JOURNAL OF VIROLOGY, July 2010, p. 6861–6865 Vol. 84, No. 13
0022-538X/10/$12.00 doi:10.1128/JVI.00335-10

NOTES

The RING Finger Domain of Varicella-Zoster Virus ORF61p Has E3 Ubiquitin Ligase Activity That Is Essential for Efficient Autoubiquitination and Dispersion of Sp100-Containing Nuclear Bodies▽

Matthew S. Walters,† Christos A. Kyratsous,† and Saul J. Silverstein*

Department of Microbiology and Immunology, College of Physicians and Surgeons, Columbia University, 701 W. 168th St., New York, New York 10032

Received 12 February 2010/Accepted 6 April 2010

Varicella zoster virus encodes an immediate-early (IE) protein termed ORF61p that is orthologous to the herpes simplex virus IE protein ICP0. Although these proteins share several functional properties, ORF61p does not fully substitute for ICP0. The greatest region of similarity between these proteins is a RING finger domain. We demonstrate that disruption of the ORF61p RING finger domain by amino acid substitution (Cys19Gly) alters ORF61p intranuclear distribution and abolishes ORF61p-mediated dispersion of Sp100-containing nuclear bodies. In addition, we demonstrate that an intact ORF61p RING finger domain is necessary for E3 ubiquitin ligase activity and is required for autoubiquitination and regulation of protein stability.

Varicella-zoster virus (VZV) and herpes simplex virus type 1 (HSV-1) are distantly related alphaherpesviruses. VZV encodes an immediate-early (IE) protein termed ORF61p that is an ortholog of ICP0, an HSV-1 IE protein (14, 18). ICP0 is a RING finger domain-containing nuclear phosphoprotein that behaves as a promiscuous activator of viral and cellular genes (2, 3, 6, 17, 19). ICP0 also functions as an E3 ubiquitin ligase to target several host proteins for proteasomal degradation. Among these targeted proteins are nuclear domain 10 (ND10) components promyelocytic leukemia protein (PML) and Sp100 (1, 5, 7, 8, 12, 16, 21). Like ICP0, ORF61p is a transcriptional activator of viral promoters (15, 24), enhances infectivity of viral DNA (15) and has E3 ubiquitin ligase activity (4), and its presence results in decreased levels of Sp100 (8, 10). Despite these similarities, ORF61p is unable to fully complement growth defects of an HSV-1 ICP0 null virus (10, 14). Unlike ICP0, ORF61p does not rescue efficient virus growth in BAG3-depleted cells and is unable to target PML for proteasomal degradation (8–10). Thus, these proteins have evolved distinct mechanisms to augment virus replication. Although there is little amino acid conservation between ICP0 and ORF61p (10% similarity at the amino acid level), ORF61p retains the conserved RING finger residues that are essential for ICP0's transcriptional activation and ND10 dissociation and degradation activities (8). Previous studies revealed that the ORF61p RING finger domain is essential for its transcriptional activation functions (13); however, little is known regarding its role in other ORF61p activities. Therefore, this study was designed to further characterize the ORF61p RING finger domain.

To extend our understanding of the ORF61p RING finger domain, a plasmid expressing amino-terminal FLAG-tagged ORF61p (pCK-flag-ORF61) (13) was used as a template to mutate amino acid Cys-19 to Gly to create pCK-flag-ORF61RF. Mutation of this residue disrupts the RING finger domain (Fig. 1A) (13). 293A cells were mock transformed or transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF to test for expression of these proteins. After 48 h, the cells were harvested in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40, and 50 mM NaF plus Complete protease inhibitor cocktail [Roche, Indianapolis, IN]) and processed for Western blot analysis as previously described (22). The electrophoretic mobilities of tagged ORF61p and ORF61RFp were examined using mouse monoclonal Anti-FLAG M2 antibody (Sigma, St. Louis, MO) (Fig. 1B). Expression of wild-type ORF61p resulted in appearance of two major protein species of approximately 64 and 70 kDa, a finding consistent with what is observed during VZV infection (23, 24). However, surprisingly, only the 64-kDa protein species was detected in cells expressing ORF61RFp. We previously demonstrated that expression of only ORF61p reduced Sp100 levels (8, 10). Therefore, we next used immunofluorescence microscopy to investigate the requirement for an intact ORF61p RING finger on this activity. MeWo cells were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF and after 48 h cells were fixed and stained for ORF61p and Sp100 using FLAG and Sp100 antibodies, as described previously (8, 23) (Fig. 1C). Expression of wild-type ORF61p results in disappearance of Sp100-containing nuclear bodies. In contrast, Sp100 persisted, and nuclear bodies remained intact in cells expressing ORF61RFp. Analysis of cells expressing either ORF61p or ORF61RFp and

