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A Major Transcriptional Regulatory Protein (ICP4) of Herpes Simplex Virus Type 1 Is Associated with Purified Virions

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Herpes simplex virus type 1 was purified by density gradient centrifugation, and the virion-associated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. By Western blot (immunoblot) analysis with an anti-ICP4 monospecific serum, the results indicated that ICP4, one of the five immediate-early proteins of herpes simplex virus type 1, was associated with the purified virions. To define the location of ICP4 within the virion, trypsin digestion experiments were performed. Purified virions were treated with trypsin in the presence or absence of detergent. The virus envelope appeared to protect ICP4 from the trypsin, since virus-associated ICP4 was sensitive to digestion only after detergent treatment. In addition, ICP4 remained associated with the virus particle when the virion-specific glycoproteins were removed after detergent treatment. Finally, ICP4 was not detected in purified preparations of type A and B capsids isolated from the nuclear fraction of virus-infected cells. The above-mentioned data suggest that detectable amounts of ICP4 are present within the tegument region of the virion.

The virion of herpes simplex virus type 1 (HSV-1) consists of four distinct morphological components: an electron-opaque core in the form of a torus which contains the viral DNA; an icosahedral-shaped capsid that encloses the core; an outer envelope or membrane with periodic projections; and electron-dense material, defined as the tegument, which is located between capsid and envelope (29, 30, 34, 36). Purified virions are reported to contain as few as 15 to as many as 33 species of structural proteins (3, 13, 26, 33); however, the specific functions of very few of these proteins are known.

The immediate-early or alpha genes of HSV-1, which are initially expressed during the infectious cycle and in the absence of prior viral protein synthesis, specify five proteins defined as infected cell polypeptides (ICP): ICP0, ICP4, ICP22, ICP27, and ICP47 (4, 14, 22). Studies of temperature-sensitive and deletion mutations in these five genes have indicated that ICP4 and ICP27 perform essential functions in HSV-1-infected cell cultures (7, 9, 24, 27, 31, 32). The immediate-early protein, ICP4 or Vmw175 (apparent molecular weight, 175,000), is a major regulatory protein which modulates viral gene expression during virus replication. It has been shown that the synthesis of ICP4 is required for the enhanced expression of early and late subsets of viral genes, the repression of ICP4 (autoregulation), and possibly other immediate-early genes (7, 9, 18). ICP4 binds to DNA directly and may recognize and interact with more than one type of DNA binding site (19).

Gibson and Roizman (12) have published data that suggest that a protein designated VP4 (apparent molecular weight, 175,000) is present as one of the structural proteins of purified virions. Whether this protein is analogous to the major transcriptional regulatory protein, ICP4 (Vmw175), has not been shown. Recent studies within our laboratory have focused on the identification of ICP4 as a component that may be associated with purified virions. The data presented in this report strongly suggest that detectable amounts of ICP4 are most likely present within the tegument region of purified virions.

MATERIALS AND METHODS

Cell culture and virus. HEp-2 cells were grown in Eagle medium supplemented with 10% newborn calf serum and 0.075% sodium bicarbonate. The KOS strain of HSV-1 was grown in human embryonic lung fibroblasts (MRC-5), and all virus titrations were conducted in African green monkey kidney (Vero) cell monolayers (1).

Infection of cells and virion purification. Monolayers of HEp-2 cells cultured in roller bottles (850 cm^2) were infected at a multiplicity of three PFU per cell. After 1 h of absorption at 37°C, maintenance medium containing 2% serum was added. All incubations were carried out at 37°C. Infected cells were labeled with [^{35}S]methionine (5 μCi/ml) from 12 to 48 h after infection. At 48 h postinfection, virions were purified by previously described methods (5). Briefly, extracellular virions were harvested from the media at 48 h postinfection and cell debris was removed by low-speed centrifugation. Virions were pelleted from the supernatant and suspended 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*. Upon completion of centrifugation, either the sucrose gradient was fractionated or 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 at a protein concentration of 100 μg/ml as determined by the modified method of Lowry et al. (23). The infectivity of purified viruses was determined by titration in Vero cell monolayers (1). The physical particle-to-PFU ratio of purified virions was approximately 100, which indicated that the purification procedure did not significantly reduce the virion infectivity. Purified viruses were stored at −80°C.

