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Association of a Major Transcriptional Regulatory Protein, ICP4, of Herpes Simplex Virus Type 1 with the Plasma Membrane of Virus-Infected Cells

FENG YAO AND RICHARD J. COURTNEY*

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, Louisiana 71130-3932

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A major transcriptional regulatory protein, ICP4, of herpes simplex virus type 1 (HSV-1) is localized primarily within the nucleus soon after its synthesis. Recent studies have shown that approximately 100 to 200 molecules of ICP4 are located in the tegument region of purified virions (F. Yao and R. J. Courtney, J. Virol. 63:3338–3344, 1989). As an extension to these studies, we present data suggesting that ICP4 may also associate with the plasma membrane of HSV-1-infected cells. The experimental approaches used included the isolation and purification of plasma membranes from HSV-1-infected cells, the isolation of purified vesicular stomatitis virus containing ICP4, and immunofluorescence of HSV-1-infected cells following selective permeabilization with detergent. The results from the above studies support the suggestion that detectable amounts of ICP4 are associated with the inner surface of the plasma membrane of HSV-1-infected cells.

The genome of herpes simplex virus type 1 (HSV-1) has the potential to encode as many as 70 viral gene products (38). The expression of these genes is coordinately regulated and has been classified into three kinetic classes, designated immediate-early, early, and late genes, based on their requirements for viral protein synthesis and DNA replication (27). Following the infection of the host cell with HSV-1, a set of five viral genes, designated immediate early or alpha, are transcribed and result in the synthesis of five proteins defined as infected-cell polypeptides: ICP0, ICP4, ICP22, ICP27, and ICP47 (5, 7, 45). The transcription of the immediate-early genes requires no prior viral DNA or protein synthesis. To date, there is no evidence indicating that ICP22 or ICP47 affects viral gene expression in transient-expression assays (11, 17), although in certain cell types ICP22 is required for efficient late-gene expression (54). In contrast, ICP0, ICP4, and ICP27 are involved in regulating immediate-early, early, and late gene expression (4, 10–12, 18, 25, 37, 41, 52, 57). ICP4 is a polypeptide with an apparent molecular weight of 175,000 as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8). ICP4 is a phosphoprotein which exists in at least three different molecular weight forms within infected cells (8, 45, 60), and these various forms may reflect different levels of phosphorylation on serine and threonine residues (19). ICP4 localizes to specific regions of the nucleus (3, 33), and recent studies have shown that 100 to 200 molecules of ICP4 are located in the tegument region of purified virions (61). Studies have clearly demonstrated that ICP4 plays an essential role in regulating the expression of all three kinetic classes of HSV-1 genes (10–12, 18, 25, 41, 48). Temperature-sensitive and deletion mutants of ICP4 are not viable in cells cultured under nonpermissive conditions (10, 12, 13, 48). Studies have shown that HSV-1-infected cells that express a defective ICP4 fail to (i) activate the transcription of early and late genes, (ii) induce viral DNA synthesis, and (iii) down regulate immediate-early gene expression (10, 12, 13, 48). More recent studies have indicated that ICP4 binds directly to viral DNA containing the consensus sequence ATCGTC (20, 21, 30, 39, 40).

Certain DNA viruses code for nonglycosylated proteins that are located in the plasma membrane of virus-infected cells. For example, approximately 5% of the simian virus 40 large T antigen is associated with the plasma membrane (55). The immediate-early gene products of vaccinia virus (14) and the latent membrane protein of Epstein-Barr virus (22) are also associated with the plasma membrane fraction of the host cell. Finally, Otto et al. (42) have reported the association of the immediate-early protein IE1 of human cytomegalovirus with the intracellular membranes of the host cell.

As mentioned above, recent studies have demonstrated that ICP4 is specifically associated with the tegumet region of purified virions (61). One may speculate that prior to the budding of the nucleocapsid from the inner nuclear membrane, molecules of ICP4 are associated with the inner surface of the nuclear membrane and are thus in a position to be incorporated into the budding virion. We have extended these studies and now report data suggesting that ICP4 is also associated with the inner surface of the plasma membrane of virus-infected cells. Several experimental approaches were used, including analysis of purified plasma membrane fractions, immunofluorescence, and the association of ICP4 into budding vesicular stomatitis virus particles. The potential role of the membrane-associated ICP4 in the cellular immune response to HSV infection is also discussed.

MATERIALS AND METHODS

Cell culture and virus. HEp-2 cells and African green monkey kidney (Vero) cells were grown in Eagle medium (containing 0.075% sodium bicarbonate) supplemented with 10% newborn calf serum and 5% fetal bovine serum, respectively. The KOS strain of HSV-1 was grown in human embryonic lung fibroblasts (MRC-5); the Indiana strain of vesicular stomatitis virus (VSV) was grown in Vero cells,

* Corresponding author.

and all virus titer determinations were conducted by using Vero cell monolayers (2).

Infection of cells and plasma membrane purification. Monolayers of HEp-2 cells cultured in roller bottles (850 cm^2) were infected with HSV-1 at a multiplicity of 10 PFU per cell. After a 1-h adsorption at 37°C, maintenance medium containing 2% serum was added and all incubations were carried out at 37°C. At 11 h postinfection, cells were removed with a rubber policeman and pelleted by low-speed centrifugation. Plasma membranes were purified by differential centrifugation. The cells were washed twice in phosphate-buffered saline (PBS) and then suspended in 1 mM $ZnCl_2$ for 15 min at room temperature and cooled in an ice bath for an additional 5 min. Cells were broken by Dounce homogenization under conditions in which more than 90% of the cells were disrupted with little visible damage to nuclei as monitored by light microscopy. Nuclei and large cell fragments were removed by centrifuging the homogenate at 1,000 × *g* for 5 min, and the supernatant was centrifuged successively at 10,000 × *g* for 20 min and 40,000 × *g* for 45 min. Pellets obtained after the 40,000 × *g* centrifugation were designated as the membrane fraction (M).

