Herpes Simplex Virus Tegument ICP0 Is Capsid Associated, and Its E3 Ubiquitin Ligase Domain Is Important for Incorporation into Virions[▽]

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Herpes simplex virus (HSV) immediate-early (IE) protein ICP0 is a multifunctional regulator of HSV infection. ICP0 that is present in the tegument layer has not been well characterized. Protein compositions of wild-type and ICP0 null virions were similar, suggesting that the absence of ICP0 does not grossly impair virion assembly. ICP0 has a RING finger domain with E3 ubiquitin ligase activity that is necessary for IE functions. Virions with mutations in this domain contained greatly reduced levels of tegument ICP0, suggesting that the domain influences the incorporation of ICP0. Virion ICP0 was resistant to removal by detergent and salt and was associated with capsids, features common to inner tegument proteins.

Herpes simplex virus (HSV) particles are composed of three morphologically distinct structural components. The icosahedral capsid is surrounded by the tegument layer, which is in turn surrounded by a host-derived lipid envelope (49). After the viral envelope fuses with the host cell membrane, the bulk of the tegument appears to remain with the cell membrane (38). Direct delivery of tegument proteins, such as VP16, can rapidly stimulate viral gene expression (2). The release of another tegument protein, vhs, leads to degradation of mRNAs (48). Tegument components can also affect pre-immediate-early (pre-IE) events, including processes that occur after penetration but prior to IE gene expression (30). The tegument layer comprises more than 19 viral proteins and trace amounts of cellular proteins (33). Tegument infected cell protein 0 (ICP0) (58) and ICP4 (57) are present in 100 to 200 copies per virion. Tegument ICP0 and ICP4 are detected by Western blotting (13, 14, 35, 45, 52, 56, 58, 59) and mass spectrometry (35) but not by protein staining (53).

ICP0 is a 110-kDa phosphoprotein that is a promiscuous transactivator of viral and cellular genes (20, 21, 47). ICP0 is expressed with IE kinetics and is needed for efficient progression to lytic infection (20, 27, 51, 54). It is required for growth during low-multiplicity infections (7) and is needed for efficient reactivation from latency (9, 28, 29, 34). ICP0 also inhibits the antiviral response to cellular interferons (42) and may help the virus bypass innate cellular repression pathways (26). Newly synthesized ICP0 induces proteasome-dependent degradation of components of nuclear subdomains called ND10, including promyelocytic leukemia antigen (PML) and Sp100 (8, 23). The N-terminal RING finger zinc-binding motif of ICP0 is required for ND10 disruption. The RING finger functions as an E3 ubiquitin ligase *in vitro* and induces colocalization of conjugated ubiquitin at ND10 (3, 19). The disruption of ND10 structures is thought to facilitate HSV gene expression (6, 37). Little is known about the ICP0 that is brought in with the infecting virion. It is not clear whether tegument ICP0 func-

tions in a manner similar to its IE counterpart. IE protein ICP27 is required for the cytoplasmic localization and the incorporation of ICP0 and ICP4 into mature virions (52). VP22 is also necessary for the efficient incorporation of ICP0. Virions assembled in the absence of VP22 have reduced amounts of ICP0, ICP4, glycoprotein E (gE), and gD (13, 14). Here, we characterize fundamental properties of ICP0 that is present in HSV virions.

We investigated whether ICP0 influences the protein composition of mature extracellular virions. All viruses were provided by R. Everett. Cell-free extracellular virions were propagated on U2OS cells and isolated as described previously (44). One microgram (Fig. 1A, left) or equivalent VP5 units (Fig. 1A, right) of virions of parental virus HSV type 1 (HSV-1) Glasgow strain 17 syn^+ (4) (herein referred to as 17^+) or virions of ICP0 null mutant dl1403 (Table 1) were analyzed by SDS-PAGE followed by Coomassie blue staining. For each structural protein of the 17^+ virions that was detectable by Coomassie blue staining, there was a counterpart protein detected in dl1403. Although several proteins were detected at various levels, the overall protein profile of virions released from U2OS cells was not grossly altered by the absence of ICP0 (Fig. 1A). Equivalent VP5 units of extracellular virions were analyzed by Western blotting with antibodies against ICP0 (mouse monoclonal antibody [MAb] 11060, a gift of R. Everett) (22), gB (MAb H1817), ICP4 (MAb HIA021), VP5 (MAb HA018), and VP16 (MAb 1-21), all from Virusys (Sykesville, MD), and gD (MAb DL6) (10) and gH-gL (rabbit polyclonal antibody R137) (46), gifts of R. Eisenberg and G. Cohen. The envelope glycoproteins and tegument proteins tested did not appear to be reduced in the absence of ICP0 (Fig. 1B). By these measures, ICP0 does not appear to grossly affect the protein content of mature virions. Based on this analysis, however, we cannot rule out that one or more structural proteins in addition to ICP0 may be missing from the ICP0 null virus.

