

**The Induction and Inhibition of an Antiviral Response
Pathway by Herpes Simplex Virus 1**

by

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The Induction and Inhibition of an Antiviral Response

Pathway by Herpes Simplex Virus 1

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University of Pittsburgh, 2006

Herpes Simplex Virus-1 (HSV-1) infection has been shown to be very resistant to the effects of the cellular interferon response. However, In the absence of viral gene expression HSV-1 has been shown to induce the expression of cellular interferon-stimulated genes, possibly through activation of the interferon-regulatory factor 3 (IRF-3) cascade. The induction of IRF-3 by HSV-1 infection has not been well characterized. Using an HSV-1 mutant deficient in the expression of viral genes, we show that HSV-1 can induce the cascade of IRF-3 activation by stimulating the phosphorylation of IRF-3 and the nuclear localization of the protein. We will show that activation of this pathway leads to the expression of interferon-stimulated genes and the production of a protective antiviral response. Further we will show that the expression of one viral gene, ICP0, is essential and sufficient to the inhibition of interferon-stimulated gene expression. An HSV-1 mutant deficient in expression of all immediate-early HSV-1 genes except ICP0 does not induce nuclear accumulation of IRF-3 and does not induce the expression of interferon-stimulated genes. This virus induces the mis-localization of the kinase (TBK-1) responsible for the phosphorylation of IRF-3. Cytoplasmically localized ICP0 expressed in wild-type infection stimulates the translocation of TBK-1 from the cytoplasm to the Golgi apparatus . This ICP0-mediated mis-localization, as well as a portion of that induced by the ICP0 expressing mutant, can be inhibited through the inhibition of the proteasome. Further, IRF-3 nuclear translocation and interferon-stimulated gene expression can be restored during infection with the ICP0-expressing mutant virus in the presence of proteasome inhibitors. This study explores the induction of the

interferon regulatory factor-3 cellular antiviral response pathway, and presents a possible mechanism for the virus' resistance to the cellular antiviral response.

TABLE OF CONTENTS

1.0	INTRODUCTION	1
1.1	HERPESVIRIDAE FAMILY.....	1
1.2	HSV PATHOGENESIS	2
1.3	HSV GENOME	4
1.4	HSV PRODUCTIVE LIFE CYCLE	5
1.4.1	Viral entry	5
1.4.2	Cascade of gene expression and replication	6
1.4.3	Host cell death	10
1.5	ROLE OF ICP0 DURING INFECTION.....	11
1.5.1	Structure and function	11
1.5.2	Cellular gene expression	12
1.5.3	Protein stability.....	12
1.6	HOST CELL ANTIVIRAL RESPONSE.....	14
1.6.1	Interferon-stimulated gene activation.....	15
1.6.2	Interferon-independent pathways	15
1.6.3	IRF-3 and TBK-1 induction cascade.....	16
1.6.4	Viral inhibition of the antiviral response	18
1.6.5	HSV escape from antiviral response.....	21
2.0	RATIONALE.....	26
2.1	SPECIFIC AIM 1	29
2.2	SPECIFIC AIM 2	30
2.3	SPECIFIC AIM 3	31
3.0	EXPRESSION OF HERPES SIMPLEX VIRUS ICP0 INDUCES THE MISLOCALIZATION OF TANK-BINDING KINASE-1	33

3.1	ABSTRACT	33
3.2	INTRODUCTION.....	34
3.3	MATERIALS AND METHODS.....	38
	3.3.1 Cells and Viruses.....	38
	3.3.2 Western blot.....	39
	3.3.3 Immunofluorescence.....	39
3.4	RESULTS	40
	3.4.1 TBK-1 is not degraded during infection in the presence of ICP0 .	40
	3.4.2 TBK-1 is mis-localized during infection in the presence but not absence of ICP0	41
	3.4.3 TBK-1 co-localizes with Golgi apparatus during productive but not unproductive infection.....	46
	3.4.4 Altered timing of ICP0 cytoplasmic translocation also delays TBK- 1 mis-localization	49
3.5	DISCUSSION.....	56
4.0	EXPRESSION OF HERPES SIMPLEX VIRUS ICP0 INHIBITS THE ACTIVATION OF INTERFERON-REGULATORY FACTOR 3.....	60
4.1	ABSTRACT	60
4.2	INTRODUCTION.....	61
4.3	MATERIALS AND METHODS.....	65
	4.3.1 Cells and Viruses.....	65
	4.3.2 Western Blot.....	65
	4.3.3 2-Dimensional Electrophoresis	66
	4.3.4 Northern blot analysis.....	67
	4.3.5 Immunofluorescence.....	68
4.4	RESULTS	68
	4.4.1 IRF-3 is not degraded during HSV-1 infection in the presence of ICP0	68
	4.4.2 IRF-3 is phosphorylated during non-productive HSV-1 infection	70
	4.4.3 IRF-3 is phosphorylated in d109 infection despite absence of DNA-PK	72

4.4.4	ICP0 inhibits the nuclear accumulation of IRF-3 in a proteasome-dependent manner	75
4.5	DISCUSSION.....	77
5.0	EXPRESSION OF HERPES SIMPLEX VIRUS ICP0 INHIBITS THE INDUCTION OF INTERFERON-STIMULATED GENES BY VIRAL INFECTION	82
5.1	ABSTRACT	82
5.2	INTRODUCTION.....	83
5.3	MATERIALS AND METHODS.....	87
5.3.1	Cells and Viruses.....	87
5.3.2	Microarray analysis	87
5.3.3	Microarray construction.....	89
5.3.4	Northern blot analysis.....	91
5.3.5	Western blot analysis.....	91
5.4	RESULTS	92
5.4.1	Expression of interferon-stimulated genes as a function of ICP0	92
5.4.2	Induction of interferon-stimulated gene expression and antiviral effect is a function of MOI	96
5.4.3	Interferon-stimulated genes are not induced by adenovirus or HSV mutants expressing ICP0	99
5.4.4	Adenovirus-expressing ICP0 inhibits induction of interferon-stimulated genes	102
5.4.5	Proteasome-inhibition reverses ICP0 suppression of interferon-stimulated gene expression	104
5.4.6	Expression of interferon-stimulated genes during non-productive infection does not require HSV nuclear entry	108
5.5	DISCUSSION.....	110
6.0	SUMMARY AND GENERAL DISCUSSION.....	115
6.1	IRF-3 ACTIVATION	115
6.2	IRF-3 INHIBITION BY ICP0	120
6.3	HYPOTHETICAL MODEL OF ICP0-MEDIATED INHIBITION OF ISG EXPRESSION	126

LIST OF TABLES

Table 1. Expression of d109 induced cellular genes by other viral backgrounds	24
Table 2. Cellular genes induced by high moi infection with d109	93

LIST OF FIGURES

Figure 1. The HSV genome.....	5
Figure 2. Interferon-stimulated gene induction by interferon-regulatory factor 3 (IRF-3)	21
Figure 3. Induction of interferon-stimulated genes by various HSV mutants.....	25
Figure 4. Effect of ICP0 expression on abundance of TBK-1 protein.....	41
Figure 5. Localization of TBK-1 as a function of ICP0 expression.....	44
Figure 6. TBK-1 peri-nuclear reorganization is associated with ICP0 cytoplasmic localization.....	46
Figure 7. TBK-1 co-localizes with GM130 in wild-type, but not d106 infection.....	49
Figure 8. A delay in ICP0 cytoplasmic localization corresponds to a delay in TBK-1 reorganization.....	54
Figure 9. IRF-3 abundance as a function of ICP0 expression.....	69
Figure 10. Infection with HSV-1 non-productive mutants induces IRF-3 phosphorylation.	72
Figure 11. IRF-3 is phosphorylated in the absence of DNA-PK.....	74
Figure 12. ICP0 inhibits the nuclear accumulation of IRF-3 in a proteasome-dependent manner.....	77

Figure 13. Infection with d109 but not d106 induces expression of interferon-stimulated genes.	95
Figure 14. Induction of interferon-stimulated genes and antiviral effect as a function of MOI.	99
Figure 15. Comparative expression of interferon-stimulated genes by adenovirus and HSV mutants.	101
Figure 16. Inhibition of induced interferon-stimulated gene expression by adenovirus-expressed ICP0.	103
Figure 17. Effect of Proteasome-inhibition on interferon-stimulated gene expression in virus-infected cells.	108
Figure 18. HSV-1 DNA entry into nucleus is not required for ISG induction.	110
Figure 19. HSV-1 induced activation and ICP0-mediated inhibition of interferon-regulatory factor 3 (IRF-3).	128

1.0 INTRODUCTION

1.1 HERPESVIRIDAE FAMILY

The name herpes comes from the Greek "herpein" meaning "to creep" because these viruses have been known to cause recurrent infections. All vertebrate classes are infected by members of the virus family Herpesviridae. Approximately 100 herpesviruses have been isolated, at least one virus in any animal that has been examined for herpesvirus. All herpesviruses have a 150-200 nm diameter virion possessing the following characteristics: a 100 nm icosahedral nucleocapsid surrounded by an amorphous tegument region with proteins that act to initiate lytic infection and promote productive infection, encased by a glycoprotein-studded lipid bilayer envelope (20). The 120-230 kb linear, double-stranded DNA genome encodes 60-170 genes (47, 198). Eight herpesviruses infecting humans have been discovered: herpes simplex virus type-1 and type-2 (HSV-1, HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpesviruses 6 and 7 (HHV-6 and 7), and Kaposi's sarcoma herpesvirus (HHV-8).

There are three subfamilies of herpesviruses, alpha-, beta-, and gamma-herpesviruses, and viruses are grouped based on tissue tropism, pathogenicity, and genome arrangement (198). The human viruses, are classified as follows: alpha-

herpesviruses: HSV-1, HSV-2, and VZV; beta-herpesviruses: HCMV, HHV6, and HHV7; and gamma-herpesviruses: EBV and HHV8 (198). Alpha-herpesviruses have a broad host range, rapid replication, visible cytopathic effects, and establish latency in neuronal cells. HSV-1 and HSV-2 commonly cause recurrent facial and genital herpetic lesions, less commonly herpetic keratitis and encephalitis. VZV causes chickenpox and shingles. The lymphotropic beta-herpesviruses have a more restricted host range and longer reproductive life cycle, and establish latency in secretory glands, lymphoreticular cells, and kidneys. HCMV causes severe infections in the immuno-compromised or immuno-suppressed and can result in congenital anomalies if a primary infection occurs during pregnancy. HHV-6 and -7 cause mild early childhood diseases. Gamma-herpesviruses exhibit slow replication and a narrow host range; while the lytic cycle can occur in differentiating epithelial cells, latency occurs in only T- or B- lymphocytes. EBV causes infectious mononucleosis, nasopharyngeal carcinoma, and Burkitt's Lymphoma. HHV-8 causes Kaposi's sarcoma, a cancer seen only in the immuno-compromised and is classified as an AIDS-defining illness (198).

1.2 HSV PATHOGENESIS

Characteristic of all herpesviruses, HSV-1 can establish either a productive, lytic, infection or an asymptomatic, latent, infection. HSV is transmitted through close personal contact of epithelia of the oropharynx or genital region during an active infection. The lytic cycle of the virus typically causes painful vesicular lesions, but can also result in herpes keratitis, which is repeated ulceration of the cornea leading to

blindness. Very rarely HSV-1 can cause encephalitis – an infection of the central nervous system that can be fatal (193, 232). During primary infection in epidermis and dermis cells, HSV replicates its DNA and produces viral progeny, which are released from the cell and infect neighboring cells (198). HSV may also infect the sensory neurons innervating the initial site of infection and undergo retrograde transport through the axon to the neuronal cell body in the dorsal root ganglia (43). The viral genome can either replicate or establish a latent state in the nucleus of these neurons (reviewed in (224)).

Latency is characterized by a genetically intact genome that persists in an inactive but stable nucleosome-associated extrachromosomal state (55, 157), expressing only the latency-associated transcript (LAT) (40, 237). The maintenance of latency is thought to result from an evolving equilibrium between HSV and the host neuron, balanced by immune surveillance from neighboring CD8⁺ T cells (126). Environmental and/or physiological stress, such as fever, ultra-violet light, menses, malnutrition, fatigue, and anxiety, as well as immuno-suppression, can result in reactivation of latent HSV (reviewed in (224)). Reactivation is thought to be facilitated by ICP0 and the de-repression of the HSV genome due to loss of CD8⁺ T cell surveillance (127). Activated HSV undergoes de novo protein synthesis and viral replication. Progeny virus travels down the nerve axon by anterograde transport back to the primary site of infection and re-establishes infection, thus "recurrent" infection (43, 237).

1.3 HSV GENOME

The HSV genome is a large (~152 kb), linear, double stranded DNA (129) packaged in the virion (20, 83). The genome has two regions covalently linked, each containing unique sequence (Figure 1) (reviewed in (155)). The unique long (U_L) region comprises 82% of the genome (108 kb) and encodes at least 56 genes. These genes have been found to code for structural components of the virion or are involved in viral gene expression and DNA replication. The 13 kb unique short (U_S) region encodes ICP22, ICP47, and glycoproteins from 12 open reading frames. Bracketing both regions are inverted repeats, the L (R_L) and S (R_S) repeat. The immediate-early protein ICP0 and the latency associated transcript are encoded by the 9 kb L repeats (R_L). The 6.6 kb S repeats (R_S) encode the immediate-early protein ICP4. As the names suggest, these repeated regions result in the genome carrying 2 total copies of the genes encoded in the repeated regions. *ab* and *b'a'* indicate the L component repeats and *a'c'* and *ca* indicate the S component repeats (197). While highly conserved as to structure, among HSV strains the *a* sequence varies in the number of sequences (250-500 bp) between the U_L and U_S region junctions and at the terminus of U_L (197). One copy of *a* is present at the terminus of U_S . The *a* sequences allow concatemerization of multiple linear strands and are used in packaging the DNA in the virion ((2, 16, 106)). Recombination of the terminal direct repeats (*ab* and *ca*) with the internal inverted repeats (*b'a'* and *a'c'*) allows the HSV genome to exist as an equimolar mixture of four isomers in which the U_L and U_S sequence invert relative to each other (51, 96). In the middle of the U_L region is an origin of replication, Ori_L . The R_S regions each have an origin of replication, Ori_S .

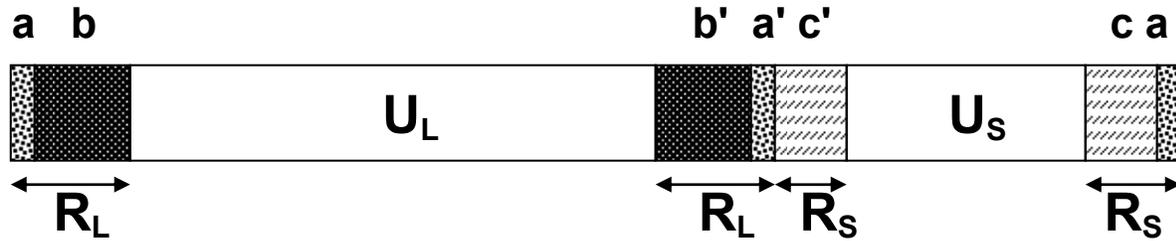


Figure 1. The HSV genome

The HSV genome is divided into two regions, the unique long (U_L) and unique short (U_S), which are separated by repeated regions R_L and R_S (arrows). The U_L is 108 kb, the U_S is 13 kb, the R_L is 9 kb, and the R_S is 6.6 kb. Direct repeats (**ab** and **ca**) are inverted (**b'a'** and **a'c'**) within the joint between the U_L and the U_S . The **a** sequences vary in number at the U_L terminus and the U_L/U_S junction. One copy of the **a** sequence is found at the terminus of U_S .

1.4 HSV PRODUCTIVE LIFE CYCLE

1.4.1 Viral entry

Multiple glycoproteins on the envelope of the virion as well as multiple cell surface receptors mediate entry of HSV into cells (reviewed in (221)). Some known cell receptors are the glycosaminoglycan heparan sulphate, HVEM of the tumor necrosis factor family, and nectin-1 and -2 of the immunoglobulin superfamily (reviewed in (221)). Glycoproteins gB and gC bind heparan sulphate and gD binds one or more of the protein receptors. Fusion of the viral envelope to the cell membrane is activated by gD receptor binding. Complete fusion requires the glycoproteins gB, gH, and gL. gC is not

required, but viral binding efficiency can be reduced along with infectivity (99). Fusion releases the capsid into the cytoplasm while the glycoprotein-studded envelope remains on the plasma membrane. The capsid traverses the cytoplasm using the intracellular transport machinery and binds to the nuclear pore complex where it ejects its DNA into the nucleus (220). The mechanism of translocation of a large DNA genome through the nuclear pore complex (NPC) is poorly understood. However it has been shown that during import the HSV genome exists as a highly condensed rod-like structure that is able to be imported by passive diffusion through the NPC much more quickly than an uncondensed DNA strand of similar length ((214).

1.4.2 Cascade of gene expression and replication

Approximately 80 genes in total are encoded by the HSV genome, but only approximately 37 are required for virus growth in cell culture. During productive infection gene expression occurs in a temporally regulated cascade with immediate early (IE or α), followed by early (E or β) and late (L or γ) gene expression (98, 109, 153, 181). In one of its first actions to inhibit host cell gene expression, the virus commandeers the host RNA polymerase II transcription machinery to transcribe its genes (222). The initiation of IE gene expression occurs through transactivation by the virion-associated protein VP16, and thus requires no *de novo* protein synthesis. VP16 forms a tri-partite complex with the cellular proteins octamer DNA-binding protein (oct-1) and host cellular factor protein (HCF). The complex binds to the TAATGARAT promoter element in all of the IE promoters (14, 29, 187) and results in detectable IE gene expression within 30 minutes of infection (108, 109). IE expression peaks between 2

and 4 hours post infection (hpi) and subsequently decreases significantly (109). Five IE genes are expressed, infected cell polypeptides (ICP) 0, 4, 22, and 27. Four of these, ICP0, 4, 22, and 27 activate and regulate E and L viral gene expression.

ICP4 is the main regulator of viral gene expression and one of the two IE genes absolutely required for productive infection. ICP4 activates both E and L gene expression (52, 69, 84, 86, 171, 190) by mediating interactions with host transcription machinery (15, 31, 44, 90, 112, 216) and E and L promoter binding sites (15, 56). E and L genes are not transcribed in the absence of ICP4 (52); IE genes, however, are over-expressed due to the absence of ICP4 mediated repression of viral gene expression (27, 52, 158, 171).

The other IE gene required for productive infection is ICP27. ICP27, like ICP4, activates E and L gene expression (154, 203, 234), but it also controls post-transcriptional modifications of both viral and cellular RNA affecting polyadenylation site selection, 3' RNA processing, mRNA nuclear export, and mRNA transcript splicing, and can even result in the shut-off of host protein synthesis (reviewed in (206)). ICP22 is not required for viral growth in many cell types, but in some cell types is required for the expression of a subset of L genes (189). It is required for the expression of certain splice variants of ICP0 (32) and mediates the phosphorylation of the RNA Pol II C-terminal domain (194, 195), which may affect viral transcription.

The IE protein ICP0 is also not required for productive infection in cell culture, however, ICP0 deletion mutants show very impaired growth and reactivation from latency (27, 39, 88, 201). ICP0 is termed a promiscuous transactivator; it is able to activate expression of IE, E and L genes (36, 153) as well as expression from a variety

of cellular promoters in transient assays (reviewed in (68, 93). ICP0 also interacts with proteins of various cellular metabolic pathways including elements of the cell cycle control (70, 103, 118, 145), proteolytic (22, 67, 70, 71, 74, 236), DNA repair (136, 180), transcriptional (124), and translational (122) pathways. ICP47, unlike the other IE proteins, does not transactivate gene expression nor is it cytotoxic in cell culture. ICP47 is one of many proteins HSV produce that help it evade or counteract the cellular immune response to infection. ICP47 binds TAP1/TAP2 and inhibits it from transporting peptide antigens to the endoplasmic reticulum, where the antigens would be incorporated into the major histocompatibility complex (MHC) class I and conveyed to the cell surface thus labeling the cell as infected and making it a target for lymphocyte recognition (82, 100, 251).

After IE gene expression, E genes are expressed, peaking between 4 and 6 h.p.i (109). Many of the early gene products contribute to DNA synthesis and viral replication. The viral DNA polymerase, single-stranded DNA binding protein (ICP8), DNA helicase-primase, UL8, UL52, and origin-binding protein (UL9) are E proteins required for DNA replication. DNA replication is believed to be involved in the shutoff of E gene expression (reviewed in (238, 244) as infection in the presence of inhibitors of replication result in continued E gene expression (137). Recently, it was proposed that the decreased expression of a cellular transcription factor, TFIIA, at late times during infection contributes to the transition from E to L gene transcription (252). TFIIA is required for E gene transactivation by ICP4, but not for L gene expression. Also, inactivation of an IE and E gene transcription factor, Sp1 by phosphorylation around 6

hours post infection may explain reduced IE and E gene expression during L gene expression (130).

Viral DNA replication begins soon after E proteins are produced and can last up to 15 hours post infection (108, 109). Any of the three origins of replication are capable sites for the initiation of DNA replication, though no one origin is specifically required (110, 217). The long accepted model of rolling circle replication (e.g. the DNA genome circularizes and DNA copies roll off of the genome as paint off a roller brush) (183) has recently been called into question by evidence that ICP0 functions very early in infection to inhibit circularization of the genome through non-homologous end joining, and only in infections destined for quiescence or in cells infected with ICP0 deletion mutants at low multiplicities does the genome circularize (114). These data provide an explanation for the multiplicity-dependence observed in replication of ICP0 deletion mutants (65, 201, 225). Thus the precise mechanism of HSV replication is unclear.

The last set of genes expressed during productive HSV infection are the late, L, genes. L gene expression peaks around 13 hours post infection (109) and consist of virion structural components, glycoproteins and capsid proteins, as well as the tegument proteins, which have various functions in virus entry and infection initiation. There are two classes of L genes, γ_1 or γ_2 , and gene products are grouped depending on their requirement for DNA replication for expression (reviewed in (238, 244)). γ_1 genes do not require DNA replication – they are expressed in the presence of replication inhibitors – but most are not detected until E gene expression begins to decline (253). True-late genes, γ_2 , are not expressed in the absence of viral DNA replication.

After the expression of L genes, in the cellular nucleus virus particles (core and capsid) are assembled. Encapsidation is initiated by formation of a terminase complex at the capsid portal, which includes the α terminal repeats. Concatemeric viral DNA is reeled into the capsid until the α terminal repeat is encountered again, representing a unit length monomers, and the DNA is cleaved (2, 16, 106). Capsids are enveloped and de-enveloped while they emerge from the nucleus into the perinuclear space, acquire the tegument proteins and then capsids are re-enveloped at the *trans*-golgi network with glycoprotein incorporation occurring simultaneously (231).

1.4.3 Host cell death

Productive HSV infection is very cytotoxic. Often, infected cells fuse and form syncytia, large multi-nucleated cells, due to the glycoproteins remaining on the cell membrane after virus fusion. While virions can exit the host cell without destroying it, the host cell eventually dies. The IE proteins themselves are cytotoxic and act to shutdown host cell metabolism while appropriating much of the host cell machinery. Cellular proteins are sequestered, bound for degradation, or directed to serve the needs of viral gene expression and replication. ICP27 alters host mRNA export (207, 208) and virion host shut-off protein (*vhs*) degrades cellular ribosome-associated mRNA (134, 176). Only about 25% of viral DNA and protein are incorporated into virions. The rest produce characteristic nuclear inclusion bodies as they accumulate in the cell. The combination of all of these factors results in the eventual and inevitable death of the cell.

1.5 ROLE OF ICP0 DURING INFECTION

The IE gene ICP0 is expressed within the first hour of infection. Without binding to promoters (117), it transactivates both viral and cellular gene expression arbitrarily. The use of HSV-1 mutants deleted for the ICP0 gene product have elucidated many properties and functions of the protein. ICP0 deletion mutants show multiplicity dependent impairment of viral growth (65, 201, 225), thus ICP0 is known to facilitate productive infection without an absolute requirement. While important for efficient reactivation of latent infections in mice (27, 39), persistent, quiescent HSV infections used to model latency require ICP0 for de-repression of gene expression (95, 202). ICP0 is thought to stimulate productive infection by inhibiting cell-mediated repression of infection. The ICP0-mediated reactivation from latency could also involve multiple mechanisms with the same goal of inhibition of cellular repression. ICP0 has been shown to be involved in the progression of the cell cycle (68, 122), transcription and translation (122, 124), proteolysis (70, 72, 74), and the structure of ND10 (Everett, 1994 #21, 152). In this study, we provide the first evidence of direct ICP0-mediated inhibition of the cellular antiviral response (60).