Isolation of nuclear fraction. HSV-1-infected cells were harvested at 12 h postinfection, pelleted, and washed twice with phosphate-buffered saline. Washed cell pellets were suspended in 1 mM $ZnCl_2$ for 15 min at room temperature

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followed by an additional 5-min incubation on ice. Cells were disrupted by Dounce homogenization. The crude lysates were centrifuged at 500 × *g* for 5 min. After the removal of supernatant, the resulting pellets (nuclear fraction) were washed twice with 1 mM $ZnCl_2$ and stored at −80°C.

Detergent treatment of purified virions. Two methods were used for treating the purified virions with detergent. First, [^{35}S]methionine-labeled purified virions were treated with 1% Triton X-100 for 15 or 30 min at 37°C, followed by 1 h of centrifugation at 70,000 × *g*, which resulted in two fractions, the supernatant and the pelleted virus particles. Second, [^{35}S]methionine-labeled virions were first treated with 0.5% deoxycholate (DOC) and 0.5% Triton X-100 for 15 min at 37°C, followed by a further 10-min incubation at 0°C with 0.01% sodium dodecyl sulfate (SDS) and either 10 or 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 HSV-1 virions were treated with 0.1 mg of trypsin per ml in either the absence or presence of 1% Triton X-100 for 5 or 15 min at 37°C. The proteolysis reaction was 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 their analysis on SDS-polyacrylamide gel electrophoresis (PAGE).

Isolation of intranuclear virus capsids. HEp-2 cells were infected with HSV-1 (strain KOS) at a multiplicity of three PFU per cell and labeled with [^{35}S]methionine (5 μCi/ml) at 4 h postinfection. Type A and B viral capsids were purified from the nuclear fraction essentially as described by Gibson and Roizman (12). Briefly, at 22 h postinfection, cells were harvested and collected by centrifugation at 1,000 × *g* for 10 min. The pellet was suspended in 0.15 M NaCl–0.01 M Tris [pH 7.2]–0.002 M $MgCl_2$ containing 1% Nonidet P-40. After incubation for 30 min at 0°C, the nuclei were pelleted from the suspension by centrifugation at 1,000 × *g* for 10 min and lysed by 0.5% DOC in the presence of 50 μg of DNase I per ml for 15 min at 37°C, followed by a further 5-min incubation at 0°C in the presence of 0.5% Brij 58 and 0.5 M urea. The extract was clarified by centrifugation at 7,000 × *g* for 10 min. The supernatant fluid was layered onto a 10 to 40% (wt/wt) linear sucrose gradient and centrifuged at 70,000 × *g* for 1 h at 4°C. After centrifugation, two bands located near the middle of the tube were collected and stored at −80°C.

SDS-PAGE, silver staining, and immunoblotting. Details of the methods used for SDS-PAGE have been previously described (25). All slab gels were 7% bisacrylamide. The slab gels were either directly visualized by silver staining (20) or dried onto filter paper and exposed to X-Omat film. Immunoblotting was performed essentially as previously described (5). Hyperimmune monospecific rabbit antisera to glycoproteins gB and gC (10), ICP4 (6), and the 65K (65,000) DNA-binding protein (17) were used for Western blot (immunoblot) analysis. The specificity of the anti-ICP4 serum has been confirmed by specific reactivity to various truncated ICP4 gene products (8), as well as by comparisons with other known anti-ICP4-specific sera (N. DeLuca, personal communication).