* Corresponding author. Mailing address: Department of Microbiology and Immunology, College of Physicians and Surgeons, Columbia University, 701 W. 168th St., New York, NY 10032. Phone: (212) 305-8149. Fax: (212) 305-5106. E-mail: sjs6@columbia.edu.
† M.S.W. and C.A.K. contributed equally to this study.
▽ Published ahead of print on 14 April 2010.

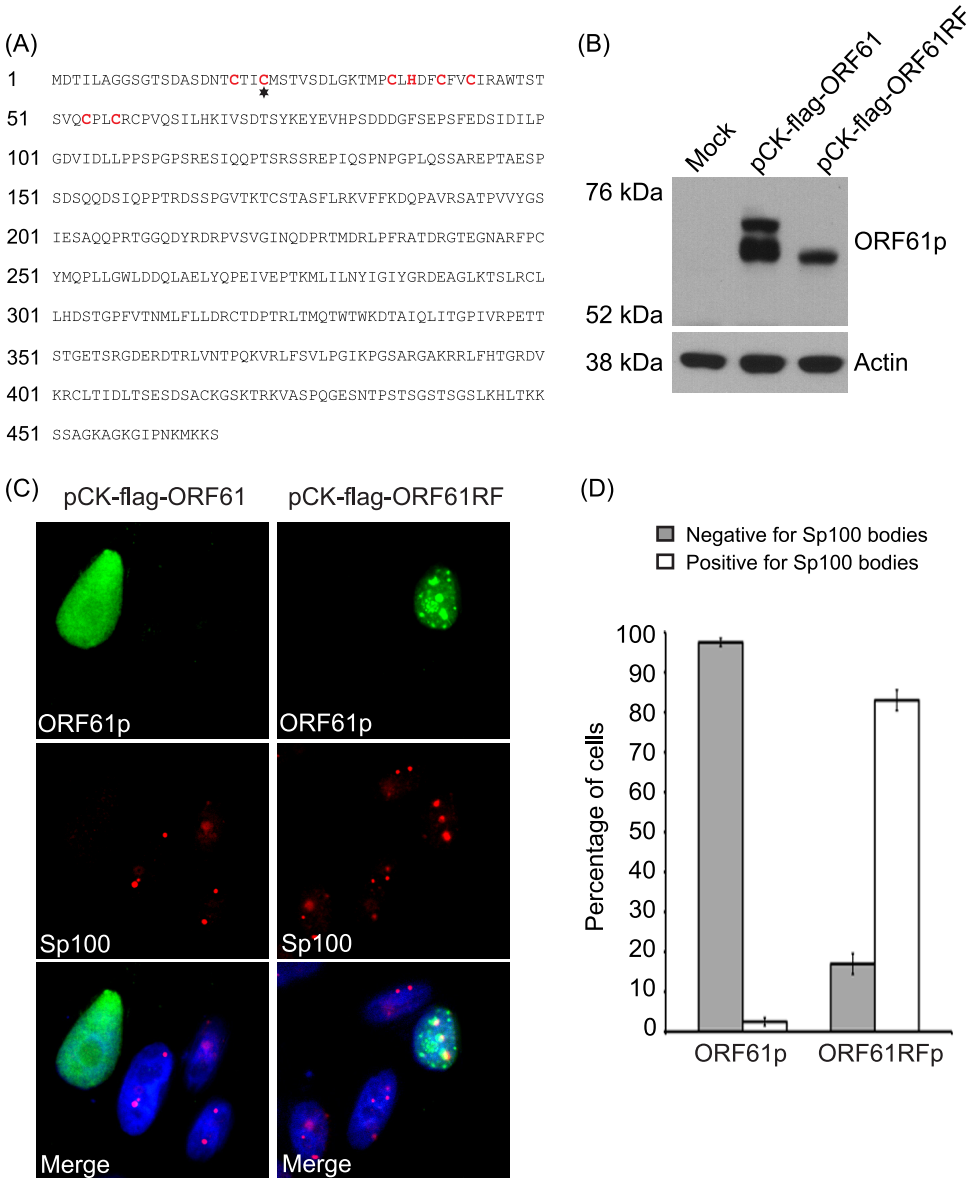

FIG. 1. Redistribution of Sp100 in ORF61p WT or ORF61RFp-expressing cells. (A) Amino acid sequence of wild type ORF61p (NCBI protein database accession number NP_040183.1). Cys and His residues of the C_3HC_4 RING finger consensus sequence are indicated in red boldface. The asterisk identifies the Cys19Gly mutation that disrupts the RING finger domain to generate ORF61RFp. (B) 293A cells were mock transformed or transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF. After 48 h, cells were harvested, and equal amounts of total protein were electrophoresed on NuPAGE 4 to 12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA) and then analyzed by Western blotting with antibodies specific for FLAG and actin. (C) MeWo cells grown on glass coverslips were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF. At 48 h posttransformation, cells were fixed, and the nuclear distribution of ORF61p (WT and RF) and Sp100 were monitored by indirect immunofluorescence microscopy using antibody specific for FLAG and Sp100, respectively. Nuclei were stained with Hoechst. Images were captured with a Zeiss Axiophot microscope using a ×100 objective lens and Open Lab software and analyzed by volume deconvolution. (D) For each sample, 50 cells were counted, and the number of ORF61p (WT and RF)-expressing cells was scored for Sp100-containing bodies. The results shown are from two independent experiments, each performed in duplicate.

