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Initial Characterization of the Membrane-Associated Form of ICP4 of Herpes Simplex Virus Type 1

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The immediate-early gene product, ICP4, of herpes simplex virus type 1 (HSV-1) is one of the major transcriptional regulatory proteins in the virus replicative process and is localized primarily within the nucleus soon after its synthesis. Earlier studies have shown that detectable amounts of ICP4 are also associated with the plasma membranes of infected cells (F. Yao and R. J. Courtney, J. Virol. 65:1516–1524, 1991). To extend our understanding of the properties of the membrane-associated ICP4, we have used various electrophoretic techniques, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis, two-dimensional gel electrophoresis, and isoelectric focusing, to compare the membrane- and nuclear-associated forms of ICP4. The data from all of these methods revealed that a single unique form of ICP4 associates with plasma membranes of HSV-1-infected cells. While multiple forms of ICP4 were detected in infected cell nuclei, the membrane-associated form of ICP4 appeared to have a lower apparent molecular weight and a more acidic pI than the various forms of ICP4 found in infected cell nuclei. These results suggest that a novel form of ICP4 may associate with plasma membranes of HSV-1-infected cells. A recombinant adenovirus, AdICP4 (encoding an ICP4 protein), was used to determine the role that other herpesvirus proteins may play in the membrane association of ICP4. The results suggest that the expression of other HSV-1 proteins is not required for the membrane association of ICP4.

ICP4 (or Vmw175) is one of five immediate-early proteins of herpes simplex virus type 1 (HSV-1) and plays an essential role in regulating the expression of all three kinetic classes of HSV-1 genes (5–7, 10, 19, 22). Temperature-sensitive and deletion mutants of ICP4 are not viable in cells cultured at nonpermissive conditions (5, 7, 8, 22). The function of ICP4 has been shown to (i) activate the transcription of early and late genes, (ii) induce viral DNA synthesis, and (iii) down regulate immediate-early gene expression (5, 7). The gene encoding ICP4 is located in the inverted repeat regions of the short component of HSV DNA and is therefore represented twice in the viral genome (18). The size of the primary translation product as predicted by the DNA sequence is 132,835 Da; however, the polypeptide migrates with an apparent molecular weight of approximately 175,000 on sodium dodecyl sulfate (SDS)-polyacrylamide gels (4). It has been suggested that this difference probably results from the atypical amino acid composition of the protein (17) as well as from posttranslational modifications (21, 30). ICP4 is a phosphoprotein which exists in at least three different molecular weight forms within virus-infected cells (4, 21, 30). A two-dimensional electrophoretic method has been used to show the heterogeneity of ICP4 in infected HEp-2 cells (1). The various forms of ICP4 may reflect different levels of phosphorylation on serine and threonine residues (9) and possibly poly(ADP) ribosylation (2, 23).

Previous studies have suggested that certain viral regulatory proteins are located in the plasma membrane of virus-infected cells. One example is the simian virus 40 large T antigen. Approximately 5% of simian virus 40 large T antigen within a cell was found associated with the plasma membrane (28). The 19-kDa product of the adenovirus type 5 E1b gene represents another example of a viral regulatory protein that associates with membranes of virus-infected cells (24). Another immediate-early protein, IE1 of human cytomegalovirus, has been reported to associate with intracellular membranes of the host cell (20). Our laboratory has shown that ICP4 is also associated with plasma membranes of virus-infected cells (32). Knowledge of the characteristics of membrane-associated viral regulatory proteins and their functional roles is extremely limited. Until now, only membrane-associated large T antigen had been investigated extensively (14, 15, 25–27). Many questions about these proteins still remain to be answered, such as the mechanism(s) involved in the targeting to the plasma membrane and possible functional roles of these proteins. In this study, we have characterized the properties of the membrane-associated form of ICP4 as our first step toward providing insight as to the role that this protein may play within the infected cell. We now report data that address (i) a comparison of the membrane-associated ICP4 with the nuclear-associated ICP4 and the HSV-1 virion-associated ICP4 and (ii) the requirement of other HSV-1 proteins for the association of ICP4 with the infected cell plasma membrane. Our results indicated that the plasma membrane-associated form of ICP4 is unique in its molecular weight and isoelectric point (pI), and the association of ICP4 with the infected cell plasma membrane does not require the presence or expression of other herpesvirus proteins.

