Novel Roles of Cytoplasmic ICP0: Proteasome-Independent Functions of the RING Finger Are Required To Block Interferon-Stimulated Gene Production but Not To Promote Viral Replication

Kathryne E. Taylor,[a] Marianne V. Chew,[b] Ali A. Ashkar,[b] Karen L. Mossman[a,b]

Departments of Biochemistry and Biomedical Sciences[a] and Pathology and Molecular Medicine,[b] McMaster Immunology Research Centre, Institute for Infectious Disease Research, McMaster University, Hamilton, Ontario, Canada

ABSTRACT

The immediate-early protein ICP0 from herpes simplex virus 1 (HSV-1) plays pleiotropic roles in promoting viral lytic replication and reactivation from latency. Most of the known actions of ICP0 occur in the nucleus and are thought to involve the E3 ubiquitin ligase activity of its RING finger domain, which targets proteins for degradation via the proteasome. Although ICP0 translocates to the cytoplasm as the infection progresses, little is known about its activities in this location. Here, we show that cytoplasmic ICP0 has two distinct functions. In primary cell cultures and in an intravaginal mouse model, cytoplasmic ICP0 promotes viral replication in the absence of an intact RING finger domain. Additionally, ICP0 blocks the activation of interferon regulatory factor 3 (IRF3), a key transcription factor of the innate antiviral response, in a mechanism that requires the RING finger domain but not the proteasome. To our knowledge, this is the first observation of a proteasome-independent function of the RING finger domain of ICP0. Collectively, these results underscore the importance of cytoplasm-localized ICP0 and the diverse nature of its activities.

IMPORTANCE

Despite ICP0 being a well-studied viral protein, the significance of its cytoplasmic localization has been largely overlooked. This is, in part, because common experimental manipulations result in the restriction of ICP0 to the nucleus. By overcoming this constraint, we both further characterize the ability of cytoplasmic ICP0 to inhibit antiviral signaling and show that ICP0 at this site has unexpected activities in promoting viral replication. This demonstrates the importance of considering location when analyzing protein function and adds a new perspective to our understanding of this multifaceted protein.

Herpes simplex virus 1 (HSV-1) is an enveloped, double-stranded DNA virus that is highly prevalent in human populations. One of the first proteins expressed during HSV-1 infection is infected-cell protein 0 (ICP0). A multifunctional protein, ICP0 is involved in regulating viral gene expression, promoting reactivation from latency, and optimizing the cellular environment to maximize replication (1). For the most part, the mechanisms used by ICP0 to achieve these varied tasks remain unclear. At present, almost all of the assorted actions of ICP0 are thought to require the E3 ubiquitin ligase activity of its RING finger domain, which has been implicated in the proteasome-dependent degradation of multiple cellular proteins (2–4). ICP0 has a nuclear localization signal (NLS) that initially causes it to translocate to the nucleus (5), and much of the work on ICP0 has focused on its roles in this location. However, as the infection progresses, ICP0 moves to the cytoplasm (6–10), and there are accumulating data suggesting that it also has important functions in this compartment (11, 12).

The innate immune response to virus infection is classically described as involving the production of type I interferon (IFN). Detection of conserved viral components by various pattern recognition receptors, including the Toll-like receptors (TLRs) and the retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs), results in the activation of a number of transcription factors, such as IFN-regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-κB), leading to the production of IFN (13). Autocrine and paracrine signaling activated by IFN culminates in the expression of IFN-stimulated genes (ISGs), the products of which work collectively to limit viral replication and spread (14). Accordingly, viruses have evolved a tremendous diversity of strategies to evade the IFN response (15). For example, ICP0 decreases IRF3 activation and ISG expression (16–20). Initial work suggested this function required both the RING finger domain and the activity of the proteasome (18, 19), yet during a wild-type (WT) HSV-1 infection, IRF3 pathway components are not degraded (12, 18), although some model systems remain contradictory in this regard (20–22). Recently, it has been found that when ICP0 is restricted to the nucleus during infection, by mutation or treatment with proteasome inhibitors, it loses its ability to block IRF3 signaling, while cytoplasmic ICP0 efficiently inhibits the antiviral response, even in the absence of a functional proteasome (12). Similarly, ectopic expression of wild-type ICP0, which causes its retention in the nucleus, fails to prevent IRF3-mediated ISG induction (12, 23). Currently, the function of cytoplasmic ICP0 in opposing IRF3 activation remains unknown.

Given that the proteasome is not required for cytoplasmic ICP0 to impede antiviral signaling, we were interested in determining whether the RING finger itself is involved in this process.

TABLE 1 ICP0 mutants used in this study

Virus	Mutation	Phenotype	Reference
17 syn	None	Wild type	112
D8	Deletion of amino acids 475–548 (NLS)	ICP0 restricted to cytoplasm	30
FXE	Deletion of amino acids 106–150 (RING)	ICP0 lacks a functional RING finger and is mainly nuclear	30
D8/FXE	Deletion of amino acids 106–150 (RING) and 475–548 (NLS)	ICP0 restricted to cytoplasm; lack of a functional RING finger	This work
D8/FXE-R	D8 and FXE mutations are repaired to wild-type sequence	Wild type	This work
dl1403	2-kb deletion in ICP0	ICP0 null	111

Typically, the E3 ubiquitin ligase activity of the RING finger is associated with proteasome-mediated degradation. However, there is mounting evidence to suggest that ubiquitin tagging does not act solely to target a protein for destruction but instead is involved in a variety of proteasome-independent signaling functions (reviewed in references 24 to 29). To investigate the role of the RING domain in the context of cytoplasmic ICP0, we generated a virus in which ICP0 is restricted to the cytoplasm and lacks the RING finger. Intriguingly, we found that despite the ability of cytoplasmic ICP0 to block ISG production in the absence of a functional proteasome, the RING finger is essential for ICP0 to inhibit the antiviral response. Unexpectedly, we also observed that cytoplasmic ICP0 has an important activity in promoting viral replication both in cell culture and in mice, even in the absence of the RING finger domain. These observations highlight two unknown aspects of ICP0: the RING finger can act in a proteasome-independent manner, and ICP0 has RING finger-independent functions in the cytoplasm.