RESULTS

Association of ICP4 with purified virions. Extracellular virions were purified from HSV-1-infected HEp-2 cells as described in Materials and Methods. The proteins of the purified virions were analyzed by SDS-PAGE and directly visualized by silver staining (Fig. 1A). The protein profile obtained for the purified virions was similar to those published by other laboratories (13, 33). Only the major structural proteins of the purified virus were detectable. The resolved proteins shown in Fig. 1A were transferred onto nitrocellulose paper, followed by immunoblotting with a polyclonal, monospecific anti-ICP4 serum (Fig. 1B). The data from Fig. 1B indicate that a protein reactive with anti-ICP4 serum, which has an approximate molecular weight of 175,000, is present in purified virions. These results suggest that ICP4 may be associated with purified virions.

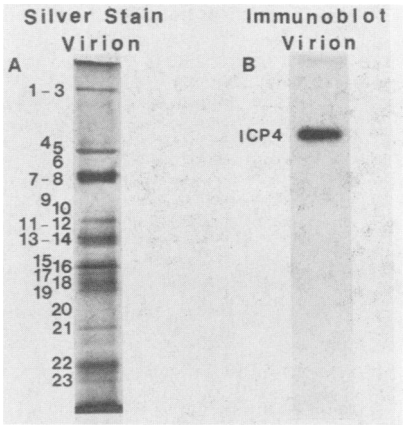

FIG. 1. Purified virions of HSV-1 contain a protein which reacts with anti-ICP4 immune serum. HEp-2 cells were infected with HSV-1 (strain KOS), and extracellular virions were harvested from the medium at 40 to 48 h postinfection. Cell debris was removed by low-speed centrifugation. Virions were pelleted from the supernatant and suspended in TNE buffer. The virus suspension was then layered onto a 20 to 60% linear sucrose gradient and centrifuged for 20 h at 20,000 rpm in a SW41 rotor. The virus band was collected from the gradient, diluted, and repelleted. The proteins of the purified virions were resolved by SDS-PAGE (7% acrylamide) and either directly visualized by silver staining (A) or transferred onto nitrocellulose paper and immunoblotted with polyclonal, monospecific anti-ICP4 serum (B).

To verify that ICP4 was associated with the purified virus particles, virions were resolved from any non-virion-associated proteins on sucrose gradients. If ICP4 is associated with purified virions, its location on the sucrose gradient should coincide with that of the major structural proteins of the virion. Extracellular virions labeled with [^{35}S]methionine were layered onto a 20 to 60% linear sucrose gradient and centrifuged as described in the Materials and Methods. Individual fractions were collected and assayed by SDS-PAGE. The gels were analyzed by autoradiography of the [^{35}S]methionine-labeled proteins (Fig. 2A) or by immunoblotting with anti-ICP4 serum (Fig. 2B). The majority of the [^{35}S]methionine-labeled HSV-1 virions was detectable in fractions 8, 9, and 10 as indicated by the presence of significant amounts of VP5, the major capsid protein, and other viral structural proteins (Fig. 2A). The same fractions contained high anti-ICP4 reactivity (Fig. 2B), which supports the observation that ICP4 is associated with purified virions. To verify that fractions that contain the ICP4-reactive antigens represent the virion peak, the titers of each fraction for infectivity from a separate sucrose gradient were determined as described in Materials and Methods. The data presented in Fig. 3 indicate that fractions 8, 9, and 10