Isolation of the nuclear fraction. The pellets resulting from the 1,000 × *g* centrifugation step during the plasma membrane purification process were resuspended in 1 mM $ZnCl_2$ and centrifuged at 500 × *g* for 5 min. After the removal of supernatant, the resulting pellets were washed once more with 1 mM $ZnCl_2$ and designated as the nuclear fraction (N).

5′-Nucleotidase and NADH diaphorase analysis. To monitor the relative purity of the membrane fraction, we performed 5′-nucleotidase assays essentially as described by Aronson and Touster (1). Briefly, 5′-nucleotidase was analyzed at 37°C for 30 min in a final volume of 0.5 ml containing 5 mM AMP, 10 mM $MgCl_2$, and 100 mM glycine–NaOH buffer (pH 9.1). The reaction was terminated by the addition of 1 ml of 12% trichloroacetic acid and centrifuged at 3,000 × *g* for 5 min at 4°C. Aliquots of the supernatant (1 ml) were diluted with 1.65 ml of water, and the P_i content was determined by the procedure of Fiske and SubbaRow as described by Aronson and Touster (1).

The NADH diaphorase assay was carried out as described by Wallach and Kamat (59). Briefly, 0.05-ml samples from the homogenate, nuclear fraction, or purified plasma membrane fraction were added to a solution containing 0.4 ml of Tris buffer (0.02 M Tris-HCl [pH 7.4]), 0.4 ml of NADH (2 mg of NADH in 10 ml Tris buffer), and 0.2 ml of 0.0033 M potassium ferricyanide solution. The solution was mixed, and oxidation of NADH was monitored spectrophotometrically at room temperature for the decrease in A_{340}.

Purification of HSV and VSV virions. For purification of VSV virions, monolayers of Vero cells cultured in roller bottles (850 cm^2) were infected with virus at a multiplicity of 20 PFU per cell. After a 1-h absorption at 37°C, maintenance medium containing 2% serum was added. Infected cells were labeled with [^{35}S]methionine (10 μCi/ml) from 2 to 18 h after infection. At 18 h postinfection, virions were purified by using basically the same methods as described for the purification of HSV-1 virions (61). Extracellular virions were harvested from the medium at 18 h postinfection, and cell debris was removed by low-speed centrifugation. Virions were pelleted from the supernatant and resuspended in TNE buffer (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA). The virus suspension was then layered onto a 20 to 60% (wt/vol) continuous sucrose gradient and centrifuged for 20 h at 50,000 × *g*. The virus band was collected by puncturing the side of the tube with a needle and syringe. The recovered virus suspension was then diluted, pelleted, and suspended to a protein concentration of 100 μg/ml as determined by the modified method of Lowry et al. (46).

For preparation of VSV containing ICP4, the following protocol was used. Monolayers of Vero cells cultured in roller bottles (850 cm^2) were initially infected with HSV-1 at a multiplicity of 3 PFU per cell in the presence of 20 μg of cytosine arabinoside (ara-C), a DNA synthesis inhibitor, per ml. Four hours after infection with HSV-1, the cells were superinfected with VSV at a multiplicity of 20 PFU per cell and labeled with [^{35}S]methionine (10 μCi/ml) at 2 h after VSV infection. Ara-C was present throughout the entire infection period. Extracellular VSV virions were harvested and purified from the medium at 18 h after VSV infection as described above. This VSV virion preparation was designated VSV4.

Detergent treatment of purified ICP4 containing VSV virions. Purified [^{35}S]methionine-labeled VSV4 virions were first treated with 0.5% Triton X-100 and 0.5% deoxycholate for 15 min at 37°C and then subjected to a further 10-min incubation at 0°C with 0.01% SDS and 50 mM urea. The reaction mixtures were separated into two fractions, the supernatant and the pelleted virus particles, by centrifugation at 70,000 × *g* for 1 h.

Trypsin treatment of purified virions. Purified VSV4 virions were treated with 20 or 40 μg/ml of trypsin in either the absence or presence of 1% Triton X-100 for 5 min at 0°C. The proteolysis reactions were terminated by the addition of 0.5 mg of soybean trypsin inhibitor per ml and 0.4 mM phenylmethylsulfonyl fluoride. Proteins were precipitated with acetone prior to SDS-PAGE analysis.

SDS-PAGE and immunoblotting. Details of the methods used for SDS-PAGE have been described previously (47). Samples were electrophoresed on 7 or 10% acrylamide gels cross-linked with *N*,*N*-methylene-bisacrylamide as indicated in the figure legends. The slab gels were either dried onto filter paper and exposed to X-Omat film or transferred onto nitrocellulose paper; they were then subjected to immunoblotting as previously described (6). Hyperimmune monospecific rabbit antisera to glycoproteins gB and gC (8, 15), and ICP4 (8) and a monoclonal antibody to the 65-kDa DNA-binding protein (35) were used for Western blot (immunoblot) analysis.

Indirect immunofluorescence. HEp-2 cells were seeded onto cover slips at an approximate density of 2 × 10^6 cells per 60-mm dish and infected with HSV-1 at a multiplicity of 3 PFU per cell. Cover slips from HSV-1-infected HEp-2 cells were harvested for immunofluorescence at 6 h postinfection. For internal immunofluorescence, cells were fixed with acetone for 5 min at room temperature and stained with a 1:10 dilution of anti-ICP4 antibody or normal rabbit serum as described previously (23). For analysis of surface or membrane immunofluorescence, cover slips were harvested and washed three times in PBS containing 0.5 mM $MgCl_2$ and then reacted with 35 μl of a 1:10 dilution of anti-ICP4 serum or normal rabbit serum. After a 30-min incubation at room temperature, cells were washed in PBS containing $MgCl_2$ as above. Next, 35 μl of a 1:40 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin were added to the cover slips, which were then incubated at room temperature for 30 min. The cover slips were washed three times in PBS containing 0.5 mM $MgCl_2$ and mounted onto a glass slide in a drop of 50% glycerol.

