Complementation of a Herpes Simplex Virus ICP0 Null Mutant by Varicella-Zoster Virus ORF61p

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The herpes simplex virus (HSV) ICP0 protein acts to overcome intrinsic cellular defenses that repress viral α gene expression. In that vein, viruses that have mutations in ICP0’s RING finger or are deleted for the gene are sensitive to interferon, as they fail to direct degradation of promyelocytic leukemia protein (PML), a component of host nuclear domain 10s. While varicella-zoster virus is also insensitive to interferon, ORF61p, its ICP0 ortholog, failed to degrade PML. A recombinant virus with each coding region of the gene for ICP0 replaced with sequences encoding ORF61p was constructed. This virus was compared to an ICP0 deletion mutant and wild-type HSV. The recombinant degraded only Sp100 and not PML and grew to higher titers than its ICP0 null parental virus, but it was sensitive to interferon, like the virus from which it was derived. This analysis permitted us to compare the activities of ICP0 and ORF61p in identical backgrounds and revealed distinct biologic roles for these proteins.

Alphaherpesviruses encode orthologs of the herpes simplex virus (HSV) α gene product ICP0. ICP0 is a nuclear phosphoprotein that behaves as a promiscuous activator of viral and cellular genes (7, 11, 28, 29). ICP0 also functions as an E3 ubiquitin ligase to target several host proteins for proteasomal degradation (4, 10, 11, 16, 26). Through this activity, ICP0 promotes degradation of components of nuclear domain 10 (ND10) bodies, including the promyelocytic leukemia protein (PML) and Sp100. These proteins are implicated in silencing of herpesvirus genomes (9, 10, 22, 34). Therefore, ICP0-mediated degradation of ND10 components may disrupt silencing of HSV genes to enable efficient gene expression. This hypothesis provides a plausible mechanistic explanation of how ICP0 induces gene activation.

Introduction of DNA encoding the ICP0 orthologs from HSV, bovine herpesvirus, equine herpesvirus, and varicella-zoster virus (VZV) can also affect nuclear structures and proteins (27). In addition, and more specific to this report, ORF61p, the VZV ortholog, activates viral promoters and enhances infectivity of viral DNA like ICP0, the prototype for this gene family (24, 25). However, we have previously demonstrated two key biological differences between the HSV and VZV orthologs. We first showed that unlike ICP0, ORF61p is unable to complement depletion of BAG3, a host cochaperone protein. As a result, VZV is affected by silencing of BAG3 (15), whereas growth of HSV is altered only when ICP0 is not expressed (17). Furthermore, we have shown that while both proteins target components of ND10s, expression of ICP0 results in degradation of both PML and Sp100, whereas ORF61p specifically reduces Sp100 levels (16). These findings suggest that these proteins have evolved separately to provide different functions for virus replication.

Virus mutants lacking the ICP0 gene have an increased particle-to-PFU ratio, a substantially lower yield, and decreased levels of α gene expression, in a multiplicity-of-infection (MOI)- and cell-type-dependent manner (2, 4, 8, 33). These mutants are also defective at degrading ND10 components (23). Depletion of PML and Sp100 accelerates virus gene expression and increases plaques efficiency of HSV ICP0-defective viruses but has no effect on wild-type virus, suggesting that PML and Sp100 are components of an intrinsic anti-HSV defense mechanism that is counteracted by ICP0’s E3 ligase activity (9, 10). Interestingly, ICP0 null viruses are also hypersensitive to interferon (IFN) (26), a property that was suggested to be mediated via PML (3).

To directly compare the activities of the two orthologs, we constructed an HSV mutant virus that expresses ORF61p in place of ICP0. The resulting chimeric virus only partially rescues the ICP0 null phenotype. Our studies emphasize the biological differences between ICP0 and ORF61p and shed light on the requirements for PML and Sp100 during infection.

MATERIALS AND METHODS

Mammalian cells. Human melanoma (MeWo), siPML (17), siSp100 (16), L7 (30), and U2OS cells were maintained as previously described (15, 35).

DNA transformation. DNAs were transformed into the appropriate cell lines using Fugene HD (Roche, Indianapolis, IN).

Drug treatment. IFN-α was purchased from PBL Biomedical (Piscataway, NJ).