MATERIALS AND METHODS

Reagents. MG132 (Sigma) or an equal volume of the vehicle control dimethyl sulfoxide (DMSO) was added to the culture medium 30 min prior to infection at a concentration of 5 μM and was maintained in the medium at this concentration throughout the experiment. Poly(I·C) (GE Healthcare) was added directly to the culture medium at a concentration of 100 μg/ml.

Cells and viruses. Human embryonic lung (HEL) fibroblasts and U2OS and Vero cells were purchased from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (HEL and U2OS) or 5% (Vero) fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. The virus strains used in this study are described in Table 1. 17 syn and D8/FXE-R were propagated on Vero cells, while all ICP0 mutants were grown on U2OS cells in the presence of 3 mM hexamethylene bisacetamide (HMBA) and purified on a 36% sucrose cushion. Viral infections were performed for 1 h in serum-free medium at 37°C and at a multiplicity of infection (MOI) of 10, unless otherwise stated.

Construction of recombinant D8/FXE and D8/FXE-R viruses. To create ICP0 containing the D8 and FXE lesions, a XhoI-KpnI fragment from p110-FXE (9), which contains the FXE lesion, was inserted into XhoI-KpnI-cut p110-D8 (9). The resulting plasmid, p110-D8/FXE, was sequenced to verify the presence of the D8 and FXE lesions. The D8/FXE virus was constructed using homologous recombination following cotransfection of U2OS cells with infectious DNA from 17 syn and the linearized p110-D8/FXE plasmid. Plaques were screened on Vero cells, which are semipermissive only for ICP0-deficient viruses, and small plaques were isolated for further characterization. Viruses containing D8/FXE ICP0 were plaque purified three times, and the presence of the D8 and FXE lesions was confirmed by Western blot analysis and sequencing. The D8/FXE-R revertant was generated by homologous recombination in U2OS cells of infectious DNA from D8/FXE with the p110 plasmid and screened on Vero cells for large plaques. Plaque purification and confirmation were done as with D8/FXE.

Preparation of cell extracts. For RIPA extracts, cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then scraped into RIPA buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 40 mM β-glycerophosphate, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 1× protease inhibitor cocktail [Sigma]), passed through a 25-gauge needle 5 times, and then centrifuged at 14,000 rpm for 20 min at 4°C. For cytoplasmic extracts, cells were washed once with ice-cold 1× PBS and once with ice-cold 0.2× PBS and then scraped into cytoplasmic buffer (10 mM HEPES, pH 7.3, 10 mM potassium chloride, 1.5 mM magnesium chloride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 1× protease inhibitor cocktail [Sigma]). The lysates were incubated on ice for 10 min, and then Triton X-100 was added to a final concentration of 1%, and samples were spun at $12,000 \times g$ for 3 min. Extracts were quantified via Bradford assay (Bio-Rad Laboratories).

Western blotting. Twenty-five micrograms of the indicated extracts were separated via electrophoresis on 10% denaturing polyacrylamide gels, transferred onto polyvinylidene difluoride (Millipore) membranes, and blocked in 5% skim milk. Blots were probed with the following primary antibodies, as indicated, at 1:1,000: anti-ICP0 (Virusys Corporation), anti-actin (Santa Cruz SC-1616), anti-ISG-56 (provided by G. Sen, Cleveland Clinic), and anti-USP7 (R2B2; provided by L. Frappier, University of Toronto). Secondary antibodies conjugated to horseradish peroxidase were used at 1:5,000, and the signal was visualized via chemiluminescence.

Immunoprecipitation. Anti-ICP0 antibody (5 μg) was incubated with 30 μl of Protein G Plus agarose (Thermo Scientific) for 1 h at 4°C. Beads were washed three times with immunoprecipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% NP-40) and incubated with 500 μg of cytoplasmic extract for an additional 4 h. The beads were washed 5 times and boiled in sample buffer containing SDS and β-mercaptoethanol.

Immunofluorescence. HEL cells were seeded onto coverslips and infected at 50% confluence. At the indicated times, the cells were fixed with 10% formalin (Sigma); permeabilized with 0.1% Triton X-100; and blocked with 3% goat serum, 3% bovine serum albumin (BSA), and 0.02% Tween 20. The cells were incubated with 1:250 anti-ICP0 and then 1:250 anti-mouse Alexa Fluor 488-conjugated secondary antibody (Invitrogen), and nuclei were stained with 1:10,000 Hoechst 33258 dye. All images were captured with a Leica DM-IRE2 inverted microscope and analyzed using Openlab software (Improvision).

Plaque assays. HEL cells were infected at the indicated MOIs and grown in 1 ml medium for 24 h, and the cells were scraped into the medium and freeze-thawed three times. Serial dilutions of the resulting samples were used to infect U2OS cells in the presence of HMBA and 2% human serum, and after 3 days, the cells were fixed with methanol and stained with Giemsa (Sigma), and the plaques were counted.