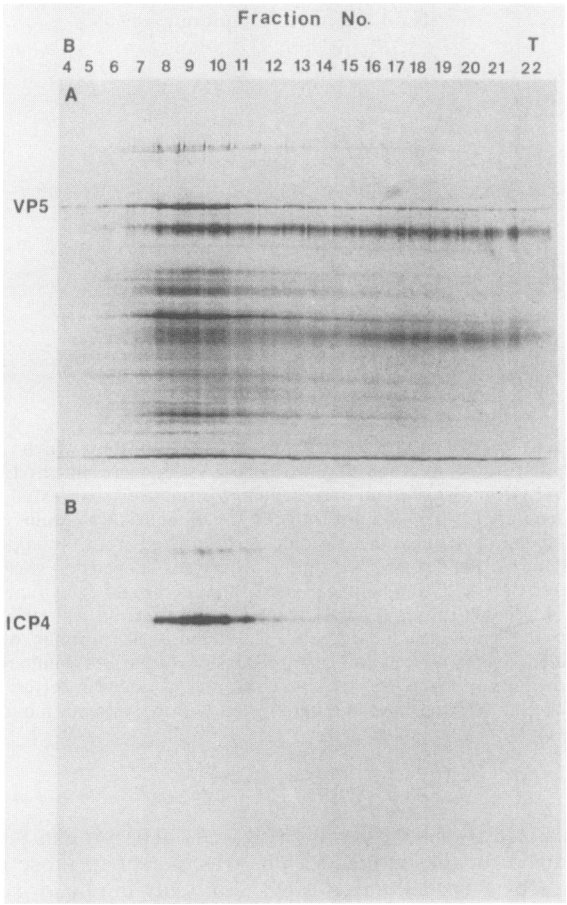

FIG. 2. Components reactive with ICP4 immune serum copurify with virus particles. [^{35}S]methionine-labeled extracellular virions were layered onto a 20 to 60% linear sucrose gradient. After 20 h of centrifugation at 50,000 × *g*, the sucrose gradient was fractionated and the individual fractions were assayed by SDS-PAGE (7% acrylamide). Figure 2A shows the autoradiography of [^{35}S]methionine-labeled protein profiles of each fraction. Figure 2B is an autoradiogram of the anti-ICP4 immunoblot of the identical samples analyzed in Fig. 2A.

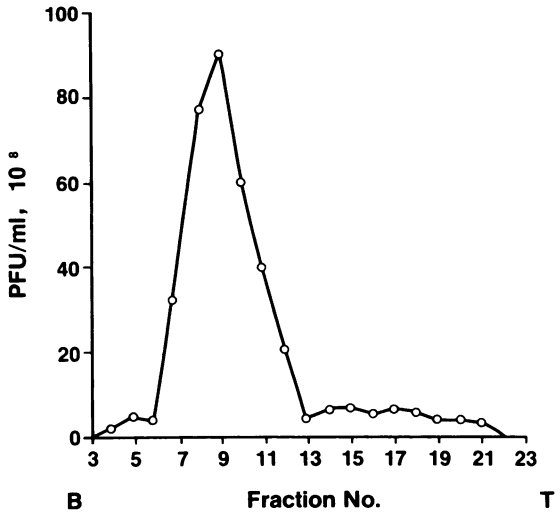

FIG. 3. Infectious HSV-1 particles copurify with ICP4-reactive components. Extracellular virions were centrifuged as described in the legend to Fig. 2. After centrifugation, individual fractions were titered for infectious virus on Vero cell monolayers (1). The same fractions were also assayed by SDS-PAGE and immunoblotted with anti-ICP4 serum.

contained the highest titer of HSV PFU. Since these same fractions contained the majority of the ICP4 detectable by immunoblotting (data not shown), these data support the observation that ICP4 is associated with purified virions.

Efforts were also made to address the possibility that the association of ICP4 with virions was due to nonspecific trapping of this nuclear-associated protein. Studies were performed to compare the association of ICP4 and the nuclear-associated 65K DNA-binding protein (11, 21) with purified virions. Samples from HSV-1-infected cell lysates, nuclear fraction, and purified virions were analyzed on SDS-PAGE and were immunoblotted with either anti-ICP4 serum (Fig. 4B) or with a monoclonal antibody specific for the 65K DNA-binding protein (Fig. 4A). The results presented in Fig. 4A demonstrate that there is no association of the 65K DNA-binding protein with purified virions, whereas ICP4 was detected in this same virion preparation. These data provide additional evidence for the selective nature of the incorporation of ICP4 into the virions.

