free medium containing 62.5 μCi of $^{32}P_i$ per ml. Cold chases were performed by rinsing the cells and incubating them with normal EMEM. Cells were harvested as described in the preceding section.

Immunoprecipitations and protein blot analysis. For immunoprecipitation reactions, 100-μl samples of cell extracts were incubated with 1 μl of the appropriate antiserum for 18 h at 4°C on a rotary shaker. The resulting complexes were precipitated by the addition of 100 μl of 10% protein A-Sepharose (Sigma Chemicals) followed by incubation at 4°C for 6 h. The immune complexes were collected by centrifugation, washed four times in RIPA buffer, resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (2% SDS, 5% β-mercaptoethanol, 50 mM Tris-HCl [pH 6.8], 5% glycerol, 0.15 mM bromophenol blue), and boiled for 2 min. The samples were then subjected to SDS-PAGE. Immunoblot analysis was performed as described before (28, 29). Bound antibodies were detected by use of an alkaline phosphatase-conjugated second antibody. Radiolabeled proteins were detected by image analysis with an ImageQuant PhosphorImager (Molecular Dynamics).

RESULTS

Stability of the UL37 protein in HSV-1-infected cells. The UL37 protein is first detected by immunoblot analysis and immunoprecipitations in HSV-1-infected cells at between 6 and 9 hpi and increases in abundance throughout the viral replication cycle (29). To determine the stability of the UL37 protein during lytic HSV replication, pulse-chase labeling experiments with [^{35}S]methionine were performed in HSV-1-infected cells. HSV-1-infected or mock-infected Vero cells were pulsed with [^{35}S]methionine for 1 h at 6, 9, 12, 15, and 18 hpi and chased in medium containing excess cold methionine for various time periods up to 24 hpi. The radiolabeled infected-cell proteins were immunoprecipitated with the UL37-specific antiserum 780, separated by SDS-PAGE, and transferred to nitrocellulose membranes.

Phosphorimage analysis of the nitrocellulose membranes is shown in Fig. 2. Two bands of 140 and 120 kDa were immunoprecipitated with the 780 antiserum from HSV-1-infected-cell extracts but not from mock-infected-cell extracts (Fig. 2). Immunoblot analysis of the nitrocellulose membranes with the 780 antiserum showed that the ^{35}S-labeled 120-kDa band was the UL37 protein (Fig. 3). As discussed below, the 140-kDa protein was identified as the large subunit of the HSV-1 ribonucleotide reductase that was nonspecifically immunoprecipitated by rabbit antisera. There were no significant differences in the rate of synthesis of the UL37 protein produced at the 6, 9, and 12 hpi time points. For example, the amount of UL37 protein produced at 6, 9, and 12 hpi and harvested 3 h later (9, 12, and 15 hpi, respectively) was similar (Fig. 2A). Likewise, the levels of radiolabeled UL37 present at 15 hpi appeared to be identical for the 6-, 9-, and 12-h pulses. There was a noticeable reduction in the rate of synthesis of the UL37 protein produced at 15 and 18 hpi and harvested at 18 and 24 hpi. This pattern of expression is in agreement with our previous data indicating that the UL37 protein belongs to the γ1 class of HSV genes (13, 29).

