

The Herpes Simplex Virus ICP0 RING Finger Domain Inhibits IRF3- and IRF7-Mediated Activation of Interferon-Stimulated Genes

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Virus infection induces a rapid cellular response in cells characterized by the induction of interferon. While interferon itself does not induce an antiviral response, it activates a number of interferon-stimulated genes that collectively function to inhibit virus replication and spread. Previously, we and others reported that herpes simplex virus type 1 (HSV-1) induces an interferon-independent antiviral response in the absence of virus replication. Here, we report that the HSV-1 proteins ICP0 and vhs function in concert to disable the host antiviral response. In particular, we show that ICP0 blocks interferon regulatory factor IRF3- and IRF7-mediated activation of interferon-stimulated genes and that the RING finger domain of ICP0 is essential for this activity. Furthermore, we demonstrate that HSV-1 modifies the IRF3 pathway in a manner different from that of the small RNA viruses most commonly studied.

Infection of cells by viruses elicits a potent and immediate cellular response aimed at limiting virus replication and spread. The hallmark of this response is the production and secretion of interferons (IFNs), a family of pleiotropic cytokines with antiviral, antiproliferative, and immune modulatory functions (37, 45, 69, 79, 80, 86, 92). Once produced, IFNs α and β are secreted from virally infected cells, bind to their cognate receptors on surrounding cells, and initiate a cascade of phosphorylation events of specific Jak/Stat molecules, culminating in translocation of a phosphorylated Stat-containing complex into the nucleus. This complex binds the IFN-stimulated response element (ISRE) within the promoter region of interferon-stimulated genes (ISGs) and initiates gene transcription (44, 46, 98).

While IFNs α and β possess multiple activities, they were initially discovered and are best known for their antiviral properties. The classical IFN-induced antiviral pathways include the double-stranded RNA-dependent protein kinase R, 2',5'-oligoadenylate synthetase, and Mx pathways, which lead to protein synthesis inhibition, RNA degradation, and inhibition of viral replication, respectively (31, 47, 77, 79). The interferon response can also induce apoptosis (see reference 31 for a review).

It has become clear over the last several years that ISGs can also be activated by viral infection or double-stranded RNA, a by-product of viral infection, in the absence of IFN production (2, 90, 94–96, 100). For example ISG56, an IFN-stimulated protein involved in translational regulation via binding to eukaryotic initiation factor 3 (33), has been shown to be upregulated by IFN, double-stranded RNA, or virus through independent pathways (34). Recent work has shown that IRF3, a

member of the interferon regulatory factor (IRF) family of transcription factors (36, 53, 78, 88), can bind directly to ISRE elements and induce ISGs in the absence of IFN production (49, 96, 97, 99).

The current model of virus-induced IRF3 activation consists of five sequential steps: (i) phosphorylation by a cellular “virus-activated” kinase(s), (ii) a conformational change resulting in protein dimerization, (iii) translocation to the nucleus, (iv) association with CBP and/or p300 coactivators, and (v) ISRE binding (41, 49, 82). Virus infection activates the cellular DNA-dependent protein kinase DNA-PK, leading to the phosphorylation of IRF3 on Thr135 followed by IRF3 nuclear localization (38). However, the role of N-terminal IRF3 phosphorylation is unclear, as other studies have found that virus-mediated IRF3 dimerization, nuclear translocation, and cofactor binding require C-terminal phosphorylation (49, 51, 99). The cellular kinases TBK-1 and IKK ϵ were found to phosphorylate IRF3 and IRF7 following infection with Sendai virus (29, 83). While multiple residues within the C-terminal region of IRF3 are phosphorylated (49, 99), to date only Ser396 has been found to be required for virus-mediated IRF3 activation *in vivo* (81).

Previously, we and others reported that herpes simplex virus type 1 (HSV-1), a large enveloped DNA virus, triggers a host cellular response characterized by the induction of a specific set of genes, primarily ISGs, resulting in the activation of an antiviral state in an IFN-independent fashion (60, 67). This cellular response is mediated by HSV-1 virion particles and is inhibited by virus replication, suggesting that a newly synthesized viral protein(s) inhibits the response. The related herpesvirus human cytomegalovirus (HCMV) induces expression of ISGs in both the presence and absence of viral gene expression, although significantly more ISGs are induced when viral gene expression is inhibited, again suggesting that a newly made virus product suppresses the host response (7, 100, 101).

Recently, the HCMV virion protein pp65 was shown to inhibit antiviral gene expression by antagonizing NF- κ B and

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IRF1 pathways (6). With HSV-1, virus binding and penetration are essential for antiviral state induction (60, 71), whereas with HCMV, soluble glycoprotein B appears sufficient to trigger the response (5, 84). The IFN-independent induction of ISGs by HSV-1 and HCMV does not require the Jak-Stat IFN pathway and appears to involve IRF3 (5, 60, 66, 67, 71).

Recently, the HSV-1 immediate-early protein ICP0 was shown to block ISG induction in a proteasome-dependent fashion (14). The mechanism by which ICP0 blocks ISG induction remains to be elucidated and is the focus of this study. ICP0 is expressed very early in infection and performs a variety of functions (21). ICP0 is a promiscuous activator of both viral and cellular promoters and appears to function synergistically with another immediate-early protein, ICP4 (28, 74, 93). During viral infection, ICP0 has been found to associate with a number of cellular proteins, including elongation factor 1 δ (39), cyclin D3 (40), kinetochore protein CENP-C (23), ubiquitin-specific protease 7 (USP7, also known as HAUSP) (26, 59), and PML, the prototypic member of nuclear domains known as ND10, PML bodies, or PODs (25, 55, 56).

Considering that many ND10 constituents, including PML, are upregulated by IFN and that a number of viruses, including HSV-1, localize to and subsequently disrupt ND10, one hypothesis has been that ND10 functions as "nuclear defense" sites (54, 57). Of particular interest is that ICP0 affects the SUMO-1 modification of ND10 components and their subsequent proteasome-mediated degradation (10, 24, 25, 55, 65). While a number of functional regions of ICP0 have been identified, an N-terminal cysteine/histidine motif termed the RING finger appears to be important for the biological activities of ICP0 (21). Although the RING finger domain resembles a zinc finger DNA binding domain, it does not appear to bind DNA (22, 27) and instead acts as a ubiquitin E3 ligase that is required for the degradation of several cellular proteins, including PML (4).