1.5.1 Structure and function

ICP0 is present in two copies in the HSV genome; the coding sequence is located within the R_L repeats bracketing the U_L region. The 2.7kb mRNA is formed by splicing together three exons and codes for a 775 amino acid protein weighing 110kDa (181, 182). The only ICP0 domain conserved among herpesvirus homologues is a RING

finger domain (37, 161, 162, 228, 246). Besides the C₃HC₄ RING finger domain, ICP0 contains other domains for protein interaction: an acidic N-terminal domain, a cyclin D3 binding domain, a herpes-associated ubiquitin specific protease (HAUSP) binding domain, a nuclear localization signal, and a C-terminal dimerization domain (64, 66, 74, 122, 165, 182). Additionally, ICP0 has many potential post-translational modification sites, including several N-terminal casein kinase II sites, two central proline rich regions, a central serine-threonine tract, and a C-terminal serine-alanine rich sequence (182).

1.5.2 Cellular gene expression

ICP0 is the only IE protein that can transactivate gene expression from all classes of viral gene promoters (28, 36, 153). From its robust performance in transient assays, ICP0 is known as a promiscuous transactivator (69, 84, 171, 190). In viral infection, ICP0 is thought to stimulate gene expression at the level of mRNA synthesis (117, 204), as it does not bind DNA (75). ICP0 expression in the absence of other IE gene expression has been found to induce a number of cellular genes, many considered p53-responsive genes (103). However, it does not induce the expression of the subset of genes termed interferon-stimulated genes (60).

1.5.3 Protein stability

ICP0 has been shown to mediate the proteasome-dependent degradation of CENP-A (146) and CENP-C (70), which are histone3-like centromere proteins; PML (71) and sp100 (179), which are components of ND10; and DNA-PK (180), which is involved in

double-strand break repair as well as the IRF-3 antiviral response cascade. The ability of ICP0 in promoting productive infection may involve its ability to induce the degradation of cellular proteins, as these proteins are theorized to act to repress lytic infection.

As mentioned above, the mediation of degradation of cellular proteins by ICP0 involves the proteasome, as proteasome inhibitors block the degradation induced by ICP0. The 26S proteasome is a normal cellular mechanism for protein turnover involving targeting of proteins by ubiquitination (reviewed in (38)). Ubiquitin is a 76 kDa protein that targets lysine residues of proteins. Transfer of ubiquitin onto proteins requires the activity of an E2 ubiquitin conjugating enzyme; polyubiquitin chains accumulate on target proteins and bind to the 26 S proteasome. The proteasome cleaves the protein rapidly, akin to a garbage disposal. Ubiquitination, however, is a reversible process catalyzed by ubiquitin specific protease.

The RING finger motif of ICP0 has also been shown to be required for the proteasome-mediated degradation of cellular proteins. E2 ubiquitin conjugating enzymes are stimulated by E3 ubiquitin ligases, which contain RING finger motifs (63). ICP0 has been shown to be an E3 ubiquitin ligase (22, 236). The proteasomal-mediated degradation of cellular proteins by ICP0 is thought to proceed through ICP0's stimulation of ubiquitin conjugation onto the target protein. ICP0's association with the ubiquitin-proteasome pathway is further strengthened by its interaction with the herpes-associated ubiquitin specific protease (HAUSP) (73, 74). Rather than saving proteins from degradation through interaction with HAUSP, ICP0 is thought to use it to scavenge ubiquitin from proteins targeted for degradation by the cell (68). While the association

of ICP0 to the ubiquitin pathway is compelling, ICP0 has never been shown *in vitro* or *in vivo* to specifically conjugate ubiquitin onto proteins, inducing their degradation.

1.6 HOST CELL ANTIVIRAL RESPONSE

The first step in the immune response to infection is the recognition of infection by a cell. Successful early recognition of a virus can lead to the induction of an intracellular antiviral response. The goal of the cellular antiviral response is the shutdown of virus replication and the communication between neighboring cells of the viral threat. The communication task is mediated by interferon. Interferons are proteins or glycoproteins that are able to be produced and secreted by most cells, and most cells can recognize them. They have antiviral, cell regulatory and immunomodulatory functions. The binding of interferon to a cell receptor stimulates an antiviral response in that cell and in many instances can make cells resistant to infection (160, 205, 212, 223, 227).

The task of inhibition of virus replication varies with cell type and from virus to virus, with virtually every stage of viral replication having been found to be inhibited (reviewed in (205, 212, 223). These inhibitory processes remain to be clarified and the antiviral actions of most ISGs are undefined. Two of the most defined antiviral pathways, the dsRNA dependent protein kinase (PKR) and the 2-5A synthetase, inhibit protein synthesis (reviewed in (205, 212, 223). PKR is a serine-threonine kinase that autophosphorylates upon binding to dsRNA. PKR phosphorylates, thereby inactivating elongation factor eIF2-alpha resulting in inhibition of translation. The ISG 2',5'-oligoadenylate synthetase produces a series of short 2',5' oligoadenylates (2-5A) that

activate the 2-5A-dependent RNase L, leading to cleavage of RNA and thus inhibition of protein synthesis.

1.6.1 Interferon-stimulated gene activation

Interferon, double-stranded RNA, and some viral infections each induce transcription of a distinct, but overlapping set of interferon-stimulated genes (ISG) (50, 53, 239). There are hundreds of ISGs; the only requirement for inclusion in the group is their transcriptional activation by interferon alpha, beta, or gamma. Some interferon stimulated genes can be induced by substances other than interferon, such as heavy metals, lipopolysaccharide, glucocorticoids, interleukin-1, poly(rI):poly(rC), double stranded viral RNA, and even the simple binding of virus to a cell (23, 81, 118, 119, 166, 240, 241).

Yet, these ISGs along with some interferon-activated proteins are responsible for the antiviral cellular response enabling cells to resist infection by inhibiting viral replication (reviewed in 205, 212, 223). The cellular antiviral response often produces inhibitory antiviral effects at every stage of viral replication: entry and uncoating (SV40), transcription (VSV), RNA stability (picornaviruses), initiation of translation (adenovirus), maturation, assembly and release (retrovirus) (205, 212, 223).

1.6.2 Interferon-independent pathways

Genes induced by IFN α / β or γ -IFN are often stimulated by viral infection and vice versa (44, 47, 227). IFN, dsRNA and viral infection induce different genes by using distinct

signaling pathways (10, 71, 84, 227, 230). The inducible genes share a similar IFN-stimulated response element, but each ISRE contains unique sequences, which could allow differential response depending on the signaling molecule induced (40, 228, 230). Interferon-regulatory factor-3 activates interferon-stimulated gene expression by an interferon-independent pathway.

1.6.3 IRF-3 and TBK-1 induction cascade

The 55kDa protein interferon-regulatory factor-3 is part of a family of essential transcription factors that regulate the expression of IFN- α /B and interferon-stimulated genes (ISG). IRF-3 is a unique member of the IRF family in that it is constitutively expressed in all tissues, and its expression is actually not induced by viral infection (102, 141, 213). Rather, viral infection stimulates the phosphorylation of IRF-3 on serine/threonine residues in its C-terminus resulting in cytoplasmic to nuclear translocation, association with the transcriptional coactivator CBP/p300, and transactivation of ISGs through DNA binding at specific interferon-stimulated response elements (ISRE) (102, 141, 213, 242, 243, 250) (Figure 2). This process is interferon-independent, occurring in cells deficient in their response to interferon (133, 243) and actually does not result in the induction of interferon in many cell types.

TANK-binding kinase 1, TBK-1 (also named NAK for NF- κ B-activating kinase) was originally discovered as a kinase that directly phosphorylates IKK, mediating the degradation of the inhibitory protein I κ B and thus activating NF- κ B through IKK β (229). However, since then it has been found to be an essential activator of IRF-3 during viral infection, independently of the NF- κ B pathway (reviewed in 101). Activation of IRF-3 as

well as the induction of certain interferon-stimulated genes does not occur in TBK-1 *-/-* mouse fibroblasts during dsRNA or LPS treatment, or viral infection (156). There are three independent pathways for TBK-1 activation that have been well characterized and that result in the phosphorylation of IRF-3; the inducers for the pathways are dsRNA, LPS, and viral RNA. The independence of these pathways and the proteins mediating TBK-1 activation in each of them may account for the downstream differences seen in the induced interferon-stimulated genes, as LPS, dsRNA, and viral infection all induces distinct, but overlapping sets of ISGs (11, 78, 92, 239, 242).

TBK-1 activation by dsRNA occurs through the Toll-like receptor 3 (TLR3). The Toll-like receptor family is characterized by extra-cellular leucine-rich repeats and a cytoplasmic Toll-IL-1R homology domain (TIR) (reviewed in 3). dsRNA recognition by TLR-3 results in tyrosine phosphorylation of the TIR and recruitment of phosphatidylinositol-3 kinase (PI3-K) (209) and Toll-IL-1R homology domain containing adaptor molecule (TICAM-1 or TRIF) (177, 247). TICAM-1 associates with NAK-associated protein (NAP1) and activates TBK-1 in such a way that it then phosphorylates IRF-3 (210). PI3-K recruitment leads to additional phosphorylation of IRF-3, but it is not clear whether PI3-K phosphorylates IRF-3 indirectly or directly or if it activates TBK-1 in some additional manner leading to further IRF-3 phosphorylation (101, 209). However, the activation of IRF-3 by dsRNA requires both PI3-K and TBK-1 (209). TBK-1 activation by LPS occurs through TLR4. As in signaling through TLR3, TICAM-1 is an essential mediator. However, TICAM-1 does not bind directly to the TIR domain of TLR4. Rather, TICAM-2 mediates activation of TICAM-1 (178). A requirement for PI3-K in TLR4 signaling through TBK-1 has not been reported.

Though TLR3 and TICAM-1 are not requirements for IRF-3 phosphorylation during viral infection (6, 107), a cytoplasmic RNA helicase called retinoic acid-inducible gene (RIG-I), was found to be essential for IRF-3 activation (249). RIG-I also contains a caspase recruitment domain (CARD) that is most likely hidden from protein interaction until the RNA helicase domain detects viral ribonucleoproteins. The CARD domain alone can lead to IRF-3 activation (249); the current model involves adaptor molecules, most likely another CARD domain, mediating the signal between RIG-I and TBK-1 or IKKe leading to IRF-3 phosphorylation and an antiviral response (138). Alternatively, the Rho GTPase Rac1 and its effector p21-activated kinase (PAK1) were found to be activated upon viral infection and act upstream of TBK-1 and IKKe to activate IRF-3 (59), but it's not clear how or if they function in concert with RIG-1. They have been implicated, however in TLR2- and TLR3-mediated signaling (9, 59, 113).

1.6.4 Viral inhibition of the antiviral response

Interferon-regulatory factor 3 activation during viral infection has been shown to be inhibited by viral products at nearly every stage of activation. Previously, Hepatitis C virus and Ebola virus were both shown to inhibit the activation of IRF-3 (12, 80). Recently, they have both been shown to act by blocking the mediation of activation by RIG-I ((24, 30), which acts to sense viral dsRNA (249). The Ebola virus protein VP35 may block the detection of dsRNA during infection by actually binding dsRNA (30). While the hepatitis C virus protease NS3/4A is able to specifically inhibit the induction of IRF-3 mediated by RIG-I, the mechanism of inhibition is unclear (24). However, a chemical inhibitor of the NS3 serine protease activity was recently found to restore IRF-

3 activity (19). Acting downstream of RIG-I, Borna disease virus P protein has been shown to inhibit TBK-1 mediated phosphorylation of IRF-3, most likely by inhibiting TBK-1 kinase activity by physically associating with it (233). The vesicular stomatitis virus was shown to block IRF-3 phosphorylation and is also hypothesized to inhibit TBK-1 (218). Similarly, the proteins NS1 and NS2 of bovine respiratory syncytial virus have been shown to inhibit the dsRNA-induced phosphorylation of IRF-3 (21). The influenza A protein NS1 has been shown to inhibit IRF-3 nuclear translocation, and is hypothesized to act by binding viral dsRNA (226); the phosphorylation of IRF-3 was not examined. Sharing only 20% homology, the NS1 protein of influenza B also inhibits IRF-3 nuclear localization (58). Downstream of IRF-3 activation, the Bunyamwera virus protein NSs actually inhibits virus-induced IRF-3-mediated cell death (132). Also acting downstream of IRF-3 activation, the human herpesvirus 8 (HHV8) protein vIRF-1 interacts with the coactivators CBP/p300 and prevents their association with IRF-3, and thus the activation of ISG (140). Another HHV8 protein vIRF3 is actually a homologue of IRF-3 and acts both as a dominant-negative mutant of IRF-3 (150) and as a transcriptional activator on genes controlled by IRF-3 (149). We and others have shown that the herpes simplex virus protein ICP0 inhibits interferon-stimulated gene expression (60, 142), most likely by inhibiting interferon-regulatory factor 3 (60, 142) through an unknown mechanism.

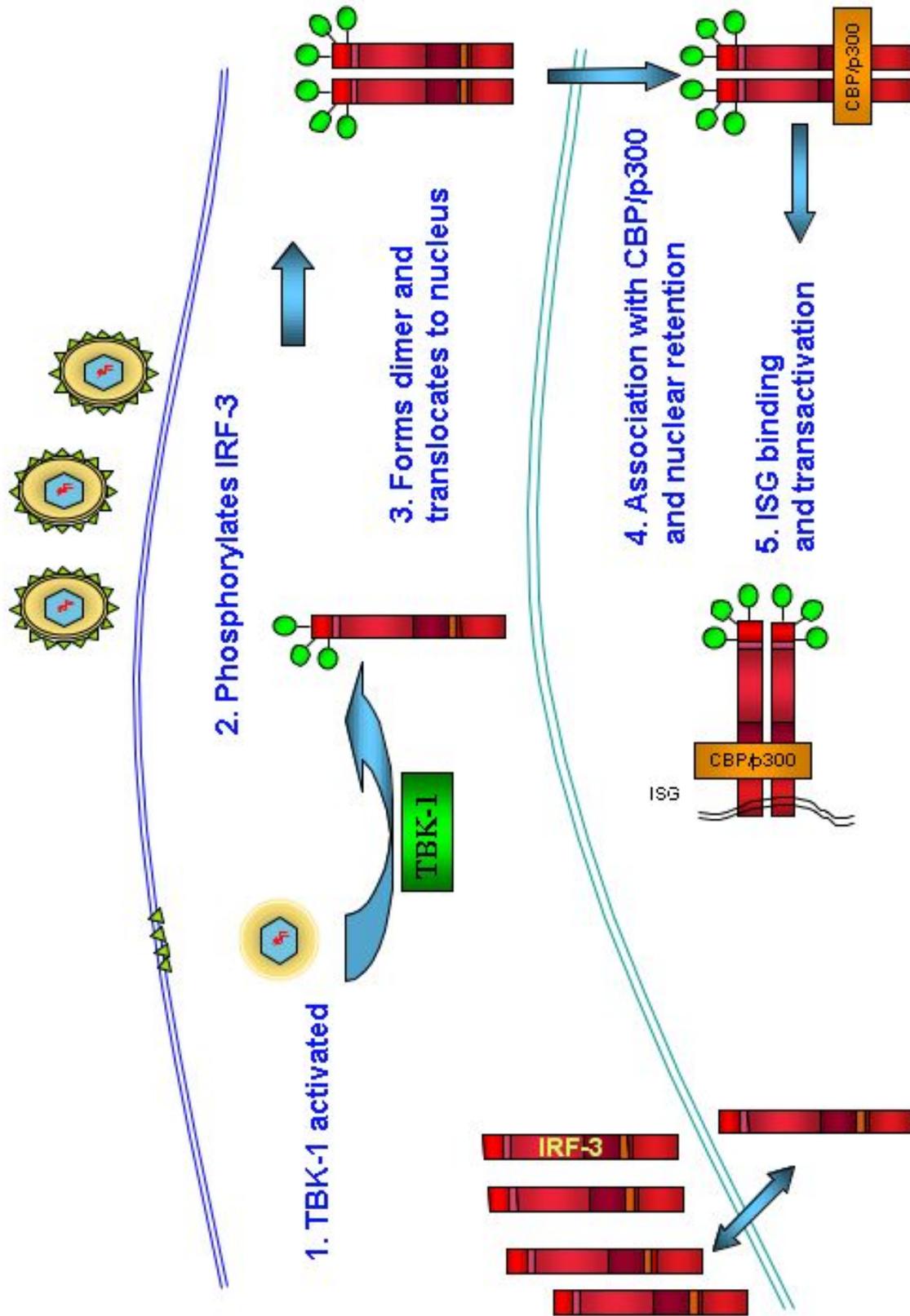


Figure 2. Interferon-stimulated gene induction by interferon-regulatory factor 3 (IRF-3)

IRF-3 is constitutively present in many cell types. It cycles between the nucleus and cytoplasm, with cytoplasmic localization dominating **1**. Viral infection activates Tank-binding kinase 1 (TBK-1) which leads to **2**. phosphorylation of IRF-3 on multiple serine/threonine sites in the C-terminus. **3**. This phosphorylated active form of IRF-3 then dimerizes and translocates to the nucleus. **4**. In the nucleus, IRF-3 forms virus-activated factor (VAF) by associating with CBP/p300, Association with the transactivators CBP/p300 blocks the nuclear export signal and allows IRF-3 to be retained in the nucleus. **5**. VAF then binds the interferon-stimulated regulatory element of an interferon-stimulated gene and transactivates its expression.

1.6.5 HSV escape from antiviral response

Many different viruses have evolved mechanisms to escape the antiviral response (205, 212, 223). HSV-1 has been shown to be very resistant to the cellular interferon response (144, 169). HSV-1 produces an inhibitor to the 2',5' –oligoadenylate synthetase-nuclease (34), and both the US11 and the γ_1 34.5 protein of HSV-1 stops the shutoff of protein synthesis by protein kinase R (33, 89, 97, 184). Protein kinase R phosphorylates eIF2-alpha and stops protein synthesis. The US11 gene product is an RNA binding protein and may associate with ribosomes; it prevents the activation of PKR (33, 186). The mechanism of the late gene γ_1 -34.5 protein of HSV-1 is to dephosphorylate eIF2-alpha, thereby circumventing phosphorylation by PKR and blocking the shutoff of protein synthesis (97, 184). However, treatment of some cell types with interferon before infection with HSV-1 has been shown to decrease the expression of IE genes in a dose-dependent manner (8, 131, 144, 160, 172, 173, 227).

It has also been shown that *d120*, which is defective for ICP4 and is restricted to the expression of IE genes (52), does not induce expression of the interferon-stimulated genes, suggesting that one of the other IE proteins may function to inhibit this response (163). Interestingly, in the same study (163), no mutant with a single mutation in any of the five IE genes induced the interferon-stimulated gene ISG54. HSV-1 viral replication is only reduced 2- to 5-fold in cells that are treated with interferon (143, 144, 164, 168). However, mutants deleted for ICP0 experience over 200-fold reduction in viral plaque formation due to the induced antiviral state (164).

Previous work in our lab found that cellular gene expression during infection by a mutant deficient for all IE gene expression except ICP0, *d106*, is perturbed to a far greater degree than in a virus that expresses no IE genes, *d109* (60, 103, 104). However, the interferon-stimulated genes induced by *d109* are not significantly induced by *d106* (Table 1), suggesting that the expression of ICP0 greatly reduced or eliminated the induction of interferon-stimulated genes (60). Microarrays of cells infected with the IE mutants *d120*- (no ICP4), *d107*- (no ICP4 or ICP27), and *-d104*- (no ICP4, ICP27, or ICP0) (characterized in (202) and (52)) showed further evidence supporting the hypothesis that the inhibition of IRF-3 activation by ICP0 results in the failure of interferon-stimulated gene expression (60). The induction of these genes is evident for the viruses that do not express ICP0 (*d104* and *d109*) and is low from the viruses that do express ICP0 (*d120*, *d107*, and *d106*) (Figure 3) (60).

In this study we will characterize the induction of the interferon-regulatory factor-3 cascade by an HSV-1 replication incompetent mutant expressing no viral genes. We will examine the activation of the tank-binding kinase-1 that mediates IRF-3

phosphorylation as well as the translocation of IRF-3 that is a hallmark of activation. Lastly we will examine the induction of interferon-stimulated genes and connect induction to a functional antiviral response. This study will also characterize the inhibition of the interferon-regulatory factor-3 pathway by ICP0. We will examine the localization and abundance of TBK-1 and IRF-3 as a function of ICP0 expression. We will also explore the level of inhibition of interferon-stimulated genes and the minimal requirements for inhibition by ICP0. With all of the studies, we will examine the function of ICP0 inhibition as it relates to the proteasome.

Table 1. Expression of d109 induced cellular genes by other viral backgrounds

Table 1. Expression of d109 induced cellular genes by other viral backgrounds.

Gene Name	Access. Num.	d109 6h	d109 24h	d106 6h	d106 24h	d105 6h	d105 24h	RJ1 6h	RJ1 24h
interferon-induced protein 56	NM_001548	1.2	22.2	1.1	2.2	1	3	1.4	1.2
interferon-induced protein 54	M14660	2.6	16.3	ND	3.2	1.6	5.4	2.1	1.7
myxovirus (influenza) resistance 1, homolog of murine (interferon-inducible protein p78)	AA477235	1.1	12.7	1.1	1.5	1.2	2	1.3	1.5
pleckstrin	X07743	-1	4.2	1.2	1.2	-1.1	ND	1.3	1.4
myxovirus (influenza) resistance 2, homolog of murine	M30818	1.1	3.6	1.1	1.1	1.1	1.5	1.2	1.4
small inducible cytokine subfamily B (Cys-X-Cys), member 10	NM_001565	-1.1	3.5	1	1.2	1.1	ND	2.5	2.6
signal transducer and activator of transcription 1, 91kD	NM_007315	-1.4	3.3	-1.4	-1.3	-1.7	-1.6	-1.1	-1.4
hypothetical protein, expressed in osteoblast	F12860	1.5	2.9	1.3	1.1	1.4	1.2	1	1.1
interferon induced transmembrane protein 1 (9-27)	J04164	-1.3	2.9	-1.2	-1.2	-1.3	-1.1	1.3	-1
serine/threonine kinase 4	NM_006282	-1	2.9	-1	1.2	-1	1.3	1.1	1.1
ESTs	AA928141	-1.2	2.8	ND	1.4	ND	ND	2.1	1.8
KIAA0129 gene product	D50919	1.1	2.7	1.2	1.1	-1	1.2	-1	-1.4
putative transmembrane protein	NM_012342	1.4	2.7	1.6	2.4	2.7	4.1	1.3	2.5
2'-5' oligoadenylate synthetase 2	M87434	-1	2.6	1.2	1.1	1.1	1.1	1.2	1.4
guanosine monophosphate reductase	M24470	1	2.6	1	1.2	1	1.2	1.2	1.2
Human clone 137308 mRNA, partial cds	U60873	1.3	2.6	1.3	-1	1.8	1.2	1.1	-1.1
interferon, gamma-inducible protein 16	M63838	-1	2.6	-1	-1	-1	1.2	-1.1	-1.3
interferon-induced protein 35	U72862	-1	2.6	-1.1	-1	-1	1.1	1.1	-1
guanylate binding protein 1, interferon-inducible, 67kD	M55542	-1.3	2.5	-1.3	1	-1.6	1.2	1.2	-1.2
apolipoprotein L	Z82215	1.1	2.4	1.2	1.4	1.3	1.3	1.1	-1.1
proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional protease 2)	A192332	-1	2.4	-1	-1	-1.2	1.3	1.3	1.2
caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	X65019	ND	2.3	-1.2	-1.1	-1.3	-1.1	1.2	1.1
nuclear receptor subfamily 1, group 1, member 3	X56199	-1.1	2.3	-1.1	1.1	-1	ND	1.6	1.6
stimulated trans-acting factor (50 kDa)	AW407653	-1	2.3	1.2	1.2	1.1	1.3	1.8	1.1
integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	A1521645	-1.3	2.2	-1.3	1.9	-2.2	2.2	-1	1.9
interferon-induced, hepatitis C-associated microtubular aggregate protein (44kD)	NM_006417	-1	2.2	1.1	1.1	1.1	1.2	-1.1	-1.2
related RAS viral (r-ras) oncogene homolog	NM_006270	1.5	2.2	1.6	1.5	1.5	1.4	1.7	2.1
Sjogren syndrome antigen A1 (52kD, ribonucleoprotein autoantigen SS-A/Ro)	M62800	1	2.2	1.2	1.4	1.1	1.6	1	1.1
uncoupling protein 1 (mitochondrial, proton carrier)	X51952	ND	2.2	1.3	1	-1.1	1.7	1.7	1.9
ESTs	AW468728	-1	2.1	1.2	-1	1.1	ND	1.8	1.8
ESTs, Weakly similar to DELTA-LIKE PROTEIN 1 PRECURSOR [H.sapiens]	AW594704	1.3	2.1	1.2	1.7	-1	1.3	1.4	2.7
interferon-induced protein with tetratricopeptide repeats 4	AF083470	1.5	2.1	1.3	1	ND	1.4	-1.4	-1
superoxide dismutase 2, mitochondrial	Y00472	1.2	2.1	1.1	3.1	-1.4	3	1.5	1.7

ND; not determined

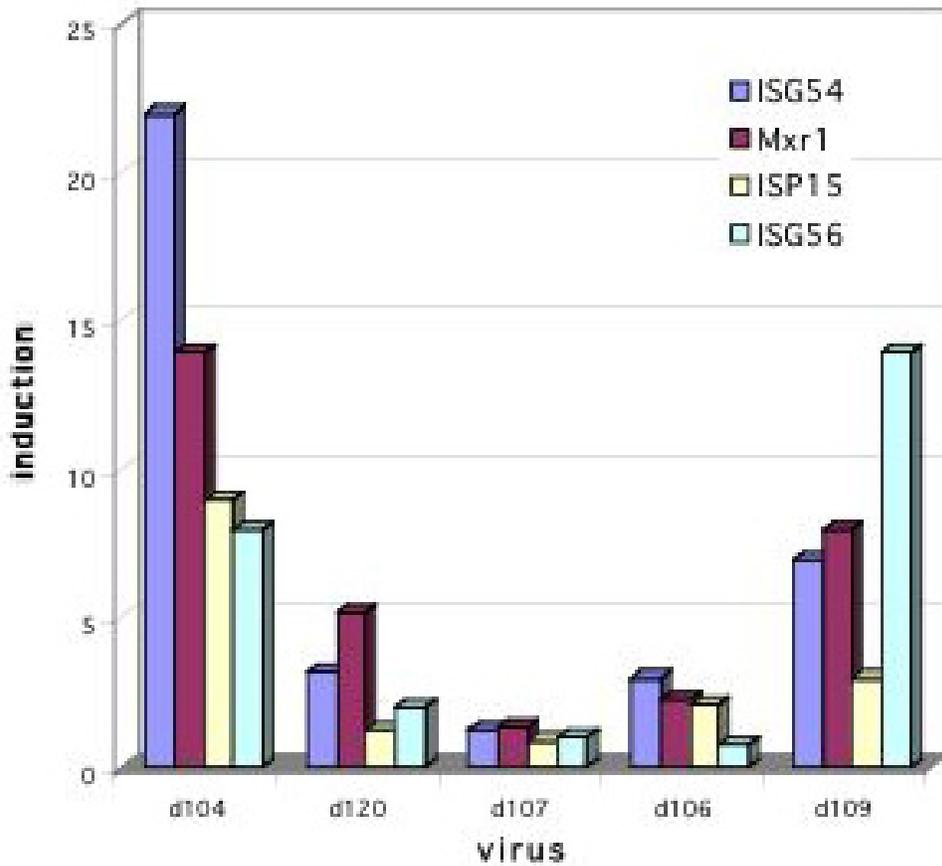


Figure 3. Induction of interferon-stimulated genes by various HSV mutants.