Quantitative reverse transcription (RT)-PCR. Six hours after infection with the indicated viruses, total RNA from HEL cells was harvested using TRIzol reagent (Invitrogen); 2.5 μg of RNA was DNase treated (DNA-free kit; Ambion), and then, 150 ng of each sample was reverse transcribed using 200 ng of a random 6-mer primer and 200 U of SuperScript II reverse transcriptase (Invitrogen). Samples were then analyzed in
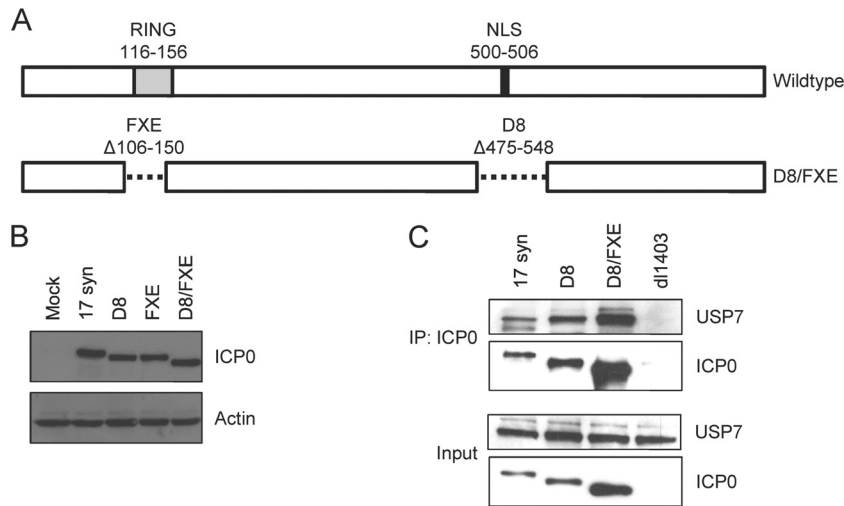
FIG 1 Generation of an HSV mutant expressing cytoplasmic ICP0 lacking the RING finger. (A) Schematic of the deletions in the *icp0* gene found in the wild-type and D8/FXE viruses. (B and C) HEL cells were infected with the indicated viruses at an MOI of 10 for 8 h. (B) Cells were harvested with RIPA extract and analyzed for ICP0 size and expression relative to actin by Western blotting. (C) Cytoplasmic extracts were obtained, and samples were immunoprecipitated with an αICP0 antibody. The eluant and input extracts were analyzed by Western blotting for ICP0 and USP7.

triplicate with universal PCR master mix and gene-specific TaqMan primers (Life Technologies): Hs01911452_s1 for IFIT1 and Hs99999905_m1 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression was normalized to GAPDH via the $\Delta\Delta C_T$ method and expressed as a fold change relative to the internal control, poly(I·C).

Mice and HSV-1 infection. Female C57BL/6 mice, 6 to 8 weeks of age, were purchased from Charles River Laboratory (Montreal, Quebec, Canada). The mice were housed under biosafety level 2 conditions in the Central Animal Facility at McMaster University. All mice were housed in level B rooms that followed a 12-h day and 12-h night schedule and were maintained under standard temperature-controlled conditions. All animal experiments were approved by the Animal Research Ethics Board (AREB) of McMaster University. To prepare mice for intravaginal infection, female mice were injected with 50 μl of 50-mg/ml Depo-Provera hormone (Pfizer Canada Inc., Kirkland, Quebec, Canada) subcutaneously 4 days prior to HSV-1 infection. To infect the mice, they were anesthetized by injectable anesthetic (150 μl of ketamine-xylazine [0.75 ml: 0.25 ml]) given intraperitoneally and infected intravaginally with 10^5 PFU of the indicated viruses in 10 μl of PBS. The mice were kept on their backs under the influence of anesthesia for 1 h to allow infection. The infected mice were followed daily for genital pathology and survival. Genital pathology was scored daily as follows: 0, no apparent infection changes; 1, redness of the external vagina; 2, severe redness and swelling of the external vagina; 3, severe redness and swelling of the external vagina and surrounding tissues and hair loss; 4, genital ulceration with severe redness, swelling, and hair loss of genital and surrounding tissues; 5, severe genital ulceration extending to surrounding tissue or any sign of hind-limb paralysis. To examine viral shedding and cytokines in the vaginal mucosa, vaginal lavage fluids were collected on days 1 to 5 postinfection by pipetting 2 doses of 30 μl of PBS into and out of the vagina six to eight times. Virus in the vaginal washes was quantified via plaque assay as described above.

ELISA. To measure IFN-γ concentrations in the vaginal lumen of infected mice, vaginal lavage fluids from several mice were pooled and assayed for IFN-γ production with the DuoSet ELISA (enzyme-linked immunosorbent assay) kit, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The plates were read using the Sapphire ELISA plate reader at 450-nm wavelength.

Statistical analysis. Analysis was performed using GraphPad Prism. Where necessary, values were first transformed via logarithmic transfor-

mation to equalize variance or arcsine-square-root transformation for proportions.

RESULTS

Generation of an HSV mutant expressing cytoplasmic ICP0 lacking the RING finger. To investigate the role of the RING finger in the cytoplasmic activity of ICP0, we generated an ICP0 construct containing both the FXE deletion (Δ106 to 150), which removes the RING finger domain, and the D8 deletion (Δ475 to 548), which disrupts the NLS (30) (Fig. 1A). This construct was introduced into the 17 syn genome via homologous recombination, and the presence of the expected mutations was verified via DNA sequencing. Western blot analysis confirmed the size reduction of the ICP0 protein expressed by the double-mutant virus, designated D8/FXE (Fig. 1B). To ensure that the presence of two deletions did not result in gross protein misfolding, we confirmed that the D8/FXE ICP0 was capable of binding to USP7, a well-characterized interaction partner whose binding site in the C terminus of ICP0 would not be directly impacted by FXE and D8 deletions (31–33) (Fig. 1C). As expected, the amount of ICP0 recovered in the cytoplasmic extracts differed among the viruses. Due to its exclusive cytoplasmic localization, a greater quantity of D8 ICP0 than of WT ICP0 was present, a portion of which was still nuclear at this time. D8/FXE ICP0 was found at the highest level, in accordance with its increased stability as a result of the loss of the RING finger (see below). However, the amount of USP7 recovered in each coimmunoprecipitation (co-IP) was proportional to the amount of ICP0 in the particular sample, demonstrating that all forms of ICP0 interact with USP7 to comparable degrees.