the disappearance of Sp100-containing bodies is shown in Fig. 1D. Thus, the RING finger domain is required for ORF61p mediated dispersion of Sp100-containing nuclear bodies. There was a distinct difference in intracellular distribution of wild-type ORF61p compared to ORF61RFp. In most cells, wild-type ORF61p displayed a diffuse nuclear staining pattern, whereas ORF61RFp demonstrated intense punctate nuclear staining.

Punctate nuclear staining of the mutant RING finger protein suggested that its aggregation might result from increased protein stability. To test this hypothesis, MeWo cells were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF and, after 48 h, the cells were fixed and stained for ORF61p using FLAG antibody as described earlier. Ten hours before fixation the cells were either treated with DMSO or 20 μM MG132. For each sample, 100 cells were counted, and the protein

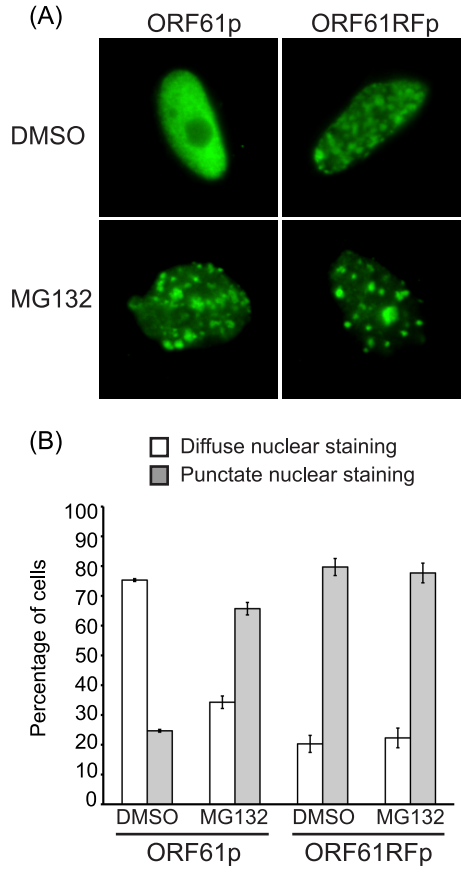

FIG. 2. Nuclear distribution of wild-type and RING finger mutant ORF61p. MeWo cells grown on glass coverslips were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF. At 38 h posttransformation, the cells were either treated with DMSO or 20 μM MG132. At 10 h posttreatment the cells were fixed, and the ORF61p (WT and RF) distribution was analyzed by indirect immunofluorescence microscopy with antibody specific for FLAG. Images were captured as described in Fig. 1. (A) Representative images for each protein and condition. (B) For each sample, 100 cells were counted, and the protein distribution was scored. The results shown are from three independent experiments.