Microarray analysis was performed comparing mock-infected HEL cells and cells infected for 24 h with the indicated mutants (MOI = 30). Shown are the induction ratios for each mutant virus infection for the four most highly induced interferon-stimulated genes (ISG54, ISG56, ISG15, and Mxr1).

2.0 RATIONALE

During HSV-1 infection, ICP0 is essential for the efficient expression of the immediate-early (IE) genes, and thus for highly productive viral replication. IE gene expression is reduced during infection in the absence of ICP0 (27, 28, 225) and in transient-transfection assays, ICP0 transactivates IE, E, and L gene expression (84, 171, 190). These observations suggest that ICP0 acts upstream of the other IE genes to ensure effective IE gene expression. Infection at a higher multiplicity of infection can help to overcome the absence of ICP0 during infection (65, 201, 225); this may indicate that ICP0 acts not only to transactivate gene expression, but to hinder a cellular antiviral mechanism that only infection with many virions can overwhelm. Previous work in our laboratory, as well as that of others, found that HSV-1 infection in the absence of gene expression triggered the cell to transactivate a group of genes termed the interferon-stimulated genes (Figure 1) (60, 163, 169) and that transactivation required the interferon-regulatory factor 3 (IRF-3) protein (41, 188). However, wild-type infection or infection with some replication-incompetent viral mutants do not result in the induction of the ISG (41, 163, 169). Through the use of a viral mutant (d106) deficient in expression of all IE gene except ICP0, we identified ICP0 as a possible mediator of the difference between ISG activation in the absence and presence of viral gene expression. We also

found that an IE deletion mutant expressing no viral genes (d109) strongly induced ISG. *Thus we hypothesize: 1. in the absence of viral gene expression, HSV-1 activates the cellular antiviral response pathway mediated by IRF-3 and 2. the expression and action of ICP0 acts to inhibit activation of the cellular antiviral response pathway mediated by IRF-3.*

The impetus for the studies described in this thesis was Incyte Genomics microarrays performed on two HSV-1 mutant viruses created in our laboratory. The d106 virus does not express the IE genes ICP4, ICP27, ICP22, or ICP47, but overexpresses ICP0 relative to wild-type virus (202). This virus has been shown to significantly affect cell survival and the abundance of many cellular transcripts, as determined by expression array analysis (103, 104, 202). The virus d109 has mutations in all five IE genes, and no viral gene expression is detectable following infection of cells in culture (202). In preliminary experiments with Incyte Genomics microarrays, which have greater than 8,000 human genes, reproducible changes were not evident in d109-infected cells at MOI of 10 PFU/HEL cell or less (data not shown). However, at multiplicities of ≥ 10 PFU/cell, reproducible changes in cellular mRNA abundance were detected. The mRNAs for 35 of the $\sim 8,000$ genes on the array were reduced in abundance > 2 -fold, while 33 were increased in abundance > 2 -fold when the cells were infected at an MOI of 30 PFU/cell, which corresponds to approximately 300 particles/cell (data not shown). The identities of the induced genes are given in Table 1. These were similar to the genes reported by Mossman et al. (163) and Nicholl et al. (169), and they represent some of the interferon-stimulated genes.

Cellular gene expression in d106-infected HEL cells is perturbed to a far greater degree than in d109-infected cells (103, 104). However, the genes that were induced by d109 were not significantly induced by d106 at 6 and 24 h postinfection (Table 1). Furthermore, d105 and RJ1 infection also did not result in the induction of interferon-stimulated genes. d105 has the same viral background as d106 except that it does not have the gene for GFP inserted into the deleted ICP27 locus (202). RJ1 has the same background as d106 except that it has a B-galactosidase gene insertion into the UL41 (vhs) locus. Therefore, neither GFP nor the action of UL41 is involved in the lack of interferon-stimulated gene expression in these backgrounds. Rather, from these studies, it appeared that the expression of ICP0, or simply expression from the viral genome, could inhibit the induction of the interferon-stimulated genes.

To examine the results of these microarrays more closely, a number of IE deletion mutants were used to infect HEL cells and arrays were used as targets for cDNA derived from the RNA of d120-, d107-, d106-, d104-, d109-, and mock-infected cells. d120 expresses all of the IE proteins except ICP4 (52). d107 does not express ICP4 and ICP27 (202). d106 does not express ICP4, ICP27, ICP22, and ICP47 (202). d104 does not express ICP4, ICP27, and ICP0 (202). d109 does not express any of the IE proteins (202). The induction ratios for the four most highly d109-induced interferon-stimulated genes are shown for each virus (Figure 3). The induction of these genes was evident for the viruses that do not express ICP0 (d104 and d109) and was low from the viruses that do express ICP0 (d120, d107, and d106). Therefore, consistent with the results in , the results depicted in Figure 3 also support the notion that expression of ICP0 inhibits the induction of interferon-stimulated genes.

During viral infection, the activation of interferon-stimulated genes is mediated by the transcription factor interferon-regulatory factor 3. Viral infection activates the serine kinase TANK-(TRAF family member-associated NF- κ B activator) binding kinase 1 (TBK-1). TBK-1 phosphorylates IRF-3 on its C-terminus resulting in cytoplasmic to nuclear translocation, association with the transcriptional coactivator CBP/p300, and transactivation of ISG (102, 141, 213, 242, 243, 250) (Figure 2).

2.1 SPECIFIC AIM 1

As we hypothesize that HSV-1 infection in the absence of gene expression induces the activation of IRF-3 and that ICP0 inhibits that activation, the first aim of our study is to examine the abundance and localization of the IRF-3 activating kinase TBK-1 as a function of ICP0.

The activation by phosphorylation of IRF-3 during viral infection was shown to be mediated by the TANK-binding kinase (TBK-1) (79, 215). Inhibition of IRF-3 by proteins produced by Borna disease virus (233) and vesicular stomatitis virus (218) is thought to occur through the inhibition of TBK-1. In this study, we will examine the abundance of TBK-1 as a function of ICP0 during infection. We will also examine the cellular localization of TBK-1 in d106 (ICP0 expressing), d109 (no IE gene expression), and wild-type HSV-1 infection, as a function of ICP0. As the cellular localization of ICP0 has been shown to be reliant on both the proteasome (76, 147) and virus-mediated post-

translational modification (4, 46), we will also examine the localization of TBK-1 as a function of ICP0 in the presence of proteasome inhibition and using a virus deleted for a kinase (UI13) that is responsible for phosphorylating ICP0 (174).

2.2 SPECIFIC AIM 2

As we hypothesize that HSV-1 infection in the absence of gene expression induces the activation of IRF-3 and that ICP0 inhibits that activation, the second aim of our study is to examine the abundance, phosphorylation state, and localization of IRF-3 as a function of ICP0.

Nicholl et al. showed in a study of wild-type HSV-1 infection in the presence of cycloheximide treatment that HSV-1 infection induced the DNA-binding activity of interferon-regulatory factor 3 (IRF-3), and the expression of interferon-stimulated genes (188). IRF-3 has been shown to be one of the most important intermediaries of the interferon-independent response to viral infection (102). Viral infection stimulates the phosphorylation of IRF-3 on serine/threonine residues in its C-terminus resulting in cytoplasmic to nuclear translocation, association with the transcriptional coactivator CBP/p300, and transactivation of ISGs through DNA binding at specific interferon-stimulated response elements (ISRE) (102, 141, 213, 242, 243, 250) (Figure 2). Therefore, we will examine the induction of interferon-regulatory factor-3 as a function of ICP0. Using Western blot analysis of d106 and d109 infected samples resolved by both one- and two-dimensional electrophoresis, we will examine changes in total amount of IRF-3 during infection, as well as the accumulation of phosphorylation states as a

function of ICP0. We will also examine the induction of IRF-3 nuclear accumulation in d106 and d109 infection, and the effect of proteasome-inhibition on IRF-3 localization in d106 infection.

2.3 SPECIFIC AIM 3

As we hypothesize that HSV-1 infection in the absence of gene expression induces ISG through the activation of IRF-3 and that ICP0 inhibits that activation, the third aim of our study is to characterize the components and functionality of, and the requirements for interferon-stimulated gene induction, as a function of ICP0.

In this study we will examine the induction of interferon-stimulated genes more fully using custom microarrays built by our laboratory. These specialized expression arrays contain a number of interferon-stimulated genes as well as genes that were found not to change with infection on the Incyte Genomics arrays. We will examine the expression of interferon-stimulated genes as a function of ICP0 using the mutants d109, which expresses no viral genes, and d106, which expresses the IE gene ICP0 but no other IE genes. We will confirm these microarray experiments with Northern blot analysis. Further, we will examine the functional significance of the d109 induced interferon-stimulated gene expression by examining the induction of cellular antiviral state. We will perform plaque assays on cells infected with d109, and then superinfected with wild-type virus. As the d106 virus over-expresses ICP0 with respect to wild-type virus ((202)), we will examine the ability of ICP0 expressed from an adenovirus vector to inhibit the induction of interferon-stimulated genes by d109. We have previously shown

that an E1⁻E3⁻E4⁻ adenovirus vector expressing ICP0 from the E4 promoter expresses 0.1% of the amount of ICP0 expressed from d106, is nontoxic to cells, and does not greatly perturb cellular gene expression despite retaining the ability to disrupt ND10 and activate quiescent d109 genomes (104). Lastly, as many actions of ICP0 have been shown to be proteasome-dependent (70, 71, 146, 179, 180), we will examine the ability of ICP0 to inhibit interferon-stimulated gene expression in the absence of a functional proteasome by infecting in the presence of a proteasome inhibitor.

Through these studies, we hope to characterize the induction of an antiviral response in cells to incoming HSV-1 virions. Using d109, we will present the first evidence that HSV-1 can induce the phosphorylation of IRF-3 as well as strongly induce its nuclear accumulation, despite reports to the contrary (41). We will show that this interferon-regulatory factor-3 mediated interferon-stimulated gene expression correlates functionally to the induction of a cellular antiviral response. We will also explore one of the earliest actions of ICP0 upon expression. For the first time, we will present evidence that ICP0 alone is essential and sufficient to inhibit the induction of a cellular antiviral response. We will also present evidence that ICP0 inhibits the induction of interferon-regulatory factor-3 in a previously undiscovered fashion thus presenting a possible ICP0-mediated mechanism for the inhibition of the cellular antiviral response during HSV-1 infection.

3.0 EXPRESSION OF HERPES SIMPLEX VIRUS ICP0 INDUCES THE MISLOCALIZATION OF TANK-BINDING KINASE-1

3.1 ABSTRACT

The induction of an antiviral response during herpes simplex virus infection in the absence of gene expression has been shown to require interferon-regulatory factor 3 (IRF-3). Activation of IRF-3 by dsRNA and RNA virus infection proceeds through activation of the tank-binding kinase-1 (TBK-1). TBK-1 phosphorylates IRF-3, stimulating its nuclear translocation and interferon-stimulated gene induction. Mutant HSV-1 viruses expressing the immediate-early protein ICP0 have been shown not to induce interferon-stimulated genes. Using an HSV-1 mutant deficient in all IE expression but ICP0, d106, we show that ICP0 does not induce the degradation of TBK-1 during infection. Infection with wild-type HSV-1 infection induces a shift in TBK-1 cellular localization from diffuse cytoplasmic localization to peri-nuclear localization, which can be correlated with the translocation of ICP0 from the nucleus to the cytoplasm. The peri-nuclear localization shows temporary co-localization with Golgi apparatus markers. d106 infection does not induce peri-nuclear Golgi-associated reorganization of TBK-1, however the protein is found to be aggregated throughout the cytoplasm. Inhibition of the proteasome results in delayed ICP0 translocation and a concurrent delay in TBK-1 reorganization during wild-type infection. In d106 infection,

proteasome inhibition decreases, but does not abrogate TBK-1 aggregation. Use of an HSV-1 mutant deficient in the expression of the UI13 protein kinase showed decreased ICP0 expression and also resulted in delayed ICP0 nuclear to cytoplasmic translocation coupled with delayed TBK-1 reorganization. Proteasome inhibition of the Δ UI13 virus resulted in almost complete inhibition of cytoplasmic ICP0 and TBK-1 reorganization. The ICP0 induced mis-localization may be a mechanism for inhibition of the activation of interferon-regulatory factor-3.

3.2 INTRODUCTION

Herpes simplex virus 1 (HSV-1) expresses its genes in a regulated cascade during infection, beginning with the induction of immediate-early (IE) genes by the virion protein VP16, continuing with early gene transcription, DNA replication, and late gene expression (14, 29, 108, 109). One of the IE genes, ICP0, is not required for viral replication, but has been found to greatly affect many different cellular proteins and processes in its quest to promote the efficiency of HSV-1 lytic infection and reactivation from latency. ICP0 has been found to be a promiscuous transactivator and alone is sufficient for HSV-1 reactivation from a quiescent state (84, 95, 171, 190, 202).

ICP0 has been found to degrade cellular proteins CENP-A ((146)), CENP-C (70), PML (71), sp100 (179), and DNA-PK (180). The degradation of CENP-A and C, as well as the induction of p53 responsive cellular genes result in cell cycle arrest and may result in inhibition of higher-order packaging of DNA (103, 145, 146). DNA-PK is involved in mediating DNA double-strand break repair (42); the degradation of DNA-PK

is thought to be part of the mechanism by which ICP0 inhibits the cell-mediated circularization of HSV-1 genomes during primary infection (114). PML and Sp100 are part of a nuclear structure termed ND10 and may partly function as a repository for antiviral proteins (87, 151, 192) and degradation of PML by ICP0 may prevent these antiviral proteins from being able to inhibit HSV-1 (35, 93).

HSV-1 mutants deficient in the expression of ICP0 have been shown to be very susceptible to interferon treatment (94, 164). Previously, others and we have shown that in the absence of viral gene expression, HSV-1 induces the expression of interferon-stimulated genes (ISG) (60, 163, 169). This induction, however, occurs independently of interferon (41, 163, 169). We have shown that the expression of ICP0 during HSV-1 infection results in severely decreased induction of interferon-stimulated genes (Table 1; Figure 3) (60).

The induction of interferon-stimulated genes by HSV-1 has been shown to proceed through and require the activation of interferon-regulatory factor 3 (IRF-3) (41, 188). IRF-3 has been shown to be one of the most important intermediaries of the interferon-independent antiviral response (102). The activation of IRF-3 during viral infection begins with phosphorylation at C-terminal serine residues by either tank-binding kinase 1 (TBK-1) or I κ B kinase- ϵ (IKK- ϵ) depending on cell type (79, 215). IRF-3 activation proceeds with dimerization, nuclear translocation, association with CBP/p300, and transactivation of the interferon-stimulated response element (ISRE) of an interferon-stimulated gene (Figure 2). (102, 141, 213, 242, 243, 250).

TBK-1 (also named NAK for NF- κ B-activating kinase) was originally discovered as a kinase that directly phosphorylates IKK, mediating the degradation of the inhibitory protein I κ B and thus activating NF- κ B through IKK β (229). However, since then it has been found to be an essential activator of IRF-3 during viral infection, independently of the NF- κ B pathway (reviewed in (101). Activation of IRF-3 as well as the induction of certain interferon-stimulated genes does not occur in TBK-1 $-/-$ mouse fibroblasts during dsRNA or LPS treatment, or viral infection (156). There are three independent pathways for TBK-1 activation that have been well characterized and that result in the phosphorylation of IRF-3; the inducers for the pathways are dsRNA, LPS, and viral RNA. The independence of these pathways and the proteins mediating TBK-1 activation in each of them may account for the downstream differences seen in the induced interferon-stimulated genes, as LPS, dsRNA, and viral infection all induces distinct, but overlapping sets of ISGs (11, 78, 92, 239, 242).

TBK-1 activation by dsRNA occurs through the Toll-like receptor 3 (TLR3). The Toll-like receptor family is characterized by extra-cellular leucine-rich repeats and a cytoplasmic Toll-IL-1R homology domain (TIR) (reviewed in (3). dsRNA recognition by TLR-3 results in tyrosine phosphorylation of the TIR and recruitment of phosphatidylinositol-3 kinase (PI3-K) (209) and Toll-IL-1R homology domain containing adaptor molecule (TICAM-1 or TRIF) (177, 247). TICAM-1 associates with NAK-associated protein (NAP1) and activates TBK-1 in such a way that it then phosphorylates IRF-3 (210). PI3-K recruitment leads to additional phosphorylation of IRF-3, but it is not clear whether PI3-K phosphorylates IRF-3 indirectly or directly or if it activates TBK-1 in some additional manner leading to further IRF-3 phosphorylation (101, 209). However, the

activation of IRF-3 by dsRNA requires both PI3-K and TBK-1 (209). TBK-1 activation by LPS occurs through TLR4. As in signaling through TLR3, TICAM-1 is an essential mediator. However, TICAM-1 does not bind directly to the TIR domain of TLR4. Rather, TICAM-2 mediates activation of TICAM-1 (178). A requirement for PI3-K in TLR4 signaling through TBK-1 has not been reported.

Though TLR3 and TICAM-1 are not requirements for IRF-3 phosphorylation during viral infection (6, 107), a cytoplasmic RNA helicase called retinoic acid-inducible gene (RIG-I), was found to be essential for IRF-3 activation (249). RIG-I also contains a caspase recruitment domain (CARD) that is most likely hidden from protein interaction until the RNA helicase domain detects viral ribonucleoproteins. The CARD domain alone can lead to IRF-3 activation (249); the current model involves adaptor molecules, most likely another CARD domain, mediating the signal between RIG-I and TBK-1 or IKK ϵ leading to IRF-3 phosphorylation and an antiviral response (138). Alternatively, the Rho GTPase Rac1 and its effector p21-activated kinase (PAK1) were found to be activated upon viral infection and act upstream of TBK-1 and IKK ϵ to activate IRF-3 (59), but it's not clear how or if they function in concert with RIG-1. They have been implicated, however in TLR2- and TLR3-mediated signaling (9, 59, 113).

While varied according to inducers, the common denominator in the activation cascade of IRF-3 is TBK-1. In this study, we examine the effect of wild-type and mutant HSV-1 on the abundance and cellular localization of TBK-1. Using a mutant of HSV-1 deficient in all immediate-early gene expression except ICP0 (d106), as well as a mutant expressing all IE genes but ICP0 (d99), we show that TBK-1 protein abundance is unaffected by ICP0. However, the cellular localization of TBK-1 was found to be

greatly affected by wild-type infection, and somewhat affected by the d106 ICP0-expressing virus. In wild-type, but not d106 infection, TBK-1 was transiently associated with the Golgi-apparatus. The mis-localization of TBK-1 was found to be dependent on the cytoplasmic, not nuclear, localization of ICP0; both ICP0 localization and TBK-1 mis-localization are proteasome-dependent. The results support a hypothesis that cytoplasmic ICP0 promotes the segregation of TBK-1, which may result in TBK-1 unable to phosphorylate IRF-3 and activate interferon-stimulated gene expression.

3.3 MATERIALS AND METHODS

3.3.1 Cells and Viruses

Human embryonic lung (HEL) fibroblasts were from American Type Culture Collection (CCL-137). Monolayer cultures were maintained by standard cell culture procedures in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum as previously described (204). The wild-type HSV-1 strain used for all experiments is KOS.

Earlier reports describe the construction and characteristics of viruses d99, d106, and d109 (103, 104, 202). The Δ UI13 virus has a GFP gene inserted into the coding region of UI13 (Dool-Boon Kim, unpublished data). All viruses were grown and titered on Vero-derived cells stably transfected with *trans*-complementing HSV-1 IE genes described previously (203, 204).

3.3.2 Western blot

Confluent monolayers of HEL cells were infected with the indicated virus at an MOI of 10 for 1, 6, or 12 hours. Total cell protein was isolated as described previously (202). Proteins were resolved on pre-cast tris-HCl polyacrylamide gels (Biorad) with SDS-containing Tris-HCl electrode buffer and transferred to polyvinylidene difluoride membranes, and Western blot analysis was performed as previously described (202) using rabbit polyclonal TBK-1 antibody (Calbiochem).

3.3.3 Immunofluorescence

Sub-confluent monolayers of HEL cells were prepared on circular coverslips. The cells were mock- or infected with the indicated virus at an MOI of 20 for the indicated time. Cells infected for 2 and 4 hours were absorbed on ice for 1 hour to synchronize infection. When specified, cells were treated with 5 μ M MG-132 for 30 minutes prior to infection and 5 μ M MG-132 was maintained during and after infection. At the indicated time post-infection, cells were fixed in -20°C Methanol for 15 minutes. Cells were rehydrated in water and stained for the indicated protein. The following antibodies were used: rabbit polyclonal TBK-1 (Calbiochem), mouse monoclonal ICP0 (192), and mouse monoclonal GM130 (BD Biosciences Pharmingen). The stained antigens were visualized by fluorescence microscopy.

3.4 RESULTS

3.4.1 TBK-1 is not degraded during infection in the presence of ICP0

ICP0 has been shown to have two regions with ubiquitin ligase activity (22, 236) and it induces the degradation of at least 5 cellular proteins: CENP-A (146), CENP-C (70), PML (71), sp100 (179), and DNA-PK (180). While Lin et al (142) have shown that TBK-1 is not degraded during wild-type infection, only one time-point was observed. To determine if TBK-1 abundance changes during infection as a function of ICP0 expression, western blot analysis was performed. d109 is a replication-incompetent HSV-1 mutant lacking all IE viral gene expression. In another replication-incompetent mutant d106, the only IE gene expressed is ICP0. HEL cells were mock-, d106-, d109-, wt-, or d99- infected at an MOI of 10 for 1, 6, or 12 hours. Whole cell extracts were made and resolved by SDS-gel electrophoresis. Western blot was performed using an antibody to TBK-1 (Figure 4). The amount of TBK-1 did not change during mock- or d109- infection. The slight decrease seen in wild-type infection may be due to cell death from high MOI, rather than a decrease in TBK-1 specifically. However, the expression of ICP0 did not appear to affect the abundance of TBK-1 as infection with the ICP0 expressing virus d106 showed similar TBK-1 levels to infection with the replication-competent ICP0 deletion mutant d99.