For the detection of ICP4 at the inner surface of HSV-1-infected cell plasma membranes, cells were fixed with paraformaldehyde and permeabilized with detergent essentially

as described by Peeples (44). HEp-2 cells were infected with HSV-1 at a multiplicity of 3 PFU per cell. At 6 h postinfection, cover slips were harvested, washed in PBS, and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. The cover slips were then washed in PBS and incubated with either 0.02 or 0.05% Triton X-100 for 15 min at room temperature. Following detergent treatment, the cover slips were washed in PBS and incubated for 30 min at room temperature with either 35 μl of a 1:10 dilution of normal rabbit serum or the same dilution of polyclonal anti-ICP4 serum. After being washed in PBS, the cover slips were treated for 30 min at room temperature with 35 μl of 10% normal goat serum. They were then washed in PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit gamma globulin as described above for the surface immunofluorescence assay. All cover slips were visualized with an Olympus microscope and a tungsten light source.

Absorption of immune and preimmune sera. Normal rabbit serum and anti-ICP4 polyclonal antibody used in internal immunofluorescence assays were preabsorbed with HEp-2 cells as described previously (23). For immunofluorescence assays of paraformaldehyde-fixed cells, normal rabbit serum and polyclonal anti-ICP4-specific antibody were also absorbed with paraformaldehyde-fixed cells as follows. Approximately 1×10^7 HEp-2 cells were seeded in 100-mm petri dishes. Cells from two dishes were harvested by scraping into the medium, pelleted, washed twice in PBS, and then suspended in 10 ml of 3% paraformaldehyde in PBS at room temperature for 40 min. Fixed cells were then pelleted, washed in PBS three times, and resuspended in 0.5 ml of a 1:10 dilution of either anti-ICP4 serum or normal rabbit serum. After a 1-h incubation at 37°C, suspensions were centrifuged at 100,000 × *g* for 30 min; the supernatant fluids (adsorbed sera) were then removed and stored at −20°C.

RESULTS

Association of ICP4 with purified plasma membranes. As an initial approach to demonstrating the association of ICP4 with the plasma membrane, cell fractionation and Western blot analysis of purified membrane fractions were used. In all of these studies HEp-2 cells were infected with HSV-1 at a multiplicity of 10 PFU per cell. At 11 h postinfection the cells were harvested, and the plasma membrane fraction was obtained by procedures described in the Materials and Methods.

Two criteria were used to document the relative purity of the plasma membrane fractions. First, 5′-nucleotidase was selected as a marker enzyme for plasma membranes and NADH-diaphorase was used as a marker enzyme for components of the endoplasmic reticulum. The 5′-nucleotidase activity detected in the plasma membrane fraction was approximately 11-fold higher than that associated with homogenate and more than 15-fold higher than that in the nuclear fraction (data not shown), indicating that the preparation was highly enriched for plasma membranes. In contrast, there was no significant increase in the NADH-diaphorase activity in the isolated plasma membrane fraction when compared with either the homogenate or the nuclear fraction. The second approach to monitoring the purity of isolated plasma membranes was the use of HSV-1 glycoproteins B and C (gB and gC) as markers. It is well established that two forms of gB and gC are detected in infected-cell lysates; the high-mannose precursor and the mature, processed form. The precursors are associated primarily with the rough endoplasmic reticulum and nuclear fraction (6), whereas the mature forms of gB and gC (120,000 and 130,000 molecular weight, respectively) appear on the plasma membranes (26, 56). Protein samples from the cell homogenate, nuclear fraction, and purified plasma membrane fraction were analyzed on SDS-PAGE and then subjected to Western blot analysis with anti-gB, anti-gC, anti-65K DNA-binding protein, and anti-ICP4 sera. The results are presented in Fig. 1. The data from Fig. 1A and B indicate that detectable amounts of pgB (110 kDa) and pgC (105 kDa) were not associated with the purified plasma membrane fraction. Only mature forms of gB and gC were detectable, which further indicates that the plasma membrane fraction obtained was highly purified. When identical samples were immunoblotted with anti-ICP4 serum, significant amounts of ICP4 were present in the purified plasma membrane fraction, suggesting that ICP4 was associated with the plasma membranes of HSV-1-infected cells (Fig. 1D). Additional proof for the specificity of the association of ICP4 with isolated plasma membranes is shown in Fig. 1C, in which the identical samples used in Fig. 1A and B were immunoblotted with the anti-65-kDa DNA-binding protein monoclonal antibody. It has been shown that the 65-kDa DNA-binding protein is localized in nuclei of infected cells (24, 43). No 65-kDa DNA-binding protein was detected in the plasma membrane preparation (Fig. 1C).

Association of ICP4 with purified VSV particles. The data presented above suggest that ICP4 was associated with purified plasma membrane fractions. A second approach was used to provide additional support that ICP4 was associated with the plasma membranes of HSV-1-infected cells. The rationale for this approach was based on selecting a virus, VSV, that acquires its envelope by budding from the plasma membrane of the infected cells (32). If molecules of ICP4 are indeed associated with the plasma membrane, the potential exists for small amounts of ICP4 to be incorporated into VSV particles as they bud from the plasma membrane. An advantage of this approach is that VSV particles can be obtained to a much higher degree of purity than is possible with plasma membrane fractions.