distribution was scored as diffuse or punctate. Representative images are shown in Fig. 2A. In MeWo cells treated with DMSO, 75% of wild-type ORF61p-expressing cells displayed diffuse nuclear staining, whereas 25% displayed punctate nuclear staining reminiscent of that observed for ORF61RFp (Fig. 2B). For ORF61RFp, 80% of cells displayed punctate nuclear staining, and 20% displayed diffuse nuclear staining. Only 34% of cells treated with MG132 and expressing wild-type ORF61p displayed a diffuse nuclear staining, while 66% displayed punctate nuclear staining. Therefore, inhibition of the proteasome alters the nuclear distribution of wild-type ORF61p to more closely resemble the ORF61RFp pattern. In contrast, proteasome inhibition had no significant effect on ORF61RFp's staining pattern with 78% of cells displaying punctate nuclear staining and 22% displaying diffuse nuclear staining. These data suggest that diffuse nuclear staining of ORF61p results from proteasome-mediated degradation, and this process requires an intact RING finger domain. Interference with proteasome activity by chemical inhibition with MG132 or disruption of the RING finger domain results in increased stability of ORF61p and subsequent aggregation and punctate nuclear staining.

ICP0's E3 ubiquitin ligase activity, which is required for autoubiquitination and targeting host proteins for proteasomal degradation, maps to its RING finger domain (1, 7) and, recently, a fragment of ORF61p encompassing its RING finger domain (amino acids 1 to 165) was shown to have E3 ubiquitin ligase activity *in vitro* (4). Based on these data, we hypothesized that an intact ORF61p RING finger domain is required for E3 ubiquitin ligase activity. To test this hypothesis, 293A cells were transformed with a plasmid expressing hemagglutinin (HA)-tagged ubiquitin (pHA-Ub) (20) alone or cotransformed with pHA-Ub and pCK-flag-ORF61 or pCK-flag-ORF61RF. At 48 h posttransformation, cell lysates were prepared, and ORF61 proteins were isolated by binding to EZview Red Anti-FLAG M2 affinity gel (Sigma, St. Louis, MO). After binding, FLAG-tagged proteins were eluted with FLAG peptide (Sigma) and analyzed by Western blotting with antibodies to FLAG and HA (Roche). Analysis with FLAG-specific antibody demonstrated that both ORF61p and ORF61RFp were expressed to equal levels, and equal amounts of protein were bound (IC) (Fig. 3A). As expected, wild-type ORF61p produced multiple species of protein, with two major species of approximately 64 and 70 kDa, whereas only a single 64-kDa protein species was present for ORF61RFp. Analysis of these proteins using HA-specific antibody demonstrated equal staining of total protein in the input lanes; however, HA staining was only observed with wild-type ORF61p. Thus, wild-type ORF61p is ubiquitinated. Indeed, staining with HA-specific antibody produced a ladder of ubiquitinated protein species ranging from 70 to >225 kDa. These higher-molecular-weight species of ubiquitinated proteins may not all correspond to ORF61p; rather, they may represent ubiquitinated cellular proteins that interact with ORF61p. However, some of these bands are identical in mobility to what is detected when we probed specifically for ORF61p with FLAG antibody. For instance, the lowest-molecular-weight species detected with HA antibody was 70 kDa, and this corresponds to the slower-migrating more-abundant species of ORF61p detected with FLAG-specific antibody. This experiment demonstrates that while the 70-kDa species of ORF61p is ubiquitinated, the 64-kDa species is not. HA staining of purified FLAG-ORF61RFp was negative.