While ICP0 may function to counteract host defense mechanisms through disruption of ND10, we and others have shown that ICP0 renders HSV-1 relatively resistant to the effects of IFN, in part by counteracting an IFN-induced block to virus transcription (35, 61, 63). In the current study, we set out to address the mechanism by which HSV-1 blocks the IFN-independent antiviral response initiated following entry of virus particles. We have found that ICP0 and vhs, the virion host shut-off protein, counteract the cellular IFN-independent antiviral response at early and late times postinfection, respectively. ICP0 specifically blocks IRF3- and IRF7-mediated cellular responses and requires the RING finger for this activity. Since HSV-1 does not appear to activate the IRF3 pathway in the same fashion as the small RNA viruses most commonly studied, the precise mechanism of ICP0 inhibition of IRF3 activity remains to be determined. These studies, however, expand our knowledge on both cellular antiviral responses to virus infection and the countermeasures adopted by viruses to ensure their successful replication and spread.

MATERIALS AND METHODS

Cells and viruses. Human embryonic lung (HEL) fibroblasts and U2OS osteosarcoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, while Vero, E5 (13), V27 (73), and A549 cells were maintained in Dulbecco's modified Eagle's medium supple-

mented with 5% fetal bovine serum. 293 HEK cells were maintained in α minimal essential medium supplemented with 10% fetal bovine serum. HSV-1 strains KOS (wild type), d22lacZ (ICP22⁻) (52), N38 (ICP47⁻) (91), Δ ICP6 (ICP6⁻) (30), and Δ sma (vhs⁻) (72) were grown in Vero cells, n212 and 7134 (ICP0⁻) (8), KM110 (ICP0⁻VP16⁻) (64), and FXE (ICP0 RING finger mutant) (18) were grown in U2OS cells, d120 (ICP4⁻) was grown on E5 cells (13), and 5dl1.2 (ICP27⁻) was grown on V27 cells (73). Newcastle disease virus (NDV, strain La Sota) and Sendai virus (strain Cantell) were kindly provided by E. Nagy and J. Hiscott, respectively. All HSV-1 infections utilized a multiplicity of infection of 10 PFU per cell (as determined in the cell line used for propagation) unless otherwise specified. NDV and Sendai virus were used at 40 hemagglutination activity units per 10⁶ cells. UV inactivation was performed with a UV Stratalinker 2400 (Stratagene) for the length of time required to reduce viral titers by a factor of 10⁵ (data not shown).

Creation and characterization of 7134 Δ sma (ICP0⁻ vhs⁻) double mutant virus. U2OS cells were coinfecting with 5 PFU per cell of both 7134 and Δ sma viruses. The ICP0 coding region in 7134 is replaced with the lacZ gene, allowing color selection with the addition of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (100 μ g per ml) in the overlay (1% agarose in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum). Individual blue plaques were isolated, and following amplification DNA was isolated from virally infected cells as previously described (64) for use in PCR and Southern blot analyses. PCR was used to detect an internal SmaI-SmaI deletion within the Δ sma vhs gene as previously described (62).

Southern blot analysis to ensure that both copies of ICP0 retained the 7134 mutation was performed as previously described (8, 64). Briefly, an ICP0 fragment spanning the entire open reading frame was hybridized to DNA digested with PstI and SstI. ICP0 resides in a genomic PstI fragment and is not cleaved by SstI. lacZ, however, contains an internal SstI site, and thus recombinant 7134 ICP0 is cleaved into two fragments, whereas wild-type ICP0 is not. Positive clones were plaque purified three times on U2OS cells, followed by a final round of PCR and Southern blot analysis.

RT-PCR and Western blot analysis. Total cellular RNA was harvested with Trizol (Gibco-BRL) according to the manufacturer's instructions. For reverse transcription (RT)-PCR, aliquots (2 μ g) were reverse transcribed with 200 ng of random hexamer primer and Superscript II reverse transcriptase (Invitrogen). PCR was subsequently performed as per the manufacturer's specifications with the following primers: human ISG56 forward and reverse (5'-ACGGTGCCCTAATTTACAGC-3' and 5'-AGTGGCTGATATCTGGGTGC-3') and human glyceraldehyde-3-phosphate dehydrogenase forward and reverse (5'-CGGAGTCAACGGATTGGTTCGTA-3' and 5'-AGCCTTCTCCATGGTGGTGAAGA C-3').

Preparation of whole-cell extracts and immunoblot analyses. For preparation of whole-cell extracts, cells were washed twice and then harvested in cold phosphate-buffered saline, followed by centrifugation at 200 \times g for 3 min at 4°C. Cell pellets were resuspended in whole-cell extract buffer [20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM β -glycerophosphate, 0.2% Triton X-100, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, and 1X protease inhibitor cocktail (Sigma)], lysed on ice for 15 min, and then centrifuged for 10 min at 13,000 \times g at 4°C. Extract concentrations were determined with a Bradford assay kit (Bio-Rad), and the indicated amounts of extracts were run on 9% polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies specific to IRF3 (Santa Cruz SC-9082), IRF7 (Santa Cruz SC-9083), β -actin (Santa Cruz SC-1616), CBP (Santa Cruz SC-369), TBK1 (Santa Cruz M-375), ICP0 (Goodwin Institute), or ISG56 (provided by G. Sen) at a dilution of 1:1,000. To visualize DNA-PK, nuclear extracts were prepared as previously described (1), and 25 μ g of extracts was run on gels containing 8% acrylamide and 0.07% bisacrylamide. Western blot analysis was performed with a 1:2,000 dilution of an anti-DNA-PK monoclonal antibody (provided by S. Lees-Miller).