To test this hypothesis, we infected Vero cells with HSV-1 in the presence of ara-C and superinfected them with VSV 4 h later as described in Materials and Methods. The doubly infected cells were labeled with [^{35}S]methionine at 2 h after VSV infection. Under these culture conditions, HSV virion production was inhibited by the presence of ara-C; however, synthesis of HSV-specific immediate-early and early proteins should not be inhibited. At 18 h after VSV infection, the extracellular VSV was harvested and purified from the medium. This virion preparation was designated VSV4. Virion preparations obtained from cells infected with only VSV were used as controls and designated VSV. The proteins of both purified virion preparations (VSV4 and VSV) were analyzed by SDS-PAGE and either directly visualized by autoradiography of [^{35}S]methionine-labeled proteins or reacted with anti-ICP4 serum; the data are presented in Fig. 2. Five [^{35}S]methionine-labeled proteins were detected in both VSV and VSV4 virion preparations (Fig. 2A). On the basis of their apparent molecular weights in SDS-gels, these five proteins represent the five structural proteins (L, N, NS, G, and M) of VSV as reported by Wagner (58) and Emerson (16). The identity of VSV G protein in the purified virion preparations was confirmed by its reactivity with a VSV G-specific polyclonal antiserum on a Western blot (data not shown). When the same proteins

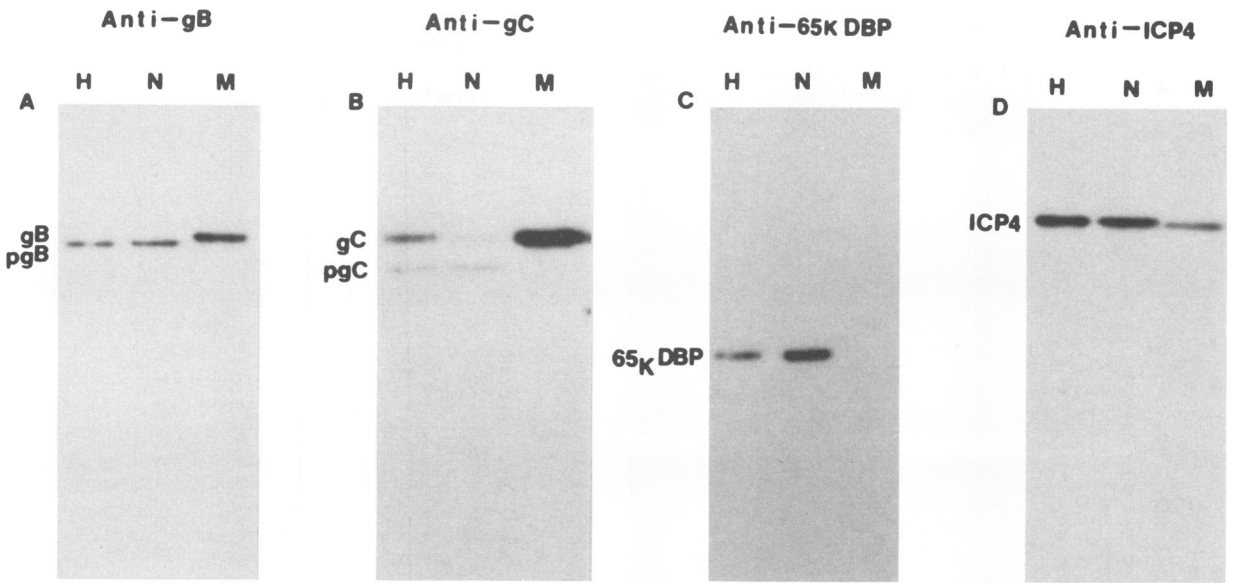

FIG. 1. Association of ICP4 with purified plasma membrane fractions of HSV-1-infected cells. Monolayers of HEp-2 cells cultured in roller bottles were infected with HSV-1 at a multiplicity of 10 PFU per cell. At 11 h postinfection, cells were removed with a rubber policeman and clarified by low-speed centrifugation, and plasma membranes were purified by differential centrifugation. Protein samples from the cell homogenate (lanes H), nuclear fraction (lanes N), and purified membrane fractions (lanes M) were analyzed by SDS-PAGE (7% acrylamide) followed by Western blot analysis with anti-gB (A), anti-gC (B), anti-65-kDa DNA-binding protein (DBP) (C), and anti-ICP4 (D) sera.

were analyzed by a Western blot by using anti-ICP4 immune serum (Fig. 2B), only the purified VSV4 contained a protein reactive with anti-ICP4. These data suggest that ICP4 was associated with purified VSV4 virions and thus provide further support that ICP4 may be associated with the plasma membranes of HSV-1-infected cells.

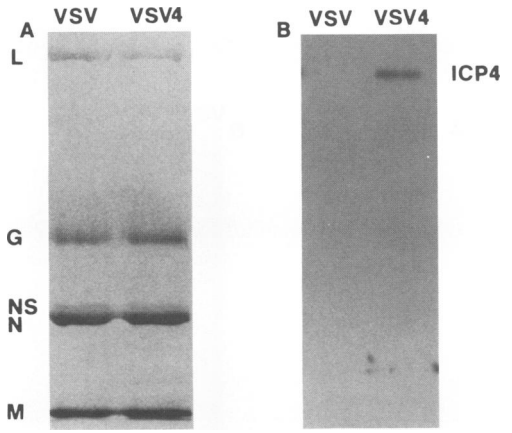

FIG. 2. Purified VSV virions from HSV-1- and VSV-superinfected Vero cells contain a protein that reacts with anti-ICP4 immune serum. Vero cells were infected with HSV-1, and 4 h later they were superinfected with VSV. The cells were labeled with 10 μCi of [^{35}S]methionine per ml at 2 h after VSV infection. Ara-C was present at 20 μg/ml throughout VSV infection to prevent HSV-1 DNA replication. The virions obtained from the superinfected cells were designated VSV4; the virions purified from VSV-infected cells were designated VSV. Extracellular VSV particles were harvested and purified from the medium at 18 h after VSV infection. The proteins of the purified virions were resolved by SDS-PAGE (7% acrylamide) and either directly visualized by autoradiography (A) or transferred to nitrocellulose paper and immunoblotted with anti-ICP4 serum (B).