Effect of detergent treatment on the association of ICP4 with purified virions. The objective of the following experiments was to determine the fate of ICP4 when the virion envelope glycoproteins were solubilized with nonionic detergents. Purified virions were treated with 1% Triton X-100 and then were subjected to high-speed centrifugation. Both the supernatant and the pelleted virus particles were analyzed by SDS-PAGE (Fig. 5). Lanes 1 and 2 of each gel shown contain virions that were incubated in the presence of Triton X-100 for 15 min. Lanes 3 and 4 contain virions that were treated with Triton X-100 for 30 min. The data in Fig. 5A show that most of the glycoproteins (gB and gC) and some tegument proteins were released from the purified virions into the supernatant after detergent treatment. Immunoblots of the same samples with anti-gC serum (Fig. 5B) revealed that over 90% of gC was solubilized from virus particles after detergent treatment. In contrast, the data presented in Fig. 5C, which is an immunoblot with anti-ICP4 serum, show that no ICP4 was detected in the supernatant fraction. All ICP4

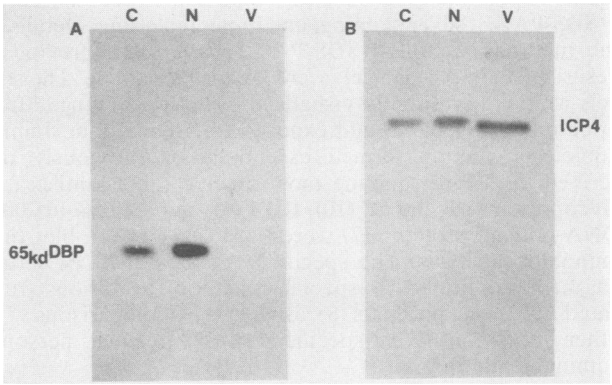

FIG. 4. Selective incorporation of ICP4 into virions. Nuclei were isolated from HSV-1-infected cells as described in Materials and Methods. The samples from HSV-1-infected cell lysates (C), nuclear fraction (N), and purified virions (V) were analyzed by SDS-PAGE and immunoblotted with either monoclonal antibody to the 65K DNA-binding protein (A) or anti-ICP4 monospecific polyclonal sera (B).

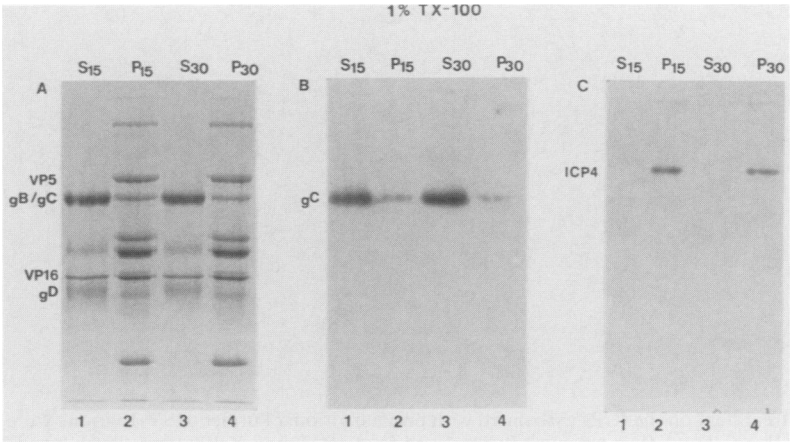

FIG. 5. Effect of detergent treatment on the association of ICP4 with purified virions. [^{35}S]methionine-labeled virions were treated with 1% Triton X-100 for 15 min (lanes 1 and 2) or 30 min (lanes 3 and 4) at 37°C, followed by 1 h of centrifugation at 70,000 × *g* which resulted in two fractions, the supernatant (S) and the pelleted virus particles (P). The supernatant and pellet fractions were analyzed by SDS-PAGE (7% acrylamide) and either they were directly visualized by autoradiography (A) or the resolved proteins were transferred onto nitrocellulose paper and blotted with anti-gC (B) or anti-ICP4 (C) serum.

remained associated with the pelleted virus particles and was not solubilized with virus envelope glycoproteins.