Luciferase assays. HEK 293 cells were transfected with pRLTK, RANTES-pGL3, or IFNB-pGL3 reporter constructs along with expression constructs encoding constitutively activated IRF3 (IRF3-5D) or IRF7 (IRF7- Δ 247-467) (48-50). At 8 h posttransfection, cells infected with 40 hemagglutination activity units of Sendai virus per 10⁶ cells or left uninfected, as indicated. Luciferase activity was analyzed 24 h posttransfection by the dual-luciferase reporter assay (Promega) as per the manufacturer's instructions. Relative luciferase activity was measured as the activation relative to the basal level of reporter gene in the presence of pFLAG-CMV2 vector after normalization with cotransfected activity. Values represent the mean \pm standard deviation for three experiments.

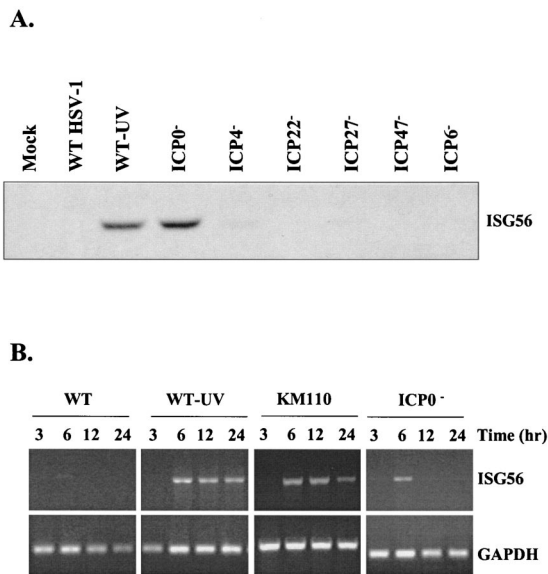


FIG. 1. Expression of ISG56 is only observed in the absence of ICP0 expression. (A) Western blot analysis of whole-cell extracts (40 μ g) of HEL fibroblasts harvested 12 h postinfection with the indicated viruses. The ISG56 protein is only visible following infection with UV-inactivated wild-type virus (strain KOS, WT-UV) or an ICP0 null mutant (n212). (B) RT-PCR analysis of total RNA harvested from HEL fibroblasts at various times postinfection with the indicated viruses. In the absence of ICP0, transient ISG56 mRNA accumulation is seen, whereas in the absence of HSV-1 replication (UV-inactivated KOS or genetically inactivated KM110), sustained ISG56 mRNA accumulation is observed. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

RESULTS

ISG induction in response to virus particles is abolished by ICP0 and vhs. Previously, we used microarray analysis to show that HSV-1 particles induce ISGs in an IFN-independent fashion (60). In these studies we found that ISG induction was masked following virus replication, yet we failed to identify a potential viral inhibitor using HSV-1 mutants individually deleted for an immediate-early gene product. Subsequently, DeLuca and colleagues determined that ICP0 was capable of inhibiting ISG induction through the use of HSV-1 mutants deleted for several immediate-early gene products along with an adenovirus expressing ICP0 (14). In our original study, we assayed mRNA accumulation by Northern blot analysis 18 to 24 h postinfection, because ISG mRNA was readily detectable at this time with either genetically inactivated virus (KM110) or UV-inactivated wild-type HSV-1.

To reconcile our results with those of DeLuca and colleagues, we assayed ISG56 mRNA and protein levels at various times postinfection with a panel of single immediate-early mutant viruses in human embryonic lung (HEL) fibroblasts. As shown in Fig. 1A, aside from UV-inactivated wild-type virus, only the virus deleted for ICP0 demonstrated ISG56 protein accumulation at 12 h postinfection, in accordance with the work of DeLuca and colleagues. Interestingly, RT-PCR analysis with the ICP0 mutant n212 consistently yielded ISG56 message at early but not late times postinfection (Fig. 1B), in accordance with our previous results. Similar results were ob-

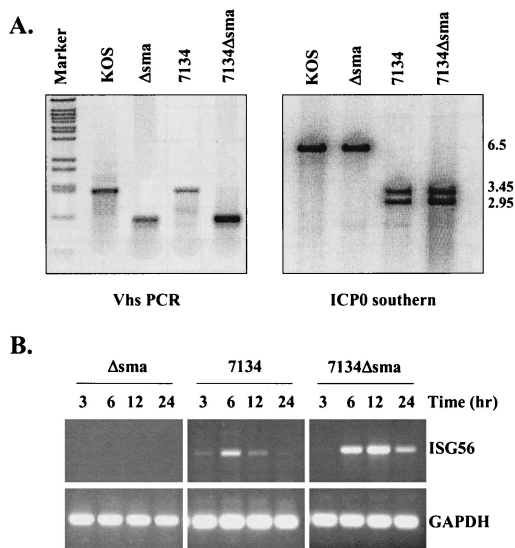


FIG. 2. Deletion of vhs and ICP0 results in sustained ISG56 mRNA accumulation. (A) Construction of a double mutant virus deleted for vhs and ICP0. U2OS cells were coinfecting with Δ sma and 7134 to create a double mutant virus as outlined in Materials and Methods. (Left panel) PCR analysis was used to confirm the internal *SmaI-SmaI* fragment deletion that defines the vhs Δ sma mutation. *BstEII*-cut lambda DNA was used as a marker. (Right panel) Southern blot analysis with a radiolabeled ICP0 probe was used to distinguish wild-type ICP0 and 7134 ICP0 sequences based on the presence of a unique *SstI* site within the *lacZ* gene that replaces the ICP0 sequence in the 7134 mutation. (B) RT-PCR analysis of total RNA harvested from HEL fibroblasts at various times postinfection with the indicated viruses. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

tained with two additional ICP0 mutants, 7134 (refer to Fig. 2) and dIX3.1 (75) (data not shown), where ISG56 mRNA accumulation peaked at 6 h postinfection and then declined rapidly. In the absence of virus gene expression, using either UV-inactivated HSV-1 or genetically inactivated KM110, we observed sustained ISG56 mRNA levels at late times postinfection. These data suggest that in the absence of ICP0, ISG56 mRNA accumulates at early times postinfection but subsequently disappears due to the presence of an additional virus-induced activity.