In an attempt to verify the specific association of ICP4 with purified VSV4 virus particles, we carried out two additional experiments. First, VSV4 virions were separated from any non-virion-associated proteins on a linear sucrose gradient as described in Materials and Methods. If ICP4 molecules are indeed incorporated into VSV virions during the budding process, they should comigrate with the major structural proteins of VSV in the sucrose gradient. [^{35}S]methionine-labeled extracellular VSV4 virions were prepared as described in Materials and Methods. After a 20-h centrifugation at 50,000 × g, the sucrose gradient was fractionated and each sample was assayed by SDS-PAGE followed by autoradiography of the [^{35}S]methionine-labeled proteins or by immunoblotting with anti-ICP4 antibody. The five VSV structural proteins were present mainly in fractions 7 to 9 (Fig. 3A). Figure 3B represents the anti-ICP4 Western blot analysis of identical samples assayed in Fig. 3A. The results demonstrate that the same fractions contained high anti-ICP4 reactivity. In an effort to demonstrate that fractions 7 to 9 represent the peak of infectious virus particles, we determined the infectivity titer of each fraction. The data (Fig. 3C) indicate that fractions 7 to 9 contained the highest titers of PFU and further support the conclusion that ICP4 was associated with the purified VSV4 virions.

In addition, a reconstruction experiment was performed to confirm that ICP4 was associated with purified VSV4 virus particles. Roller bottles (850 cm^2) of Vero cells were infected with either VSV at a multiplicity of 20 PFU per cell or HSV-1 at a multiplicity of 3 PFU per cell in the presence of 20 μg of ara-C per ml. Cells were labeled with [^{35}S]methionine at 2 h postinfection. After 18 h of VSV infection and 22 h of HSV-1 infection, the extracellular media from all cultures were harvested and combined. The VSV viruses were purified from the combined culture supernatants, and

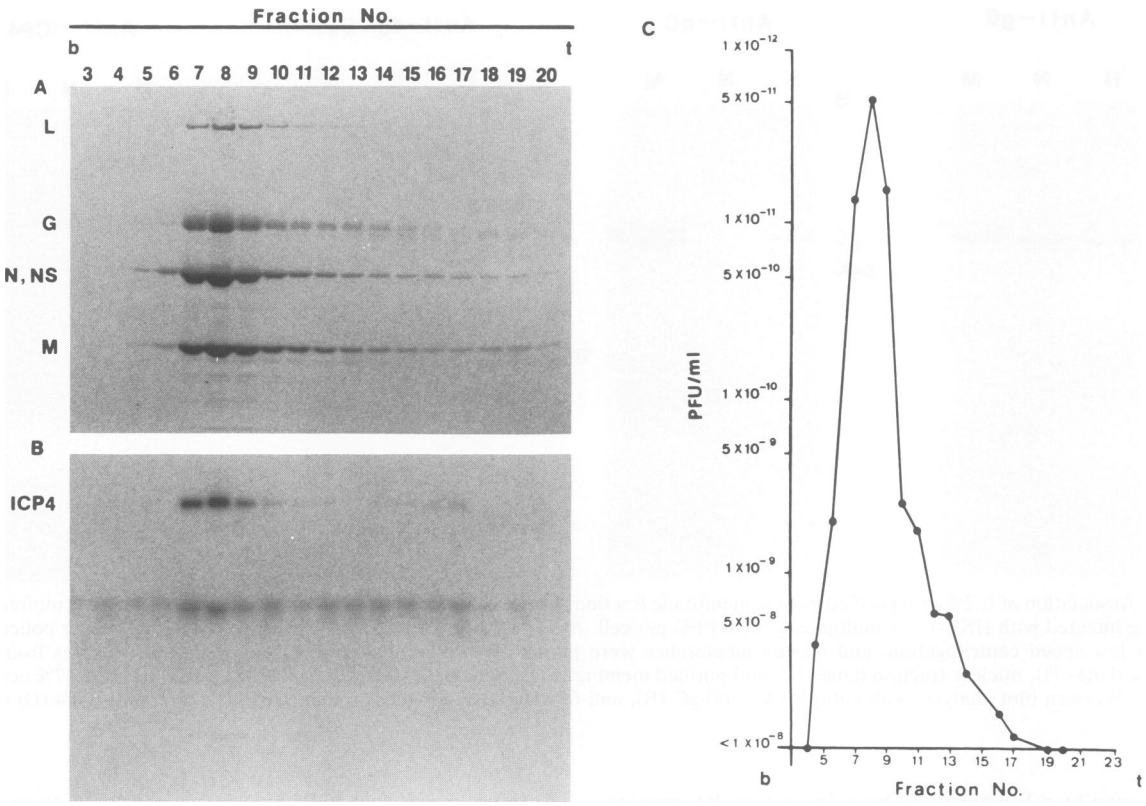

FIG. 3. Proteins reactive with anti-ICP4 serum copurify with VSV4 infectious virus particles. [^{35}S]methionine-labeled VSV4 virions were produced in Vero cells doubly infected with HSV-1 and VSV as described in the legend to Fig. 2. The extracellular virions were layered onto a 20 to 60% linear sucrose gradient and centrifuged at 50,000 × *g* for 20 h. Twenty fractions were collected from the gradient and analyzed by SDS-PAGE. (A) Autoradiogram of the [^{35}S]methionine-labeled protein profiles of each fraction. (B) Autoradiogram of an immunoblot of the identical samples shown in panel A reacted with the anti-ICP4 serum. (C) VSV infectivity titers of each fraction from the gradient. Titers of infectious virus in the individual fractions were determined in Vero cell monolayers (2).

the purified VSV particles were designated VSV′. Proteins from both VSV4 and VSV′ virions were analyzed by SDS-PAGE (Fig. 4). Figure 4A is an autoradiography of resolved [^{35}S]methionine-labeled protein profiles, and Fig. 4B represents the autoradiography of the identical sample in Fig. 4A blotted with anti-ICP4 serum. ICP4 was detected only in VSV4 virions and not in the VSV′ virion preparation (Fig. 4B). These results suggest that the association of ICP4 with VSV4 is not due to contamination of non-VSV ICP4-containing membrane vesicles or HSV-1 virions.