UL37 protein phosphorylated in infected cells. To deter-

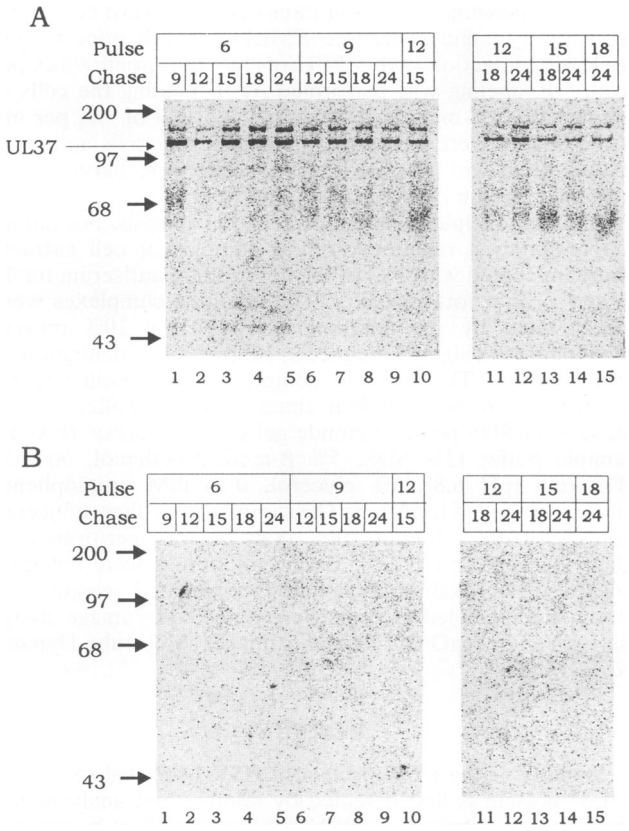

FIG. 2. Synthesis and stability of the UL37 protein in HSV-1-infected cells. Immunoprecipitates of (A) HSV-1-infected- and (B) mock-infected-cell extracts were pulse labeled with [^{35}S]methionine. HSV-1-infected Vero cells were pulse labeled and harvested at the times indicated (in hours postinfection) as described in Materials and Methods. Proteins from aliquots taken at each time point were immunoprecipitated with the UL37-specific antiserum 780, separated by SDS-PAGE, and transferred to nitrocellulose membranes. ^{35}S-labeled proteins were detected by phosphorimage analysis of the membranes. Positions of size markers are indicated on the left (in kilodaltons).

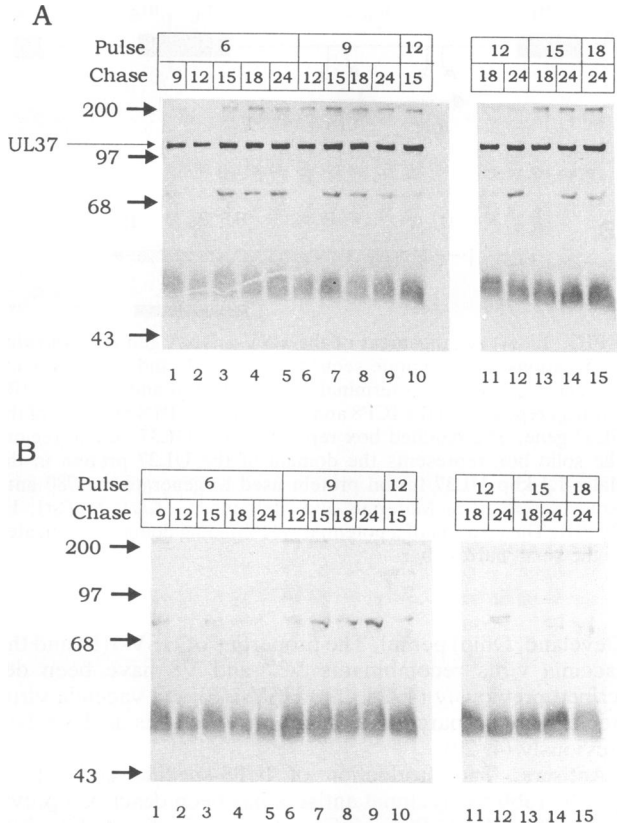

FIG. 3. Identification of the UL37 protein. Photograph of immunoblot analysis of (A) HSV-1-infected- and (B) mock-infected-cell proteins. Proteins processed as described in the legend to Fig. 2 were subjected to immunoblot analysis with the 780 antiserum and goat anti-rabbit immunoglobulin antiserum conjugated to alkaline phosphatase.