We next investigated the localization of D8/FXE ICP0 via immunofluorescence microscopy (Fig. 2). Unlike the FXE mutant, which was found predominantly in the nucleus, D8/FXE ICP0 was found exclusively in the cytoplasm. This localization pattern was confirmed in primary mouse fibroblasts and primary human genital epithelial cells (data not shown).
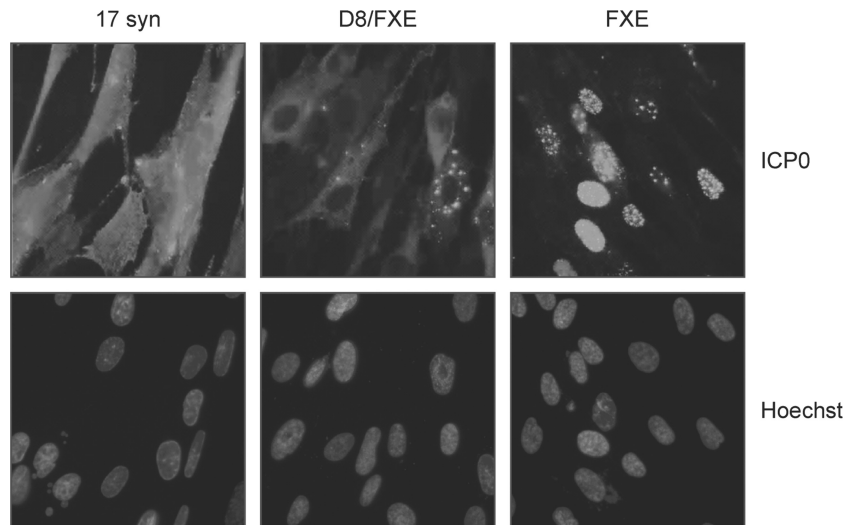
FIG 2 An ICP0 NLS mutant lacking the RING finger localizes to the cytoplasm. HEL cells were infected with the indicated viruses at an MOI of 10 for 8 h, and immunofluorescence microscopy was performed to determine the subcellular location of ICP0. Nuclei were stained with Hoechst dye. Magnification, ×400.

Cytoplasmic ICP0 promotes virus replication in cell culture in the absence of the RING finger. Since most of the known roles of ICP0 are thought to occur in the nucleus and require an intact RING finger domain, we expected the D8/FXE virus to be highly attenuated. HEL fibroblast cells were infected with D8/FXE, the single mutant D8 or FXE, the ICP0-null dl1403, and the wild-type parent strain 17 syn at both high (10) and low (0.1) MOIs for 24 h. The titer of the virus on U2OS cells in the presence of HMBA was then determined. Remarkably, the D8/FXE virus, as well as the D8 virus, grew significantly better than either the dl1403 or the FXE mutant at both high and low MOIs (Fig. 3A and C). Although neither D8/FXE nor D8 replicated as well as 17 syn, both viruses caused a productive infection at an MOI of 10, reaching titers approximately 8-fold higher than that of dl1403, which produced little more than the input virus. A similar pattern was observed even at the low MOI of 0.1, indicating that cytoplasmic ICP0 is still able to promote viral replication, even in the absence of a functional RING finger domain. The particle-to-PFU ratios of D8, D8/FXE, and dl1403 were comparable to, though higher than, those of 17 syn, as expected (data not shown). Therefore, differences in particle numbers cannot explain these results. To confirm that the increased growth of D8/FXE was not due to secondary site mutations, we generated the revertant virus D8/FXE-R, in which mutant ICP0 is replaced with WT ICP0. As expected, D8/FXE-R replicated to titers similar to those of 17 syn at both high and low MOIs (Fig. 3B and D).

To further characterize the point in the replication cycle affected by cytoplasmic ICP0, the expression of the immediate-early protein ICP4 was determined via immunofluorescence microscopy after infection with 17syn, D8, D8/FXE, or dl1403. The number of cells positive for ICP4 was determined relative to the total number of cells in each field of view (Fig. 3E). The pattern of ICP4 expression mirrored the observations for viral replication, with both D8 and D8/FXE showing a significantly greater number of cells expressing ICP4 than dl1403.

In terms of both titer and ICP4 expression, D8/FXE showed a slight but reproducible improvement in replication over D8. This may be explained by ICP0 autoubiquitination and subsequent degradation (34), which would be expected to occur in D8 but not the RING-deficient D8/FXE. In support of this possibility, D8/FXE ICP0 was found to accumulate to higher levels than D8, but the addition of MG132 normalized the levels of the two cytoplasmic ICP0 mutants (Fig. 3F). Together, these data suggest that cytoplasmic ICP0 stimulates the replication of HSV-1 in cell culture through a mechanism independent of the RING finger.

Cytoplasmic ICP0 cannot block antiviral signaling in the absence of the RING finger. We next investigated whether the increase in viral replication observed with cytoplasmic mutants of ICP0 could be explained by their ability to block antiviral signaling. HEL cells were infected with various HSV-1 mutants in the presence or absence of the proteasome inhibitor MG132, and antiviral state induction was monitored by determining the accumulation of ISG-56 message at 8 h postinfection via quantitative RT-PCR (Fig. 4A). 17 syn efficiently blocked the accumulation of ISG-56 message when the proteasome was active but lost the ability after treatment with MG132, while D8 blocked ISG-56 accumulation even when the proteasome was inhibited, in accordance with previous results (12). Intriguingly, D8/FXE was unable to prevent ISG-56 accumulation, regardless of the status of the proteasome, suggesting that cytoplasmic ICP0 requires the RING finger, but not the proteasome, for its ability to block antiviral signaling. We confirmed that the inability of D8/FXE to impede ISG-56 induction was due to the absence of the ICP0 RING finger domain, as the repaired strain, D8/FXE-R, efficiently prevented the activation of innate signaling (Fig. 4B). Similarly, examining ISG-56 protein levels via Western blot analysis confirmed that D8 efficiently blocked the activation of the antiviral response, while D8/FXE could not (Fig. 4C). Collectively, these data suggest that cytoplasmic ICP0 plays distinct roles in viral replication and antiviral state inhibition.