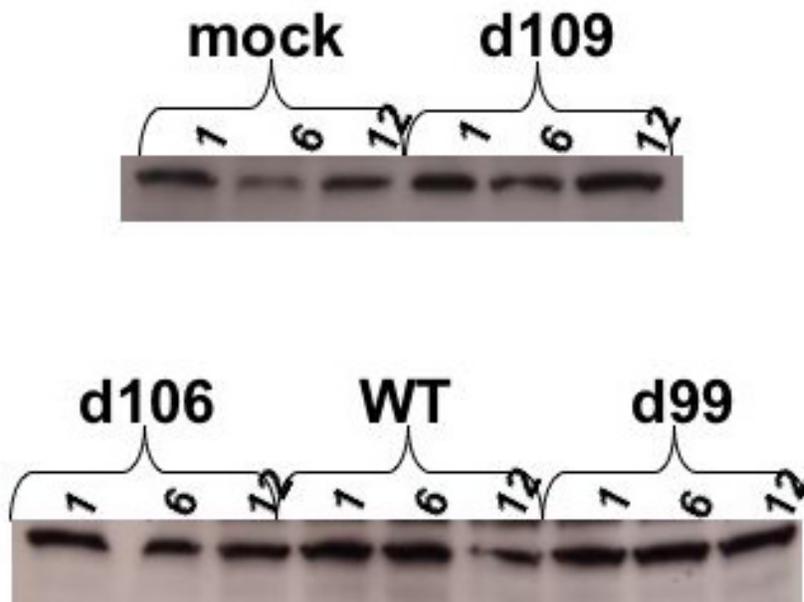


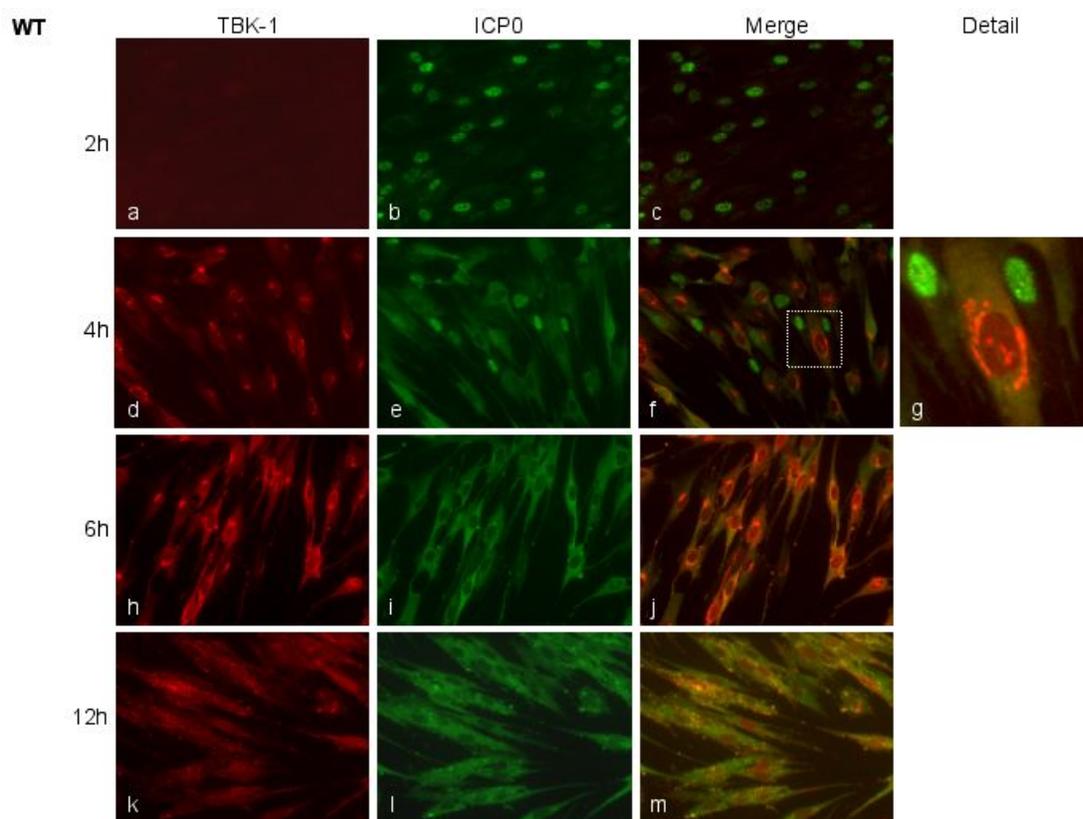
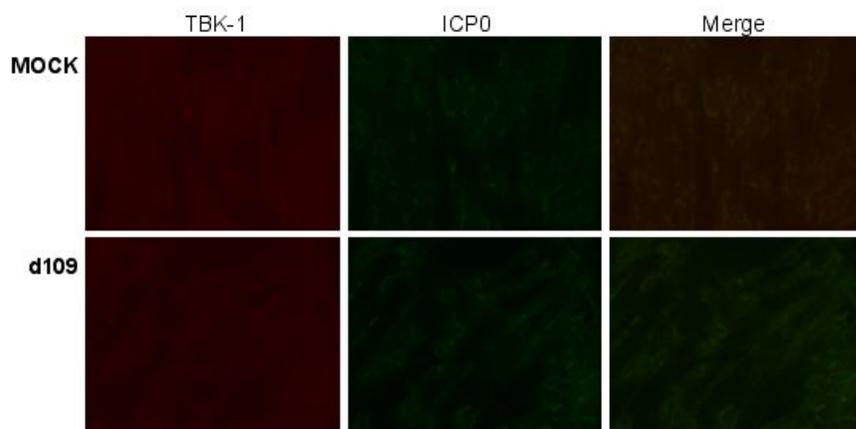
Figure 4. Effect of ICP0 expression on abundance of TBK-1 protein.

HEL cells were infected with the indicated virus for 1, 6, and 12 hours before total protein was isolated and examined by Western blot analysis using an antibody to TBK-1. TBK-1 protein abundance does not change during infection in the presence of ICP0.

3.4.2 TBK-1 is mis-localized during infection in the presence but not absence of ICP0

ICP0 was recently shown to be able to induce the sequestration of the protein ATRIP, which is a binding partner of the ATM and Rad3-related phosphoinositide-3-kinase-related protein kinase (ATR) (245). ATRIP does not appear to be degraded, but is rather mis-localized and unable to trigger the ATR pathway involved in DNA double-strand break repair. To determine if the localization of TBK-1 changes as a function of ICP0, immunofluorescence microscopy was performed. HEL cells were mock-, d106-, d109-, or wt- infected for 2, 4, 6, or 12 hours. Cells were fixed with cold methanol and immunofluorescence was performed using antibodies to TBK-1 (red) and ICP0 (green).

At 1 hour post infection, TBK-1 in all infected cells is not organized in any sub-cellular compartment (not shown). Throughout the time course in mock- and d109- infected cells TBK-1 does not change localization and remains diffusely cytoplasmic (mock, d109; Figure 5). ICP0 in wild-type infection at 2 hours is localized exclusively in the nucleus (WT, b; Figure 5). At this time, TBK-1 is diffusely cytoplasmic (WT, a; Figure 5). At 4 hours post infection, the ICP0 in wild-type HSV-1-infected cells begins to translocate from the nucleus to the cytoplasm (WT, e-g; Figure 5). Cells in which this shift has occurred also show TBK-1 reorganization to the peri-nuclear region (WT, d-g; Figure 5). By 6 hours, most wild-type infected cells showed exclusively cytoplasmic ICP0 localization (WT, i; Figure 5), and those cells demonstrate the peri-nuclear TBK-1 localization (WT, h-j; Figure 5). During d106 infection, ICP0 remains predominantly in the nucleus, although low levels of cytoplasmic ICP0 are detectable as early as 2 hours post infection (d106, o,r,v,y; Figure 5). TBK-1 appears to aggregate in the cytoplasm throughout d106 infection at all time points (d106, n,q,u,x; Figure 5), although the localization is not peri-nuclear. At later times in infection this reorganization becomes more apparent (d106, u, x; Figure 5).



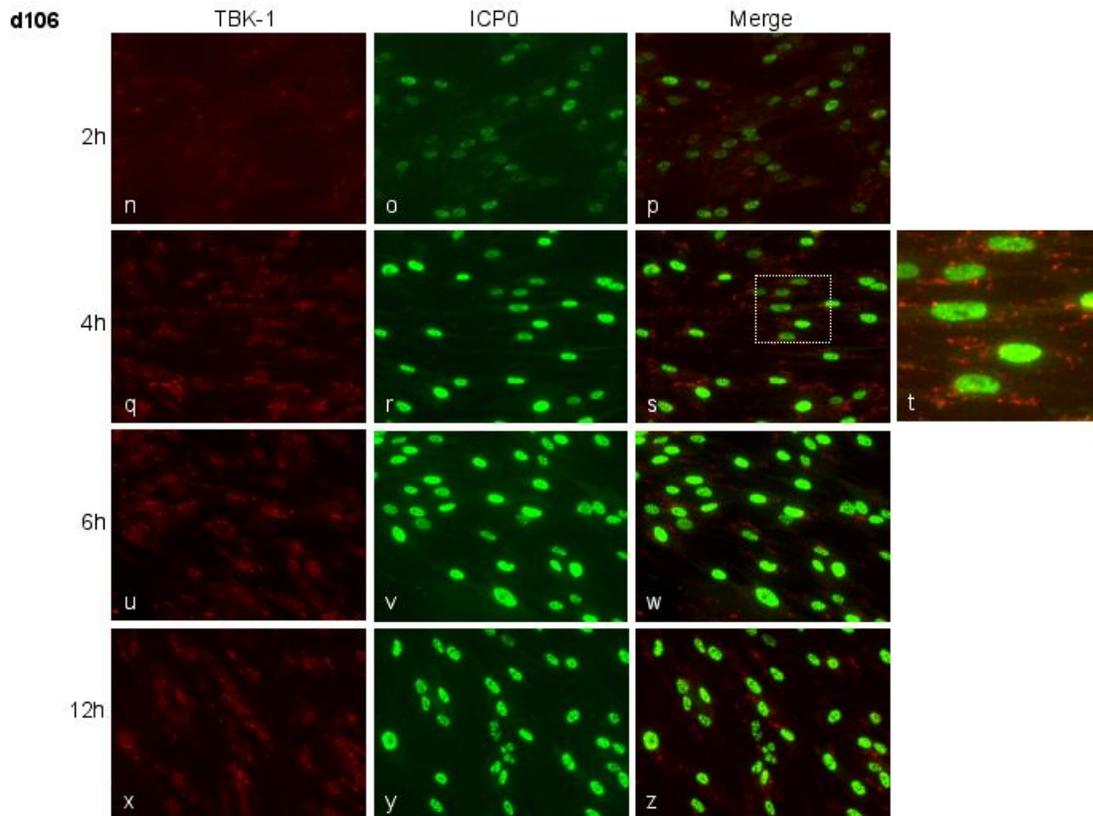


Figure 5. Localization of TBK-1 as a function of ICP0 expression.

HEL cells were mock-, or infected with d109, wild-type (WT, a-m), or d106 (n-z) at an moi of 20 prior to fixation and indirect immunofluorescence (IF) staining at 2 hours (mock; d109; WT, a-c; d106, n-p), 4 hours (d-g; q-t), 6 hours (h-j; u-w), or 12 hours (k-m; x-z) post infection. TBK-1 (red) and ICP0 (green). TBK-1 reorganizes in the presence of ICP0 (WT, inset g; d106, inset t), but not absence (d109) of ICP0.

To determine the association between ICP0 cytoplasmic localization and TBK-1 peri-nuclear localization, a 2X2 contingency table was created and chi-square analysis was performed. Cells from four separate immunofluorescence experiments examining the localization of TBK-1 (red) and ICP0 (green) were counted – two experiments each of 4 and 6 hour infections; one counted field is shown in Figure 6A. To avoid mislabeling of auto-fluorescence as cytoplasmic ICP0, only cells in which ICP0 was

completely excluded from the nucleus were counted as positive (white dot). Cells exhibiting any level of nuclear ICP0, regardless of cytoplasmic ICP0, were not counted as a positive (white X). The 2X2 contingency table for this example cell count is Figure 6B. The table for the entire set is Figure 6C. Cytoplasmic ICP0 was found to be very highly associated with TBK-1 peri-nuclear reorganization by the chi-square test ($p < .0001$). The odds ratio of finding TBK-1 peri-nuclear organization in cells demonstrating exclusively cytoplasmic ICP0 was 143 to 1. These results suggest that the presence of ICP0 in the cytoplasm induces a distinct change in the localization of TBK-1. As TBK-1 localization does not change during infection with a virus, d109, known to induce interferon-stimulated genes most likely through the IRF-3 pathway, the peri-nuclear reorganization of TBK-1 does not correlate with functionality.

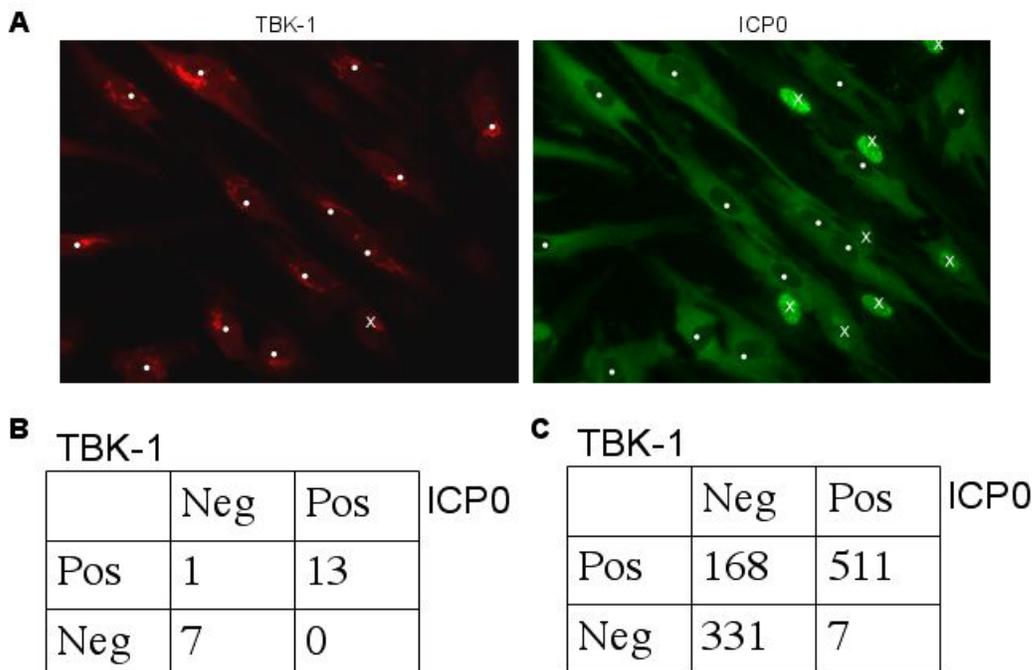


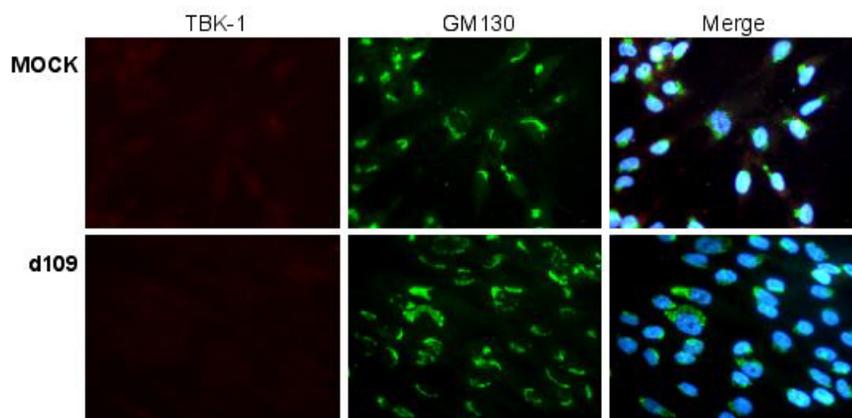
Figure 6. TBK-1 peri-nuclear reorganization is associated with ICP0 cytoplasmic localization.

HEL cells from four separate immunofluorescence experiments examining the localization of TBK-1 (red) and ICP0 (green) were counted – two experiments each of 4 and 6 hour infections. An example of one counted set is shown in **A**. Cells positive for exclusively cytoplasmic ICP0. Cells negative for exclusively cytoplasmic ICP0 are labeled with a white x. Only matched cells are labeled in TBK-1. **B**. An example 2X2 contingency table for the cell count in **A**. **C**. The 2X2 contingency table for the entire experiment. Chi-square analysis for this table gave a significance for association between exclusively cytoplasmic ICP0 and reorganized TBK-1 at $p < .0001$.

3.4.3 TBK-1 co-localizes with Golgi apparatus during productive but not unproductive infection

The perinuclear organization of TBK-1 induced during wild-type infection resembles the morphology of the Golgi apparatus organelle. The Golgi apparatus is a very complex, multi-functional organelle with 3 major roles: the processing and post-translational modification of secretory proteins (77), protein sorting and transport to and from the ER to the plasma membrane (199), and as a membrane scaffold for sorting and cytoskeleton proteins (7, 49, 57). Additionally, signaling molecules, including PI3-K, have also been found to associate with the Golgi, (48, 57, 85). To determine if TBK-1 is localizing to the Golgi apparatus in d106 or wild-type infection, immunofluorescence microscopy was performed on 2, 5, 8, and 18 hour d106-, d109- and wt-infections. Antibodies to the Golgi network marker GM130 (green) and to TBK-1 (red) were used. Dapi was employed to stain nuclei. Throughout the time course of mock- and infection

with the mutant expressing no IE genes, d109, the localization of TBK-1 and GM130 did not change (mock, d109; Figure 7). As before, at 2 hours post wild-type virus infection TBK-1 is diffusely cytoplasmic (WT, a; Figure 7), but by 5 hours post infection, the perinuclear TBK-1 localization is again apparent (WT, d; Figure 7). At 5 hours post-infection, this TBK-1 is co-localizing with the *cis*-Golgi marker GM130 (WT, e-g; Figure 7). By 8 hours post wild-type infection, however, the co-localization is no longer observed, but the TBK-1 is still organized in the cytoplasm as previously seen (WT, h-k; Figure 7). Throughout d106 infection, TBK-1 remains aggregated as seen before (d106, o, r, v, z; Figure 7), but TBK-1 and the Golgi marker GM130 are never co-localized (d106, q, t-u, x-y, β ; Figure 7). The differences seen between TBK-1 localization during wild-type HSV-1 infection versus d106 infection implicate other HSV-1 proteins or processes in the complete mis-localization of TBK-1. However, the TBK-1 aggregation observed during d106 infection as well as the association between ICP0 in the cytoplasm and Golgi-associated TBK-1 clearly connects ICP0 to the process.



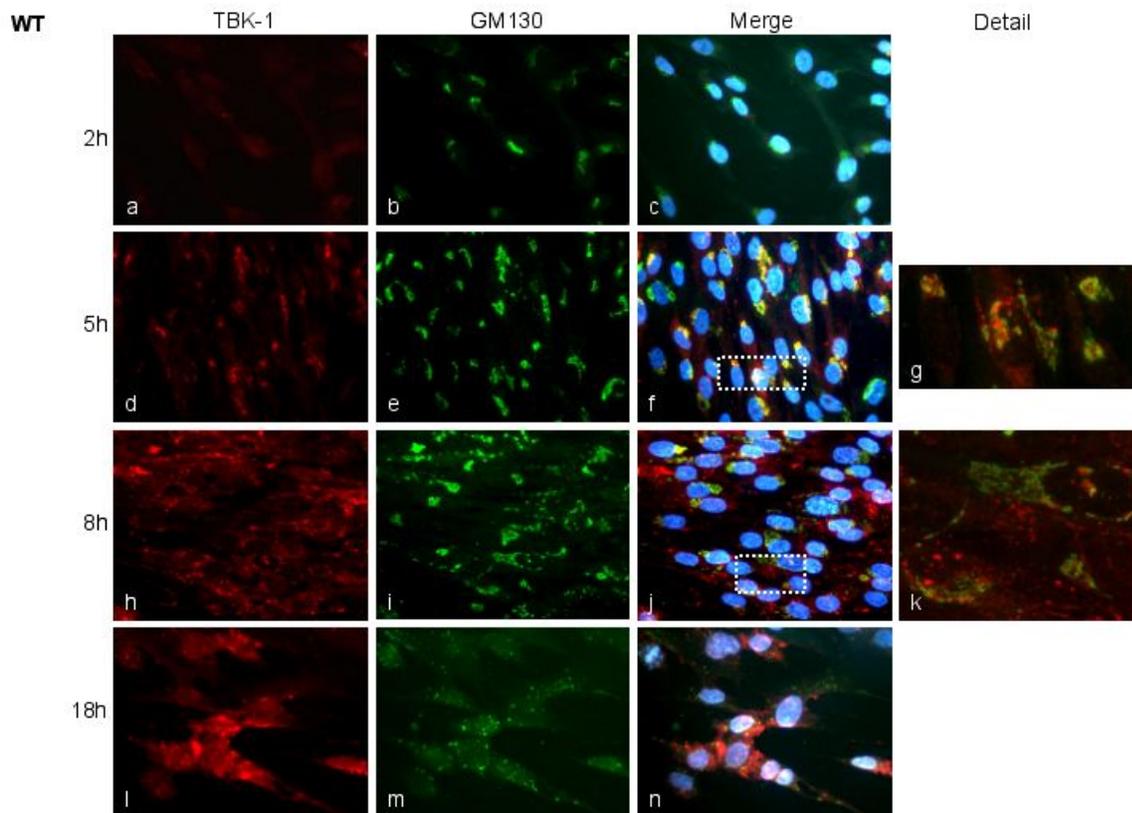
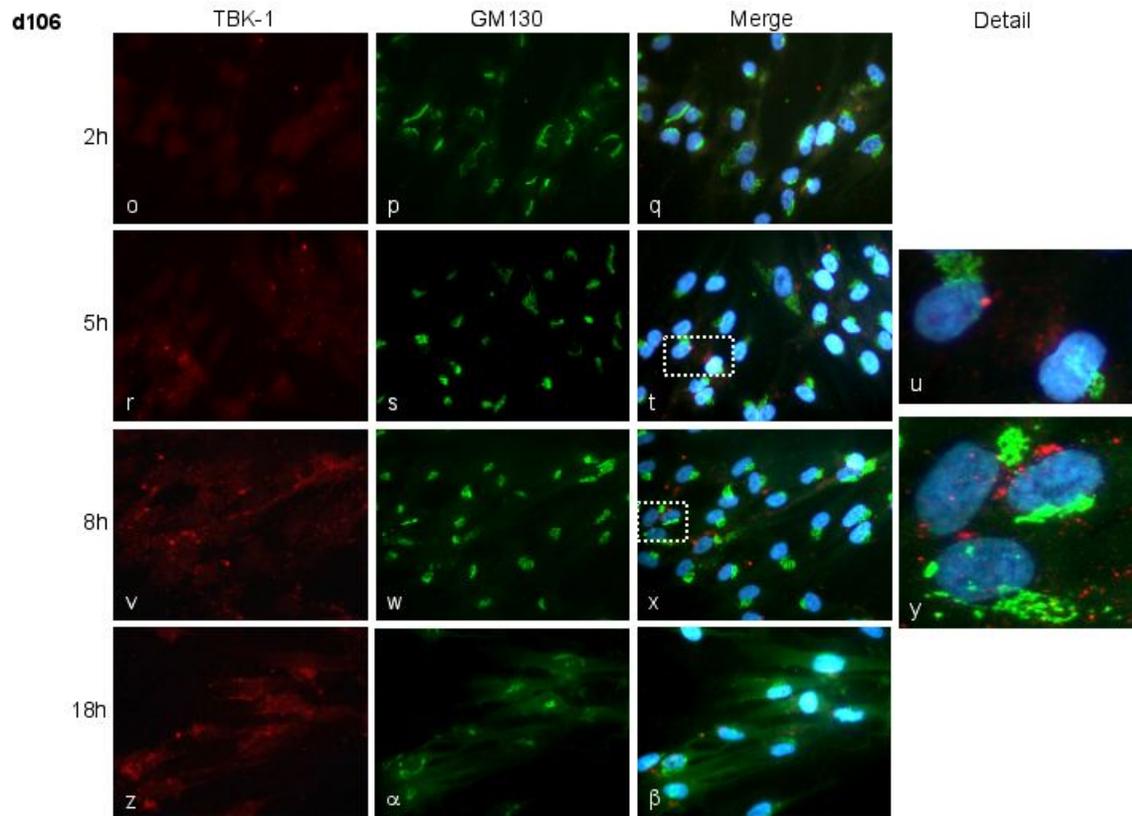


Figure 7. TBK-1 co-localizes with GM130 in wild-type, but not d106 infection.

HEL cells were **mock-**, infected with d109, wild-type (WT, **a-n**), or d106 (**o-β**) at an moi of 20 prior to fixation and indirect immunofluorescence (IF) staining at 2 hours (mock; d109; WT, **a-c**; d106, **o-q**), 5 hours (**d-g, r-u**), 8 hours (**h-k, v-y**) or 18 hours (**l-n, z-β**) post infection. TBK-1 (red), GM130 (green), Dapi (blue). Co-localization is shown in yellow. TBK-1 co-localizes with GM130 at early times in WT infection (**f, g**), but not later (**j, k, n**). TBK-1 in d106 infection does not co-localize with GM130 (**q, t, u, x, y, β**).