The purpose of our first series of experiments was to compare ICP4 isolated from infected cell membranes, nuclear fractions, and purified virions by using different electrophoretic techniques. The experimental design was based on the heterogeneity of ICP4 in infected cells which had been clearly shown either by SDS-polyacrylamide gel electrophoresis (PAGE) or two-dimensional electrophoresis (1, 4, 21, 30). To compare the various forms of ICP4 isolated from different fractions, infected HEp-2 cell plasma membrane and nuclear fractions were prepared. HSV-1 virions were purified by sucrose gradi-

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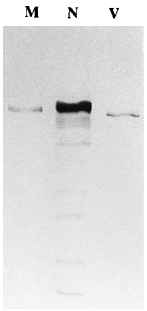

FIG. 1. SDS-PAGE analysis of ICP4 associated with membrane fractions, nuclear fractions, and HSV-1 virions. Monolayers of HEp-2 cells were infected with HSV-1 at a multiplicity of infection of 10 PFU per cell. The infected cells were harvested at 11 h postinfection. The nuclear and membrane fractions were prepared by differential centrifugation (32). HSV-1 virions were purified by sucrose gradient centrifugation (3). Proteins from the nuclear fraction (N), purified membrane fraction (M), and HSV-1 virions (V) were resolved by SDS-PAGE (6% acrylamide gel) followed by Western blot analysis with an anti-ICP4 antiserum.

ent centrifugation. Samples from purified plasma membranes, nuclear fractions, and purified virions were analyzed by SDS-PAGE and then subjected to Western blot (immunoblot) analysis with anti-ICP4 sera. The data presented in Fig. 1 show that virion-associated ICP4 migrates faster than both membrane-associated ICP4 and nuclear-associated ICP4 in the SDS-polyacrylamide gel. The difference in migration between membrane- and nuclear-associated ICP4 was not significant on the SDS-polyacrylamide gel. Since ICP4 is a large molecule with an estimated molecular weight of 175,000 by SDS-PAGE, certain posttranslational modifications might not change its molecular weight significantly.

Two-dimensional electrophoresis was used to show the differences of membrane- and nuclear-associated ICP4 molecules. The method, which separates proteins on the basis of their pI values in the first dimension followed by second-dimensional separation on the basis of their molecular weights, is able to differentiate very small differences between two similar polypeptides. Protein samples were separated first by isoelectrofocusing (IEF) and then by SDS-PAGE in the second dimension. The proteins in the gel were transferred to a nitrocellulose membrane and blotted with anti-ICP4 sera. The data presented in Fig. 2 show that there were multiple species of ICP4 in the nuclear fraction (Fig. 2A), while only a single species of ICP4 was detected in the membrane fraction (Fig. 2B). The single species of ICP4 found in the membrane fractions did not comigrate with any species detected in the nuclear fractions. The pI value of membrane-associated ICP4 was more acidic than that of the nuclear-associated forms of ICP4. Multiple experiments showed consistently that the pI values of the membrane-associated and nuclear-associated forms of ICP4 differed by at least 0.1 pH units. One of the approaches used to show the association of ICP4 with the plasma membrane in our previous studies (32) was the incorporation of ICP4 into virions of vesicular stomatitis virus (VSV). The rationale for this approach was based on selecting a virus, such as VSV, that acquires its envelope by budding from the plasma membrane of the infected cells (16). If VSV particles can indeed incorporate the membrane-associated species of ICP4, the ICP4 molecules in these virions (VSV4 virions) should be the same species as the membrane-associated ICP4. The results confirmed our assumption by showing that ICP4 in VSV4 virions migrated similarly in the two-dimensional gel to ICP4 from plasma membrane fractions (Fig. 2C). In other words,