mine whether the UL37 protein is phosphorylated, HSV-1-infected cells were labeled with $^{32}P_i$ from either 6 to 12 or 12 to 24 hpi. Immunoprecipitation of the radiolabeled proteins with the 780 antiserum detected the presence of a 120-kDa phosphoprotein that was not immunoprecipitated with either ICP8-specific or normal rabbit antiserum (Fig. 4). To determine whether the UL37 protein was phosphorylated by either a viral or cellular kinase, ^{32}P-labeled extracts from V37-infected cells were immunoprecipitated with the UL37-specific, ICP8-specific, and normal rabbit antisera. V37 is a recombinant vaccinia virus that expresses the UL37 protein (29). As shown in Fig. 4, a 120-kDa phosphoprotein was immunoprecipitated with the 780 antiserum but not with the ICP8-specific or normal rabbit antiserum, suggesting that phosphorylation was the result of a cellular kinase, although at this point we cannot rule out the possibility that a protein kinase encoded by vaccinia virus is responsible for UL37 phosphorylation in the V37-infected cells.

As a control to ensure that phosphorylation of UL37 in the V37-infected cells was protein specific, ^{32}P-labeled V8-infected-cell protein extracts were also immunoprecipitated with the UL37-specific, ICP8-specific, and normal rabbit antisera. V8 is a recombinant vaccinia virus that expresses the ICP8 protein, which has been reported to have no posttranslational modifications in HSV-1-infected cells (17, 28). As shown in Fig. 4, immunoprecipitations of the ^{32}P-labeled V8-infected-cell extracts failed to detect any phosphoprotein.

To demonstrate that the 120-kDa phosphoprotein immunoprecipitated from HSV-1- and V37-infected-cell extracts was UL37, the nitrocellulose membranes were probed with either UL37- or ICP8-specific antiserum and then with goat

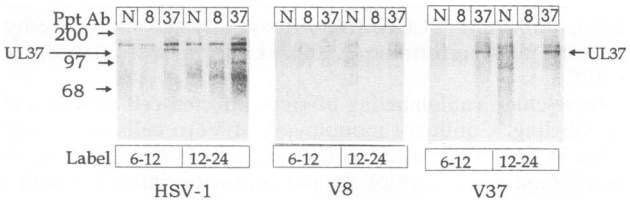

FIG. 4. Identification of the UL37 protein as a phosphoprotein. ^{32}P-labeled protein extracts from HSV-1-, V8-, and V37-infected cells were immunoprecipitated with either normal rabbit serum (lanes N), ICP8-specific antiserum (lanes 8), or UL37-specific antiserum (lanes 37). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Radiolabeled proteins were detected by phosphorimage analysis.