HSV-1 particles contain within their tegument the virion host shut-off protein (vhs) that either is, or is part of, an RNase that causes the degradation of both viral and cellular mRNAs (15, 72, 87). Thus, we assayed whether this protein was responsible for the decline in ISG56 mRNA levels at later times postinfection. For this experiment, we created a double mutant virus by recombining the 7134 ICP0 null virus (8) with the Δ sma vhs-deleted virus (72) (Fig. 2). Due to an internal deletion of a *SmaI-SmaI* fragment, the Δ sma and wild-type vhs alleles are easily distinguishable by PCR analysis, while Southern blot analysis distinguishes wild-type and 7134 ICP0 alleles based on the introduction of an *SstI* restriction site within the *lacZ* gene that replaces ICP0 coding sequences in the 7134 mutant (8) (Fig. 2A).

An RT-PCR time course analysis of HEL fibroblasts infected with Δ sma, 7134, or 7134 Δ sma was performed in order to determine the contributions of ICP0 and vhs activities in the accumulation of ISG message. As demonstrated above with

n212 (Fig. 1B), ISG56 mRNA accumulation following infection with 7134 was transient, peaking at approximately 6 h postinfection (Fig. 2B). In contrast, sustained ISG56 mRNA was observed upon deletion of both ICP0 and vhs, indicating that vhs activity contributes to the loss of ISG56 mRNA at late times postinfection. This vhs-induced activity was only observed in the absence of ICP0, consistent with the observation that ICP0 prevents ISG induction following virus entry (14). Accordingly, we failed to detect ISG56 mRNA accumulation in fibroblasts infected with Δ sma, as this viral mutant contains wild-type ICP0. Similar results were found by RT-PCR with primers specific for other ISGs originally identified by microarray analysis (data not shown).

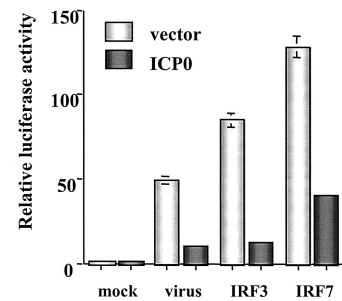
ICP0 RING finger inhibits IRF3- and IRF7-mediated ISG induction. IFN-independent ISG induction following virus infection may occur through the activation of the constitutively expressed transcription factor IRF3 due to its ability to bind to the ISRE element within ISG promoters (32, 36). In addition, Preston and colleagues showed that in the absence of virus gene expression, HSV-1 induces the DNA binding activity of IRF3 (71). In a series of transient-transfection assays with a luciferase reporter under the control of two different ISRE-containing ISG promoters, RANTES and IFN- β , we found that expression of ICP0 significantly dampened ISG promoter activation in response to Sendai virus infection and expression of constitutively activated forms of both IRF3 [IRF-3(Δ 5D)] and IRF7 [IRF-7(Δ 247-467)] (48–50) (Fig. 3). In all transient transfection assays, a dose-response curve was performed with the ICP0 plasmid (data not shown).

To determine which region of ICP0 was responsible for this inhibition, constructs containing ICP0 deletion, truncation, and point mutations were utilized (Fig. 4). We first assayed a series of ICP0 truncation mutants for their ability to inhibit the activation of the IFN- β promoter by Sendai virus or constitutively activated forms of IRF3 and IRF7. As shown in Fig. 5A, successive truncation of the C terminus to residue 312 had no effect on the inhibitory activity of ICP0. However, the truncation mutant 1-241 failed to inhibit activation of the IFN- β promoter by virus, IRF3, or IRF7. Along with the observation that mutant 105-519 effectively repressed IFN- β promoter activation, these data suggest that the region between residues 105 and 312 is important for ICP0-mediated inhibition.

Within this region of ICP0 is the RING finger motif, which has been shown to be responsible for many of the biological activities of ICP0. The RING finger is a zinc-stabilized fold that requires a histidine and several cysteine residues (of which the first and last are at residues 116 and 156) to form a structure that has catalytic E3 ligase activity (3, 4, 20). To assess the role of the RING finger motif in inhibiting IRF3- and IRF7-mediated IFN- β promoter activation, we next tested a series of ICP0 mutants harboring deletions, insertions, and point mutations both within and exterior to the RING finger domain (Fig. 5B).

Deletion mutant Δ 106-149 (the lesion in virus FXE) removes several of the essential metal-coordinating residues and thus abolishes the RING finger activity, while the mutation in mutant ins150 affects a critical alpha helix within the RING fold with similar functional consequences (4, 20). Mutation K144E lies within the same helix and causes reduced catalytic activity in transfected and infected cells (20). Deletion Δ 162-

A. RANTES promoter



B. IFN β promoter

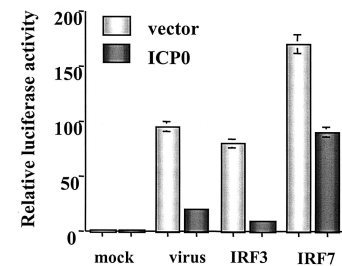


FIG. 3. ICP0 blocks virus-, IRF3-, and IRF7-mediated activation of ISG-specific promoters. Luciferase assays in 293 cells were used to measure activation of the RANTES (A) or IFN- β (B) promoters in response to infection with the paramyxovirus Sendai virus or expression of constitutively activated forms of IRF3 [IRF-3(Δ 5D)] and IRF7 [IRF-7(Δ 247-467)] in the presence or absence of full-length ICP0. Results represent the mean \pm standard deviation of three independent experiments.

188 removes sequences that are part of the RING domain fold (3) and produces a phenotype that is indistinguishable in all available assays from that of a core RING deletion (20). These four ICP0 mutants failed to inhibit virus-, IRF3-, and IRF7-mediated activation of the IFN- β promoter. Consistent with the data in Fig. 5A, deletions (Δ 9-76 and Δ 594-632) and point mutations (R623L/K624I) outside of the RING finger region had no adverse effect on the inhibitory activity of ICP0. Similar results were obtained with the RANTES promoter construct (data not shown).