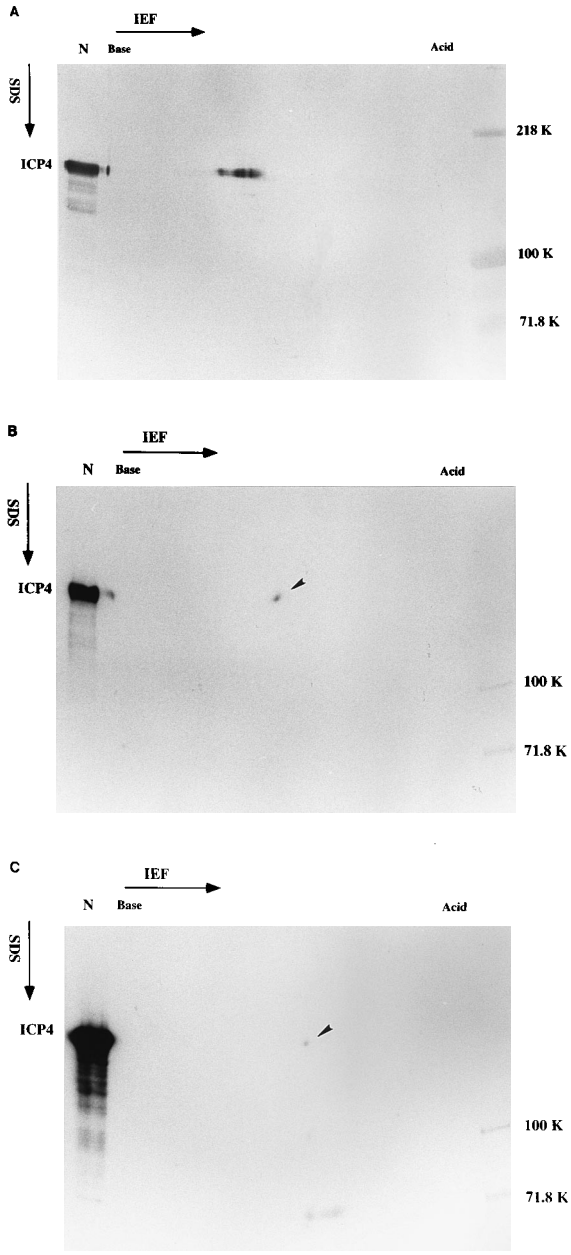

FIG. 2. Two-dimensional gel electrophoresis of ICP4 associated with membrane and nuclear fractions of HSV-1-infected HEp-2 cells and purified VSV4. The nuclear and membrane fractions of HSV-1-infected cells were prepared by differential centrifugation (32). VSV4 virions were purified by sucrose gradient centrifugation (32) and designated VSV4. Protein samples from isolated plasma membrane and nuclear fractions from HSV-1-infected HEp-2 cells as well as VSV4 virions were solubilized in urea buffer (9 M urea, 4% [wt/vol] ethylphenylpolyethylene glycol [Nonidet P-40], 2% ampholines [preblended, pH 3.5 to 9.5; Sigma Chemical Co.], 2% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin per ml, 1 mM lysine). After a 2-h incubation at room temperature, samples were cleared by centrifugation and loaded onto an isoelectrophoresis gel. Upon completion of isoelectrophoresis, the gel was soaked in equilibration buffer containing SDS and Tris-glycine (pH 6.8) for 30 min. The resolved proteins were then electrophoresed in the second dimension on an SDS-polyacrylamide gel. An aliquot of the HSV-1-infected cell nuclear fraction (N) and molecular weight markers were loaded on either side of the gel. Immunoblot analysis with anti-ICP4 was performed following the transfer of proteins to the nitrocellulose membrane. (A) Nuclear fraction of HSV-1-infected HEp-2 cells; (B) membrane fraction of HSV-1-infected HEp-2 cells; (C) VSV4 virions.

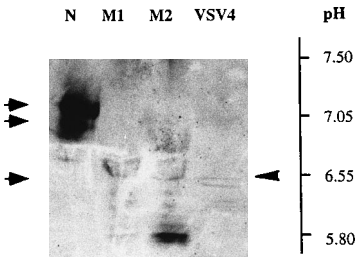

FIG. 3. Vertical slab IEF analysis of ICP4 associated with HSV-1-infected HEp-2 cell nuclear and plasma membrane fractions and VSV4 virions. Vertical slab IEF was performed with a Bio-Rad minigel unit (Mini-PROTEAN II Dual Slab Cell) (11). The nuclear (N) and membrane (M1 and M2) fractions of HSV-1-infected HEp-2 cells were prepared by differential centrifugation (32). VSV4 virions were purified by sucrose gradient centrifugation (32). Samples were solubilized in lysis buffer (9 M urea, 4% [wt/vol] Nonidet P-40, 2% ampholines [preblended, pH 5 to 8; Sigma], 2% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 5 μg of aprotinin per ml, 1 mM lysine) (1). After 2 h of incubation at room temperature, the samples were cleared by centrifugation and loaded onto the isoelectrophoresis gel. The gels were run at 250 V for 30 min and then at 400 V for 12 h at 4°C. Upon completion of isoelectrophoresis, a slice of the gel was cut and divided into 10 pieces for pH measurement. The remaining gel was soaked in equilibration buffer (0.12 M Tris-HCl [pH 6.8], 10% glycerol, 10% SDS, 2% β-mercaptoethanol) for 30 min. The proteins on the gel were transferred to a nitrocellulose membrane and blotted with an anti-ICP4 antibody. Approximately twice the concentration of proteins was loaded in the M2 lane as in the M1 lane.