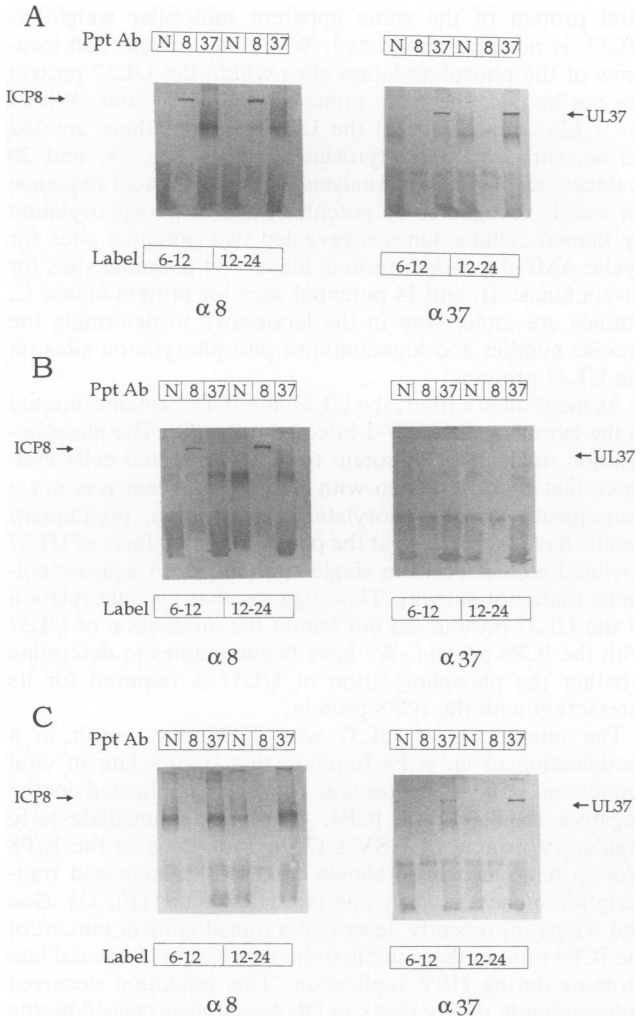

FIG. 5. Detection of UL37 and ICP8 proteins. Duplicates of the gels shown in Fig. 4 were subjected to immunoblot analysis with either ICP8-specific or UL37-specific antiserum and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin antiserum. For each panel, the blot on the left was probed with ICP8-specific antiserum (α8) and the blot on the right was probed with UL37-specific antiserum (α37). (A) HSV-1-infected-cell proteins; (B) V8-infected-cell proteins; (C) V37-infected-cell proteins.

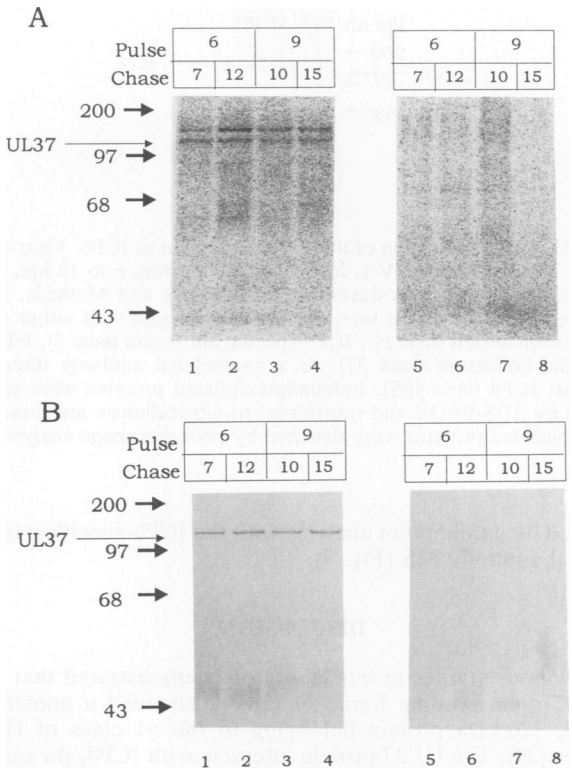

FIG. 6. ^{32}P pulse-chase experiment. Immunoprecipitations of HSV-1-infected-cell (lanes 1 to 4) or mock-infected-cell (lanes 5 to 8) protein extracts pulse labeled with $^{32}P_i$. HSV-1-infected Vero cells were pulse labeled and harvested at the times indicated (in hours postinfection) as described in Materials and Methods. Proteins from aliquots taken at each time point were immunoprecipitated with the UL37-specific antiserum 780, separated by SDS-PAGE, and transferred to nitrocellulose membranes. (A) ^{32}P-labeled proteins were detected by phosphorimage analysis of the nitrocellulose membranes. (B) Nitrocellulose membranes were subjected to immunoblot analysis with UL37-specific antiserum and alkaline phosphatase-conjugated goat antiserum.

anti-rabbit immunoglobulin antiserum conjugated to alkaline phosphatase. As shown in Fig. 5, the UL37-specific antiserum immunoprecipitated UL37 protein from HSV-1- and V37-infected cells, while the ICP8-specific antiserum immunoprecipitated the ICP8 protein from HSV-1- and V8-infected cells. The 120-kDa phosphorylated proteins in Fig. 4 aligned with the UL37 protein.