To further confirm that an intact RING finger domain is required for ORF61p E3 ubiquitin ligase activity, we performed *in vitro* ubiquitination assays. Briefly, 293T cells were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF. After 48 h, cell lysates were prepared in lysis buffer and incubated for 1 h with Anti-FLAG M2 affinity gel at 4°C. Bound proteins were either directly resolved or incubated with UBE1, His-UbcH3, UbcH5c, and ubiquitin (Boston Biochem, Cambridge, MA) at 30°C for 2 h. Proteins were detected by Western blotting with FLAG-specific antibody (Fig. 3B). Wild-type ORF61p possessed E3 ubiquitin ligase activity producing multiple higher-molecular-weight species indicative of polyubiquitin chains. However, ORF61RFp displayed significantly reduced levels of E3 ubiquitin ligase activity, resulting in a large reduction of higher-molecular-weight species of protein. The low level of E3 ubiquitin ligase activity associated with

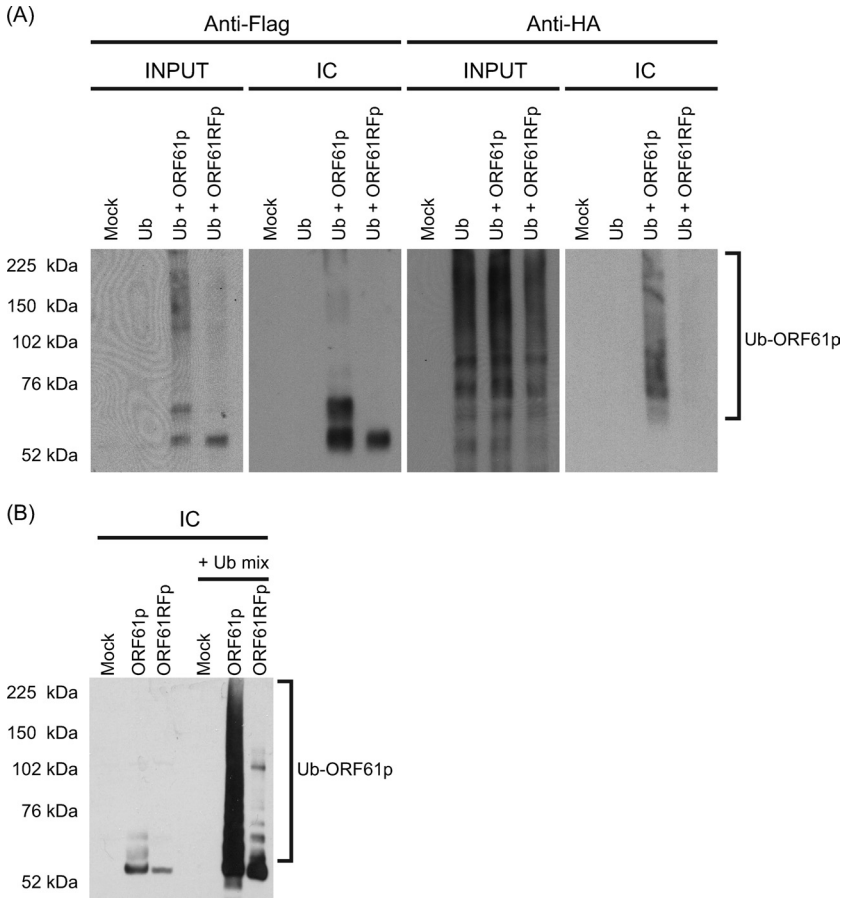

FIG. 3. Analysis of ORF61p ubiquitination. (A) 293A cells were mock transformed, transformed with pHA-Ub alone, or cotransformed with pHA-Ub and pCK-flag-ORF61 or pCK-flag-ORF61RF. After 48 h, cell lysates were prepared in lysis buffer and incubated overnight with EZview Red Anti-FLAG M2 affinity gel at 4°C. After incubation, immunocaptured (IC) proteins were eluted with FLAG peptide. Input and immunoprecipitated (IP) fractions were resolved on NuPAGE 4 to 12% Bis-Tris gradient gels and analyzed by Western blotting with antibody specific for FLAG and HA. (B) 293T cells were mock transformed or transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF. After 48 h, cell lysates were prepared in lysis buffer and incubated for 1 h with EZview Red Anti-FLAG M2 affinity gel at 4°C. Bound (IC) proteins were either directly resolved on NuPAGE 4 to 12% Bis-Tris gradient gels (Invitrogen) or incubated with UBE1, His-UbcH3, UbcH5c, and ubiquitin (Boston Biochem) at 30°C for 2 h (+ Ub mix). Proteins were detected by Western blotting with antibody specific for FLAG.