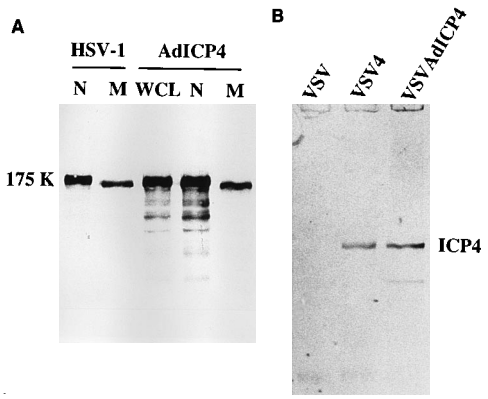

FIG. 4. (A) Expression of ICP4 in AdICP4-infected HEp-2 cells. HEp-2 cells were infected with AdICP4 at a multiplicity of infection of 100 PFU per cell. Two days after infection, cells were washed twice with cold phosphate-buffered saline and harvested. Membrane and nuclear fractions were prepared by differential centrifugation (32). Protein samples from the membrane fraction (M), nuclear fraction (N), and whole cell lysates (WCL) of AdICP4-infected HEp-2 cells were analyzed by SDS-PAGE followed by Western blot analysis with an anti-ICP4 antiserum. As a control, protein samples from membrane and nuclear fractions of wild-type HSV-1-infected HEp-2 cells were also analyzed in the same gel. (B) Incorporation of ICP4 expressed in AdICP4-infected cells into VSV virions. Vero cells were first infected with AdICP4 at a multiplicity of infection of 150 PFU per cell, with the addition of cytosine arabinoside in the medium. After 30 h of incubation, cells were superinfected with VSV at a multiplicity of infection of 20 PFU per cell. Extracellular VSV (VSVAdICP4) virions were harvested from the medium at 18 h after VSV infection and purified by sucrose gradient centrifugation. SDS-PAGE and Western blotting were used to detect the incorporation of ICP4 into the virions. VSV virions (VSV) prepared from VSV-infected Vero cells were included as a negative control. VSV4 virions prepared from Vero cells doubly infected with wild-type HSV-1 and VSV were used as a positive control.

ICP4 molecules incorporated into VSV virions most probably represent membrane-associated forms of ICP4.

In addition to two-dimensional electrophoresis, vertical slab gel IEF was performed to provide a direct comparison of various forms of ICP4 on the same gel. HSV-1-infected cell nuclear and plasma membrane fractions as well as VSV4 virions were solubilized in lysis buffer. Upon completion of isoelectrophoresis, the gel was soaked in equilibration buffer and proteins on the gel were transferred to a nitrocellulose membrane. Western blotting was performed to detect ICP4 with an anti-ICP4 antibody. As shown in Fig. 3, the nucleus-associated ICP4 has multiple forms which migrated at a higher pH range than the ICP4 associated with the membrane fraction or VSV particles. The migration of the plasma membrane-associated ICP4 and VSV ICP4 at a lower pI than ICP4 associated with the nuclear fraction is supportive of the data obtained by two-dimensional gel analysis (Fig. 2). It is possible that some of the ICP4 in our samples was degraded during the 2-h incubation period at room temperature in lysis buffer. Since isoelectrofocused proteins are resolved on the basis of their pI values, the degradation products detected on the IEF gel may not be detected in the two-dimensional gel.