Viruses. (i) HSV. The strains used were wild-type HSV type 1 (HSV-1) (Glasgow strain 17) and an ICP0 null virus derivative of strain 17 (dl1403) (33).

(ii) HSV expressing VZV ORF61p (HSV-ORF61). dl1403 nucleocapsids were cotransfected with linearized pCPC-061 into MeWo cells. Large plaques were picked and screened for recombinant viruses by PCR. Plaques that were positive for ORF61p but not for ICP0 coding sequence were plaque purified five times.

(iii) Virus growth assays. (i) Plaque assays. Confluent monolayers of MeWo, siPML, siSp100, L7, or U2OS cells were infected with 10-fold serial dilutions of virus stocks, the monolayers were fixed and stained, and plaques were counted.

(ii) Growth curves. The titers of all HSV stocks were determined prior to analysis by titration on the ICP0-complementing cell line L7. Virus yield was determined as previously described (17).
**RESULTS**

**Generation of a VZV-HSV recombinant expressing ORF61p.** Coinfection with VZV complemented growth of an HSV-ICP0 mutant (33). Subsequently, a cell line that conditionally expressed ORF61p was used to complement an ICP0 null mutant (24). The latter experiment suggested that these virus orthologs shared some biological activities. However, these proteins differentially affected ND10 components, and wild-type VZV, but not HSV, showed a distinct requirement for these components (16). Therefore, to further dissect the function of ORF61p, we asked if it might substitute for HSV ICP0 when it replaced the duplicated IE-0 loci.

To replace the loci encoding ICP0 HSV, dl1403 was used as the viral backbone. dl1403 encodes the first 105 amino acids and an additional 56 amino acids that are derived from an out-of-frame fusion of the second and third exons of the IE-0 gene. ORF61p-coding sequences were amplified and inserted into the viral backbone.

**Antibodies.** Polyclonal antibodies to ICP0 were described previously (20). Monoclonal antibodies to tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against PML and ORF61p were described previously (16). Monoclonal antibodies to ICP0 and ICP4 were purchased from the Rumbaugh-Goodwin Institute (Plantation, FL). Polyclonal antibodies against PML and Sp100 were purchased from Chemicon (Temecula, CA). Goat anti-rabbit and anti-mouse antibodies conjugated to horseradish peroxidase for immunoblotting were from KPL (Gaithersburg, MD).

**SDS-polyacrylamide gel electrophoresis and Western blotting.** Infected or biochemically transformed cells were washed twice with cold phosphate-buffered saline (PBS), lysed in 1.5% Tris-HCl [pH 6.8], 150 mM dithiothreitol, 3% SDS, 0.15% bromophenol blue, 15% glycerol, and boiled, and host and viral proteins were subjected to SDS-polyacrylamide gel electrophoresis (18). Proteins were transferred to nitrocellulose membranes before Western blotting. After membranes were blocked in 5% nonfat milk in PBS-Tween 20 (PBST), immobilized proteins were reacted with the appropriate antibodies in 1% nonfat milk in PBST. Membranes were washed three times for 5 min each with PBST, incubated with an anti-rabbit or anti-mouse antibody conjugated to horseradish peroxidase, and washed again three times for 5 min with PBST and twice with PBS. Antibodies were visualized by addition of LumiGLO substrate (KPL) and exposure to X-ray film.
when ORF61p was expressed (Fig. 3). As expected, ICP0 and ORF61p were detected only in cells infected with viruses that expressed these proteins. These data, consistent with studies of plaquing efficiency and growth (Fig. 2), demonstrated that ORF61p did not fully phenocopy the biological properties of ICP0, although it clearly boosted replication of an ICP0 null virus.

**Fate of ND10 components following infection.** ICP0 is necessary and sufficient to dissociate ND10s and target their two major components, PML and Sp100, for proteasomal degra-

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**FIG. 1.** Construction of an HSV expressing VZV ORF61p. (A) Schematic diagram of the ICP0 locus. UTR, untranslated region. (B) pCPC-061 and Hirt DNA prepared from dl1403- or HSV-ORF61-infected cells was amplified using the primer sets specified in Materials and Methods. (C) MeWo cells were either mock treated or infected with wild-type HSV-1, dl1403, or HSV-ORF61 at an MOI of 5. At 8 hpi cells were harvested, and Western blotting using the antibodies described in Materials and Methods was used to monitor the abundance of ICP0, ICP4, ORF61p and tubulin.