ICP4 is expressed with IE kinetics and is a major transactivator of early and late genes (12). IE ICP0 and ICP4 interact and have a synergistic effect on gene expression (25, 47). We used Western blot analysis to determine whether the absence of ICP0 affected the incorporation of ICP4. ICP4 was detected
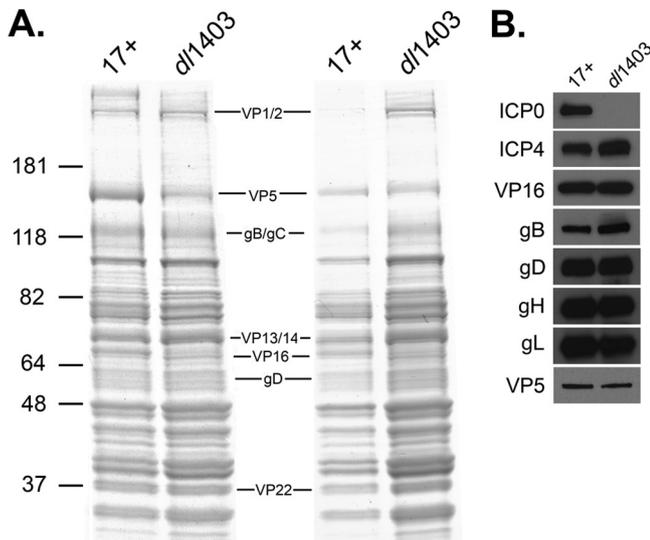
FIG. 1. Protein composition of herpes simplex virions in the absence of ICP0. (A) The HSV-1 wild type (17⁺) or ΔICP0 strain (dl1403) was analyzed by SDS-PAGE followed by Coomassie blue staining. One microgram (left) or equivalent VP5 units (right) of each virus were loaded. In the center, locations of several HSV structural proteins are indicated. Numbers to the left are molecular mass markers in kilodaltons. (B) Equivalent VP5 units of extracellular virions were analyzed by SDS-PAGE followed by Western blotting with antibodies against the indicated tegument protein or glycoprotein.
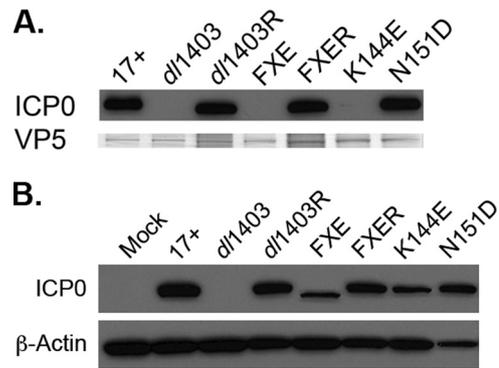

FIG. 2. Effect of RING finger mutations on the incorporation of ICP0 into virions. (A) Incorporation of tegument ICP0 into HSV-1 mutants. The indicated extracellular virions (identified by relevant mutations where applicable) were analyzed by SDS-PAGE followed by Western blotting with the 11060 antibody to ICP0. In parallel, VP5 was detected by Coomassie staining to demonstrate equivalent levels of particle loading. dl1403R and FXER, dl1403 and FXE rescuants carrying a restored ICP0 gene. (B) Expression of ICP0 in cells infected with wild-type or mutant viruses. U2OS cells were infected with the indicated virus (MOI = 1) for 18 h. Cell lysates were analyzed by SDS-PAGE followed by Western blotting with MAb H1A027 to ICP0.

in dl1403 virions (Fig. 1B), suggesting that it can be incorporated into virions independently of ICP0. Note that these results do not rule out a role for ICP0 in proper virion assembly. Tegument assembly involves a complex series of protein-protein interactions, and individual tegument proteins may serve redundant functions (40). Other tegument proteins in dl1403 virions may substitute for a structural or assembly role of tegument ICP0.