ICP0 does not induce the degradation of known IRF3 pathway components. DeLuca and colleagues demonstrated that the proteasome inhibitor MG132 prevented ICP0 from inhibiting ISG induction (14), suggesting that the function of ICP0 is to induce the proteasomal degradation of a member(s) of the ISG induction pathway. To determine whether ICP0 blocks ISG induction by inducing the degradation of known IRF3 pathway components, we determined the levels of endogenous TBK-1, IRF3, IRF7, and CBP protein following infection of HEL fibroblasts for 10 h with wild-type HSV-1 (strain KOS), n212, and the RING finger mutant FXE (Fig. 6). Due to the Δ 106-149 deletion of ICP0 within the FXE virus, the ICP0 protein migrates slightly faster than its wild-type counterpart (top panel). ISG56 protein was readily observed following infection with n212 and FXE, consistent with previous results (Fig. 1A and 5B, respectively). Actin served as a control for

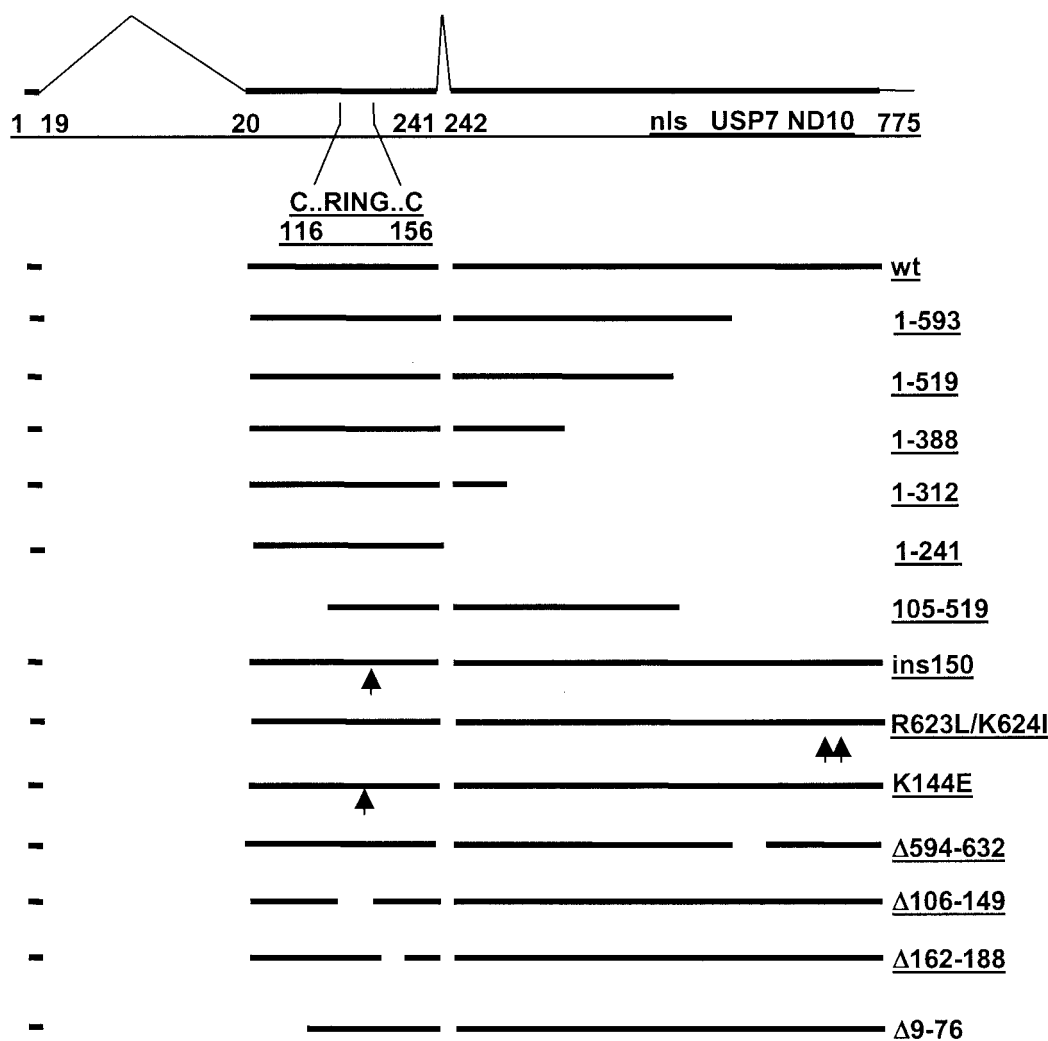


FIG. 4. Schematic representation of ICP0 functional domains and mutations used in this study. The uppermost line depicts the 775-amino-acid ICP0 open reading frame (thick line) with the positions of the introns (thin angled lines) and the amino acid coordinates at the intron-exon junctions marked. These sequences are contained within the ICP0 expression plasmid p111 (19). Also marked are the positions of the first and last cysteine residues of the RING finger, the nuclear localization signal (nls) (residues 501 to 510) (17), the USP7 interaction domain (residues 594 to 633) (58), and the sequences required for self-multimerization and efficient localization at ND10 (residues 633 to 720) (11, 55, 58). Deletion mutants 1-519, 1-388, and 1-312 express C-terminally truncated proteins and were constructed by utilizing the *Mlu*I, *Xma*I, and *Nru*I restriction sites at the positions of these 3' truncations. Mutant 105-519 expresses the coding region between the *Xho*I and *Mlu*I sites. Mutant *ins*150 contains a 4-amino-acid insertion in codon 150 (p110F1) (19), mutant K144E has a lysine to glutamic acid substitution in the helix region of the RING finger (16), and deletion mutants Δ594-632, Δ106-149, Δ162-188, and Δ9-76 have the stated deletions (p110D12, p110FXE, p110D22, and p110D1, respectively) (17). Truncation mutant 1-593 has stop codons inserted into codon 594 (p110E52X) (58). Finally, mutant 1-241 contains only the sequences encoded in exons 1 and 2 (p110262) (25). Mutants Δ106-149, Δ162-188, and *ins*150 have lost ubiquitin E3 ligase activity both in vitro and in vivo, while mutant K144E retains in vitro activity but appears to have reduced activity in vivo (4, 20).

protein loading and nonspecific protein degradation, while DNA-PK served as a positive control for ICP0-mediated degradation, as this nuclear protein was shown to be specifically degraded by wild-type ICP0 (43).