**FIG. 2.** Growth analysis of HSV expressing VZV ORF61p. (A) Vero or L7 cells were infected with serial dilutions of wild-type HSV-1, dl1403, or HSV-ORF61. At 3 days postinfection, monolayers were fixed and stained and plaques were counted. The relative plaquing efficiency is the titer on L7 cells/titer on Vero cells × 100. The error bars indicate standard deviations from four independent experiments, each performed in duplicate. (B) MeWo cells were infected with wild-type HSV-1, dl1403, or HSV-ORF61 at an MOI of 0.1. At 2, 12, 24, and 48 hpi, infected cells were harvested and subjected to three rounds of freeze-thawing, and yields were calculated after titration on L7 cells.
dation. In contrast, ORF61p does not degrade PML but decreases Sp100 levels (16). Accordingly, the fate of PML and Sp100 was followed during an infection with HSV-ORF61 and compared with what occurred in cells infected with wild-type or dl1403 virus. Western blot analysis revealed that degradation of PML was detected in cells infected with wild-type virus as early as 2 hpi. In contrast, PML levels were not altered in cells infected with either dl1403 or HSV-ORF61 (Fig. 4A).

These results corroborated our previous findings and demonstrated that ORF61p did not affect the steady-state level of PML even when other HSV immediate-early or early proteins were present. Sp100 is another major component of ND10s, and it is well known that it is efficiently degraded following infection with HSV in an ICP0-dependent fashion (4). Here we demonstrated that in the context of HSV gene expression, ORF61p effectively directed degradation of Sp100 (Fig. 4A).

Because wild-type HSV and VZV are differentially affected by depletion of PML or Sp100 (16), we asked if HSV-ORF61 virus was affected by downregulation of these host proteins. To do this, we measured the relative plaquing efficiency of HSV-ORF61 in siPML and siSp100 cells and compared it to the efficiencies of wild-type HSV and dl1403. As previously reported, wild-type virus plaquing efficiency was not affected when titrated on cells from which PML (siPML) or Sp100 (siSp100) was depleted (Fig. 4B). In contrast, dl1403 was partially complemented in the absence of these ND10 components (Fig. 4B). The relative plaquing efficiency of HSV-ORF61 phenocopied VZV (16). More specifically, the virus titer increased in siPML cells, whereas it remained unchanged in siSp100 cells (Fig. 4B).

Effect of IFN on virus replication. Previous studies suggested that HSV’s IFN sensitivity is mediated via PML and proposed that an ICP0 null virus is hypersensitive in part because it fails to degrade this cellular protein. Having shown that HSV-ORF61 was unable to degrade PML, we asked how IFN treatment would affect the growth of this mutant virus. We compared the plaquing efficiencies of HSV-ORF61, wild-type HSV-1, and dl1403 on MeWo, Vero (which respond to but do not express IFN [6]), and U2OS (a cell line that complements ICP0 mutant viruses [36]) cells in the presence and absence of IFN. The plaquing efficiencies of both dl1403 and HSV-ORF61 in MeWo and Vero cells were affected by IFN-stimulated gene products presumably synthesized in response to IFN (Fig. 5). The small difference (four to fivefold) in sensitivity seen with wild-type virus on MeWo and Vero cells was not a result of differences in absolute plaquing efficiency but rather reflected greater sensitivity of all viruses to the effects of IFN in Vero cells (Table 1). In support of this, we note the increased sensitivity of dl1403 to IFN in Vero cells. As previously described (26), U2OS cells rescued the sensitivity of ICP0 mutants to IFN. In a similar fashion, the plaquing efficiency of HSV-ORF61 following treatment with IFN was also rescued (Fig. 5). These analyses revealed that while VZV ORF61p

FIG. 3. Time course of expression of virus-specified proteins. MeWo cells were infected with the wild type (HSV-1), ICP0 null virus (dl1403), and HSV-ORF61 at an MOI of 0.2. Infected cells were harvested at the indicated times and examined for the synthesis and abundance of ICP4, ICP27, ICP0, and ORF61p by Western blotting. All lanes were stained with antitubulin antibody to demonstrate that equivalent amounts of cell protein were loaded in each lane.