We determined whether the RING finger domain influences ICP0 incorporation into the tegument layer. First, the relative amounts of VP5 in various ICP0 mutant virions (Table 1)

TABLE 1. Details of ICP0 proteins expressed by viruses

Virus	ICP0 structure[f]	Description
17⁺[a]	1–775	Wild type
dl1403[b]	1–105	ICP0 null mutant
dl1403R[c]	1–775	ICP0⁺ rescuant
FXE[d]	1–105::150–775	RING finger domain deletion mutant
FXER[c]	1–775	Rescuant with wild-type RING finger
K144E mutant[e]	Point mutation at amino acid 144	Mutant with substitution in RING finger helix
N151D mutant[e]	Point mutation at amino acid 151	Mutant with substitution in RING finger helix

[a] Parental virus HSV-1 Glasgow strain 17 syn⁺ (4).
[b] Has a 2-kb lesion in both inverted repeat copies of the ICP0 gene (54).
[c] Rescued virus was obtained by cotransfection with mutant virion DNA and a plasmid containing a fragment comprising the ICP0 gene (18, 50).
[d] Has a defined lesion in the ICP0 RING finger domain yielding a deletion of 45 amino acids (16-18, 24).
[e] Has a single amino acid substitution in the alpha helix of the ICP0 RING finger (15).
[f] Amino acids included or relevant mutation in ICP0 variant.

propagated on U2OS cells were determined for each preparation of virus (Fig. 2A). The ICP0 contents of virions were then analyzed by SDS-PAGE and Western blotting with MAb 11060 to ICP0. As expected, the wild-type strain 17⁺ contained tegument ICP0 (Fig. 2A, lane 17⁺) while the dl1403 mutant did not (Fig. 2A, lane dl1403). Strain FXE virions did not contain ICP0 (Fig. 2A, lane FXE), suggesting that the RING finger domain is important for the association of ICP0 with mature virions. ICP0 was detected in dl1403 and FXE rescuants (Fig. 2A, lanes dl1403R and FXER), both of which bear the restored ICP0 gene. Interestingly, K144E virions had greatly reduced levels of tegument ICP0 whereas N151D virions had wild-type levels (Fig. 2A, lanes K144E and N151D). Trace amounts of ICP0 were reproducibly detected in K144E virions upon longer exposure to film. To confirm that the reduction in tegument ICP0 in FXE and K144E viruses was not due to lack of expression of mutant ICP0 in infected cells, the expression of ICP0 during mutant virus infection was compared to that during wild-type 17⁺ infection. U2OS cells were infected with equivalent VP5 units, corresponding to a multiplicity of infection (MOI) of 1 for 17⁺. Cell lysates were prepared 18 h postinfection. Samples were analyzed by SDS-PAGE and Western blotting with antibody to ICP0 (MAb H1A027; Virusys, Sykesville, MD) or to β-actin (MAb AC-74; Sigma, St. Louis, MO) to demonstrate equivalent levels of loading. Mutant ICP0 was readily detected in cells infected with FXE or the K144E mutant (Fig. 2B) as reported previously (15, 18, 54). Thus, the RING finger plays an important role in the incorporation of ICP0 into virions.

While K144E and N151D mutations in the RING alpha helix both decrease the ability of ICP0 to activate gene expression, the K144E phenotype is more pronounced (3, 15). Also, K144E and N151D mutants both fail to induce the colocalization of conjugated ubiquitin, but ICP0 with the N151D mutation may still interact with the ubiquitin machinery (19). It is not clear whether these activities relate to the inclusion of the