The next set of experiments was designed to release ICP4 by somewhat harsher solubilization procedures. Purified virions were treated with 0.5% DOC and 0.5% Triton X-100 for 15 min, followed by a further 10-min incubation with 0.01% SDS and 10 or 50 mM urea. The reaction mixtures were separated into the supernatant and pelleted virus particles by high-speed centrifugation as described above. The samples were assayed by SDS-PAGE and immunoblotted with anti-ICP4 (Fig. 6A) or anti-gC (Fig. 6B) serum. Lanes 1 and 2 in Fig. 6A and B contain fractions derived from purified virions treated with detergent in the presence of 10 mM urea, while the samples in lanes 3 and 4 were prepared from virus treated with detergent and 50 mM urea. Under these solubilization conditions, gC was completely released from the virus particles as indicated by the absence of any immunoreactivity with the pelleted material. In contrast, data presented in Fig. 6A illustrate that ICP4 was not released into the supernatant, which indicates that ICP4 remains associated with virus particles under conditions that solubilize the envelope glycoproteins.

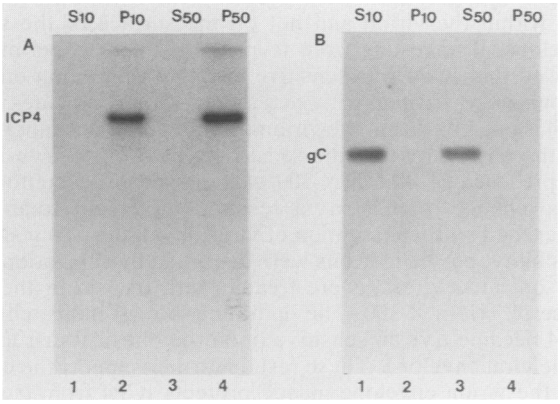

FIG. 6. Effect of detergent plus urea treatment on the ICP4 associated with purified virions. Purified virus particles were treated with 0.5% DOC and 0.5% Triton X-100 for 15 min at 37°C, followed by a further 10-min incubation with 0.01% SDS and either 10 (lanes 1 and 2) or 50 (lanes 3 and 4) mM urea at 0°C. The reaction mixtures were separated into supernatant (S) and pelleted virus particles (P) by centrifugation at 70,000 × *g* for 1 h. The samples were resolved by SDS-PAGE (7% acrylamide) and immunoblotted with either anti-ICP4 (panel A) or anti-gC (panel B) serum.

Effect of trypsin treatment on the ICP4 associated with purified virus particles. The studies described above showed that, unlike the virion glycoproteins, ICP4 could not be solubilized with detergent treatment, which suggests that virion-associated ICP4 is located internally. If the ICP4 is present within the virion and not on the surface, then trypsin treatment of purified virions should not affect the virion-associated ICP4. To test this, purified virions were treated with trypsin in either the presence or absence of detergent as described in the Materials and Methods. The proteolysis reaction was terminated by the addition of trypsin inhibitors, and the proteins were precipitated with acetone. The samples were resolved by SDS-PAGE, transferred onto nitrocellulose paper, and immunoblotted with anti-gB, anti-gC, and anti-ICP4 sera. The results presented in Fig. 7 show that glycoproteins gB (Fig. 7B) and gC (Fig. 7C) were sensitive to trypsin treatment both in the presence (lanes 3 and 5) and absence (lanes 2 and 4) of detergent. In fact, no components of gB were detectable after trypsin treatment. Glycoprotein C was less sensitive to trypsin and always yielded two detectable trypsin digestion products (Fig. 7C). In contrast, the data presented in Fig. 7A indicate that ICP4 was sensitive to trypsin treatment only if the virions were also treated with detergent (lanes 3 and 5). Trypsin treatment of the virions in the absence of detergent yielded amounts of ICP4 that were similar to those yielded by the mock-treated virion control (Fig. 7A, lane 1). These results suggest that the virus envelope protects ICP4 from trypsin digestion, and, therefore, ICP4 is apparently located within the virion.