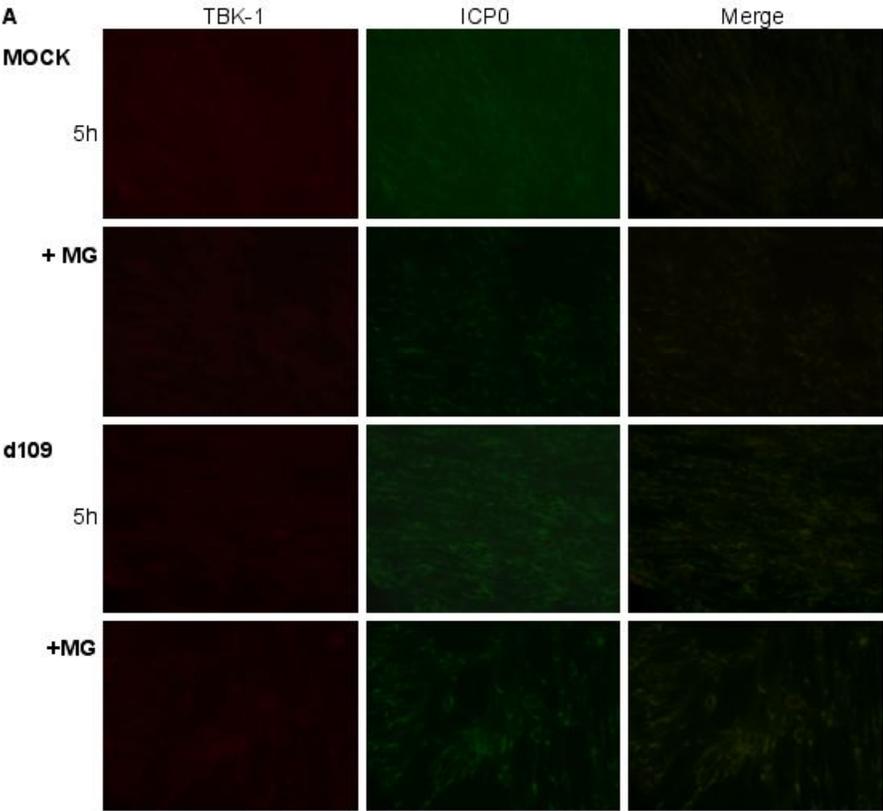
3.4.4 Altered timing of ICP0 cytoplasmic translocation also delays TBK-1 mis-localization

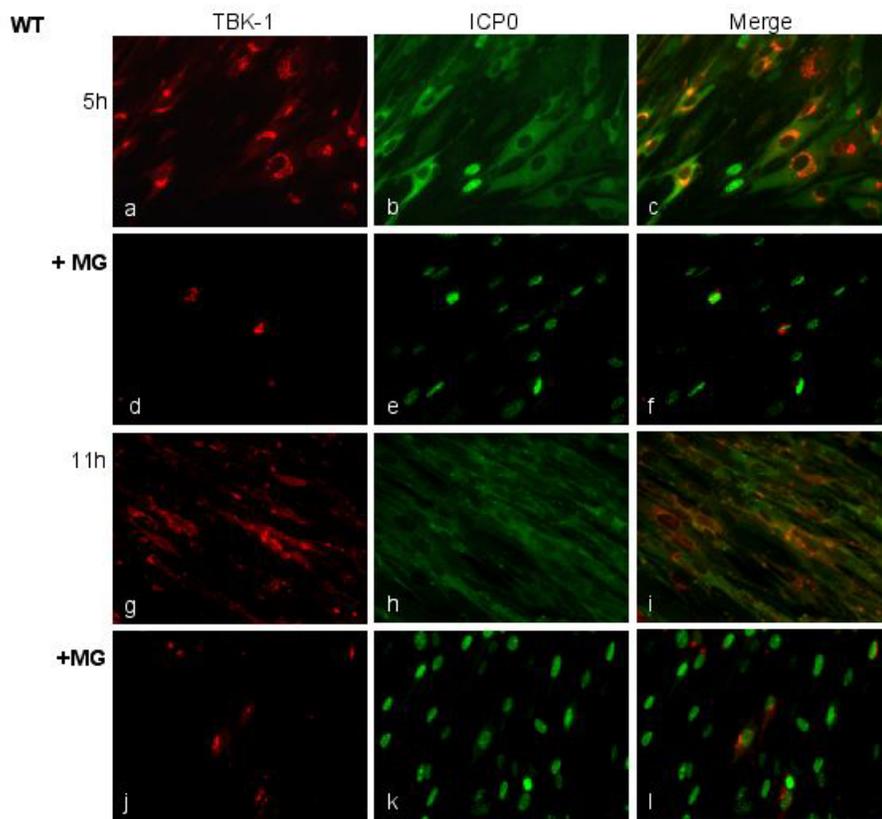
Because of the differences in TBK-1 localization during d106 infection and wild-type infection, we inhibited ICP0 nuclear to cytoplasmic translocation to examine the effect on TBK-1 mis-localization in wild-type and d106 infection. Use of the proteasome-inhibitor MG132 has been shown to inhibit ICP0 nuclear to cytoplasmic translocation due to proteasomal involvement in ICP0 nuclear egress (76). To examine the effect of inhibiting ICP0 cytoplasmic localization on TBK-1 organization, immunofluorescence microscopy was performed on mock-, d106-, d109-, wild-type-, and Δ UI13- infected cells in the presence or absence of the proteasome-inhibitor MG132. Antibodies to TBK-1

(red) and ICP0 (green) were used. Again mock and d109-infected cells showed no differences in TBK-1 localization and proteasome inhibition did not have an effect (Mock, d109; Figure 8A). The effect of MG-132 treatment on TBK-1 localization in cells infected with the ICP0-expressing IE mutant d106 was not conclusive. At both the 5 and 11 hour time points in untreated d106-infected cells, aggregation of TBK-1 was observed (d106, m, s; Figure 8A). Proteasome-inhibition resulted only in decreased, not abrogated aggregation of TBK-1 at both time points in d106-infection (d106 +MG, p, v; Figure 8A). However, the mechanism and timing of ICP0 cytoplasmic localization during infection with non-replicating viruses differs from that of productive infection and may be only partly proteasome-independent (147).

The effect of proteasome-inhibition during wild-type infection was more dramatic than that observed during d106 infection. As before, at 5 hours post-wild-type infection cytoplasmic ICP0 was found in cells in which TBK-1 mis-localization had taken place (WT, a-c; Figure 8A). However, 5 hours of wild-type infection in the presence of the proteasome inhibitor MG-132 resulted in complete inhibition of visible cytoplasmic ICP0 (WT+MG, e; Figure 8A). Mirroring this, very few cells exhibit the peri-nuclear TBK-1 (WT+MG, d, f; Figure 8A). By 11 hours of MG-132 treatment, a few wild-type- infected cells showed cytoplasmic ICP0 localization and TBK-1 was reorganized in these cells (WT+MG, j-l; Figure 8A). As with late time points seen previously, the 11 hour untreated wild-type- infected cells showed widespread cytoplasmic ICP0 and TBK-1 reorganization (WT, g-l; Figure 8A). The link between ICP0 in the cytoplasm and TBK-1 Golgi-associated mis-localization shown previously, while dramatic, could have been coincidental with other events occurring during wild-type HSV-1 infection. However, by

inhibiting ICP0 translocation and seeing a concurrent inhibition of TBK-1 mis-localization, the connection between ICP0 and TBK-1 becomes more convincing.





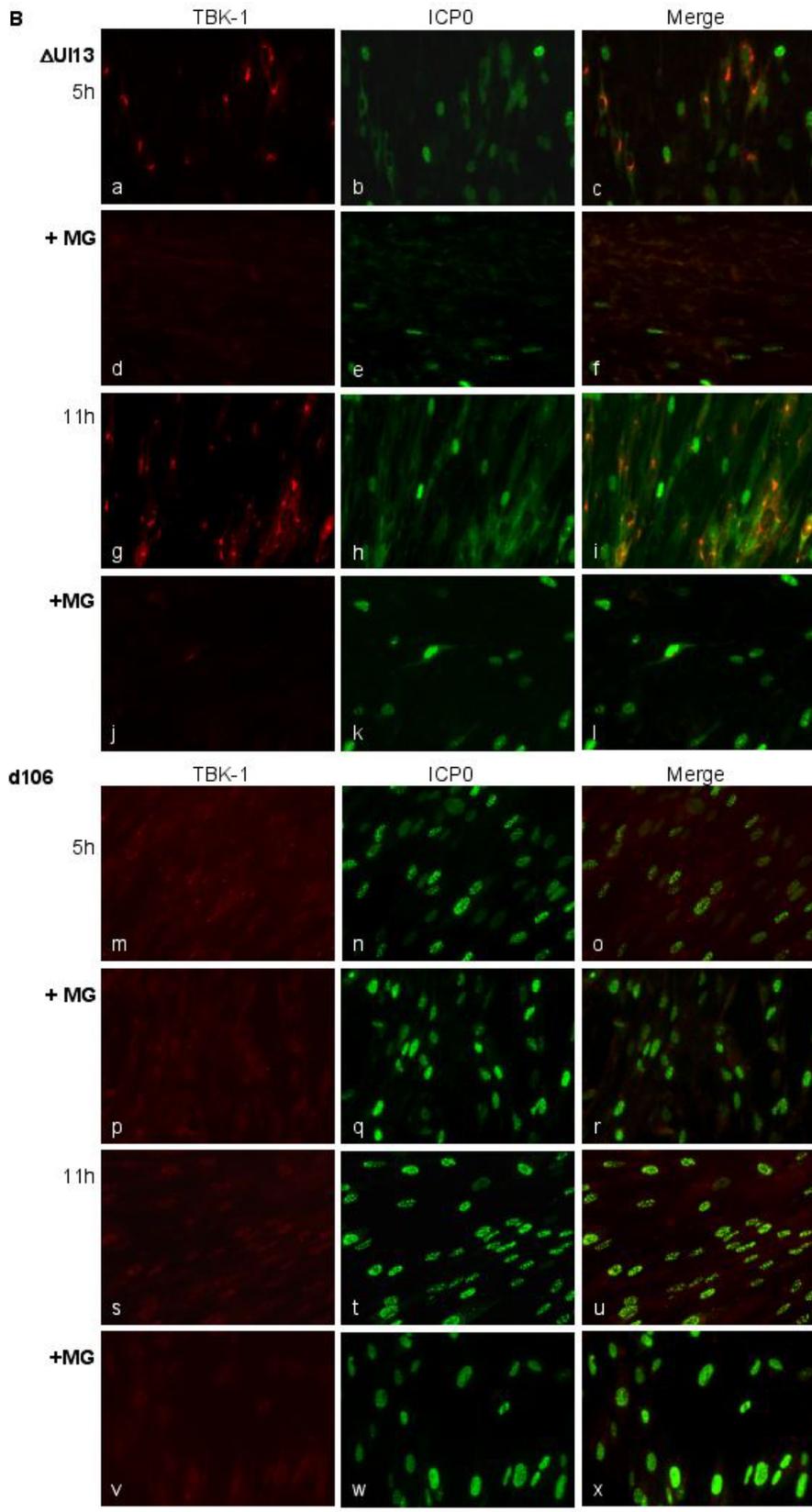


Figure 8. A delay in ICP0 cytoplasmic localization corresponds to a delay in TBK-1 reorganization.

HEL cells were treated as indicated with MG132 and then **A.** mock-, infected with d109, wild-type (WT, **a-l**), d106 (**m-x**) or **B.** Δ UI13 (**a-l**) at an moi of 20 prior to fixation and indirect immunofluorescence (IF) staining. TBK-1 (red) and ICP0 (green). **A.** Cells were infected for 5 hours (mock; d109; WT, **a-c**; d106, **m-o**), 5 hours + MG132 (Mock +MG; d109 +MG; WT, **d-f**; d106, **p-r**), 11 hours (**g-i**; **s-u**), or 11 hours + MG132 (**j-l**; **v-x**). MG132 treatment caused a delay in both ICP0 cytoplasmic localization and TBK-1 reorganization in WT infected cells. d106 infection with MG132 treatment showed less TBK-1 reorganization. **B.** Cells were infected for 5 hours (**a-c**), 5 hours + MG132 (**d-f**), 11 hours (**g-i**), or 11 hours + MG132 (**j-l**). Infection with Δ UI13 caused a delay in ICP0 cytoplasmic localization and TBK-1 reorganization. MG132 treatment abrogated both ICP0 nuclear localization and TBK-1 reorganization.

The HSV-1 tegument protein UI13 protein kinase has been shown to phosphorylate several viral proteins including ICP0 (121, 159, 167, 174). UL13 deletion mutants also show reduced mRNA levels of ICP0 (189). To determine the effect of UL13 on ICP0 cellular localization, and thus TBK-1 peri-nuclear reorganization, we used a virus deleted for the UL13 gene product, Δ UI13. This virus is replication competent, however the phenotype resembles that of an ICP22 deletion mutant and results in reduced accumulations of a subset of late genes. During infection with the Δ UI13 mutant, a delay in ICP0 expression was observed (Δ UI13, b; Figure 8B) compared to wild-type infection at 5 hours post infection (WT, b; Figure 8A). Despite infecting at an MOI of 20, sufficient to infect all cells, at 5 hours post infection ICP0 was not detectable in all cells. Moreover, fewer infected cells exhibited cytoplasmic ICP0 (Δ UI13, b; Figure 8B) than in the wild-type infected cells (WT, b; Figure 8A), demonstrating a delay in

ICP0 nuclear to cytoplasmic translocation; it is not clear if the delay was a result of slower expression of ICP0 or reflects a defect in the ability of ICP0 to translocate. Regardless, only in cells in which ICP0 had translocated to the cytoplasm was TBK-1 mis-localized to the expected peri-nuclear Golgi region (Δ UI13, a-c; Figure 8B). By 11 hours post Δ UI13 infection, all cells exhibited ICP0 staining (Δ UI13, h; Figure 8B). However, many cells still exhibited nuclear ICP0 localization, similar to the proportion observed at 5 hours post wild-type virus infection (WT, b; Figure 8A). Despite this delay, the cells in which cytoplasmic ICP0 was observed demonstrated mis-localized TBK-1 (Δ UI13, g-i; Figure 8B). Proteasome inhibition during Δ UI13 infection resulted in an even more reduced amount of ICP0 at 5 hours post infection (Δ UI13 +MG, e; Figure 8B). In a few cells, nuclear ICP0 was detectable and no cytoplasmic ICP0 or peri-nuclear TBK-1 was observed (Δ UI13 +MG, d-f; Figure 8B). By 11 hours post infection during MG-132 treatment only about 1/3 of total cells demonstrated ICP0 protein (Δ UI13 +MG, k; Figure 8B). Detectable cytoplasmic ICP0 was present in very few cells, but those cells displayed TBK-1 peri-nuclear reorganization (Δ UI13 +MG, j-l; Figure 8B). This experiment provides more evidence that the TBK-1 mis-localization to the Golgi apparatus is mediated by a function of ICP0.

3.5 DISCUSSION

The proteasome mediates many of ICP0's functions during infection. The ability of ICP0 to act as a ubiquitin E3 ligase (22, 236) may be involved in its proteasome-dependent degradation of CENP-A, CENP-C, PML, sp100, and DNA-PK. The degradation of PML and Sp100 is mediated by a ubiquitin conjugating enzyme (91). Additionally, the proteasome regulates the localization of ICP0 during infection. Proteasome inhibition early in infection inhibits nuclear to cytoplasmic ICP0 translocation (76). Proteasome inhibitors given later in infection actually cause cytoplasmic ICP0 to move back into the nucleus (147). ICP0 localization is also controlled by phosphorylation by cellular and viral kinases (4, 46). Post-translational modification of ICP0 at specific sites may also control the ICP0 localization function of the proteasome (4).

In our study, we established that ICP0 mediates the proteasome-dependent mis-localization of TBK-1. The association between cytoplasmic ICP0 and peri-nuclear TBK-1 during wild-type infection was found to be highly significant ($p < .0001$). The association between the two may be even more significant however, as our analysis did not differentiate between cells showing exclusively nuclear ICP0 and those with both nuclear and cytoplasmic ICP0. Many cells demonstrating both nuclear and cytoplasmic ICP0 contained peri-nuclear TBK-1, but these cells were not included in the positive group for chi-square analysis due to the subjective differentiation between cytoplasmic ICP0 and auto-fluorescence of cells. By delaying the timing of ICP0 translocation to the cytoplasm using a proteasome-inhibitor, we were able to further substantiate the association when a concurrent delay in TBK-1 mis-localization was observed. This association was again strengthened when use of a Δ UI13 mutant resulted in delayed

ICP0 expression and cytoplasmic localization. Again, only cells in which ICP0 had translocated to the cytoplasm showed peri-nuclear reorganization of TBK-1.

The mis-localization of TBK-1 observed during infection with the IE deletion mutant expressing only ICP0, d106, was less dramatic than that seen with wild-type infection. Rather than localizing to the Golgi-associated peri-nuclear region, TBK-1 only aggregated in the cytoplasm in response to d106 infection. Also, during infection in the presence of proteasome inhibitors, some TBK-1 was still found aggregated in the cytoplasm. ICP0 is extensively post-translationally modified by HSV-1 proteins (1, 17, 18, 159) and this modification of ICP0 may also play a part in its cellular localization (4, 46). Likewise, the localization of ICP0 is thought to play a part in its post-translational modification, i.e. if it is not present in the cytoplasm it is not phosphorylated by a cytoplasmically-localized kinase (46). However, it is not clear what responsibility the proteasome has in the translocation of ICP0 from the nucleus in replication-incompetent viruses. Lopez et al. (147) observed cytoplasmic ICP0 during infection with the replication incompetent ICP4 deletion mutant d120, but proteasome-inhibition repressed egress of ICP0 in only 67% of cells. The differential requirement of the proteasome for ICP0's cellular localization in wild-type virus versus the ICP4 deletion mutant d120 observed by Lopez et al (147) may be due to decreased or abrogated expression of a viral kinase in d120 infection. In d106 infection, ICP0 is greatly over-expressed, but besides ICP6, few other, if any viral genes are expressed (202). The absence or reduced expression of a viral kinase responsible for phosphorylating ICP0 may affect

both ICP0's cellular localization and the ability of the proteasome to mediate its localization. The continued, while decreased, aggregation of TBK-1 observed in d106 infection in the presence of proteasome-inhibitors may be a result of a small amount of proteasome-independently localized i.e. "leaky" ICP0, as is observed during d120 infection (147).

Recently, TBK-1 was shown to be a client of the cytosolic chaperone protein Hsp90 (248). Yang et al show interaction with Hsp90 to be essential to the stability of TBK-1; failure of Hsp90 interaction resulted in the proteasome-mediated degradation of TBK-1 (248). They also found that by forming a complex, Hsp90 facilitates interaction between IRF-3 and TBK-1. While we and others do not observe degradation of TBK-1 during HSV-1 infection (142), the ICP0 cytoplasmic requirement for TBK-1 mis-localization suggests that ICP0 may interfere with the stability of the Hsp90/TBK-1 complex, resulting in TBK-1 being sequestered in the Golgi with other kinases (48, 57, 85). The lack of TBK-1 degradation may simply be due to appropriation of the cellular proteasome by ICP0 for the degradation of more vitally inhibitory proteins. Separating Hsp90 and TBK-1 may be sufficient to preclude IRF-3 activation. While it is unlikely that Hsp90 is proteasomally degraded by ICP0 given that HSV-1 DNA polymerase nuclear localization requires Hsp90 (25), the recently discovered localization of Hsp90 to VICE domains (25) may be mediated by ICP0. ICP0 is essential and sufficient for the formation of VICE domains (26), which include other molecular chaperone/heat-shock proteins Hsc70/Hsp70 as well as proteasome machinery (25, 26, 67). ICP0 may also mediate the localization of Hsp90 to the VICE domains, resulting in the dissociation of TBK-1 and Hsp90, and subsequently the mis-localization of TBK-1.

Many of the mediators of the activation of TBK-1 by dsRNA and RNA viral infection have been identified. However, mediators required for one induction pathway may be dispensable for another. The induction of TBK-1 during DNA virus infections has not been characterized. This study does not demonstrate any changes in TBK-1 during d109 infection, which is known to induce interferon-stimulated genes by a pathway thought to include the TBK-1 substrate IRF-3. Nevertheless, we do show an inhibitor of ISG expression, ICP0, affecting the sub-cellular localization of TBK-1.

4.0 EXPRESSION OF HERPES SIMPLEX VIRUS ICP0 INHIBITS THE ACTIVATION OF INTERFERON-REGULATORY FACTOR 3

4.1 ABSTRACT

The induction of interferon-stimulated genes during herpes simplex virus infection in the absence of gene expression has been shown to require the transactivator Interferon-Regulatory Factor 3 (IRF-3) (41). The activation of IRF-3 comprises of phosphorylation, dimerization, and nuclear translocation. Infection with an HSV-1 mutant, d109, that does not express any of the immediate-early (IE) proteins results in IRF-3 phosphorylation. Infection with an HSV-1 mutant, d106, deficient in the expression of all IE genes except ICP0 induces phosphorylation of IRF-3 at early time points, but at later time points, phosphorylated IRF-3 is not detectable. Infection with both d106 and d109 induces phosphorylation and interferon-stimulated gene expression in two glioblastoma multiforme cell lines differing in their expression of DNA-PK. However, d109 infection in the DNA-PK expressing cell line (MO59-K) showed an additional IRF-3 isoform not present during d106 infection and the ISG54 expression was greater in d109- than in d106- infection. IRF-3 nuclear translocation was discernable in d109 infection, but not d106 infection. The addition of the proteasome-inhibitor MG-132 during d106-infection resulted in the restoration of IRF-3 nuclear localization at levels similar to those seen

during d109-infection. Thus ICP0 may function through the proteasome to inhibit the activation of IRF-3 by inhibiting phosphorylation and preventing the resultant nuclear translocation.

4.2 INTRODUCTION

Herpes-simplex virus 1 has been shown to have multiple mechanisms to both prevent the induction of and inhibit the actions of a protective antiviral response in infected cells. The HSV-1 tegument protein virion host-shutoff protein (vhs) has been found to degrade host mRNA's (135, 191) and thus may degrade any stimulated antiviral genes. The IE gene ICP47 inhibits the TAP transporter blocking MHC antigen presentation on the surface of infected cells (5, 100, 230). Also many viral infections activate the constitutively expressed protein PKR, which shuts off protein synthesis machinery by phosphorylating eIF2-alpha. However HSV-1 has developed two means of PKR inhibition: the late protein US11 prevents PKR activation (33, 89) and gamma₁-34.5 of HSV-1 dephosphorylates eIF2-alpha, circumventing phosphorylation by PKR, thus blocking the shutoff of protein synthesis (97, 184).

ICP0 is one of the first proteins to be expressed during HSV-1 infection, and has been shown to greatly perturb the expression of many cellular genes and inhibits a variety of cellular processes (reviewed in (93). We have shown that the induction of interferon-stimulated genes during HSV-1 infection is severely curtailed in the presence of ICP0 (60) (**Table 1**). Further, ICP0 has been shown to mediate HSV-1 resistance to

interferon treatment (94). Interferon-stimulated genes are not detected in wild-type infection, except in transient amounts very early in infection (163, 169, 188) lending credence to the hypothesis that ICP0 inhibits ISG expression very early during wild-type infection. As HSV-1 infection in the presence of cycloheximide induced the binding of interferon-regulatory factor 3 (IRF-3) to an interferon-stimulated response element of ISG15 (188), the role of IRF-3 as a mediator of the induction of interferon-stimulated genes in non-productive HSV-1 infection is being explored. Recently, the stimulation of an antiviral response during infection with UV-inactivated HSV-1 was shown to require the presence of interferon-regulatory factor 3 (IRF-3) (41). Thus, the effect of ICP0 on IRF-3 activation is hypothesized to be responsible for the difference in ISG activation in productive versus non-productive infection.

The 55kDa protein IRF-3 is part of a family of essential transcription factors that regulate the expression of IFN- α /B and interferon-stimulated genes (ISG). IRF-3 is a unique member of the IRF family in that it is constitutively expressed in all tissues, and its expression is actually not induced by viral infection (102, 141, 213). Rather, viral infection stimulates the phosphorylation of IRF-3 on serine/threonine residues in its C-terminus resulting in cytoplasmic to nuclear translocation, association with the transcriptional coactivator CBP/p300, and transactivation of ISGs through DNA binding at specific interferon-stimulated response elements (ISRE) (102, 141, 213, 242, 243, 250) (Figure 2). This process is interferon-independent, occurring in cells deficient in their response to interferon (133, 243) and actually does not result in the induction of interferon in many cell types.