The next series of experiments were designed to characterize the association of ICP4 with VSV particles. First, VSV4 particles were treated with detergents to determine whether ICP4 was released from the particles. Purified virions were treated with detergent and urea as described in Materials and Methods. Following high-speed centrifugation, two fractions were obtained, and both the supernatant and the pelleted virus particles were resolved by SDS-PAGE (Fig. 5). Figure 5A represents the [^{35}S]methionine-labeled virus particles, and Fig. 5B contains the identical samples analyzed by immunoblotting with anti-ICP4 serum. The data show that detergent treatment results in the complete and partial solubilization of VSV proteins G and M, respectively, while no ICP4 was detected in the supernatant fraction.

The studies described above suggested that, unlike the VSV G protein that was solubilized with detergent treatment, ICP4 remained associated with the virus particle. If

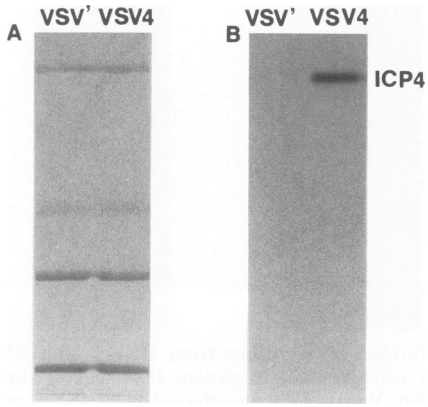

FIG. 4. Specific association of ICP4 with the purified VSV4 virus particles. Vero cells were infected with either VSV or HSV-1 in the presence of 20 μg of ara-C per ml. Cells were labeled with [^{35}S]methionine at 2 h postinfection. After 18 h of VSV infection and 22 h of HSV-1 infection, the extracellular media from both cultures were harvested and combined. VSV were purified from the combined culture supernatants, and the purified VSV particles were designated VSV′. Proteins from both VSV4 and VSV′ virions were resolved by SDS-PAGE (7% acrylamide) and either directly visualized by autoradiography (A) or transferred to nitrocellulose paper and immunoblotted with anti-ICP4 serum (B).

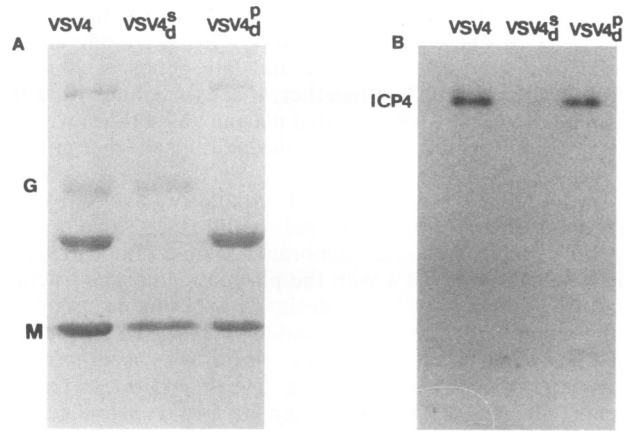

FIG. 5. Effect of treatment with detergent plus urea on the ICP4 associated with purified VSV4 virions. Purified VSV4 particles were treated with 0.5% deoxycholate and 0.5% Triton X-100 for 15 min at 37°C and then subjected to a further 10-min incubation with 0.01% SDS and 50 mM urea at 0°C. The reaction mixtures were separated into two fractions, the supernatant (VSV4$_d^s$) and the pelleted virus particles (VSV4$_d^p$) by centrifugation at 70,000g for 1 h. The samples were resolved by SDS-PAGE (10% acrylamide). The mock detergent-treated control virus is designated VSV4. (A) [^{35}S]methionine-labeled protein profile; (B) anti-ICP4 immunoblot of the identical samples in panel A.

ICP4 was located within the virion, it should be insensitive to trypsin treatment. Purified VSV4 virions were treated with trypsin in either the presence or absence of detergent. The proteolysis reactions were terminated by the addition of trypsin inhibitors, and the proteins were precipitated with acetone. The samples were resolved by SDS-PAGE and immunoblotted with anti-ICP4 serum (Fig. 6). The results indicate that the virion-associated ICP4 was sensitive to trypsin digestion only if VSV4 particles were also treated with detergent. Therefore, ICP4 is protected from trypsin

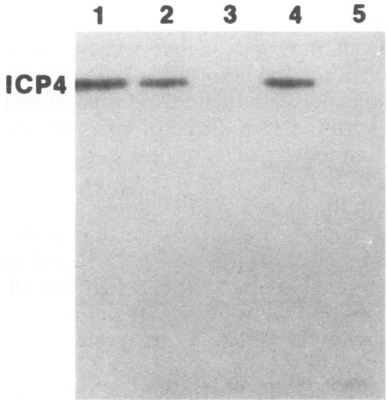

FIG. 6. Effect of trypsin treatment on the ICP4 associated with purified VSV4 virions. Purified virions were treated with 20 μg (lanes 2 and 3) or 40 μg (lanes 4 and 5) of trypsin per ml in either the absence (lanes 2 and 4) or presence of 1% Triton X-100 (lanes 3 and 5) for 5 min at 0°C. The proteolysis reactions were terminated by the addition of 0.5 mg of soybean trypsin inhibitor per ml and 0.4 mM phenylmethylsulfonyl fluoride followed by acetone precipitation. The samples were analyzed by SDS-PAGE (10% acrylamide) and immunoblotted with anti-ICP4 serum. Lane 1 contains mock-treated purified VSV4 virions.

treatment by the viral envelope and is located within the virion. Because of the insensitivity of VSV4-associated ICP4 to trypsin digestion in the absence of detergent, the results suggest that ICP4 is probably associated with the inner surface of the plasma membrane of HSV-1-infected cells.