Stability of UL37 phosphorylation. To determine the stability of the phosphate groups on the phosphorylated UL37 protein, HSV-1-infected and mock-infected Vero cells were pulse labeled with $^{32}P_i$ for 1 h at 6 and 9 hpi and either harvested immediately or chased with medium containing cold phosphate for an additional 5 h. The radiolabeled proteins were immunoprecipitated with the 780 antiserum, separated by SDS-PAGE, and transferred to nitrocellulose membranes. As shown in Fig. 6, the amount of phosphorylated UL37 protein did not change between the 1-h pulse and the subsequent 5-h cold chase. Thus, the phosphorylation of UL37 does not appear to cycle on and off but rather appears to occur soon after translation, and the phosphates remain with the protein throughout the viral replication cycle.

Identification of the 140-kDa protein. The UL37-specific 780 antiserum immunoprecipitated a 140-kDa protein from ^{35}S-labeled, HSV-1-infected cells but not from mock-infected cells (Fig. 2). The 780 antiserum failed to bind the 140-kDa band in immunoblot experiments, indicating that it was not the UL37 protein (Fig. 3). Similarly, a 140-kDa phosphoprotein was immunoprecipitated from HSV-1-infected cells with either UL37-specific, ICP8-specific, or normal rabbit antiserum (Fig. 4). These results suggested that the 140-kDa band represented an HSV-1 phosphoprotein that was immunoprecipitated by rabbit antiserum. The most likely candidate for this protein is the 140-kDa HSV-1 ribonucleotide reductase (ICP6) encoded by the UL39 gene product. ICP6 is one of the major HSV-1 phosphoproteins and shows, at the amino acid level, a strong homology with ribonucleotide reductases encoded in other systems (33, 34). ICP6 exhibits 38% relatedness at the amino acid level with the large subunit of ribonucleotide reductase from *E. coli* (33). The identity of the 140-kDa band as ICP6 was con-

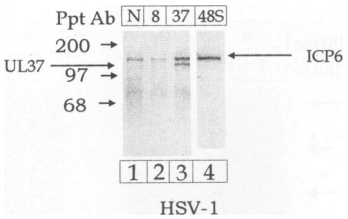

FIG. 7. Identification of the 140-kDa protein as ICP6. Vero cells were infected with HSV-1, labeled with $^{32}P_i$ from 6 to 12 hpi, and harvested at 12 hpi as described in Materials and Methods. ^{32}P-labeled protein extracts were immunoprecipitated with either normal rabbit serum (lane N), ICP8-specific antiserum (lane 8), UL37-specific antiserum (lane 37), or a monoclonal antibody directed against ICP6 (lane 48S). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Radiolabeled proteins were detected by phosphorimage analysis.

firmed by immunoblot analysis with the ICP6-specific monoclonal antibody 38S (Fig. 7).

DISCUSSION

Previous studies in our laboratory demonstrated that the UL37 open reading frame of HSV-1 encoded a nonstructural, 120-kDa protein belonging to the γ1 class of HSV genes (29). The UL37 protein interacts with ICP8, the major HSV-1 DNA-binding protein, in the cytoplasm of infected cells. In a recent study, we demonstrated that two results of this interaction were transport of the UL37 protein to the nucleus of infected cells and coelution of the UL37 protein with ICP8 from both single- and double-stranded DNA columns (28). In this article, we report that the UL37 protein exhibited little or no apparent turnover during HSV replication. As a result of this stability, the UL37 protein accumulated in the cell during viral replication. We have also shown that the UL37 protein was phosphorylated in both HSV-1-infected and V37-infected cells. The phosphate groups were added soon after translation and remained on the protein throughout the viral replication cycle and therefore did not appear to cycle on and off as has been reported for other HSV phosphoproteins (34). Furthermore, the state of phosphorylation of the UL37 protein relative to the rate of UL37 synthesis did not appear to increase or decrease during HSV-1 replication.