ORF61RFp may result from residual activity since only a single residue within the RING finger domain was altered. Alternatively, this activity may result from cellular E3 ligases that interact with ORF61p and coprecipitated with it. Overall, these data confirm that an intact RING finger domain is essential for ORF61p E3 ubiquitin ligase activity and autoubiquitination.

Pulse-chase analysis was used to measure the half-life ($t_{1/2}$) of ubiquitinated and nonubiquitinated forms of ORF61p. 293A cells were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF and after 24 h incubated with 100 μg of cycloheximide/ml. At intervals, cells were harvested by addition of Laemmli SDS-PAGE sample buffer (11), and equal volumes from each sample were analyzed by Western blotting with antibody specific for FLAG. For wild-type ORF61p, the nonubiquitinated 64-kDa form had a $t_{1/2}$ of 4 h, whereas the major 70-kDa ubiquitinated form had a $t_{1/2}$ of <2 h (Fig. 4). The $t_{1/2}$ of ORF61RFp was 4 h, which is identical to that observed for nonubiquitinated ORF61p. Therefore, RING finger-dependent ubiquitination of ORF61p results in faster turnover.

In summary, we demonstrate here that VZV ORF61p E3 ubiquitin ligase activity requires an intact RING finger do-

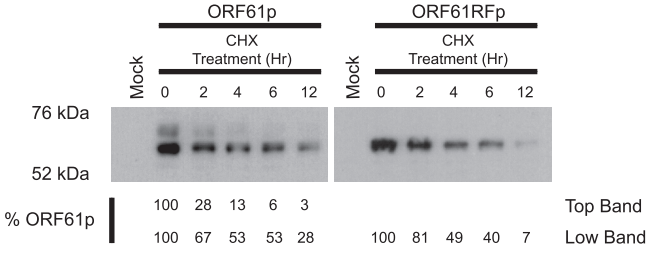

FIG. 4. Protein half-life analysis of wild type and RING finger mutant ORF61p. 293A cells were transformed with pCK-flag-ORF61 or pCK-flag-ORF61RF and after 24 h treated with 100 μg of cycloheximide (CHX)/ml. At various times posttreatment, cells were harvested directly by addition of Laemmli SDS-PAGE sample buffer, and equal volumes for each sample were run on NuPAGE 4 to 12% Bis-Tris gradient gels and analyzed by Western blotting with antibody specific for FLAG. Band intensity was quantified using ImageJ software.

main. The E3 ubiquitin ligase activity of ORF61p is essential for regulating its own stability and nuclear distribution and also for ORF61p-mediated dispersion of Sp100-containing nuclear bodies.

This study was supported by grant AI-024021 from the Public Health Service to S.J.S.