Is ICP4 associated with virus capsids isolated from the nuclear fraction? The experiments described above suggest that virion-associated ICP4 is not part of the viral envelope. Since ICP4 is a DNA-binding protein (19), it might be located within the core of the nucleocapsid. To test this, virus capsids were purified from the nuclear fraction of HSV-1-infected HEp-2 cells by a method previously described by Gibson and Roizman (12). Proteins of purified type B viral

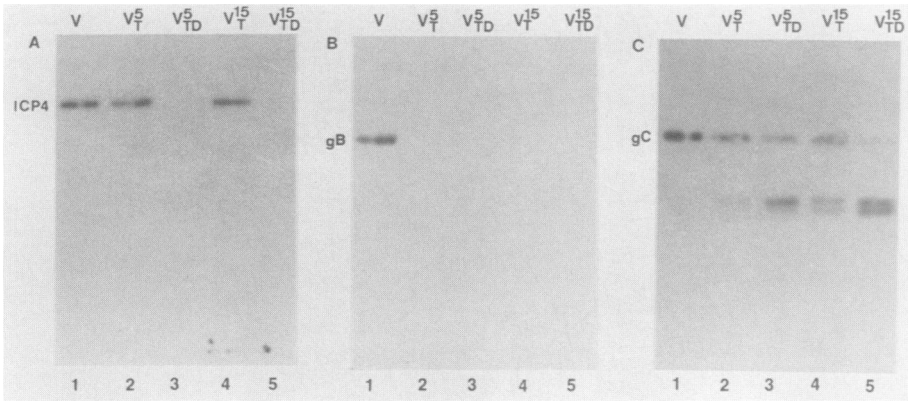

FIG. 7. Effect of trypsin treatment on the ICP4 associated with purified virions. Purified HSV-1 virions were treated with 0.1 mg of trypsin per ml in either the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 1% Triton X-100 for 5 (lanes 2 and 3) or 15 (lanes 4 and 5) min at 37°C. The proteolysis 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 (7% acrylamide) and immunoblotted with anti-ICP4 (A), anti-gB (B) or anti-gC (C) serum. Lane 1 in each gel contains mock-treated purified virions.

capsids and virions labeled with [^{35}S]methionine were resolved by SDS-PAGE, and the gels were analyzed either by autoradiography or by immunoblotting with anti-ICP4 serum. Nearly equal amounts of the [^{35}S]methionine-labeled major capsid polypeptide (VP5) were detected in both the purified virions and the type B nucleocapsid preparations (Fig. 8A), which indicates that a similar number of virus particles was present in both samples. In addition, the type B virus capsids contained a protein designated VP22a, which is characteristic of type B nucleocapsids (12). Figure 8B clearly demonstrates that little to no ICP4 was detectable in the purified type B capsids obtained from the nuclear fraction. In addition, ICP4 was not detected in purified preparations of type A capsids isolated from the nuclei of infected cells (data not shown). These results suggest that ICP4 is not located within the viral nucleocapsid core and thus may be associated with the tegument portion of the virion.

In an effort to quantitate the amount of ICP4 within the virion, HSV-1-infected Vero cells were labeled with ^{14}C-amino acid mixture (1 μl/ml) at 2 h postinfection, and extracellular virions were purified 24 h after infection, as described in Materials and Methods. The purified virions were resolved on SDS-PAGE, analyzed by autoradiography of the ^{14}C-amino acid-labeled proteins, and quantitated by densitometric scanning with a laser densitometer (data not shown). The number of molecules of ICP4 per virion was estimated according to a method described by Heine et al. (13), and the results indicate that each HSV-1 virion contains approximately 100 molecules of ICP4.