The phosphorylation of a UL37 protein expressed by a recombinant vaccinia virus (V37) suggests that the protein kinase responsible for phosphorylation was a cellular enzyme. The identity of the cellular kinase responsible for this phosphorylation is not known.

We have also demonstrated that the HSV-1 ICP8 protein is not phosphorylated in either HSV-1-infected or V8-infected cells. This is an important point regarding phosphorylation specificity, since the UL37 protein is not one of the major HSV phosphoproteins. The UL37 and ICP8 proteins have a similar number of amino acid residues (1,123 for UL37 and 1,196 for ICP8), and we have previously demonstrated that the ICP8 and UL37 proteins comigrate on SDS-PAGE, with identical apparent molecular weights (28). The phosphorylation of UL37 but not ICP8 in both HSV-1-infected cells and cells infected with vaccinia viruses expressing each protein (V37 and V8) suggests that the phosphorylation of UL37 is not due to an adventitious phosphorylation of a relatively large protein, since ICP8, a viral protein of the same apparent molecular weight as UL37, is not phosphorylated. While the number and locations of the phosphorylation sites within the UL37 protein are not known, there are numerous potential sites. Within the 1,123 amino acids of the UL37 protein, there are 162 serine, threonine, and tyrosine residues (71, 71, and 20 residues, respectively). Analysis of the amino acid sequence for motifs recognized as potential sites of phosphorylation by known cellular kinases revealed two potential sites for cyclic AMP-dependent protein kinase, 14 potential sites for casein kinase II, and 14 potential sites for protein kinase C. Studies are under way in the laboratory to determine the precise number and location(s) of phosphorylation sites on the UL37 protein.

As mentioned earlier, the UL37 and ICP8 proteins interact in the cytoplasm of HSV-1-infected cells (28). The phosphorylation of the UL37 protein from V37-infected cells indicates that the interaction with the ICP8 protein was not a prerequisite for phosphorylation. In addition, preliminary results have indicated that the phosphorylated form of UL37 coeluted with ICP8 from single-stranded DNA-agarose columns (data not shown). This suggests that phosphorylation of the UL37 protein did not inhibit the interaction of UL37 with the ICP8 protein. We have begun studies to determine whether the phosphorylation of UL37 is required for its interaction with the ICP8 protein.

The interaction of UL37 with ICP8 may result in a modification of an ICP8 function that occurs late in viral replication. The ICP8 protein has been implicated in the negative regulation of ICP4, the major immediate-early regulatory protein of HSV-1 (7–9). Mutations in the ICP8 protein have also been shown to result in increased transcription of several early and late HSV genes (10, 11). Gao and Knipe (8) recently described a transdominant mutant of the ICP8 protein that inhibited the expression of several late proteins during HSV replication. This inhibition occurred independently of any block in DNA synthesis caused by the mutant ICP8 protein. These results have led to the hypothesis that at late times in infection, the ICP8 protein binds either to small single-stranded DNA regions, keeping promoter regions open for transcription, or to specific structures or sequences in HSV-1 late-gene promoters.

In order for ICP8 to recognize these late-gene promoters, it may require direct interactions with other viral and/or cellular proteins. From the results of our earlier studies, we postulated that the UL37 protein would be a reasonable candidate for such a viral protein (28). Previous studies in several laboratories have demonstrated that protein phosphorylation can affect nuclear localization, DNA binding, and transactivation and repression (12, 19, 23). While the exact role of phosphorylation in the function of the UL37 protein in viral replication has yet to be determined, it is interesting to speculate that it might be involved in the interaction with the ICP8 protein. Studies are in progress in our laboratory to determine whether the phosphorylation of UL37 is involved in the protein's nuclear localization or its interaction with the ICP8 protein.

ACKNOWLEDGMENTS

We thank William T. Ruyechan and Kathryn V. Holmes for a critical reading of the manuscript.

This investigation was supported by USUHS grant R07396.

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