Use of indirect immunofluorescence to localize the membrane-associated ICP4. An additional approach used to confirm that molecules of ICP4 are associated with the plasma membrane of HSV-1-infected cells included the use of indirect immunofluorescence assays. First, anti-ICP4 serum was used to stain unfixed cells in a membrane immunofluorescence assay. No specific ICP4 reactivity was detected on the surface of the virus-infected cells (Fig. 7C and D).

The next approach was to use acetone-fixed cells that are reacted with anti-ICP4 antibody. As expected, these cells exhibited intense staining of the nuclei, with little to no staining detectable in the cytoplasmic regions of the infected cell (Fig. 7A and B).

The final approach was to use a selective fixation procedure which would permit the detection of ICP4 associated with the inner surface of the plasma membrane. HSV-1-infected cells were fixed with paraformaldehyde, a cross-linking reagent that does not remove the lipid bilayer or cause the loss of membrane associated proteins. Following fixation, the cells were selectively permeabilized with either 0.02 or 0.05% Triton X-100 and then stained with anti-ICP4 serum. Cells treated with 0.02% detergent exhibited a distinct positive cytoplasmic-type fluorescence (Fig. 7E). However, if the detergent concentration was increased to 0.05%, thus increasing the permeabilization of the membranes, increasing numbers of cells with nuclear fluorescence were observed (Fig. 7G). When detergent concentrations of greater than 0.05% were used, all infected cells showed a nuclear fluorescence (data not shown). Taken together, the lack of cytoplasmic immunofluorescence in acetone-fixed cells and a positive immunofluorescence in selectively permeabilized cells add further support to the notion that ICP4 may be localized at the inner surface of the plasma membrane of HSV-1-infected cells.

DISCUSSION

The results presented in this paper show that detectable amounts of ICP4, the major transcriptional regulatory protein of HSV-1, are associated with the plasma membrane of HSV-1-infected cells. Evidence for the presence of membrane-associated forms of ICP4 includes the following. First, enriched plasma membrane fractions contained ICP4. We successfully isolated plasma membranes from HSV-1-infected HEp-2 cells by using differential centrifugation techniques. A limitation to this approach is that one cannot rule out the possibility that the detection of ICP4 in isolated membrane preparations was due to nonspecific copurification of ICP4 or HSV-1 virions with the plasma membranes. There are several lines of evidence against such possibilities. (i) Recent data have shown that only the low-molecular-weight form of ICP4 was detected in purified plasma membranes, whereas in infected cells, ICP4 exists predominantly as a high-molecular-weight form (data not shown). (ii) Our preliminary studies with HSV-1 ICP4 nonsense and deletion mutants have shown that although certain truncated forms of ICP4 are localized mainly in the cytoplasm of infected cells, increasing amounts of ICP4 were not detected in the isolated plasma membrane fraction compared with the plasma membranes of wild-type virus-infected cells (data not shown). (iii) Detectable amounts of ICP4 molecules are present within