IRF-3 has been shown to be phosphorylated by three cellular kinases: I κ B kinase- ϵ (Ikk ϵ), tank-binding kinase (TBK-1) and DNA-dependent protein kinase (DNA-PK) (79, 120, 215). Only phosphorylation by Ikk ϵ and TBK-1 has been shown to induce IRF-3 nuclear translocation and gene activation. DNA-PK phosphorylation occurs in the nucleus and acts to protect activated IRF-3 from proteasome-mediated degradation (120). The activation of IRF-3 during RNA viral infection does not occur in TBK-1 $-/-$ fibroblasts (156). Three independent pathways for IRF-3 activation through TBK-1 have been characterized; while they share common mediators, the pathways are induced by dsRNA, LPS, and viral RNA. The cellular recognition proteins that induce TBK-1 activation are TLR-3, TLR-4, and RIG-1, respectively. None of these cellular proteins have been shown to recognize or respond to DNA virus infection.

The transactivational ability of IRF-3 has been found to be inhibited by a variety of viruses and mechanisms. Vaccinia virus protein E3L ((218))inhibits IRF-3 phosphorylation as does Ebola virus protein VP35 (12), Influenza A protein NS1 inhibits IRF-3 nuclear retention (226), and activation of the IFN α and IFN- β promoters are inhibited by an HHV8 IRF-3 protein homolog, vIRF-3 (150) and Bovine Viral Diarrhea Virus (10), respectively. Others and we have shown that the herpes simplex virus protein ICP0 inhibits interferon-stimulated gene expression (60, 142), most likely by inhibiting interferon-regulatory factor 3 (60, 142) through an unknown mechanism.

Recently, DNA-PK was found to phosphorylate IRF-3 on the N-terminal threonine 135 residue (120). DNA-PK is located primarily in the nucleus, so DNA-PK mediated phosphorylation of IRF-3 is thought to occur after initial C-terminal activation of IRF-3 in the cytoplasm, which triggers the cytoplasmic to nuclear change in IRF-3 localization

(120). The N-terminal phosphorylation occurs near the hydrophobic nuclear export signal; experimentally, IRF-3 remains in the nucleus longer in the presence of DNA-PK (120). In the absence of DNA-PK, activated IRF-3 was found to be vulnerable to cell-mediated proteasome-dependent degradation (120). As ICP0 has been found to direct the proteasome-mediated degradation of DNA-PK during infection (136, 180), the role of DNA-PK in activation of IRF-3 during HSV-1 infection as well as the subsequent ICP0-mediated inhibition of the antiviral effect will be addressed.

In this study, we show that non-productive infection by both the IE-deficient virus d109 and the ICP0-expressing virus d106 induce phosphorylation of IRF-3 at early time points. Yet, in d106 infection, IRF-3 never reaches the level of phosphorylation observed in d109 infection, and further, at later time points, the phosphorylated IRF-3 seen in d106 disappears. While DNA-PK is thought to play a role in the activation of IRF-3 in non-productive HSV-1 infection (60, 142), our studies show that even in the absence of the DNA-PK protein, IRF-3 is phosphorylated during d106 and d109 infection and interferon-stimulated gene expression is induced. However, despite phosphorylation at early time points by d106, IRF-3 nuclear translocation is not observed in d106 infection, while in d109 infection nuclear translocation is readily seen. The proteasome-dependence of ICP0's ability to inhibit IRF-3 nuclear translocation is demonstrated by reversal of inhibition during d106 infection in the presence of the proteasome-inhibitor MG-132.

4.3 MATERIALS AND METHODS

4.3.1 Cells and Viruses

Human embryonic lung (HEL) fibroblasts were from American Type Culture Collection (CCL-137). Monolayer cultures were maintained by standard cell culture procedures in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum as previously described (204). MO59 J and MO59K, both from American Type Culture Collection, are cell lines isolated from the same human glioblastoma tumor. Monolayer cultures were maintained in a 1:1 mixture of DMEM and Ham's F12 medium with 2.5 mM L-glutamine and supplemented with 0.5 mM sodium pyruvate, 0.05 mM non-essential amino acids, and 10% FBS.

Earlier reports describe the construction and characteristics of viruses d105, d106, and d109 (103, 104, 202). All viruses were grown and titered on Vero-derived cells stably transfected with *trans*-complementing HSV-1 IE genes described previously (203, 204).

4.3.2 Western Blot

Confluent monolayers of HEL cells were infected with the indicated virus at an MOI of 30 for 12 or 24 hours. Total cell protein was isolated as described previously (202). Nuclear extracts were isolated as follows. Monolayer cell cultures were washed in TBS + 0.2mM PMSF, scraped and cells were pelleted by centrifugation at 1.5 K for 5 minutes. Cell pellets were washed in hypotonic buffer at a volume 5 times the packed

cell volume (hypotonic buffer: 10mM HEPES, pH 7.9, 1.5mM MgCl₂, 10mM KCl, 0.2mM PMSF, 0.5mM DTT) and then resuspended at 3 times the pcv and swelled on ice for 10 minutes. Cells were lysed with a glass Dounce homogenizer to 90% cell lysis. Nuclei were collected by centrifugation at 2.5 K for 15 minutes. Nuclei were lysed in SDS-sample buffer. Proteins were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, and Western blot analysis was performed as previously described (202) using rabbit polyclonal IRF-3 antibody 1:1000 (Santa Cruz).

4.3.3 2-Dimensional Electrophoresis

For all infections, confluent monolayers of HEL, MO59J or MO59K cells were infected with the indicated MOI of the HSV mutants. For comparisons to uninfected cells, cells were simultaneously mock infected and incubated for the same time as the infected cell sample. Mock- or infected confluent monolayer of cells were washed and scraped into in TBS + 0.2mM PMSF, and pelleted by centrifugation at 1.5 K for 5 min. Cell pellets were frozen in liquid nitrogen and kept at -80°C until used. Thawed cell pellets were resuspended in multiple surfactant solution (5M Urea, 2M Thiourea, 2% CHAPS, 2% SB 3-10, and 0.0002% Bromophenol Blue) supplemented to 2mM TBP for 20 minutes at room temperature. Cell extracts were centrifuged at 15,300 rpm for 20 minutes at 18°C and extracts were separated from viscous DNA pellet. Extracts were treated with 100 U Benzonase (Novagen) for 20 minutes at room temperature. 35 µL of each extract was combined with 110 µL of multiple surfactant solution and samples were supplemented to 2mM and 0.2% carrier ampholyte (2:1 ratio of pH range 4.7-5.9: 3-10). IPG strip, pH range 4-7 (Biorad), were rehydrated with samples for 15-17 hours. Isoelectric focusing

was performed with the following conditions: rapid ramping for 15 minutes with a voltage limit of 250 V, slow ramping for 60 minutes with a voltage limit of 4000 V, and rapid ramping with a voltage limit of 4000 volts until 20,000 V-hours was reached. All IEF strips were run with a current limit of 50 μ A/strip.

After iso-electric focusing, IEF strips were equilibrated in two separated rinses in equilibration base buffer (6M Urea, 2% SDS, 0.05M Tris/HCl, pH 8.8 gel buffer, 20% glycerol) supplemented to 2% DTT followed by 2.5 % Iodoacetamide for 10 minutes each. Strips were quickly rinsed in Tris-HCl electrode buffer and placed in IEF-strip comb of 10% Tris-Cl polyacrylamide gel (Biorad) and immobilized in 1% agarose. SDS-polyacrylamide gel electrophoresis was performed at 200 V for 35 minutes and transferred to polyvinylidene difluoride membranes. Western blot analysis was performed as described previously (202) using rabbit polyclonal IRF-3 antibody 1:100 (Santa Cruz). Detection reagent used was the ECL Plus kit (Amersham Pharmaceuticals).

4.3.4 Northern blot analysis

For all infections confluent monolayers of MO59 J and K cells were infected with an MOI of 30 of the HSV-1 mutants. For comparisons to uninfected cells, MO59J and K cells were simultaneously mock infected and incubated for the same time as the infected cell sample. Total RNA was isolated as described above and 10 μ g of each sample was resolved by denaturing formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose membranes, and probed as previously described (111). Probe fragments were generated by PCR amplification of the appropriate human cDNA clone obtained

from Incyte Genomics, Inc., or Research Genetics, Inc. ³²P-labeled probes were generated from purified PCR fragments by nick translation using [α -³²P]dCTP and [α -³²P]dGTP.

4.3.5 Immunofluorescence

Sub-confluent monolayers of HEL cells were prepared on circular coverslips. The cells were mock- or infected with the indicated virus at an MOI of 30 for 12 hours. When specified, cells were treated with 5 μ M MG-132 for 30 minutes prior to infection and 5 μ M MG-132 was maintained during and after infection. At 12 hours post-infection, cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 and stained for interferon-regulatory factor 3 with rabbit polyclonal IRF-3 antibody at 1:500 dilution (Santa Cruz). The stained antigens were visualized by fluorescence microscopy.

4.4 RESULTS

4.4.1 IRF-3 is not degraded during HSV-1 infection in the presence of ICP0

As ICP0 has been shown to be a ubiquitin ligase and directs the degradation of cellular proteins, such as DNA-PK and PML, we examined the protein levels of IRF-3 during infection in the presence and absence of ICP0. Whole cell and nuclear extracts

were isolated from d106- and d109-infected cells at 12 and 24 hours post-infection. Our western blot analysis of whole cell extracts show no difference in amount of IRF-3 between mock-, d106-, or d109- infected cells (Figure 9). Nuclear extracts however show greater IRF-3 levels in d109-infected cells, and appear to show a lower mobility band that may represent the activated phosphorylated form of IRF-3. The difference in nuclear localization of d109- versus d106-infected IRF-3 is indicative of a difference in IRF-3 activation levels during the two infections.

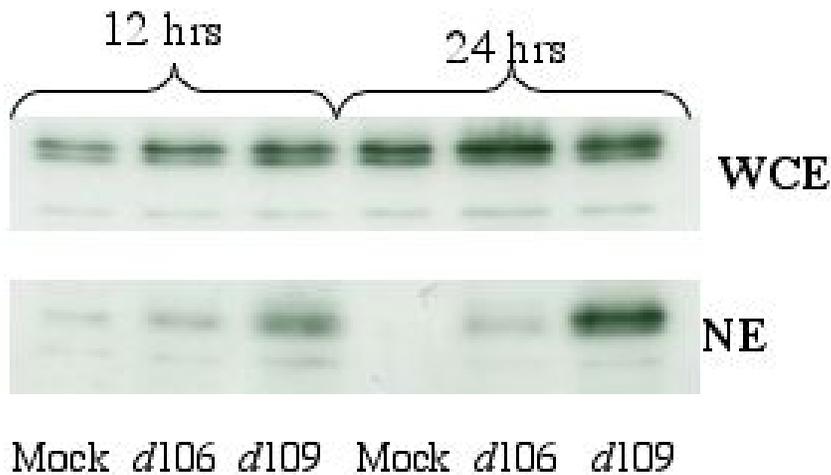


Figure 9. IRF-3 abundance as a function of ICP0 expression.

HEL cells were mock-, d106- (ICP0), or d109- infected at an MOI of 30 and at the indicated times post infection whole cell extracts (WCE) or nuclear extracts (NE) were isolated as described in Materials and Methods and Western blot analysis was performed using an antibody to IRF-3. IRF-3 is not degraded during infection with an ICP0 expressing virus (d106). d109 infection induces nuclear accumulation as well as an additional lower mobility band.

4.4.2 IRF-3 is phosphorylated during non-productive HSV-1 infection

IRF-3 has been shown by 1- and 2 -dimensional SDS-PAGE and immunoblot to be phosphorylated in response to many RNA virus infections (102, 141, 209, 213, 250). However, this change in phosphorylation has not been shown in response to DNA virus infections such as HSV-1. In order to fully examine the changes in IRF-3 phosphorylation during HSV-1 infections, we performed two-dimensional gel electrophoresis and IRF-3 immunoblot. HEL cells were mock- infected; infected with d106 virus, which expresses ICP0; or infected with d109, which does not express any IE genes. Infections were for 4, 8, 12, or 18 hours. Two-dimensional gel electrophoresis was performed, followed by IRF-3 immunoblot. When describing the IRF-3 isoforms, they will be lettered from the most positive isoelectric point (pI) (more negative pH (-)) to the least positive and thus more phosphorylated (more positive pH (+)).

In general, during the first 8 hours of infection, IRF-3 in d106-infected cells undergoes a more rapid change in phosphorylation state than that in the d109-infected cells. A more phosphorylated isoform becomes visible in d106-infected cells by 8 hours post infection, while it is barely visible at the same time point in d109 infection (e; Figure 10). However, by 12 hours the e isoform in d106-infected cells has begun to disappear and by 18 hours is undetectable (e; Figure 10). Over the same time interval in d109-infected cells, the 5th isoform becomes more visible and by 18 hours a 6th isoform is also visible (e, f; Figure 10). From these immunoblots of two dimensional gels, it is apparent that IRF-3 is phosphorylated in response to non-productive HSV infection. By later time points, in the presence of ICP0, this phosphorylation is inhibited and/or reversed.

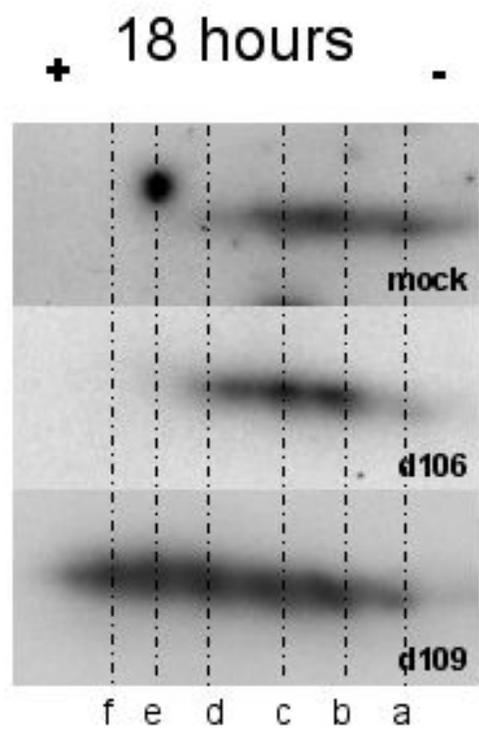
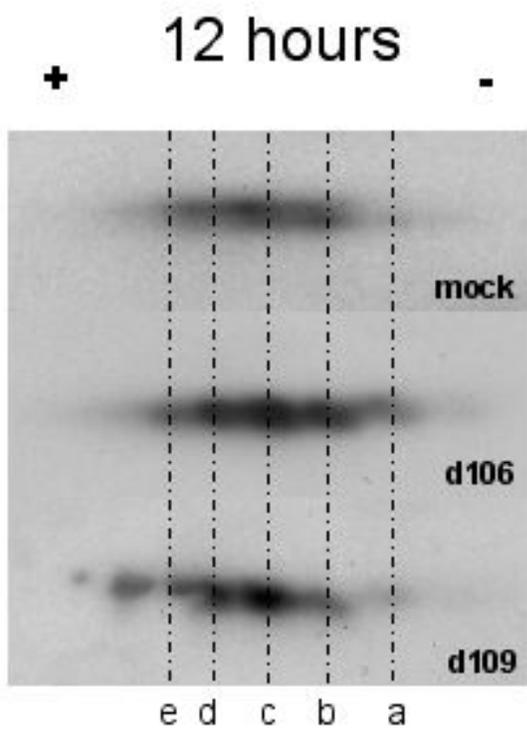
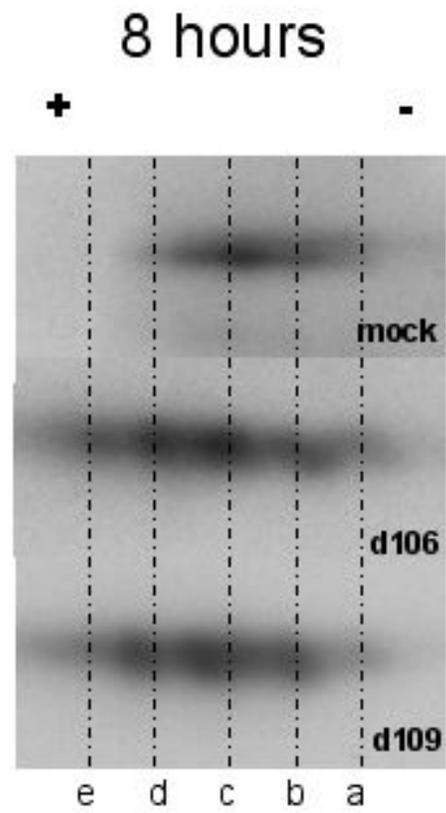
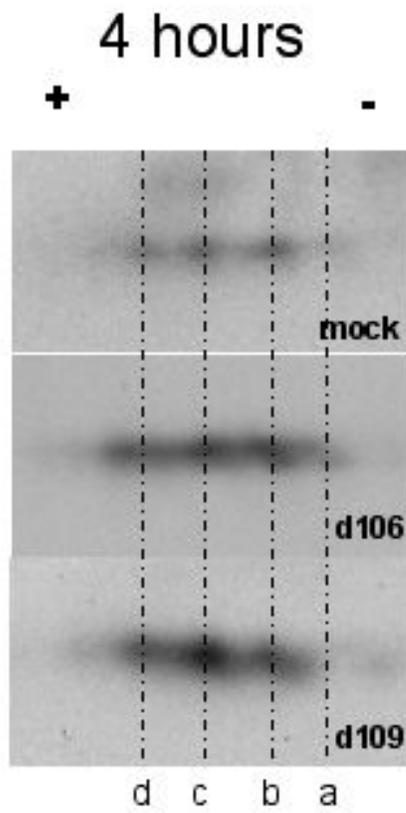


Figure 10. Infection with HSV-1 non-productive mutants induces IRF-3 phosphorylation.

HEL cells were mock-, d106- (ICP0), or d109- infected at an MOI of 30. At the indicated times post infection, total protein was isolated as described in Materials and Methods and resolved by two-dimensional electrophoresis. Western blot analysis was performed with an antibody to IRF-3. IRF-3 isoforms are lettered from least phosphorylated (least positive pI - a) to most phosphorylated (most positive pI - f). d106 induces IRF-3 phosphorylation at early time points (8 hours, e), but it is not observed at later time points (18 hours, e). d109 induces IRF-3 phosphorylation by 12 hours (12 hours, e) and a second isoform by 18 hours (18 hours, f).

4.4.3 IRF-3 is phosphorylated in d109 infection despite absence of DNA-PK

In addition to the TBK-1 mediated C-terminal phosphorylation of IRF-3 examined in Figure 10, DNA-dependent protein kinase (DNA-PK) has been shown to phosphorylate IRF-3 near its N-terminus (120). This phosphorylation may help stabilize the activated IRF-3. As ICP0 is known to degrade DNA-PK (136, 180), we wanted to determine if DNA-PK has a role in the differing phosphorylation states of d109- and d106- infected cells. We used MO59 J (DNA-PK -) and MO59 K (DNA-PK +) cells, two cell lines from the same glioblastoma multiforme tumor, but differing in their expression of DNA-PK. After an 18 hour mock-, d106-, or d109- infection we performed IRF-3 immunoblot on two dimension electrophoresed samples (Figure 11A). Both cell types show multiple phosphorylation states of IRF-3. Mock- and d106-infected MO59K (DNAPK+) cells each have 6 isoforms, with the fourth and fifth isoforms predominant. d109-infected cells however have 7 isoforms, with the 3 most phosphorylated isoforms predominant, perhaps reflecting phosphorylation by DNA-PK. The IRF-3 in d106- and d109- infected

MO59 J (DNA PK-) cells has 7 isoforms with the fourth and fifth forms predominant. Only 6 isoforms are detectable in mock-infected MO59 J cells.

The multiple phosphorylation states of IRF-3 in DNAPK + and - cells is reflective of the induction of ISG54 during mock, d106, and d109 infection. Cells were mock- d106- or d109- infected for 24 hours, RNA was harvested, electrophoresis gel run, and Northern blot was performed using ISG54 as a probe. All samples showed ISG54 induction (Figure 11B). In MO59K (DNAPK +) cells, ISG54 induction was greater during d106 infection than in the mock samples, with the greatest induction seen during d109 infection. This pattern was identical in the MO59J (DNAPK -) cells, but a greater level of induction was seen overall. These experiments show that DNA-PK alone does not account for the activated state of IRF-3 during infection.

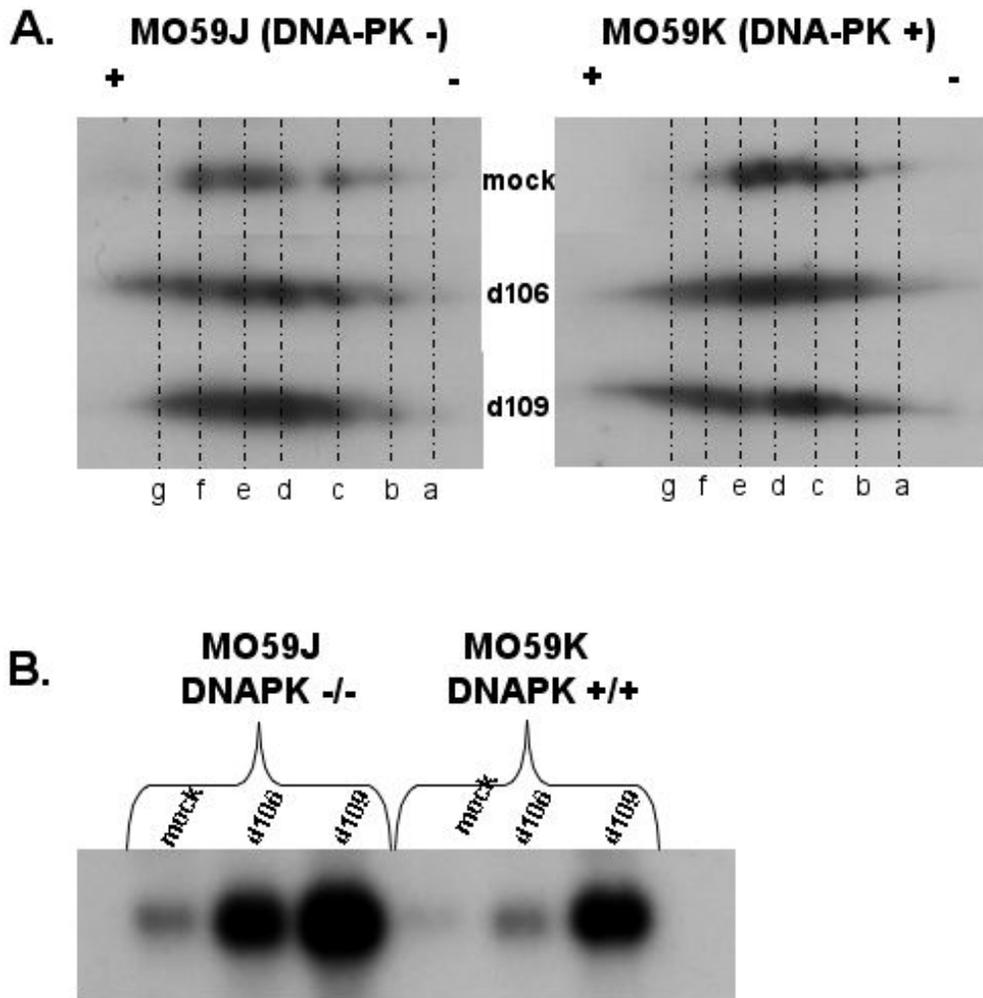


Figure 11. IRF-3 is phosphorylated in the absence of DNA-PK.

A. MO59J (DNA-PK -) and MO59K (DNA-PK +) cells were mock-, d106- (ICP0), and d109- infected for 18 hours. Total protein was isolated as described in Materials and Methods and resolved by two-dimensional gel electrophoresis. Western blot analysis was performed using an antibody to IRF-3. IRF-3 isoforms are lettered from least phosphorylated (least positive pI - a) to most phosphorylated (most positive pI - g). IRF-3 is phosphorylated in both MO59K (DNA-PK +) cells and MO59J (DNA-PK -) cells as a result of non-productive HSV-1 mutant infection (d106, d109) (e, f, g). Mock-infected cells show a constitutive level of phosphorylated IRF-3 (e). **B.** MO59 J (DNA-PK -) cells and MO59K (DNA-PK +) cells were mock-, d106- (ICP0), and d109- infected for 24 hours. Total RNA was isolated as described in Materials and Methods and resolved by

formaldehyde gel electrophoresis. Northern blot analysis was performed using a 32p-labeled probe to ISG54. ISG54 is expressed even in the absence of DNA-PK (MO59J). ISG54 expression is induced by both d106 (ICP0) and d109 infection. Greater levels of induction are observed overall in the MO59J cells (DNA-PK -).