Cytoplasmic ICP0 does not require the RING finger to promote virus replication *in vivo*. To determine whether the differences in viral replication observed in cell culture were reproducible *in vivo*, a mouse intravaginal model was used. Despite increasing clinical data linking HSV-1 with genital infections, few murine studies utilize HSV-1 in intravaginal inoculation. Thus, in
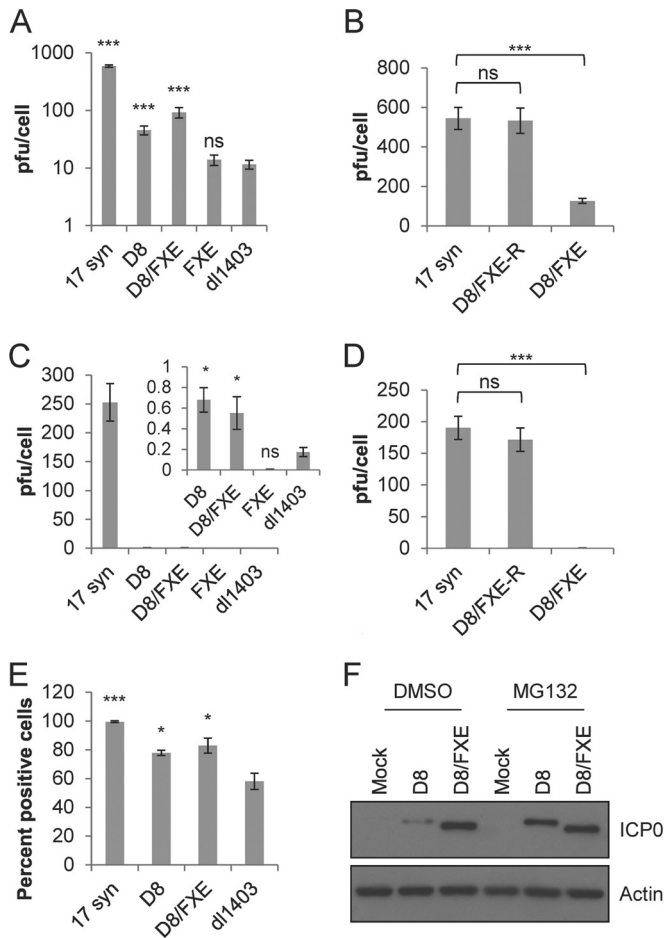
FIG 3 Cytoplasmic ICP0 promotes viral replication in the absence of the RING finger domain in primary fibroblasts. (A to D) HEL cells were infected with the indicated viruses at a high MOI of 10 (A and B) or a low MOI of 0.1 (C and D) for 24 h. Cells and supernatant were harvested and sent through 3 freeze-thaw cycles, and the titer of U20S cells in the presence of HMBA was determined. (E) HEL cells were infected with the indicated viruses at an MOI of 10 for 12 h, and immunofluorescence microscopy was performed to determine the number of cells expressing ICP4 relative to the total number of cells, as determined by staining nuclei with Hoechst dye. The data are the averages of three independent experiments ± standard errors of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's posttest (B and D) or Dunnett's posttest (A and C) relative to dl1403. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant. (F) HEL cells were infected with the indicated viruses at an MOI of 10 for 8 h in the presence of MG132 or the carrier DMSO and then harvested via cytoplasmic extract. Western blot analysis was performed to determine the levels of ICP0 and actin.

preliminary studies, 17 syn and dl1403 were tested in parental C57BL/6 (B6) and IRF3$^{-/-}$ mice (Fig. 5). One day postinfection (Fig. 5A), there was a trend toward increased viral titers in the vaginal washes for both 17 syn and dl1403 in IRF3$^{-/-}$ mice compared to B6-infected mice, suggesting that IRF3 contributes to the host response to vaginal HSV challenge. While this general trend was maintained at day 2 postinfection (Fig. 5B), we found that dl1403 was being cleared in both strains of mice. Of note, the ICP0-null virus did not reach wild-type titers in IRF3-deficient mice, indicating that ICP0 contributes important activities in addition to blocking IRF3.

From our *in vitro* data, we were particularly intrigued to ob-

serve that cytoplasmic ICP0 contributes to HSV replication in the absence of a RING finger. Since D8/FXE cannot block the IRF3-mediated antiviral response, while D8 can (Fig. 4), we chose to assess its ability to support virus replication *in vivo* in mice deficient for IRF3. Thus, we challenged IRF3-deficient mice with 17 syn, D8, D8/FXE, or dl1403, and viral titers in the vaginal washes were determined 2 days postinfection (Fig. 6A). Similar to *in vitro* findings, both D8 and D8/FXE replicated to significantly higher titers than dl1403, with the enhanced stability of ICP0 in D8/FXE likely responsible for the increase in titers over D8. Vaginal pathology results correlated closely with viral titers (Fig. 6B), and a similar pattern was also observed when levels of the cytokine IFN-γ were quantified in the vaginal washes (Fig. 6C). Collectively, these data show that *in vivo*, as well as in cell culture, cytoplasmic ICP0 has a growth-promoting activity that is distinct from its ability to block antiviral signaling and that does not require the RING finger.

DISCUSSION

This study demonstrates that while ICP0 mediates significant functions in the nucleus, its cytoplasmic roles also have a largely unappreciated importance in viral replication. We show that ICP0 restricted to the cytoplasm can promote viral growth both in cell culture and in mice and, surprisingly, that it can achieve this equally well in the presence or absence of a functional RING finger domain. We also demonstrate that the RING finger contributes to blocking antiviral signaling in a proteasome-independent fashion.