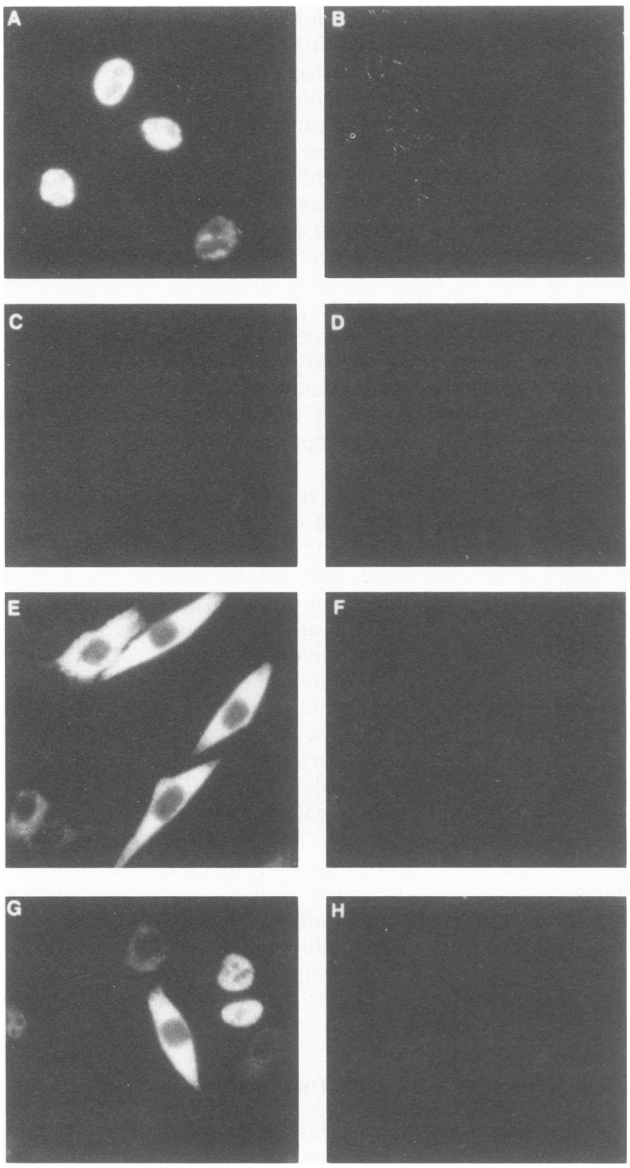

FIG. 7. Association of ICP4 with the inner surface of plasma membranes of HSV-1-infected cells. HEp-2 cells were seeded onto cover slips at an approximate density of 2×10^6 cells per 60-mm dish and infected with HSV-1. Cover slips were harvested for immunofluorescence at 6 h postinfection. For internal immunofluorescence, cells were fixed with acetone for 5 min at room temperature and stained with a 1:10 dilution of anti-ICP4 antibody (A) or normal rabbit serum (B) as described previously (23). For analysis of surface or membrane immunofluorescence, cover slips were washed in PBS containing 0.5 mM $MgCl_2$ and then reacted with a 1:10 dilution of anti-ICP4 serum (C) or normal rabbit serum (D). After a 30-min incubation at room temperature, the cells were washed as above and reacted with a 1:40 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit serum at room temperature for 30 min. Cover slips were washed three times in PBS containing 0.5 mM $MgCl_2$ and mounted onto a glass slide in a drop of 50% glycerol. For the detection of ICP4 at the inner surface of the HSV-1-infected cell plasma membrane, cover slips were washed in PBS and fixed with 3% paraformaldehyde. The cover slips were then washed in PBS and incubated with either 0.02% (E and F) or 0.05% (G and H) Triton X-100 for 15 min at room temperature. Following detergent treatment, the cover slips were washed in PBS and incubated with either a 1:10 dilution of anti-ICP4 antibody (E and G) or the same dilution of normal rabbit serum (F and H). After the cover slips had been washed in PBS, they were treated with 10% normal goat serum for 30 min at room temperature. They were then washed in PBS and reacted with fluorescein isothiocyanate-labeled goat anti-rabbit serum as described for the surface immunofluorescence assay.

purified plasma membrane fractions isolated from cells treated with cycloheximide followed by dactinomycin, in which the expression of early and late genes is inhibited (data not shown). Taken together, it seems unlikely that the association of ICP4 with isolated plasma membranes was the result of nonspecific interaction during the cell fractionation process.

Second, ICP4 was associated with VSV, a virus which replicates in the cytoplasm and acquires its envelope by budding from the plasma membrane. In an attempt to verify the association of ICP4 with the plasma membrane fraction of HSV-1-infected cells, we designed experiments to obtain ICP4 containing VSV, designated VSV4. To study the location of ICP4 in the VSV4 virion, we used two approaches. (i) The data from the trypsin treatment experiments indicated that ICP4 was sensitive to trypsin digestion only if VSV4 virions were also treated with detergent, suggesting that ICP4 was not present on the viral envelope. (ii) The results from detergent treatment experiments demonstrated that ICP4 could not be released from the VSV4 virions under the same conditions in which VSV G protein was completely released, suggesting that ICP4 is within the VSV4 virions. These results also suggest that ICP4 is unlikely to be exposed on the cell surface of HSV-1-infected cells. In addition, trypsin digestion of intact HSV-1-infected HEp-2 cell monolayers and surface iodination to HSV-1-infected cell monolayers both suggest that ICP4 is not present on the cell surface of HSV-1-infected cells (data not shown).

Third, immunofluorescent staining of HSV-1-infected cells that were partially permeabilized enabled the detection of a non-nucleus-associated form of ICP4. Although cytoplasmic immunofluorescence staining was not detectable in acetone-fixed HSV-1-infected cells, ICP4-specific cytoplasmic immunofluorescence was observed in paraformaldehyde-fixed cells permeablized with 0.02% Triton X-100. It has been shown that fixation with paraformaldehyde does not remove the lipid bilayer, whereas fixation with acetone does result in the loss of the lipid bilayer during the process. Therefore, we believe that the failure to detect the cytoplasmic ICP4 immunofluorescence in acetone-fixed cells is the result of removal of the lipid bilayer during fixation of the cells. The conclusion of the results discussed above in which three different experimental approaches were used suggests that ICP4 is located at the inner surface of the plasma membrane.

Several studies have indicated the involvement of regulatory proteins with the membrane of virus-infected cells. For example, the immediate-early proteins of both human cytomegalovirus (34, 49) and herpes simplex virus (35, 36) can serve as targets for cytotoxic T lymphocytes. It is generally believed that cytotoxic T cells recognize peptides or proteins which have been processed intracellularly and are presented on the cell surface in association with the class I major histocompatibility antigens. Whether the interaction of ICP4 with plasma membrane is in any way related to its potential to serve as a cytotoxic target antigen (36) is unknown. In addition, Otto et al. (42) have shown that the immediate-early antigen of human cytomegalovirus is associated with the intracellular membranes of the virus-infected cells. How-

ever, the authors provided no evidence for association of the protein with the plasma membrane.

Finally, studies with simian virus 40 T antigen have shown that, in addition to its well-known role in transcriptional control, approximately 5% of simian virus 40 T antigen is present in the plasma membrane (55). The fraction of T antigen that is membrane associated is modified by palmitylation; in contrast, the nuclear forms of T antigen are not palmitylated (31). Using in vitro translation and translocation analyses, Jarvis et al. (28) demonstrated that the transport of T antigens to the plasma membrane is not mediated by the normal secretory pathway. Whether palmitylation leads to plasma membrane association of T antigen or vice versa is not known. However, there are examples in which modification is thought to play a critical role in the localization and function of a protein, as in the myristoylation of $pp60^{src}$ and certain retrovirus *gag* polypeptides (9, 29, 50, 51, 53). At present we know little about the membrane-associated form of ICP4 and whether it is modified by fatty acid acylation or phosphorylation. It is also of interest to determine whether other immediate-early gene products are associated with the plasma membrane of HSV-1-infected cells. These aspects are currently under study.

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