FIG. 4. Fate and requirement of PML and Sp100 during infection with HSV expressing VZV ORF61p. (A) MeWo cells were either mock treated or infected with wild-type HSV-1, dl1403, or HSV-ORF61 at an MOI of 10. At 2 and 4 hpi, cells were harvested and Western blotting was used to monitor the abundance of PML, Sp100, ICP0, ORF61p, and tubulin. (B) Empty, siPML, and siSp100 cells were infected with serial dilutions of wild-type HSV-1, dl1403, or HSV-ORF61. At 3 days postinfection, monolayers were fixed and stained and plaques were counted and compared to the number formed in empty cells. The error bars represent standard deviations from three independent experiments, each performed in duplicate.
substituted for some of ICP0's functions, it was clear that it did not complement all of the defects in d1403, as evidenced by HSV-ORF61's failure to recapitulate the wild-type IFN-resistant phenotype (Fig. 5).

**DISCUSSION**

HSV ICP0 is a RING finger protein that acts as a strong and promiscuous transcriptional activator of gene expression. Orthologs of ICP0 exist in other members of the alphaherpesvirus family. These proteins are related to ICP0 by virtue of their location within the virus genome and ability to influence gene expression. Sequence similarities are limited, with the exception of a RING finger close to the N termini in all orthologs. Specifically, the ICP0 ortholog in VZV, ORF61p, accelerates replication of an ICP0 null virus when coexpressed and also influences gene expression (24, 25). In spite of these similarities, we previously emphasized the lack of homologous ICP0 orthologs of ICP0 exist in other members of the alphaherpesvirus family. These proteins are related to ICP0 by virtue of their promiscuous transcriptional activator of gene expression. Or- thologs expressed in an identical genetic background, and any differences in the different cell lines, one might expect that an HSV expressing ORF61p in place of ICP0 would phenocopy VZV.

We therefore compared the plaquing efficiency of HSV-ORF61's failure to recapitulate the wild-type IFN-resis- tant phenotype, replication of HSV-ORF61 is less efficient than replication of wild-type virus. There is, of course, a possibility that these proteins are expressed to different levels, have distinct half-lives, and interact with HSV proteins differently, and this might affect the growth phenotype of HSV- ORF61. Nevertheless, we concluded that ORF61p lacks some of ICP0's functions.

ICP0 expressed from an adeno-virus causes efficient depletion of two major ND10 components, PML and Sp100, whereas an ORF61p-expressing adeno-virus reduced only Sp100 levels (16). Here, we compared the effects of these proteins on ND10 component abundance during virus replication and observed that even when other HSV proteins are expressed, ORF61p specifically decreases Sp100 with no effect on PML.

ND10s have been suggested to provide a nuclear form of innate immunity. Specifically, ND10 components act to repress expression of herpesvirus and other DNA virus genomes. In that vein, it is interesting that the replication and plaquing efficiency of d1403 but not wild-type virus are augmented in cells that lack PML or Sp100 (Fig. 4) (9, 10). In contrast, replication and plaquing efficiency of wild-type VZV are unaffected by depletion of Sp100 and augmented in siPML cells (16). Based on these observations and assuming that only ICP0 and ORF61p are necessary for the observed plaquing efficiencies in the different cell lines, one might expect that an HSV expressing ORF61p in place of ICP0 would phenocopy VZV. We therefore compared the plaquing efficiency of HSV- ORF61 on siPML and siSp100 cells to that on their parental