REFERENCES

1. **Boutell, C., S. Sadis, and R. D. Everett.** 2002. Herpes simplex virus type 1 immediate-early protein ICP0 and is isolated RING finger domain act as ubiquitin E3 ligases in vitro. J. Virol. **76:**841–850.
2. **Chen, J., and S. Silverstein.** 1992. Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. J. Virol. **66:**2916–2927.
3. **Everett, R. D.** 1985. Activation of cellular promoters during herpesvirus infection of biochemically transformed cells. EMBO J. **4:**1973–1980.
4. **Everett, R. D., C. Boutell, C. McNair, L. Grant, and A. Orr.** 2010. Comparison of the biological and biochemical activities of several members of the alphaherpesvirus ICP0 family of proteins. J. Virol. doi: 10.1128/JVI.02544–09.
5. **Everett, R. D., S. Rechter, P. Papior, N. Tavalai, T. Stamminger, and A. Orr.** 2006. PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0. J. Virol. **80:**7995–8005.
6. **Gelman, I. H., and S. Silverstein.** 1985. Identification of immediate-early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. Proc. Natl. Acad. Sci. U. S. A. **82:**5265–5269.
7. **Hagglund, R., C. Van Sant, P. Lopez, and B. Roizman.** 2002. Herpes simplex virus 1-infected cell protein 0 contains two E3 ubiquitin ligase sites specific for different E2 ubiquitin-conjugating enzymes. Proc. Natl. Acad. Sci. U. S. A. **99:**631–636.
8. **Kyratsous, C. A., and S. J. Silverstein.** 2009. Components of nuclear domain 10 bodies regulate varicella-zoster virus replication. J. Virol. **83:**4262–4274.
9. **Kyratsous, C. A., and S. J. Silverstein.** 2008. The co-chaperone BAG3 regulates herpes simplex virus replication. Proc. Natl. Acad. Sci. U. S. A. **105:**20912–20917.
10. **Kyratsous, C. A., M. S. Walters, C. A. Panagiotidis, and S. J. Silverstein.** 2009. Complementation of a herpes simplex virus ICP0 null mutant by varicella-zoster virus ORF61p. J. Virol. **83:**10637–10643.
11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
12. **Maul, G. G.** 1998. Nuclear domain 10, the site of DNA virus transcription and replication. Bioessays **20:**660–667.
13. **Moriuchi, H., M. Moriuchi, and J. I. Cohen.** 1994. The RING finger domain of the varicella-zoster virus open reading frame 61 protein is required for its transregulatory functions. Virology **205:**238–246.
14. **Moriuchi, H., M. Moriuchi, H. A. Smith, S. E. Straus, and J. I. Cohen.** 1992. Varicella-zoster virus open reading frame 61 protein is functionally homologous to herpes simplex virus type 1 ICP0. J. Virol. **66:**7303–7308.
15. **Moriuchi, H., M. Moriuchi, S. E. Straus, and J. I. Cohen.** 1993. Varicella-zoster virus (VZV) open reading frame 61 protein transactivates VZV gene promoters and enhances the infectivity of VZV DNA. J. Virol. **67:**4290–4295.
16. **Mossman, K. L., H. A. Saffran, and J. R. Smiley.** 2000. Herpes simplex virus ICP0 mutants are hypersensitive to interferon. J. Virol. **74:**2052–2056.
17. **Pereira, L., M. H. Wolff, M. Fenwick, and B. Roizman.** 1977. Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virology **77:**733–749.
18. **Perry, L. J., F. J. Rixon, R. D. Everett, M. C. Frame, and D. J. McGeoch.** 1986. Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. **67**(Pt. 11)**:**2365–2380.
19. **Quinlan, M. P., and D. M. Knipe.** 1985. Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. **5:**957–963.
20. **Stallings, C. L., and S. J. Silverstein.** 2006. Posttranslational modification and cell type-specific degradation of varicella-zoster virus ORF29p. J. Virol. **80:**10836–10846.
21. **Tavalai, N., P. Papior, S. Rechter, M. Leis, and T. Stamminger.** 2006. Evidence for a role of the cellular ND10 protein PML in mediating intrinsic immunity against human cytomegalovirus infections. J. Virol. **80:**8006–8018.
22. **Walters, M. S., A. Erazo, P. R. Kinchington, and S. Silverstein.** 2009. Histone deacetylases 1 and 2 are phosphorylated at novel sites during varicella-zoster virus infection. J. Virol. **83:**11502–11513.
23. **Walters, M. S., C. A. Kyratsous, S. Wan, and S. Silverstein.** 2008. Nuclear import of the varicella-zoster virus latency-associated protein ORF63 in primary neurons requires expression of the lytic protein ORF61 and occurs in a proteasome-dependent manner. J. Virol. **82:**8673–8686.
24. **Wang, L., M. Sommer, J. Rajamani, and A. M. Arvin.** 2009. Regulation of the ORF61 promoter and ORF61 functions in varicella-zoster virus replication and pathogenesis. J. Virol. **83:**7560–7572.