Partial or complete restriction of ICP0 to the nucleus results
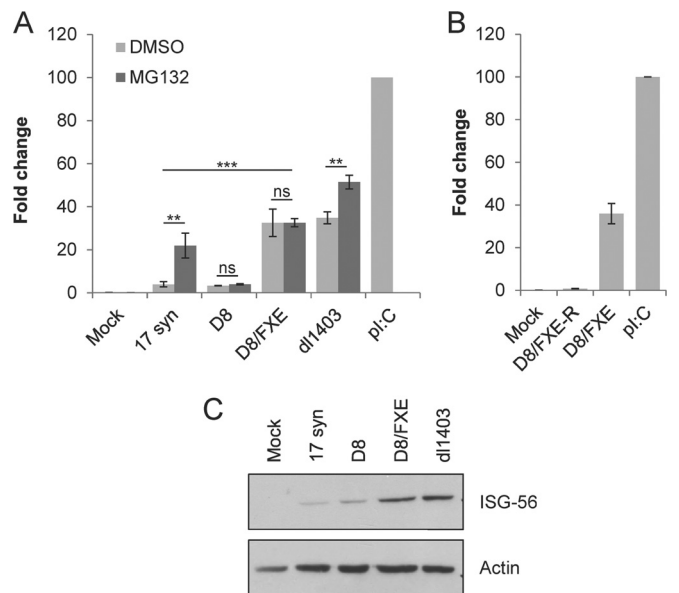
FIG 4 Cytoplasmic ICP0 requires the RING finger but not the proteasome to block ISG expression. HEL cells were infected with the indicated viruses at an MOI of 10. (A) Infections were performed in the presence or absence of the proteasome inhibitor MG132, as indicated. (A and B) RNA was harvested after 6 h, and the expression of ISG-56 relative to GAPDH was determined using the TaqMan system of quantitative RT-PCR. Values are reported relative to poly(I·C) treated cells, whose fold change was set to 100. The data are the averages of three independent experiments ± SEM. Statistical analysis was performed using one-way ANOVA and Tukey's posttest. **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. (C) Protein was harvested after 8 h and analyzed for ISG-56 and actin expression by Western blotting.
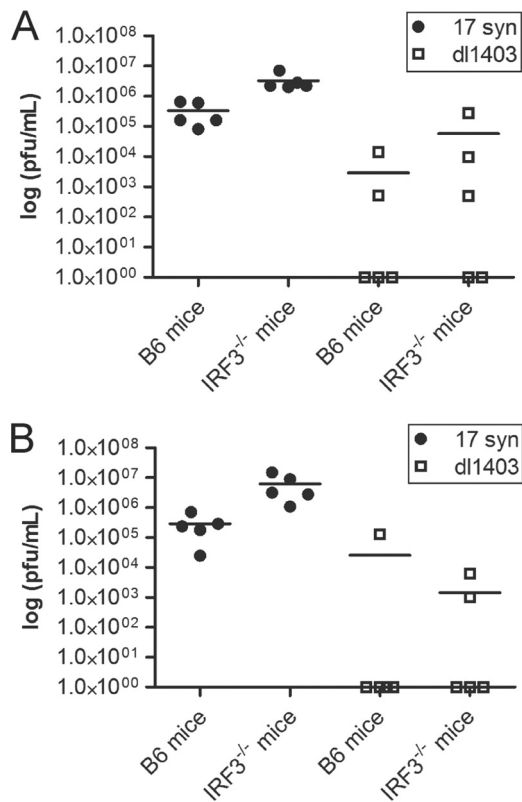
FIG 5 IRF3 deficiency cannot compensate for the loss of ICP0 in a mouse model of genital HSV infection. Wild-type B6 or *irf3*$^{-/-}$ mice were intravaginally infected with 1×10^5 PFU of the indicated viruses. Vaginal washes were collected after 1 day (A) and 2 days (B), and the titer on U2OS cells was determined in the presence of HMBA. Scatter plot and mean values are shown.

from a wide variety of experimental manipulations (6, 35–40), including commonly used strategies, such as DNA transfection prior to infection, the use of proteasome inhibitors, or ICP0 expression in the absence of other viral proteins. Thus, unintentionally, many studies have exclusively considered the roles of nuclear ICP0. Similarly, as shown here (Fig. 2) and elsewhere (37), RING finger mutations cause ICP0 to be largely restricted to the nucleus. This may be explained by the previously suggested hypothesis that ICP0 must complete its nuclear functions before it can translocate to the cytoplasm (35), so that preventing ICP0 from performing these activities by disrupting its RING finger results in its nuclear retention. Our results are consistent with the vast amount of literature demonstrating that disrupting the RING finger of ICP0 results in a virus as attenuated as an ICP0-null strain (30, 41–45) and suggest that ICP0 absolutely requires the RING finger to perform its functions specifically within the nucleus. However, most studies using RING finger mutants conclude that ICP0 requires a RING finger for all of its biological roles, without taking the effect of ICP0 localization into consideration. By including the additional D8 deletion to remove the NLS and create D8/FXE, we have overcome the nuclear restriction of FXE, allowing us to investigate the activity of RING-deficient ICP0 in the cytoplasm. It is important to note that although immunofluorescence studies cannot conclusively demonstrate that a small amount of our ICP0 with the NLS deleted is not reaching the nucleus, we have previously shown that the D8 virus does not degrade nuclear PML (12). Since

it is well established that even a small amount of nuclear ICP0 leads to the loss of PML (43), it can be concluded that this mutation prevents a biologically relevant amount of ICP0 from localizing to the nucleus. Additionally, since FXE is mainly nuclear and yet is inactive, a small amount of D8/FXE reaching the nucleus cannot explain our results.

How cytoplasmic ICP0 induces virus replication, particularly in the absence of the RING finger domain, is currently unclear. ICP0 has been found to bind to EF-1δ, a cytoplasmic elongation factor involved in translation (7), although little is known about the significance of this interaction. Other potential cytoplasmic activities identified for ICP0, such as the degradation of IκBα (46) or the disruption of the cellular microtubule network (11), appear to require RING-dependent ubiquitination. It has also been found that ICP0 must be cytoplasmic in order to be packaged into the viral tegument (47, 48), and capsids from viruses lacking tegument ICP0 have been found to have disrupted transport to the nucleus (49). Therefore, the cytoplasmic localization of D8 and D8/FXE may allow their packaging into the tegument, resulting in more direct capsid transport and greater efficiency of replication. Finally, ICP0 has been found to bind the cellular deubiquitinating enzyme USP7 (31–33) and to transport it from the nucleus to the cytoplasm in a RING-independent but NLS-dependent manner (50). Conversely, USP7 translocation can occur after TLR stimulation in the absence of ICP0 (50), and we observed that ICP0 restricted to the cytoplasm can still interact with USP7. Loss of USP7 binding by ICP0 has been found by some groups to decrease viral growth (51, 52), while others have found that such viruses show increased gene expression, though decreased cell-to-cell spread (53). While USP7 binding has been found to stabilize ICP0 (34), the exact function of this interaction in viral replication is unknown.