We failed to detect a significant decrease in the level of endogenous TBK-1, one of two kinases shown to phosphorylate IRF3 following virus infection (29, 83). As expression of the second kinase, IKKε, is thought to be restricted to activated leukocytes (70), we did not test for endogenous levels of this kinase in HEL fibroblasts. However, we did observe that expression of wild-type ICP0 along with IKKε in a transient

transfection assay failed to decrease levels of the IKKε protein (data not shown). We also failed to detect a decrease in the endogenous levels of IRF3 and CBP. IRF7 is not constitutively expressed in cells, and we failed to detect significant induction of this protein at the time of harvest (10 h postinfection). However, in subsequent time course analyses, we frequently detected low levels of IRF7 at late times (12 to 24 h) postinfection with wild-type HSV-1 (data not shown). Infection with an adenovirus overexpressing IRF7 was used as a positive control for IRF7 detection. Thus, ICP0 does not appear to block ISG induction by inducing the degradation of known

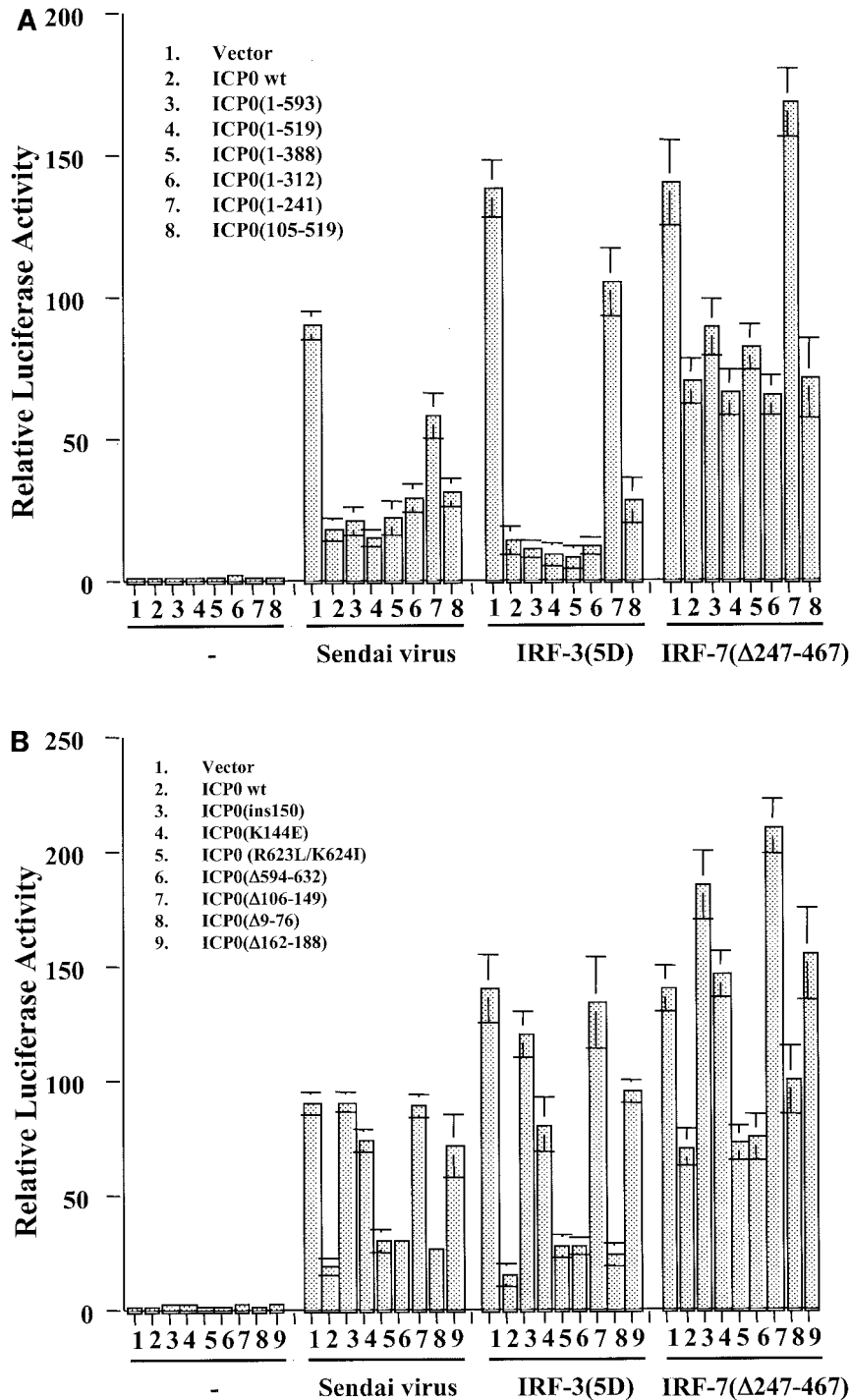


FIG. 5. ICP0 RING finger is both necessary and sufficient to block virus-, IRF7-, and IRF3-mediated ISG promoter activation. Luciferase assays in 293 cells were used to measure the activation of the IFN- β promoter following Sendai virus infection or expression of constitutively activated IRF3 [IRF-3(5D)] and IRF7 [IRF-7(Δ 247-467)] in the presence or absence of full-length and mutant forms of ICP0 (refer to Fig. 4). Results represent the mean \pm standard deviation of three independent experiments.

IRF3 pathway constituents. Further work is required to identify the mechanism of ICP0 action with respect to the proteasome and ISG inhibition.