NaCl

ICP0

VP1-2

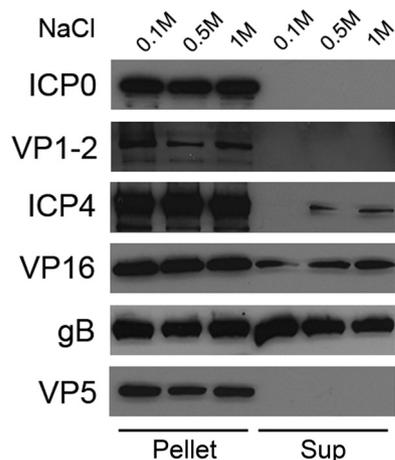ICP4

VP16

gB

VP5

Pellet   Sup

FIG. 3. Sensitivity of tegument ICP0 to detergent lysis and salt treatment. Extracellular virions were treated with 1% Triton X-100 in the presence of 0.1, 0.5, or 1 M NaCl. The reaction mixtures were then centrifuged through a 35% sucrose cushion. Pelleted (pellet) and released (sup) fractions were analyzed by SDS-PAGE and immunoblotting with antibodies to ICP0, VP1-2, ICP4, VP16, gB, or VP5.

N151D mutant protein and the exclusion of the K144E mutant protein from the tegument. Late in infection, newly synthesized ICP0 shuttles from the nucleus to the cytosol (32). ICP0 may be incorporated into the teguments of virions in the cytosol (14, 52). RING finger mutation causes ICP0 to be more readily retained in the nucleus at late time points in infection (36). Future studies will investigate the role that the RING domain plays in the relationship between the cellular localization of ICP0 and the incorporation of ICP0 into the tegument layer.

To determine the position of ICP0 within the tegument layer, a tegument release assay was employed (1, 41, 55). This is a measure of how readily detergent and salt can remove a structural protein from the virion. Typically, outer tegument proteins are partially released from virions following treatment with Triton X-100 and up to 1 M NaCl, but capsid-associated or inner tegument proteins are resistant to such treatment. Extracellular virions (3×10^9 PFU) were resuspended in 0.2 ml lysis buffer (50 mM HEPES [pH 7.4] and 1% Triton X-100) containing 0.1, 0.5, or 1 M NaCl and incubated for 30 min at 37°C. The reaction mixtures were then layered onto 0.5 ml of a 35% sucrose solution (containing 50 mM HEPES [pH 7.4] and 0.1 M NaCl) and centrifuged at $21,000 \times g$ for 10 min. The 200-μl supernatant above the sucrose cushion was recovered. Released proteins in the supernatant were heat precipitated and resuspended in Laemmli buffer. The sucrose cushion was removed, and the pellet, containing capsids and capsid-associated tegument proteins, was resuspended in Laemmli buffer. Pelleted and released samples were separated by SDS-PAGE and blotted onto nitrocellulose. Western blots were probed with antibodies against ICP0 (MAb H1A027), gB (MAb H1817), ICP4 (MAb HIA021), VP5 (MAb HA018), VP16 (MAb 1-21; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or VP1-2 (polyclonal antibody R27B4, a gift of R. Courtney) (39).

As expected, gB was detected in the supernatant, indicating that it was removed along with the virion envelope (Fig. 3). In contrast, ICP0 remained in the pellet along with VP5, the major capsid component, even at high salt concentrations (Fig. 3). ICP0 was not detected in the supernatant even after longer exposures to X-ray film (data not shown). VP1-2 is a known inner tegument protein and remained associated with capsids in the pellet (Fig. 3) (5, 41, 55). In contrast, a fraction of VP16 was readily removed by 1% Triton X-100, consistent with the presence of VP16 in the outer tegument (41, 43, 55). Interestingly, detectable amounts of ICP4 were released by detergent and increasing salt concentrations (Fig. 3), suggesting that ICP4 is an outer tegument component. The presence of tegument ICP4 near the viral envelope may facilitate rapid release into the cytoplasm upon viral penetration and play a pre-IE role in HSV infection. In contrast, ICP0 is capsid associated. Although specific functions have not yet been ascribed to virion ICP0 and ICP4 proteins, it is tempting to speculate that their different positions in the tegument layer reflect distinct roles in pre-IE events.

We previously showed that HSV entry requires 26S proteasome activity at a postpenetration step, likely the transport of incoming capsids to the nucleus (11). We are currently investigating whether tegument ICP0 and its E3 ubiquitin ligase domain play a role in proteasome-dependent entry of HSV. There is also potential for functional interaction of ICP0 with the inner tegument protein VP1-2, which has an N-terminal ubiquitin-specific protease domain (31). Ongoing studies are aimed at delineating the fate and function of tegument ICP0 during viral entry.

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