A role for cytoplasmic ICP0 in promoting viral replication via increased translation, capsid transport, or USP7 binding would be a departure from the current paradigm in ICP0 biology, which suggests that ICP0 stimulates virus replication by increasing the probability that an incoming genome will launch a productive infection (reviewed in reference 1). In the absence of ICP0, the viral genome is more likely to remain in a quiescent state, suggestive of latency, though in the infrequent cells where a productive infection is launched, the replication cycle proceeds normally (30). ICP0 is thought to overcome the intrinsic cellular resistance provided by several constituents of specific subnuclear domains known as ND10, including PML, which are degraded by ICP0 (54–56). ICP0 has also been suggested to act as a DNA template remodeler (57), decreasing repressive and increasing active histone modifications and counteracting the formation of heterochromatin (58–61). The mechanism behind this activity is currently unclear (62) and may involve a variety of factors (63–67), including the disruption of the coREST-REST repressor complex (68, 69). However, it is difficult to understand how cytoplasmic ICP0 could affect such nuclear components. Interestingly, our results are supported by a recent study demonstrating that a virus expressing a RING finger mutant of ICP0 that was retained exclusively at ND10 domains was more highly attenuated than a virus encoding a RING domain mutant with additional deletions allowing it to disperse away from ND10, suggesting that RING-independent functions of ICP0, in a location-dependent context, can promote viral replication (70).

Although D8/FXE was as capable as D8 in promoting viral
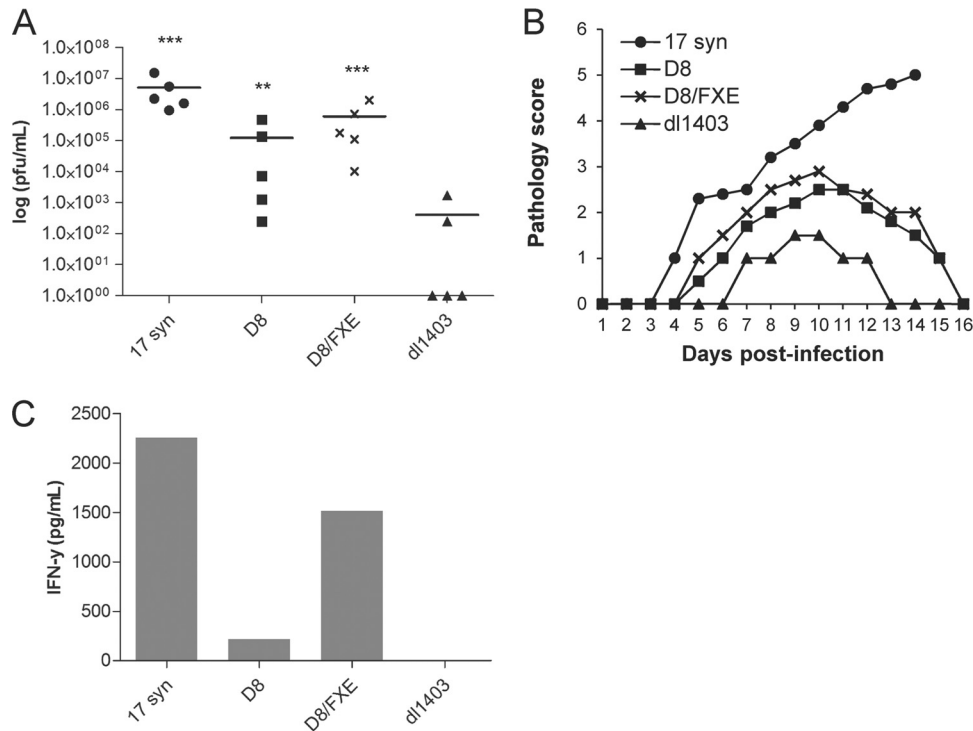
FIG 6 Cytoplasmic ICP0 promotes viral replication in the absence of the RING finger domain in a mouse genital model of HSV infection. The indicated viruses (1×10^5 PFU) were used to intravaginally infect $irf3^{-/-}$ mice. (A) Vaginal washes were collected after 2 days, and the titer on U2OS cells was determined in the presence of HMBA. Statistical analysis was performed using one-way ANOVA and Dunnett's posttest relative to dl1403. **, $P < 0.01$; ***, $P < 0.001$. Scatter plot and mean values are shown. (B) Vaginal pathology was monitored daily and scored on a 5-point scale. (C) Vaginal washes collected after 2 days were pooled, and ELISA was used to measure IFN-γ levels.

replication, cytoplasmic ICP0 failed to block antiviral signaling in the absence of the RING domain. The mechanism through which ICP0 blocks IRF3 activation remains controversial. We have consistently found that cytoplasmic ICP0 can prevent IRF3 signaling in the absence of a functional proteasome (12), and others have demonstrated that cytoplasmic bICP0 from the related bovine herpesvirus 1 also inhibits IRF3 activation (71). In contrast, ICP0 is unable to block IRF3 activation within the nucleus (12, 23), although it plays a role in inhibiting cellular responsiveness to IFN (23), likely via its disruption of PML (72). However, it has been alternatively reported in a Sendai virus coinfection model that wild-type HSV-1 can both enhance IRF3 degradation and sequester nuclear IRF3 away from its target genes in comparison to an ICP0-null virus (21, 22). It has also been suggested that nuclear ICP0 blocks IRF3 activation by degrading the nuclear DNA sensor IFI16 in a RING- and proteasome-dependent manner (20). However, IFI16 may be more important in epigenetic regulation of DNA expression as opposed to IFN production (67, 73). Importantly, under conditions allowing efficient viral replication, IFI16 has been found to be degraded during infection with an ICP0-null virus (74). This suggests that ICP0 simply promotes the expression of an additional viral product that is directly responsible for the loss of IFI16, though ICP0 does appear to block the recruitment of IFI16 to the viral genome even without directing the degradation of IFI16 (74).