HSV-1 does not induce the hyperphosphorylation of IRF3.
In the current model of virus-mediated IRF3 activation, viral

infection induces the phosphorylation on multiple C-terminal serine and threonine residues of IRF3, followed by nuclear translocation and proteasome-mediated degradation (49, 82, 99). These studies have primarily utilized small, related RNA viruses such as Sendai virus and Newcastle disease virus, mem-

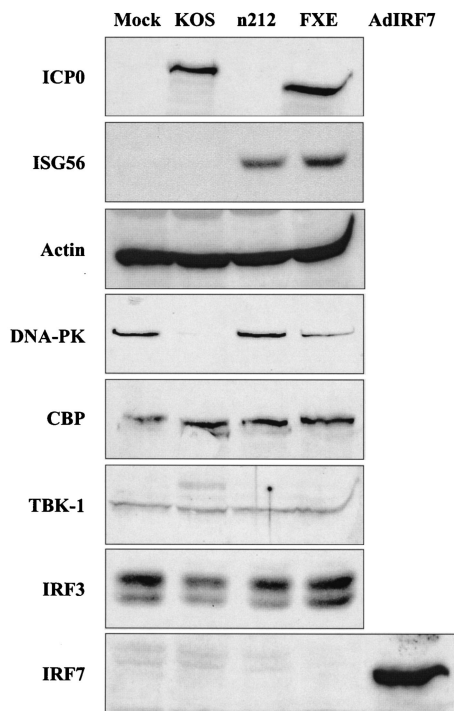


FIG. 6. ICP0 fails to degrade known components of the IRF3 pathway. Western blot analysis of whole-cell extracts (40 μ g) or nuclear extracts (25 μ g, for DNA-PK) of HEL fibroblasts harvested 10 h postinfection with the indicated viruses. Wild-type ICP0 fails to induce the degradation of IRF3, CBP, or TBK-1, components of the cellular antiviral pathway, whereas DNA-PK is specifically degraded by wild-type but not mutant ICP0. In the absence of wild-type ICP0, ISG56 protein is observed, whereas expression of IRF7 is only detected with a recombinant adenovirus expressing human IRF7 (AdIRF7). Actin was used as an internal loading control.

bers of the paramyxovirus family of negative, single-stranded RNA viruses, in transformed cell lines such as A549 lung epithelial cells. In the previous experiment, we failed to detect significant degradation of IRF3 following HSV-1 infection.

To determine if replicating HSV-1 induces the hyperphosphorylation of IRF3, we performed Western blot analysis on whole-cell extracts harvested from A549 cells and HEL fibroblasts infected with KOS, UV-inactivated KOS, n212, and NDV. Results from A549 cells are presented in Fig. 7. New-

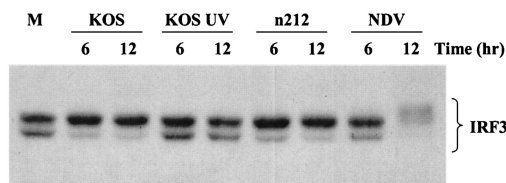


FIG. 7. HSV-1 fails to hyperphosphorylate IRF3. Western blot analysis of whole-cell extracts (40 μ g) of A549 cells harvested 6 and 12 h postinfection with the indicated viruses. Endogenous IRF3 is present as two species and becomes hyperphosphorylated and subsequently degraded following infection with the paramyxovirus Newcastle disease virus (NDV). A shift from the lower to the upper form of IRF3 is readily observed following infection with wild-type and ICP0 null viruses but not in the absence of virus gene expression.

castle disease virus induced the hyperphosphorylation of IRF3 between 6 and 12 h postinfection, illustrated by the appearance of slower-migrating species of IRF3. We also noted the accompanying degradation of IRF3 following hyperphosphorylation in response to Newcastle disease virus infection, as previously reported in the literature (49). Following HSV-1 infection, however, we failed to detect a similar level of IRF3 hyperphosphorylation. Instead, we routinely observed a shift from the lower to the upper form of IRF3. While this shift was routinely observed in both A549 cells and HEL fibroblasts, the shift was consistently easier to detect in A549 cells and was primarily observed at later times (18 to 24 h postinfection) in HEL fibroblasts (data not shown).

The apparent lack of hyperphosphorylation characteristic of small RNA viruses was not due to the action of ICP0, since n212 failed to demonstrate a similar level of IRF3 phosphorylation. Of interest, although UV-inactivated KOS consistently failed to elicit a similar shift from the lower to the upper form of IRF3, n212 mediated this posttranslational modification with kinetics similar to the wild-type virus. A similar shift was observed with three additional ICP0 mutant viruses (data not shown). Thus, HSV-1 replication induces a cellular function that is distinct from that mediated by RNA viruses but does not rely on the activity of ICP0. Studies are currently under way to delineate the mechanism by which HSV-1 modifies IRF3 following productive infection.

DISCUSSION

Our current working hypothesis of HSV-1 pathogenesis is that incoming virus particles stimulate an antiviral response that induces the direct induction of ISGs through the activation of IRF3 (12). Following virus replication, HSV-1 abolishes this cellular response through the activities of ICP0 and vhs. At early times postinfection, ICP0 appears to specifically inhibit the IRF3 pathway via the RING finger domain, while vhs presumably functions in a nonspecific fashion later in infection. Since vhs mutants that produce ICP0 fail to accumulate detectable levels of ISG mRNA, while ICP0 mutants that produce vhs transiently accumulate ISG mRNA, it is likely that ICP0 represents the primary mechanism utilized by HSV-1 to counteract the cellular antiviral response to incoming virus particles. A related herpesvirus, HCMV, appears to function in a similar fashion. Using microarray analysis, Shenk and colleagues demonstrated that while both wild-type and UV-inactivated HCMV induce ISGs, significantly more ISGs were induced following infection with UV-inactivated virus, indicating that virus replication produces a protein that functions to suppress the host antiviral response (100, 101). Furthermore, both HSV-1 and HCMV particles were found to induce the DNA binding activity of IRF3 (66, 71).