4.4.4 ICP0 inhibits the nuclear accumulation of IRF-3 in a proteasome-dependent manner

Once IRF-3 is phosphorylated in response to viral infection, IRF-3 translocates to the nucleus where it associates with interferon-stimulated response element to transactivate the expression of interferon-stimulated genes (141) (Figure 2). To determine the localization of IRF-3 in HEL cells during HSV infection, immunofluorescence microscopy was performed. HEL cells were mock-, d105-, and d109- infected for 12 hours. d105, like d106, expresses ICP0, but unlike d106, does not have the GFP gene (202). Cells were fixed in 4% paraformaldehyde and immunofluorescence was performed with an antibody to IRF-3. Mock-infected cells do not show IRF-3 nuclear accumulation (Mock; Figure 12A). d105-infected cells show almost no IRF-3 nuclear accumulation (d105; Figure 12A). For contrast, the field chosen contains one of the only d105-infected cells to demonstrate IRF-3 nuclear accumulation. d109-infected cells however show IRF-3 nuclear accumulation to a great extent (d109; Figure 12A).

Inhibiting the proteasome can inhibit many of the actions of ICP0. ICP0 mediated degradation of CENP-A (146), CENP-C (70), PML (71), sp100 (179), and DNA-PK (180) are inhibited by use of proteasome inhibitors. Proteasome inhibition also interferes with ICP0's ability to stimulate viral infection and reactivation from quiescent

infections (76). ICP0 has been shown to interact with proteasomes and possesses E3 ubiquitin-ligase activity (22, 236). Further, the proteasome-inhibitor MG-132 has been shown to dramatically affect ICP0 cellular localization: preventing nuclear to cytoplasmic translocation when used early in infection (76). As proteasome inhibition was also observed to inhibit the ICP0 mediated mis-localization of TBK-1 (Figure 5), we examined the effect of the proteasome inhibitor MG132 on the nuclear localization of IRF-3 during d106- and d109- infection. HEL cells were mock-, d105-, and d109- infected for 12 hours in the presence or absence of 5 μ M MG132. MG132 treatment was begun 30 minutes prior to infection in treated cells. Proteasome-inhibition did not affect IRF-3 localization in mock or d109-infected cells. As before, mock-infected cells show no nuclear accumulation, while d109-infected cells show IRF-3 nuclear accumulation to the same extent as without MG-132 (Figure 12B). Proteasome inhibition reverses inhibition of IRF-3 nuclear localization observed in d105 infection, and IRF-3 localizes to the nucleus to a similar extent as that seen in d109 infection (Figure 12B).

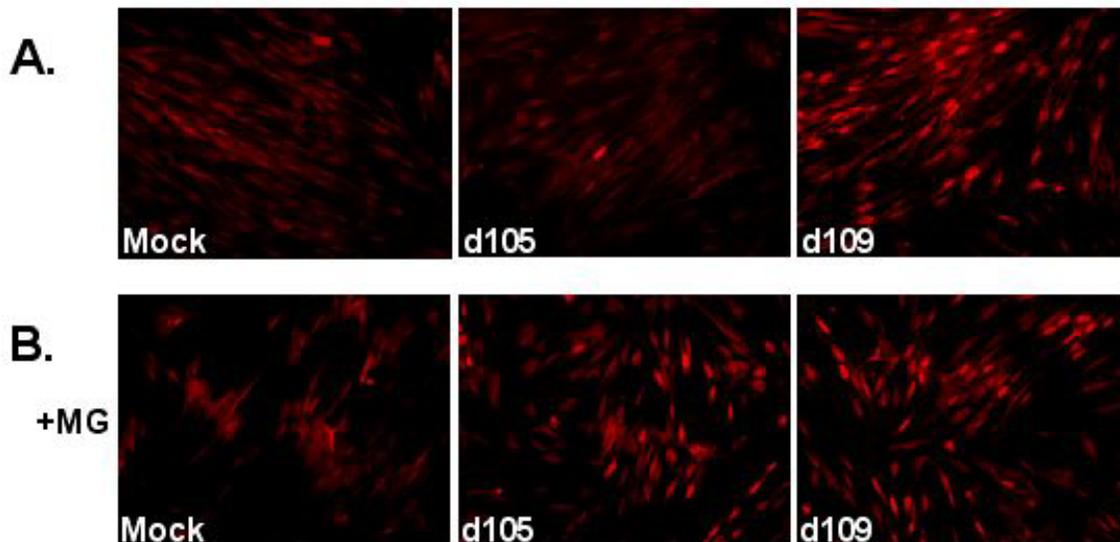


Figure 12. ICP0 inhibits the nuclear accumulation of IRF-3 in a proteasome-dependent manner.

HEL cells were mock-, d105- (ICP0), and d109- infected in the absence (A.) or presence (B.) of the proteasome-inhibitor MG-132 at an moi of 30 prior to fixation and indirect immunofluorescence (IF) staining. TBK-1(red). A. IRF-3 accumulates in the nucleus in the absence (d109) but not presence (d105) of ICP0. B. Proteasome-inhibition abrogates the ICP0-mediated inhibition of IRF-3 nuclear accumulation in d105 infection.

4.5 DISCUSSION

The activation of IRF-3 by RNA virus infections is very easy to detect. TBK-1-mediated phosphorylation results in a slower mobility band of sufficiently different size from normal IRF-3 to be detected by Western blot of one-dimensional SDS-PAGE (215). While the C-terminal phosphorylation at the serine 396 residue has been shown to be required for association with CBP and thus ISG transactivation (141), there are six other serine phosphoacceptor sites on the C-terminus of IRF-3; phosphorylation of four sites (serine 398, 402, 404, and 405) enhance IRF-3's induction ability *in vitro* (213). The phosphorylation of all of these sites is thought to account for the mobility shift in activated IRF-3. Recent evidence indicates that this hyperphosphorylation is not indicative of IRF-3 activation, but rather robust viral replication (41). Evidently, the dsRNA product of RNA virus infections triggers Toll-like receptor 3 (TLR3), which activates the phosphatidylinositol-3 kinase (PI3K) leading to differential phosphorylation from that mediated by virus-activated TBK-1 (209). It is not clear whether PI3K phosphorylates IRF-3 directly or activates TBK-1 to an additional level. Regardless, the

PI3K-mediated hyperphosphorylation is not seen during infection with HSV-1, and the TBK-1 mediated phosphorylation of serine 396 is not detectable by one-dimensional SDS-PAGE.

In this study a change in IRF-3 phosphorylation as a result of infection with the HSV-1 mutants d106 and d109 was observed by two-dimensional gel electrophoresis in two different cell types. The five isoforms of IRF-3 that were detected by 8 and 12 hours in d106 and d109 infection resemble five isoforms detected in the two-dimensional gel analysis of IRF-3 by Sarkar et al. (209); The fifth isoform was a result of dsRNA treatment in cells that were unable to mediate TLR3 activation and cells in which PI3K was inhibited. dsRNA treated cells with PI3K inhibition were also found by one-dimensional SDS-PAGE to induce serine 396 phosphorylation and immunofluorescence to induce IRF-3 nuclear translocation (209). While we failed to detect serine 396 phosphorylation using a phosphospecific antibody (data not shown), we have found that the detection of IRF-3 by western blot of two-dimensional electrophoresis requires a very sensitive and highly concentrated antibody. Thus it may be possible that the 5th isoform of IRF-3 detected during d109 infection represents phosphorylation at serine 396.

While the Sarkar et al study did not find ISG56 induction in the cells in which PI3K was inhibited, our lab found d109 to very highly induce ISG56, as well as ISG54 and ISG15 in HEL cells by microarray analysis (Table 1) (60). Further Preston et al. found IRF-3 to associate with CBP on the promoter of ISG15 in cells from an HSV-1 infection in the presence of cycloheximide (188) and Collins et al. showed ISG56 mRNA to be expressed in response to UV-inactivated HSV-1 (41). However, no studies

published to date have shown IRF-3 phosphorylation or IRF-3 nuclear translocation resulting from any form of HSV-1 infection. In addition to the induced IRF-3 phosphorylation seen in our studies, we found IRF-3 to be very distinctly translocated to the nucleus in d109, but not d106 infection. The restoration of IRF-3 nuclear translocation in d106 infection in the presence of the proteasome-inhibitor MG-132 reflects the proteasome-dependence illustrated by other ICP0 functions, such as the degradation of DNA-PK and PML (71, 180).

The role of DNA-PK in IRF-3 activation in the context of HSV-1 infection has not been elucidated. In productive RNA virus infections, N-terminal IRF-3 phosphorylation by DNA-PK acts to stabilize the C-terminal phosphorylated IRF-3, preventing its proteasome-mediated degradation. The constitutive ISG54 expression and IRF-3 phosphorylation seen in MO59J and K cells leave the role of DNAPK in the activation of IRF-3 unclear. No difference in the number of phosphorylated IRF-3 isoforms is observed between d106- and d109- infected MO59J (DNAPK -) cells, only a greater amount of the most phosphorylated isoform is seen in d109 infection. The additional isoform seen in d109-infected MO59K (DNAPK +) cells versus MO59J cells may be indicative of DNA-PK catalyzed IRF-3 N-terminal phosphorylation as this isoform is not observed in d106-infected MO59K cells, in which ICP0 mediates DNAPK degradation. The irregularity of these cells in regard to ISG54 expression and other characteristics make drawing direct comparisons to the IRF-3 phosphorylation state in HEL cells impractical. However, as ISG54 is expressed even in uninfected MO59J (DNAPK -) cells, with coincident IRF-3 phosphorylation, it is unlikely that the changes in IRF-3 phosphorylation and subsequent ICP0 inhibition seen in HEL cells can be attributed

solely to the actions of DNAPK. DNAPK activity in HEL cells may only account for one isoform of the phosphorylated IRF-3 seen in d109 infection.

As ICP0 has been found to degrade DNA-PK, it could be hypothesized that ICP0 inhibits interferon-stimulated gene expression by promoting the degradation of activated IRF-3. However, simple IRF-3 degradation as the mechanism for ISG inhibition does not account for all of the data we present. The cell-mediated degradation of activated IRF-3 is a slow process – in a study by Karpova et al. MO59 J (DNA-PK⁻) cell nuclei show considerable activated IRF-3 present at 6 hours post infection with some remaining even at 12 hours (120). In contrast, at 12 hours post d106 infection, activated IRF-3 is not present in the nucleus of HEL cells. In the same study, western blot of MO59 J cells detected no IRF-3 by 15 hours post infection; our two-dimensional gel analysis of MO59J cells was performed on extracts from 18 hours post infection and d106- and d109- infection showed similar amounts of IRF-3. Additionally, the cell-mediated proteasome-dependent degradation of IRF-3 is hypothesized to only ensue following IRF-3 *hyperphosphorylation* (141), which is not seen in d106- or d109- infection. Regardless, were IRF-3 phosphorylation not inhibited by ICP0, then the robust nuclear translocation observed in d109-infection would be expected at early time points in d106-infection, and would be followed by a precipitous decline in total IRF-3 levels as is seen during IRF-3 activation by other viruses (120, 141). This is not the case in our studies. Differences in overall IRF-3 protein levels in d106 versus d109 infections are not detectable, thus the phosphorylation observed in d106 infection at early time points must represent a small fraction of the total IRF-3 or the lack of DNA-PK mediated phosphorylation does not result in cell-mediated degradation.

The activation of IRF-3 by viral infection is a very complex process: activation by dsRNA occurs through TLR3, but can be independent of TLR3 during viral infection; activation is required for interferon production in some cell types, but many times does not result in the induction of interferon; activation is accomplished through at least 2 and perhaps 4 different kinases, but phosphorylation on only one serine residue is required for ISG induction. While inhibition of IRF-3 activation during HSV-1 infection is proteasome-dependent, neither IRF-3 nor its serine/threonine kinase TBK-1 are degraded during infection in the presence of ICP0 and the IRF-3 phosphorylating kinase whose degradation is mediated by ICP0, DNA-PK, does not appear to be required for ISG induction. However, the continuing elusiveness of ICP0's inhibitory mechanism does not detract from the clarity of its actions – ICP0 prevents lasting IRF-3 activation and thus inhibits the induction of an antiviral response.

5.0 EXPRESSION OF HERPES SIMPLEX VIRUS ICP0 INHIBITS THE INDUCTION OF INTERFERON-STIMULATED GENES BY VIRAL INFECTION

5.1 ABSTRACT

The herpes simplex virus type 1 (HSV-1) mutant *d109* does not express any of the immediate-early (IE) proteins, and persists in cells for a prolonged length of time. Previously we showed that mutants defective for sets of IE genes established that the lack of ICP0 expression was necessary for high levels of interferon-stimulated gene expression in HEL cells (60). We have shown that *d109*, but not a similar virus expressing the IE gene ICP0, *d106*, induces activation of IRF-3. As has been shown by Nicholl et al. (169) and Mossman et al. (163) using other mutants defective for IE gene expression, infection with *d109* induced the expression of a number of interferon-stimulated genes. Induction of these genes was significantly greater at multiplicities of infection (MOI) of 10 PFU/cell or greater, and the resulting antiviral effect was only seen at MOIs greater than 10 PFU/cell. The induction of interferon-stimulated genes by *d109* could also be inhibited by infection with an E1-:E3-:E4- adenovirus expressing levels of ICP0 that are comparable to those expressed within the first hour of wild-type virus infection. The addition of the proteasome inhibitor MG132 to cells infected with a mutant that expresses ICP0, *d106*, also resulted in the induction of interferon-stimulated

genes. Lastly, a temperature-sensitive mutant of HSV-1, tsB7, (13) that fails to eject its DNA into the nucleus at the non-permissive temperature induced interferon-stimulated gene expression. Thus, ICP0 may function through the proteasome very early in HSV infection to inhibit a cellular antiviral response induced by the virion, but not DNA replication.

5.2 INTRODUCTION

Gene expression during lytic infection with herpes simplex virus type 1 (HSV-1) progresses in a regulated cascade, beginning with the induction of immediate-early (IE) genes ICP0, ICP4, ICP22, ICP27, and ICP47 by the virion protein VP16 (14, 29, 108, 109). Expression continues with early gene transcription, followed by DNA replication and subsequently the expression of late genes (108, 109). The five IE proteins, except ICP47, act as the principal regulators for the efficient and coordinated expression of early and late genes. ICP4- and ICP27- mutants have demonstrated the necessity of these proteins for virus replication (52, 154, 200). ICP0- mutants show impaired growth and poor reactivation from latency (27, 39, 88, 201). The mutant *d*109, deficient in all IE gene expression, does not express any viral proteins and is nontoxic to cells at very high multiplicities of infection (MOI) (202).

HSV-1 mutants deficient in IE gene expression have been shown to induce the expression of interferon-stimulated genes (163, 169). Interferons are proteins or glycoproteins secreted by various cells in response to viral infection or other stimuli. They have antiviral, cell regulatory, and immunomodulatory functions. In many

instances, interferon can make cells resistant to infection (160, 164, 205, 212, 223, 227). The cellular response to interferon, i.e., the induction of interferon-stimulated genes, often produces inhibitory antiviral effects at different stages of viral replication: entry and uncoating (simian virus 40), transcription (vesicular stomatitis virus), RNA stability (picornaviruses), initiation of translation (adenovirus), maturation, and assembly and release (retrovirus) (reviewed in references (205, 212, 223)).

Some interferon-stimulated genes can be induced by substances other than interferon, including heavy metals, lipopolysaccharide, glucocorticoids, interleukin-1, poly(rI)-poly(rC), double-stranded viral RNA, and absorption of virus to the cell surface (23, 81, 118, 119, 166, 240, 241). Genes induced by alpha/beta interferon or gamma interferon are often stimulated by viral infection and vice versa (50, 53, 239). Interferon, double-stranded RNA and viral infection induce different sets of interferon-stimulated genes by using distinct signaling pathways (11, 78, 92, 239, 242). The inducible genes have a similar interferon-stimulated response element, but each interferon-stimulated response element contains unique sequence, which could allow differential responses depending on the signaling molecule induced (45, 139, 240, 242).

Interferon-stimulated gene expression has also been detected in cells infected with wild-type HSV-1 when uv-irradiated or treated with cycloheximide, but little to no interferon-stimulated gene induction was detected during wild-type virus infection (41, 169, 188). It has also been shown that virions must enter cells to induce the expression of interferon-stimulated genes (163). In microarray experiments performed by our lab, mRNAs of 33 genes were increased in abundance during d109 infection (Table 1) (60). These are similar to the genes reported by Mossman et al. ((163)) and Nicholl et al.

(169), and they represent some of the interferon-stimulated genes. Together, the existing data support the idea that some component of the virion or an event that occurs before viral gene expression results in the induction of interferon-stimulated genes through interferon regulatory factor 3 (IRF-3) (41, 188) and that wild-type virus must therefore express a gene product(s) that inhibits this response.

Many different viruses have evolved mechanisms to escape the antiviral response (reviewed in references (205, 212, 223)). HSV-1 has been shown to be very resistant to the cellular interferon response (144, 168). Both the US11 and the $\gamma_134.5$ protein of HSV-1 stops the shutoff of protein synthesis by protein kinase R (33, 89, 97, 184). However, treatment of some cell types with interferon before infection with HSV-1 has been shown to decrease the expression of IE genes in a dose-dependent manner (8, 131, 144, 160, 172, 173, 227). It has also been shown that *d120*, which is defective for ICP4 and is restricted to the expression of IE genes (52), does not induce expression of the interferon-stimulated genes, suggesting that one of the other IE proteins may function to inhibit this response (163). Interestingly, in the same study (163), no mutant with a single mutation in any of the five IE genes induced the interferon-stimulated gene ISG54. However, previous studies have shown that pre-treatment of cells with interferon severely reduces the plaque-forming ability of ICP0 mutants (164).

Previously, we found that cellular gene expression during infection by the ICP0-expressing mutant *d106* is perturbed to a far greater degree than in *d109*-infected cells (60, 103, 104). However, the interferon-stimulated genes induced by *d109* are not significantly induced by *d106* (Table 1), suggesting that the expression of ICP0 greatly

reduced or eliminated the induction of interferon-stimulated genes (60). Microarrays of cells infected with the IE mutants d120-, d107-, and -d104- (characterized in (202) showed further evidence supporting the hypothesis that the inhibition of IRF-3 activation by ICP0 results in the failure of interferon-stimulated gene expression (60). The induction of these genes is evident for the viruses that do not express ICP0 (d104 and d109) and is low from the viruses that do express ICP0 (d120, d107, and d106) (Figure 3) (60).

In this study, we show that infection by the IE-deficient virus *d109*, which possesses functional VP16 in the virion and activates IRF-3, also results in the induction of interferon-stimulated genes and an antiviral state. As ICP0 gene expression has been shown to inhibit the activation of interferon-regulatory factor-3, which is required for the induction of an antiviral state in non-productive HSV-1 infection (41), we used an ICP0 expressing mutant defective in all other IE genes along with an ICP0-expressing adenovirus to examine the consequences of ICP0-mediated inhibition of IRF-3 on cellular gene expression. It was established that the expression of ICP0 could inhibit the induction of interferon-stimulated genes and that the activity of the proteasome was involved. Further, the induction of interferon-stimulated genes in non-productive infection did not require the presence of the viral genome in the nucleus. The results support a hypothesis that the interferon-stimulated gene expression and antiviral responses induced by the virion are inhibited very early in infection by an activity of ICP0.

5.3 MATERIALS AND METHODS

5.3.1 Cells and Viruses

Human embryonic lung (HEL) fibroblasts and Vero cells were from American Type Culture Collection (CCL-137). Monolayer cultures were maintained by standard cell culture procedures in Dulbecco's modified Eagle's medium with 10% fetal bovine serum as previously described (194). The wild-type HSV-1 strains used for all experiments is KOS, except where indicated wild-type strain HFEM was used.

Earlier reports recount the construction and description of viruses *d*106, and *d*109 (95, 96, 192) all are derived from HSV-1 (KOS). All viruses were grown and titered on Vero-derived cells stably transfected with *trans*-complementing HSV-1 IE genes as described previously (193, 194). Adenovirus mutants AdS.10 (E1-:E3-) AdS.11D (E1-:E3-:E4-), and AdS.11E4(ICP0) (E1-:E3-:E4-:ICP0) were constructed at GenVec Inc., Gaithersburg, MD. (96). The construction and characterization of the temperature-sensitive mutant of the wild-type HFEM HSV strain, tsB7, provided to us by Preshant Desai, has been previously described (13).

5.3.2 Microarray analysis

For all of the following, confluent monolayers of HEL cells were infected with the indicated MOI of the HSV mutants. All AdS.10, AdS.11D, and AdS.11E4(ICP0) infections were carried out at 1,000 particles per cell. For comparisons to uninfected cells, HEL cells were simultaneously mock infected and incubated for the same time as

the infected cell sample. Total RNA was isolated at the indicated time postinfection using Ultraspec RNA reagent (Biotex) as per the manufacturer's protocols.

For experiments using arrays constructed in this study, RNA samples from the two conditions to be compared on each array were differentially labeled with indocarbocyanine (Cy3) and indodicarbocyanine (Cy5). In comparisons involving infected and uninfected cell RNA, mock-derived cDNA was labeled with Cy5, while infected-cell-derived cDNA was labeled with Cy3.

Two methods of labeling were employed. In one of the methods, total RNA from the two samples in a given comparison was reverse transcribed and subsequently differentially labeled following hybridization to the arrays with Cy3 and Cy5 using a Tyramide signal amplification kit (Perkin Elmer) following the manufacturer's protocol. Alternatively, Cy3 and Cy5 (Amersham) were also directly incorporated into cDNA reverse transcribed from poly(A)⁺RNA of the two samples in a comparison prior to hybridization, as previously described (211), with minor modifications. Three micrograms of poly(A)⁺ mRNA was reverse transcribed into Cy3- or Cy5-labeled cDNA using an oligo(dT)₁₂₋₁₈ (Amersham) as the primer. After labeling, the probes were passed over a Centrisep column (Princeton Separations) to remove unincorporated nucleotides. For both methods, an *Arabidopsis thaliana* mRNA mix (Stratagene) was spiked into the labeling reaction in order to later normalize the data. The two methods yielded similar results.

cDNA probes were resuspended in ultrapure H₂O (Gibco-BRL). The cDNA probe preparations were denatured prior to hybridization at 65°C for 2 min and then chilled on ice. The probes were suspended in 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M

sodium citrate) and 0.2% sodium dodecyl sulfate (SDS) along with 0.5 μg of pdA(12-18) (Amersham) and 0.5 μg of tRNA per μl . They were then dispensed onto a 20-mm array containing target sequences generated as described below and covered with a 22-mm by 22-mm hydrophobic cover slip (Grace Biolabs). Slides were hybridized in a hybridization cassette (Telechem) at 62°C overnight. Twenty-five microliters of 5x SSC and 0.2% SDS was added to both wells in the hybridization cassette to maintain humidity. The posthybridization washes were done at room temperature as follows: 1x SSC and 0.2% SDS for 5 min, two washes of 0.1X SSC and 0.2% SDS for 5 min each, followed by two washes in 0.1 X SSC for 30 s each.

Following hybridization and washing, the slides were scanned by an Affymetrix 418 array scanner and quantified using Imagene 4.1 (Biodiscovery). Normalization and analysis were done using Genesight 2.0 (Biodiscovery) and Excel spreadsheets. Prior to inserting gene identifiers into the data, the raw data was processed in the following manner: irregular spots or spots with high background were eliminated, local backgrounds were determined and subtracted from the total signals, a lower limit for signal was established, replicate spots were averaged, the Cy3 and Cy5 signals were normalized using the spike controls, and differential expression ratios were established.

5.3.3 Microarray construction

All clones were obtained from a Resgen sequence-validated human cDNA library (Research Genetics). Each clone to be incorporated on the array was amplified in a 96-well plate by adding 5 μl of the corresponding transformed *Escherichia coli* to a 100- μl PCR mixture along with 7.5 U of Yield Ace DNA polymerase (Stratagene), 1 μM each of

the universal forward and reverse primers, and 300 μ M each nucleotide. The universal forward and reverse primers were: forward, 5'-CTGCAAGGCGATTAAGTTGGGTAAC, and reverse, 5'-GTGAGCGGATAACAATTTACACAGGAAACAGC. Primers were synthesized with a C6 amino modifier (Glen Research) for covalent attachment to the slides.

PCR was cycled as follows: 2 min at 92°C, followed by 10 cycles of 20 s at 95°C, 58°C for 20 s, and 72°C for 2.5 min. The 10 cycles were followed by 20 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 2.5 min (extension time increases by 10 s each cycle). A final extension of 7 min was done at 72°C. PCR products were purified using a 96-well purification kit (Telechem). The DNA concentrations of the purified amplified products were quantified using Picogreen (Molecular Probes) and determined to be in the range of 150 to 250 ng/ μ l. All PCR products were also electrophoresed on 0.8% agarose gels to examine whether the amplified fragment length corresponds to the insert size as given in the Resgen library for each clone.