At first glance, it is difficult to understand how the RING finger, with its ubiquitin ligase activity, could be required for the ability of ICP0 to prevent antiviral signaling while the proteasome itself is dispensable. This observation is consistent with our previous work demonstrating the requirement for the RING finger in antiviral inhibition (18) and that no factor involved in IRF3 activation has been unequivocally identified as being degraded by ICP0 during HSV-1 infection (12, 18), though some have been suggested (20–22). In recent years, there has been a tremendous increase in our understanding of the proteasome-independent activities of ubiquitin modifications (reviewed in references 24 to 29 and 75). The traditional signal for proteasomal degradation consists of ubiquitin moieties conjugated into chains via their lysine residues at position 48 (Lys 48), yet ubiquitin can also be linked in a variety of atypical manners via other lysine residues, and these alternative chains have been associated with nondegradative signaling roles. Atypical linkages result in chains that adopt a variety of conformations, which can be recognized by different ubiquitin-binding domains (UBDs), found in a wide diversity of proteins, in a linkage-specific manner. Thus, ubiquitin modification can control protein-protein interactions and is therefore involved in cellular processes ranging from receptor endocytosis to DNA repair. Of particular interest is the fact that Lys 63 linkage, one of the best characterized of these alternative chains, plays an extensive role in antiviral signaling (76–80). For example, Lys 63-linked chains, but not Lys 48-linked chains, are essential for the activation of IRF3 (76); Lys 63 chains are important in RLR activation (reviewed in reference 81); and NF-κB activation is extensively regulated by atypical ubiquitin modification of signal transduction proteins (reviewed in reference 82).

Though the activity of the RING finger of ICP0 as a ubiquitin

ligase has been confirmed *in vitro* (2, 83), as well as *in vivo* (3), and the RING domain has been implicated in the proteasome-dependent degradation of a tremendous variety of targets (20, 46, 51, 66, 83–92), to our knowledge, the linkage type found in the ubiquitin chains produced by ICP0 has never been determined. The loss of ICP0-targeted proteins from the cell implicates Lys 48 linkages and proteasomal degradation; however, the RING finger may also generate alternatively linked chains involved in proteasome-independent signaling roles, a possibility that we are currently investigating. Alternatively, the RING finger may act in a ubiquitin-independent manner. Recently, it has been found that the cellular retrovirus restriction factor TRIM-CypA is able to inhibit virus infection by a mechanism that requires its RING domain but neither the proteasome nor ubiquitin conjugation (93). Finally, we cannot rule out the possibility that HUL-1, the second region of ICP0 with E3 ubiquitin ligase activity (94), plays a role in the replication-promoting activities of D8/FXE in the absence of the RING, though to date only one target of HUL-1 has been identified. We are currently investigating potential proteasome-independent functions of the ICP0 RING finger domain.

While unable to control the antiviral response, D8/FXE grows as well as D8 in cell culture. This observation is consistent with previous studies demonstrating that the depletion of neither IRF3 nor STAT-1 in cultured cells could improve the replication of an ICP0-null virus (95). In contrast, the type I IFN response is crucial in controlling HSV replication in mouse models (96–100). Conversely, IRF3-deficient mice survive intravenous infection with wild-type HSV-1 (101) and show no increased viral replication in peripheral tissues (101–103), though augmented replication was observed in the central nervous system (102, 103). While ICP0-null mutants are attenuated in wild-type mice (96, 104, 105), viruses lacking ICP0 have not been studied in IRF3-deficient mice. Here, we used IRF3$^{-/-}$ mice to investigate the replication of our ICP0 mutants, using an intravaginal model of infection, as HSV-1 is now responsible for at least 50% of new genital herpes episodes in developed countries (reviewed in reference 106). We found that while there was a trend toward higher titers in IRF3$^{-/-}$ mice for both 17 syn and dl1403, it was not statistically significant. It is probable that this results from the compensatory role of IRF7 in IRF3$^{-/-}$ mice, as these mice continue to express IFN-α via IRF7 in plasmacytoid dendritic cells and thus survive infection with wild-type HSV-1, while IRF7$^{-/-}$ mice lack IFN-α production and succumb (101). This is analogous to previous work demonstrating that ICP0-null viruses show augmented replication in STAT1$^{-/-}$ and IFNAR$^{-/-}$ mice (96, 97) but remain attenuated in PML$^{-/-}$ mice (96). Therefore, IRF3$^{-/-}$ mice are useful for these studies, allowing us to compare our two cytoplasmic ICP0 mutants without the confounding effects of IRF3 activation, which D8 controls and D8/FXE cannot. However, these mice are not so deficient as to eliminate the requirement for ICP0 to achieve maximal viral replication.

Our observations in IRF3$^{-/-}$ mice confirm the ability of cytoplasmic ICP0 to support virus growth in a RING-independent manner. Virus growth is mirrored by the observed pathology in these mice and further confirmed by measurement of levels of IFN-γ, a cytokine produced by NK cells that represents a characteristic feature of the innate immune response to replicating genital HSV (107–109). Consistent with our data, previous work in lymphocyte-deficient *rag2*$^{-/-}$ mice demonstrated that an HSV-2 virus expressing an NLS mutant of ICP0 was lethal in

80% of mice compared to only 20% infected with a RING finger mutant (110). Surprisingly, D8/FXE reaches titers even higher than those of D8 and also induces higher levels of IFN-γ. This may be explained by the increased stability of RING finger mutants (34, 41), as ICP0 undergoes RING-dependent auto-ubiquitination. Indeed, we observed that ICP0 accumulates to higher levels in D8/FXE than in D8.

In conclusion, our work demonstrates that cytoplasmic ICP0 has two independent activities: blocking ISG production in a mechanism that involves the RING finger but not the proteasome and promoting virus replication in a RING-independent manner. These observations underscore the importance of cytoplasmic ICP0 and suggest alternative functions for the RING finger domain, opening new avenues for the investigation of this multifaceted viral protein.

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