To associate with the plasma membrane, ICP4 may need to interact with other herpesvirus proteins or, alternatively, there may be no requirement for the presence of other herpesvirus proteins for the membrane association of ICP4. If ICP4 could associate with the plasma membrane in the absence of other herpesvirus proteins, one experiment to test this possibility would be to express ICP4 as the only HSV-1 protein in a host cell and to determine the association of ICP4 with the plasma membrane. A recombinant adenovirus encoding ICP4, AdICP4, generously provided by Silvia Bacchetti (McMaster University), was chosen for the experiment. The advantage of using a virus vector instead of transfection to transfer a gene is the high efficiency of infection possible. AdICP4 has an inserted ICP4 gene with its own promoter in an E1a site and hence could not encode E1a and E1b (29). A human 293 cell line is required to grow AdICP4, since the cell line can complement both E1a and E1b functions (12). AdICP4-infected Vero and HEp-2 cells were first analyzed by SDS-PAGE to determine if ICP4 was expressed in AdICP4-infected cells. Plasma membrane fractions and nuclear fractions were prepared from AdICP4-infected HEp-2 cells, and protein samples were analyzed by SDS-PAGE shown in Fig. 4A. The results demonstrated that detectable amounts of ICP4 were expressed in the cells infected with AdICP4, and ICP4 could be detected in the membrane fractions. We could also detect ICP4 in AdICP4-infected Vero cells (data not shown). Experiments were conducted to determine if ICP4 expressed in AdICP4-infected cells was incorporated into VSV virions. Since this method could provide highly purified VSV virions, we could reduce the risk of contamination of nonmembrane proteins. The recombinant VSVAdICP4 was prepared and analyzed by SDS-PAGE. The proteins were then transferred to a nitrocellulose membrane and blotted with anti-ICP4 sera. The results indicate that ICP4 molecules expressed in AdICP4-infected cells were incorporated into VSV virions (Fig. 4B). Since the only herpesvirus gene transferred into the cells was the gene for ICP4, the results strongly suggest that the membrane association of ICP4 does not require the presence of other herpesvirus proteins.

The majority of ICP4 molecules expressed in infected cells are modified (phosphorylation) and transported into the nucleus. A different modification may occur on membrane-associated ICP4 molecules and enhance their localization at the cell's plasma membrane. Our data suggest that a specific species of ICP4 was detected in HSV-infected cell plasma membrane fractions. On an SDS-polyacrylamide gel, membrane-associated ICP4 migrates slightly faster than the nuclear ICP4 but more slowly than HSV-1 virion-associated ICP4. The pI value of membrane-associated ICP4 determined by both IEF

and two-dimensional electrophoresis is more acidic than that of nuclear-associated ICP4. Only a single species of ICP4 could be detected in infected cell membrane fractions, while multiple species of ICP4 exist in nuclear fractions. Because of the insolubility of HSV-1 virion-associated ICP4 in the IEF sample buffer, we were unable to analyze HSV-1 virion-associated ICP4 (31) by two-dimensional gel electrophoresis. ICP4 associated with purified plasma membrane fractions and ICP4 associated with VSV4 virions exhibited similar patterns when assayed by two-dimensional electrophoresis. These results support our earlier hypothesis that membrane-associated ICP4 was incorporated into VSV virions as they bud through infected cell plasma membranes. The differences between membrane-associated and nucleus-associated ICP4 might account for the differences in cellular location.

The finding of membrane-associated ICP4 is reminiscent of studies with the simian virus 40 large T antigen, in which small amounts of large T antigen were found associated with the plasma membrane of both virus-infected and transformed cells (28). Analyses by Klockmann and Deppert (15) indicated that the membrane-associated form of T antigen was modified by palmitylation. Moreover, both the nuclear and the membrane-associated forms of T antigen appeared to be glycosylated (13). Our attempts to metabolically label the membrane-associated ICP4 with [^{3}H]palmitate have been unsuccessful, possibly because of the small quantities of membrane-associated ICP4 or a different fatty acid modification of ICP4. Further studies are needed to determine the modifications of ICP4 that are responsible for its association with membranes.

A recombinant adenovirus encoding ICP4, AdICP4, was used to introduce ICP4 gene into the majority of the recipient cells with extremely high efficiency. Such an approach allowed us to examine the possible association of these expressed ICP4 molecules with plasma membranes. The incorporation of ICP4 expressed in AdICP4-infected cells into VSV virions suggests that no other herpesvirus proteins are required for the membrane association of ICP4. Though our data suggest that other herpesvirus proteins are not required for the membrane association of ICP4, we still do not know whether certain host proteins are involved in this process. Using a homobifunctional cross-linker, bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, we were able to show that membrane-associated ICP4 was cross-linked and formed a protein complex which did not enter the separating gel used for SDS-PAGE (data not shown). This finding suggests that membrane-associated ICP4 may interact with one or more membrane proteins. The mechanism involved in the targeting of ICP4 to the plasma membranes remains to be identified. Certain posttranslational modifications, including phosphorylation or acylation, might be important for the targeting of ICP4 to the plasma membrane. These modifications may either enhance the hydrophobicity of ICP4 (as acylation) or mask the nuclear localization signal of ICP4. These modifications could lead to the retention of ICP4 molecules in the cytosol and may create a signal for the membrane association of ICP4. The unique pattern of migration of membrane-associated ICP4 in SDS-PAGE and two-dimensional gel electrophoresis seems to support the existence of such modifications of the membrane-associated ICP4.

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