### TABLE 1. Virus titers

<table>
<thead>
<tr>
<th>Cells and IFN-α treatment</th>
<th>Virus titer (PFU), mean ± SD</th>
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<tbody>
<tr>
<td></td>
<td>HSV-17</td>
</tr>
<tr>
<td>MeWo</td>
<td></td>
</tr>
<tr>
<td>-IFN-α</td>
<td>1.65 x 10^6 ± 7.07 x 10^6</td>
</tr>
<tr>
<td>+IFN-α</td>
<td>1.10 x 10^7 ± 1.41 x 10^7</td>
</tr>
<tr>
<td>Vero</td>
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<tr>
<td>-IFN-α</td>
<td>1.30 x 10^6 ± 2.83 x 10^7</td>
</tr>
<tr>
<td>+IFN-α</td>
<td>3.00 x 10^7</td>
</tr>
<tr>
<td>U2OS</td>
<td></td>
</tr>
<tr>
<td>-IFN-α</td>
<td>1.23 x 10^6 ± 3.89 x 10^7</td>
</tr>
<tr>
<td>+IFN-α</td>
<td>4.00 x 10^7 ± 1.41 x 10^7</td>
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control cell lines and observed that although virus replication was partially complemented when PML was depleted, a reduction of Sp100 levels did not affect virus replication. This is a further demonstration that ORF61p has evolved to titrate Sp100 levels to allow efficient virus replication. However, unlike ICP0, ORF61p does not target PML; therefore, when only ORF61p is expressed, this host protein remains available to repress virus growth and replication.

Viruses expressing ICP0 or ORF61p differentially affect Sp100 degradation. Sp100 is normally resolved as three species during SDS electrophoresis (13, 32). The species recognized by our antibody in terms of rate of electrophoretic migration are Sp100A, Sp100A-SUMO, and Sp100-HMG. In cells infected with wild-type virus, the higher-molecular-weight species of Sp100 gradually disappeared, whereas Sp100A was stabilized (Fig. 4). This electrophoretic pattern of Sp100 mimics what is observed in cells depleted of PML by small interfering RNA (9, 16). In contrast, when ORF61p is expressed in place of ICP0, all isoforms and modified species of Sp100 gradually disappeared during infection, with no apparent difference in PML’s electrophoretic pattern. This is in agreement with experiments with small interfering RNA, which show that, unlike depletion of PML, downregulation of Sp100 has no effect on other components of ND10 bodies (9, 10, 16). Furthermore, we previously observed that infection of cells with an adenovirus expressing ICP0 resulted in disappearance of PML and Sp100 species except for Sp100A, whereas an ORF61p-expressing adenovirus reduced all Sp100 forms with no effect on PML (16).

These results lead us to propose that while these alphaherpesvirus orthologs target components of ND10, they do so in distinct ways. HSV ICP0 targets PML for proteasomal degradation. The reduction of PML levels results in disappearance of Sp100 species, except Sp100A. Therefore, by targeting PML, ICP0 directly or indirectly targets both major ND10 components. In contrast, ORF61p independently targets Sp100 for degradation. Unlike with ICP0, a reduction of Sp100 levels has no apparent effect on other ND10 proteins. We believe that differential targeting of ND10 proteins by these orthologs may account for at least some of the observed differences in their biological activities.

The precise role of PML during virus infection remains elusive. It is known, however, that HSV mutants lacking ICP0 and VZV mutants lacking ORF63p are hypersensitive to IFN (1, 26), and this effect is mediated by PML (3). In contrast, wild-type HSV (26) and VZV (1) are less sensitive to IFN. These data, along with our observation that PML is not degraded during VZV infection (16), suggest that IFN inhibits replication of these two human alphaherpesviruses by distinct mechanisms and that these viruses have evolved different and specific countermeasures. As a result, in contrast to HSV, it is likely that VZV does not require degradation of PML to overcome inhibition by IFN.

Our studies provide a basis for a molecular understanding of the functional differences between HSV ICP0 and VZV ORF61p. Our unpublished observations are consistent with previous reports that in spite of its functional handicap, ORF61p still activates both VZV and HSV promoters. However, as noted above, ORF61p lacks the immune regulatory activities of ICP0. Based on these observations, it is likely that Sp100 depletion is required to boost virus gene expression, whereas the activities that affect commandeerung of the IFN arm of the innate immune system might be mediated solely via PML. Therefore, further dissection of the functions of these orthologs might provide insight into the role of ND10 components during infection.

It has not escaped our notice that these results might also aid in development of HSV backbones as vaccine vectors (19). In these instances expression of ICP0 is required for chromatin modification and remodeling to allow efficient expression of virus genes (5, 12, 19). However, expression of ICP0 interferes with innate immunity. Moreover, deletion of ICP0 results in decreased virus titer. Thus, the use of HSV-ORF61 as the basis for a backbone provides an alternative to current herpesvirus-based vectors.

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