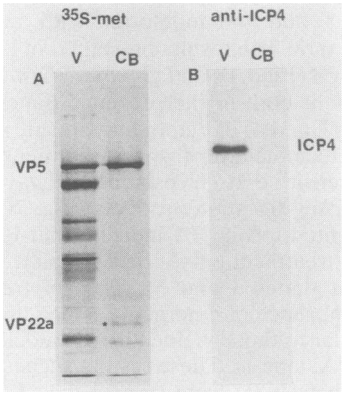

FIG. 8. Association of ICP4 with capsids isolated from the nuclear fraction. Type B viral capsids were purified from the nuclear fraction of HSV-1-infected HEp-2 cells by a method described by Gibson and Roizman (12). The [^{35}S]methionine-labeled proteins (^{35}S-met) of purified capsids and virions were resolved by SDS-PAGE (7% acrylamide) and were either directly visualized by autoradiography (A) or transferred onto nitrocellulose paper and immunoblotted with anti-ICP4 serum (B). V, Purified virions; C_B, purified type B viral capsids. *, Type B virus capsid-specific protein, VP22a.

DISCUSSION

In the studies described above, we have shown that detectable amounts of the major transcriptional regulatory protein of HSV-1, ICP4, are associated with purified virions. Furthermore, the results suggest that the virus-associated ICP4 is located within the tegument region of the virus particles. To our knowledge, this is the first report of an association of a HSV-1 immediate-early protein with purified virions.

Several approaches were used to demonstrate that ICP4 was within the virion and not on the surface of the virus envelope. The results from trypsin treatment experiments showed that ICP4 was sensitive to trypsin digestion only if virions were treated with detergent, which indicates that ICP4 is present within the virions. However, one cannot rule out the possibility that the sensitivity of ICP4 to trypsin in the presence of Triton X-100 was caused by a conformational change in ICP4 as a result of detergent treatment rather than by the disruption of virus envelopes. To test this possibility, purified virions were disrupted by sonication and the sonicated virions were treated with trypsin in the absence of Triton X-100. The data (not shown) indicated that ICP4 became trypsin sensitive upon the physical disruption of the viral envelope. These results further support the claim that the virion envelope indeed protects ICP4 from trypsin digestion and, therefore, ICP4 is present within the purified virion.

A series of experiments were conducted in an attempt to release ICP4 from virus particles. For example, purified virions were treated with 0.5% Triton X-100 and DOC in the presence of 0.01% SDS and 10 or 50 mM urea (Fig. 6). Alternatively, virions were treated with 0.5 M urea, 0.5% Brij 58, conditions similar to those used to prepare type B

nucleocapsids, and 0.25 to 1 M KCl. By using either approach, ICP4 was never detected in the supernatant fraction (data not shown). In contrast, significant amounts of the virus tegument protein, VP16 or Vmw65, were released into the supernatant under these two treatment conditions (data not shown). These data suggest that ICP4 is tightly associated with the viral capsid.

It is of interest to consider at what stage in the virion morphogenesis ICP4 becomes associated with the virus particle. It has been reported that at late times after infection ICP4 is located at the inner lamella of the nuclear membrane (2). It is possible that ICP4 is incorporated into the tegument as the nucleocapsid buds from the inner nuclear membrane. Recent preliminary results indicate that the lower-molecular-weight species of ICP4 is present in purified virions, while the predominate high-molecular-weight form is associated with purified nuclei (data not shown). Whether its incorporation into the virus tegument is a specific or nonspecific process is currently under study.

The most important question yet to be answered concerns the functional role that virion-associated ICP4 may play during the viral replication cycle. Three possibilities exist: (i) ICP4 may be inadvertently incorporated into the virus particle and has no functional role; (ii) the virion-associated ICP4 may be required as a minor structural component of the virus tegument or matrix region; (iii) the virion-associated ICP4 may be involved in enhancing the expression of immediate-early genes. The best example of a virion component acting as a *trans*-inducing factor is VP16, or Vmw65, which is required for the enhanced expression of immediate-early genes (15, 16, 28, 35). Whether the virion-associated ICP4 may also play a role in the *trans*-induction of specific immediate-early genes is currently under study.

ACKNOWLEDGMENTS

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