ICP0 is a multifunctional protein to which diverse activities have been ascribed (21). With respect to virus pathogenesis and the antiviral response, ICP0 appears to play an important role in both IFN-dependent and -independent pathways. Classical antiviral pathways involve the production of IFNs, primarily IFN- α and IFN- β . Along with ICP34.5, ICP0 renders HSV-1 relatively resistant to the effects of these IFNs, in part by overcoming an IFN-induced block to virus transcription (35,

61, 63). The cellular antiviral response to incoming particles, however, was shown to be IFN independent (60, 67).

Recent studies with the IRF family of transcription factors have found that IRF3 and IRF7 are involved not only in virus-induced production of type I IFNs but also in the direct induction of ISGs through binding to ISRE elements in ISG promoters (36, 88). Given the observations that HSV-1 particles induce IRF3 DNA-binding activity and that ICP0 blocks ISG induction, it was not surprising that ICP0 was found to specifically block IRF3- and IRF7-mediated activation of two ISRE-containing ISG promoters. The ICP0 inhibitory effect on activated IRF7 was not as great as that observed following virus infection or with activated IRF3. Since IRF3 is constitutively expressed while IRF7 induction is dependent on IFN signaling (88), the preferential ability of ICP0 to inhibit IRF3 would be predicted given its apparent role in overcoming early antiviral responses, including those not mediated by IFN.

However, it remains to be determined exactly how ICP0 blocks IRF3-mediated antiviral responses. The initial work of DeLuca and colleagues found that ICP0 functions via a proteasome-dependent pathway (14). Known components of the virus-induced IRF3 pathway, including TBK-1, IKK ϵ , IRF3, and CBP, were not degraded following the production of ICP0. During these studies, however, we discovered that HSV-1 does not appear to activate the IRF3 pathway in the same fashion as the RNA viruses most commonly studied. First, several groups studying paramyxovirus infection have reported that virus replication is required for IRF3 and/or IRF7 C-terminal hyperphosphorylation and subsequent activation (85, 89). Not only does HSV-1 replication mask ISG induction, we failed to observe IRF3 hyperphosphorylation following infection with either wild-type or ICP0 null viruses. In contrast, we routinely observed a shift from the lower band to the upper band of the endogenous IRF3 doublet, which has been observed previously in the context of N-terminal phosphorylation of IRF3.

Work from the laboratories of Hiscott and Howley demonstrated that N-terminal phosphorylation occurs in response to stress stimuli and involves DNA-PK (38, 82), a cellular kinase involved in DNA double-strand break repair and V(D)J recombination (42). However, the role of N-terminal IRF3 phosphorylation during virus infection remains to be determined, as the majority of published data suggest that C-terminal IRF3 phosphorylation is essential (49, 51, 81, 99).

Interestingly, ICP0, through its RING finger domain, has been found to induce the proteasome-dependent degradation of DNA-PK (43, 68). It is unclear at this time, however, how ICP0, DNA-PK, and IRF3 may be related, particularly with respect to the IFN-independent antiviral state. Also unclear are the differences in the activation of the IRF3 pathway between large DNA and small RNA viruses. Specifically, although we failed to detect IRF3 hyperphosphorylation characteristic of infection with paramyxoviruses, replicating HSV-1 appeared to posttranslationally modify IRF3 in some fashion in an ICP0-independent manner. Additionally, in the transient transfection assays, ICP0 was able to block activation of two ISRE-containing ISG promoters in response to paramyxovirus infection. Thus, although HSV-1 and paramyxovirus infection may differentially modify components of the IRF3 pathway, a common event in this pathway is inhibited by ICP0.

While the precise mechanism of blocking IRF3-mediated

activation remains to be elucidated, it is clear that the RING finger motif of ICP0 is critical. While sequences in the C-terminal region of ICP0, including the nuclear localization signal, the USP7-binding domain and the ND10 localization region, are important for ICP0 function, the N-terminal RING finger domain appears to be important for most biological activities of ICP0 (21). In this study, we found that the region containing the RING finger domain appears to be both necessary and sufficient for the ICP0-mediated suppression of the IRF3 pathway, at least within our experimental design.

Although similar to zinc finger DNA binding motifs, the ICP0 RING finger fails to bind DNA in solution, either alone or in the context of full-length ICP0 (22, 27) and it is now clear that the RING finger of ICP0 constitutes a typical ubiquitin E3 ligase domain (4). The RING finger is essential, however, for localization to and the subsequent disruption of ND10 (25, 55). Since many ND10 constituents are upregulated by IFN, one hypothesis is that ND10 serve as an intracellular defense mechanism and that ICP0 functions to overcome this defense. Recent work with mouse fibroblasts that are deficient in PML has indicated that, in contrast to the PML-positive control cells, pretreatment of PML^{-/-} cells with IFN had little effect on the replication of ICP0-negative HSV-1 (9). Of interest, CBP has been found to localize to ND10 via its interaction with PML (76).

Although ICP0 induces the proteasome-dependent degradation of both SUMO-1 modified and unmodified forms of PML, the levels of CBP appear to remain constant throughout infection. We have found, however, that following infection with wild-type virus or transfection with full-length ICP0, the punctate nuclear staining of CBP, characteristic of ND10 constituents, changes to a diffuse nuclear staining (K. Laryea and K. Mossman, unpublished data). This difference in nuclear localization is not observed, however, with ICP0 null or RING finger mutant virus infection. How these observations collectively fit in to the mechanism of ICP0 suppression of the cellular antiviral response is unclear at the moment, but warrants further investigation.

In conclusion, we have shown that HSV-1 responds to the IRF3-mediated cellular response to incoming virus particles by subverting the IRF3 pathway through an ICP0-specific mechanism. This mechanism involves the ICP0 RING finger and the proteasome but does not appear to induce the degradation of known signaling components. In addition, the RNase activity associated with vhs further ensures that ISG mRNA fails to accumulate, due to its ability to nondiscriminately degrade both viral and cellular mRNA. Thus, HSV-1 elicits at least two mechanisms to ensure successful virus replication in light of the rapid induction of an immune response, characterized by the direct upregulation of both intracellular and secreted immune modulatory functions. These studies serve to increase our knowledge of both the immune response to pathogen infection and the strategies used by viruses to overcome these barriers and will ultimately permit the design of effective therapeutics to suppress viral infection.

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