Arrays were printed on Superaldehyde slides (Telechem) using a Affymetrix 417 arrayer. Targets were printed in 0.5x microspotting solution (Telechem) in replicates of 5 or 10 with spot spacing of 375 μ m and spot diameter of approximately 175 μ m. For quality control and normalization purposes, viral DNA, green fluorescent protein (GFP), and Spot Report 10 (Stratagene) were printed in addition to the genes of interest. Slides were processed as described (211). In addition, the slides were UV cross-linked in a Stratalinker (Stratagene) before processing.

5.3.4 Northern blot analysis

For all infections confluent monolayers of HEL cells were infected with the indicated MOI of the HSV mutants. For comparisons to uninfected cells, HEL cells were simultaneously mock infected and incubated for the same time as the infected cell sample. Total RNA was isolated as described above and 10 μ g of each sample was resolved by denaturing formaldehyde-agarose gel electrophoresis, transferred to nitrocellulose membranes, and probed as previously described (111). Probe fragments were generated by PCR amplification of the appropriate human cDNA clone obtained from Incyte Genomics, Inc., or Research Genetics, Inc. ³²P-labeled probes were generated from purified PCR fragments by nick translation using [α -³²P]dCTP and [α -³²P]dGTP.

5.3.5 Western blot analysis

Total protein was isolated as described previously (202). Proteins were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes, and Western blot analysis was performed as previously described (202). Detection reagent used was the ECL Plus kit (Amersham Pharmaceuticals).

5.4 RESULTS

The virus *d109* has mutations in all five IE genes, and no viral gene expression is detectable following infection of cells in culture (202). In previous studies using small-filter arrays to assess the effects of *d109* infection on cellular gene expression, no significant changes were detectable (103). In contrast, the virus *d106* does not express ICP4, ICP27, ICP22, or ICP47 but over-expresses ICP0 relative to wild-type virus. This virus has been shown to significantly affect cell survival and the abundance of many cellular transcripts, as determined by expression array analysis (103, 104, 202).

5.4.1 Expression of interferon-stimulated genes as a function of ICP0

In order to practically conduct more detailed analysis of the induction of interferon-stimulated genes by HSV than that performed using Incyte Genomics arrays (Table 1 & Figure 3) (60), specialized expression arrays were constructed that contained interferon-stimulated genes. A number of interferon-stimulated genes as well as genes that were found not to change with infection on the Incyte Genomics arrays were printed 10 times each on glass slides along with targets for internal spike controls. RNA from HEL cells infected with *d109* at an MOI of 30, which corresponds to approximately 300 particles/cell (data not shown), was analyzed using these arrays, and *d109* was found to induce the expression of interferon-stimulated genes (Table 2). The resulting pattern of induction on the custom arrays was similar to that seen with the Incyte Genomics arrays despite the fact that different clones representing these genes were used in the construction of the two arrays.

Table 2. Cellular genes induced by high moi infection with d109

GeneName	Acc. Num.	d109/mock
interferon-induced protein 56	NM_001548	93.5
interferon-induced protein 54	M14660	36.5
myxovirus (influenza) resistance 1, homolog of murine (interferon-inducible protein p78)	AA477235	32.4
small inducible cytokine subfamily B (Cys-X-Cys), member 10	NM_001565	17.1
activating transcription factor 3	N39944	14.2
phorbol-12-myristate-13-acetate-induced protein 1	D90070	9.5
2'-5'-oligoadenylate synthetase 2	M87434	8.6
2',5'-oligoadenylate synthetase 1	AA732702	8.2
putative transmembrane protein	NM_012342	7.8
interferon, gamma-inducible protein 16	M63838	7.4
proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional protease 2)	AI923532	7.4
stimulated trans-acting factor (50 kDa)	AW407653	7.3
signal transducer and activator of transcription 1, 91kD	NM_007315	7.3
myxovirus (influenza) resistance 2, homolog of murine	M30818	7.3
interferon induced transmembrane protein 1 (9-27)	J04164	7.1
interferon-induced protein 75, 52kD {Incyte PD:769541}	L22343	6.7
interferon-induced protein with tetratricopeptide repeats 4	AF083470	6.5
guanylate binding protein 1, interferon-inducible, 67kD	M55542	5.9
Sjogren syndrome antigen A1 (52kD, ribonucleoprotein autoantigen SS-A/Ro)	M62800	5.5
interferon-induced protein 35	U72882	5.5
ATP-binding cassette, sub-family B (MDR/TAP), member 2	L21208	5.3
superoxide dismutase 2, mitochondrial	Y00472	5.2
ESTs	AI826495	5.1
caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase)	X65019	4.9
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 5	NM_003601	4.8
ESTs, Weakly similar to DY3.6 [C.elegans]	AW051824	4.7
hypothetical protein, expressed in osteoblast	F12860	4.4
phospholipid scramblase 1	AB006746	4.4
tumor necrosis factor receptor superfamily, member 6	M67454	4.4
natural killer cell group 7 sequence	S69115	4.3
ras homolog gene family, member E	W03441	4.2

guanosine monophosphate reductase	M24470	4.1
apolipoprotein L	Z82215	4.1
protein kinase, interferon-inducible double stranded RNA dependent	M85294	4
retinoid X receptor, gamma	NM_006917	3.8
interferon regulatory factor 2	NM_002199	3.7
interferon stimulated gene (20kD)	X89773	3.6
ring finger protein 15	U90547	3.4
butyrophilin, subfamily 3, member A2	AL021917	3.4
E74-like factor 1 (ets domain transcription factor)	AW503166	3.4
tryptophanyl-tRNA synthetase	X59892	3.3
Homo sapiens clone 25071 and 25177 mRNA sequences	AF131742	3.3
P311 protein	NM_004772	3.3
Homo sapiens cig5 mRNA, partial sequence	AF026941	3.2
downregulated in ovarian cancer 1	U53445	3.2
2,3-bisphosphoglycerate mutase	NM_001724	3.2
spermidine/spermine N1-acetyltransferase	Z14136	3.1
ESTs, Weakly similar to CGI-82 protein [H.sapiens]	AI309334	3.1
interferon, alpha-inducible protein 27	AA302123	3.1
nuclear antigen Sp100	AI918021	3.1
ESTs	AA281719	3.1

To confirm the induction of the most highly expressed interferon-stimulated genes by d109 infection and examine the induction of another gene (the promyelocytic leukemia [PML] gene) not included on the custom microarray, Northern blot analysis was performed (Figure 13). As with the custom array, d109 infection was found to highly induce expression of ISG54, ISG56, ISG15, and myxovirus resistance gene 1 (Mrx1), ISGs all known to be transactivated by IRF-3 (41, 188, 209). Additionally, d109 was also found to induce expression of PML. Infection with d106 did not significantly induce expression of any of these genes by Northern blot analysis.

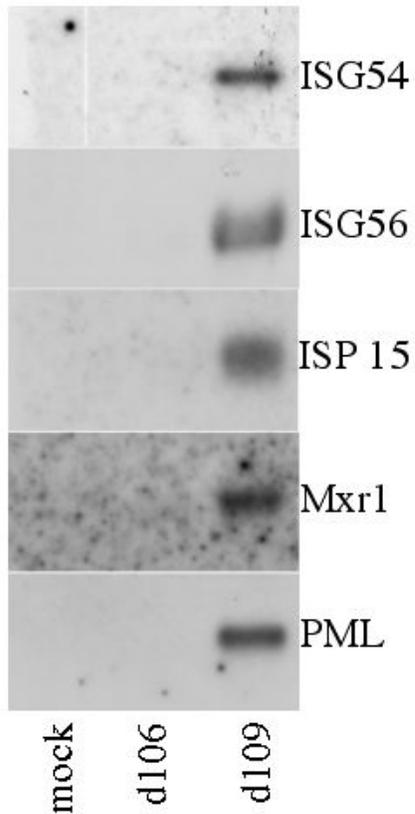


Figure 13. Infection with d109 but not d106 induces expression of interferon-stimulated genes.

Total RNA was isolated from mock-, d106- (ICP0), and d109- infected cells at 24 hours post infection as described in Materials and Methods. RNA was resolved by formaldehyde gel electrophoresis and Northern blot analysis was performed probing for the indicated genes as described in Materials and Methods.

5.4.2 Induction of interferon-stimulated gene expression and antiviral effect is a function of MOI

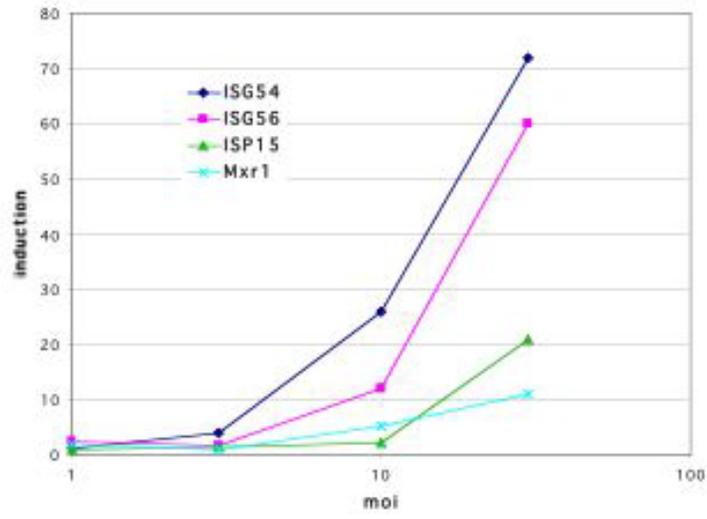
From the previous experiments, it was clear that d109 induced the interferon-stimulated genes at high MOI. Previous studies documented the induction of ISG56, ISG54, myxovirus resistance gene 1, and ISG15 by IE-deficient viruses defective in VP16 activation function (163, 169). However, both used viruses at an MOI of 5, which was insufficient to induce a response measurable by our array experiments. Therefore, we examined the induction of interferon-stimulated genes by d109 as a function of MOI by microarray analysis.

An increase in the abundance of the most highly induced interferon-stimulated genes became detectable at an MOI of 10 and more pronounced at an MOI of 30 (Figure 14A). This analysis failed to detect reproducible differential expression of most interferon-stimulated genes during d109 infection at a lower MOI. Because small changes are often difficult to observe using microarray analysis, Northern blot analysis of HEL cells infected with d109 at MOI of 2.5, 5, 7.5, 10, and 30 was performed using a probe for the most highly expressed interferon-stimulated gene, ISG54 (Figure 14B). d109 induced low levels of ISG54 at an MOI of 5 and barely detectable levels at an MOI of 2.5. Similar to the microarray, the induction of ISG 54 increased dramatically with multiplicities of infection greater than 10 PFU/cell. In contrast, the amount of GFP RNA increased significantly only between MOI of 2.5 and 5 and did not increase further with increasing MOI.

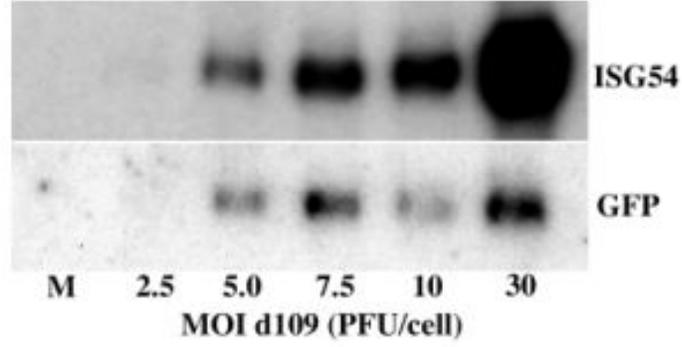
It has been shown previously that the induction of interferon-stimulated genes in cells renders them refractory to wild-type virus plaque-forming ability (163). Induction of

interferon-stimulated genes, however, does not always correlate with an effective antiviral response in cells. Previously, IRF-3 was found to be required for induction of an antiviral response during infection with UV-irradiated HSV (41). Thus, we examined the ability of different multiplicities of the IRF-3 activating mutant d109 to inhibit plaque formation of wild-type virus on HEL cells (Figure 14C). Inhibition of wild-type virus plaque-forming ability, like interferon-stimulated gene induction, increased with MOI. The inhibition of plaque-forming ability was modest at an MOI of 10 but became substantial at an MOI of 30 PFU/cell. There was no antiviral effect at lower MOI. Although it is not clear which interferon-stimulated gene products are responsible for the protective antiviral response, inhibition of plaque-forming ability appears to require a substantial induction of the interferon-stimulated genes by d109.

A.



B.



C.

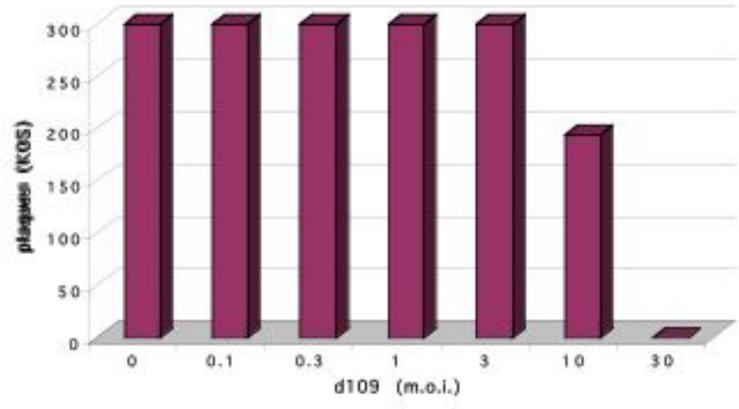


Figure 14. Induction of interferon-stimulated genes and antiviral effect as a function of MOI.

A. Microarray analysis was performed comparing mock-infected cells and cells infected with d109 at the indicated MOI as described in Materials and Methods. Shown are the induction ratios as a function of MOI for the four most highly induced interferon-stimulated genes. B. Abundance of ISG54 and GFP in cells infected with d109 at various MOI was examined by Northern blot analysis. Total RNA was isolated from mock-infected cells and cells infected with the indicated MOI of d109 at 24 hours post infection. C. The antiviral effect of d109 is shown as a function of MOI. Monolayers of HEL cells were infected with the indicated MOI of d109 and 24 hours later were used in a plaque assay with 300 PFU of KOS. Shown are the resulting numbers of plaques that developed 3 days later.

5.4.3 Interferon-stimulated genes are not induced by adenovirus or HSV mutants expressing ICP0

The results of the previous experiment indicate that the expression of ICP0 may preclude the induction of interferon-stimulated genes. However, all of the ICP0-expressing viruses used above over-express ICP0 relative to wild-type virus. We have previously shown that an E1⁻E3⁻E4⁻ adenovirus vector expressing ICP0 from the E4 promoter expresses 0.1% of the amount of ICP0 expressed from d106, is nontoxic to cells, and does not greatly perturb cellular gene expression despite retaining the ability to disrupt ND10 and activate quiescent d109 genomes (104). Therefore, we also examined the ability of this virus to inhibit the d109 induction of the interferon-stimulated genes.

HEL cells were infected with the indicated virus for 24 h and then processed for array analysis. Figure 3 show four comparisons. The first (Figure 15A) compares an E1⁻ E3⁻ adenovirus with an E1⁻ E3⁻ E4⁻ adenovirus. There were very few differences seen in this study. Only STAT1 and α -glucoside were induced in the E1⁻ E3⁻ virus relative to the E1⁻ E3⁻ E4⁻ virus. There were no significant differences seen in the comparison of the E1⁻ E3⁻ E4⁻ virus and the E1⁻ E3⁻ E4⁻ ICP0⁺ virus (Figure 15B).

In the comparison of d106-infected to uninfected cells (Figure 15C), the only gene that was substantially differentially expressed in d106-infected cells was GFP (circled). GFP is expressed from the human cytomegalovirus promoter on the d106 genome. Consistent with the previous data, the set of genes differentially expressed in d109-infected cells corresponded to the interferon-stimulated genes (Figure 15D). The identities of the induced genes and the magnitudes of their induction are listed in Figure 16.

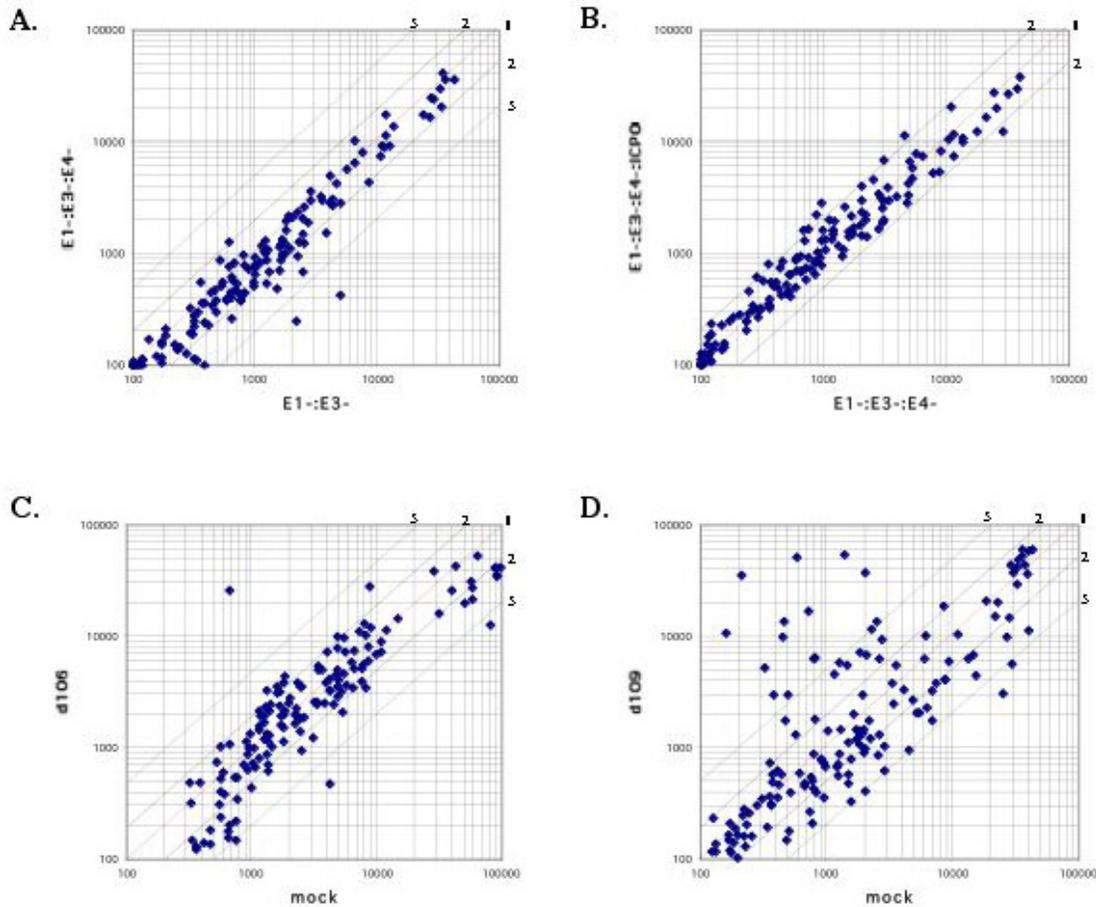


Figure 15. Comparative expression of interferon-stimulated genes by adenovirus and HSV mutants.

The effect of infection by AdS.11D($E1^{-} E3^{-} E4^{-}$), AdS.11E4(ICP0) ($E1^{-} E3^{-} E4^{-} ICP0^{+}$), AdS.10 ($E1^{-} E3^{-}$), d106, and d109 on cellular gene expression was examined by microarray analysis. Total RNA was isolated from infected and mock-infected HEL cells at 24 hours post infection. RNA was labeled and analyzed as described in Material and Methods. Log-log scale scatter plots of fluorescent intensity are shown, with relative fold increase or decrease represented by diagonal lines. Each spot represents a single gene. The circled spot represents the signal for GFP. A. Comparative expression in $E1^{-} E3^{-} E4^{-}$ and $E1^{-} E3^{-}$ adenovirus-infected cells. B. Comparative expression in $E1^{-} E3^{-} E4^{-} ICP0^{+}$ and $E1^{-} E3^{-} E4^{-}$ adenovirus-infected cells. C. Comparison of d106 and mock-infected cells. D. Comparison of d109- and mock- infected cells.

5.4.4 Adenovirus-expresssed ICP0 inhibits induction of interferon-stimulated genes

In a parallel set of infections, HEL cells were first infected with the E1⁻ E3⁻ E4⁻ adenovirus or the E1⁻ E3⁻ E4⁻ ICP0⁺ adenovirus at an MOI of 1,000 particles per cell and incubated for 24 h. The data in Figure 15B demonstrate that no gene on the array is differentially expressed between these two infections. Both sets of adenovirus-infected cells were then infected with d109, and 24 h later RNA was isolated from both cultures, which were used in a single comparison on the array depicted in Figure 16. The abundance of all interferon-stimulated genes induced greater than threefold by d109 in Figure 15D was always greater in the d109-infected sample previously infected with the E1⁻ E3⁻ E4⁻ virus than in that infected with the E1⁻ E3⁻ E4⁻ ICP0⁺ (Figure 16). Conversely, GFP RNA abundance was 500 times greater in the d109-infected sample that was previously infected with the E1⁻ E3⁻ E4⁻ ICP0⁺ virus than in the d109-infected sample that was previously infected with the E1⁻ E3⁻ E4⁻ virus. Therefore, the prior expression of ICP0 from the adenovirus is sufficient to both activate gene expression from the d109 genome and inhibit or reduce the induction of the interferon-stimulated genes. Further, it is sufficient to abrogate the antiviral response induced by d109 infection (data not shown).

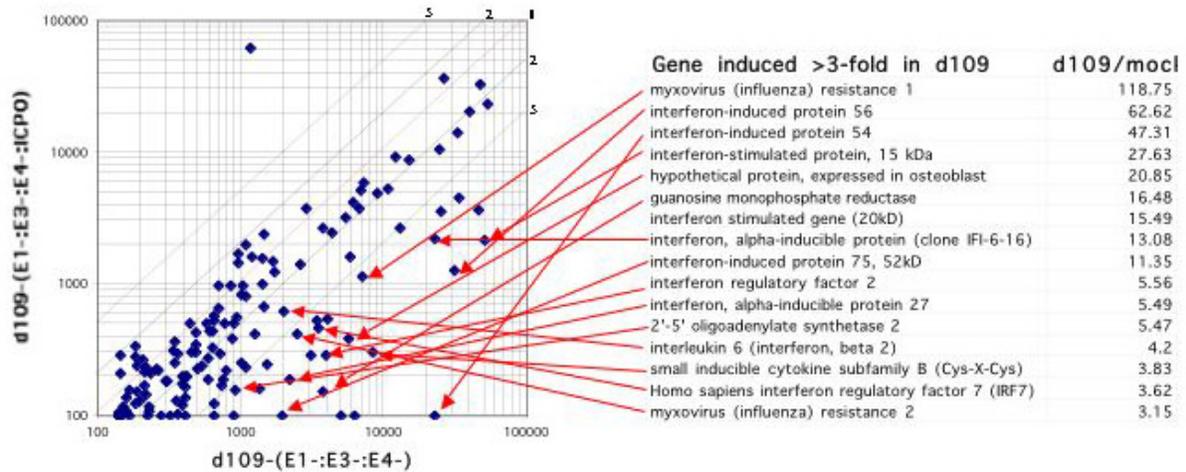


Figure 16. Inhibition of induced interferon-stimulated gene expression by adenovirus-expressed ICP0.

HEL cells were infected with AdS.11D (E1⁻ E3⁻ E4⁻) or AdS.11E4 (ICP0) (E1⁻ E3⁻ E4⁻ ICP0⁺) adenovirus. At 24 hours post infection, the cells were superinfected with d109. Total RNA was isolated 24 hours later, and microarray analysis was performed as in Figure 15. A log-log scale scatter plot shows the relative expression of the interferon-stimulated genes in d109-infected cells that were previously infected with the E1⁻ E3⁻ E4⁻ or E1⁻ E3⁻ E4⁻ ICP0⁺ adenovirus. Each spot represents a single gene. The relative fold increase or decrease is represented by diagonal lines. The circled gene represents GFP expression. The genes induced greater than threefold by d109 relative to mock-infected cells in Figure 15D are listed along with the fold induction ratios. The arrows extend from the indicated genes on this list to their corresponding signals on the scatter plot.

5.4.5 Proteasome-inhibition reverses ICP0 suppression of interferon-stimulated gene expression

During infection, ICP0 may function by targeting specific cellular proteins for degradation through the proteasome-dependent degradation pathway (71, 74). ICP0 itself has been shown to possess ubiquitin ligase activity (22, 236). Many groups have shown that some consequences of ICP0 function can be blocked by addition of the proteasome inhibitor MG132 (71, 72, 146, 179, 180). Further, it has been previously shown by Everett et al. (76) that MG132 inhibits ICP0 nuclear to cytoplasmic translocation due to proteasomal involvement in ICP0 nuclear egress. Earlier we showed that proteasome-inhibition reversed ICP0-mediated effects on the localization of two proteins involved in the interferon-stimulated gene induction pathway TBK-1 (Figure 8) and IRF-3 (Figure 12B). It follows that if the proteasome is involved in the inhibition of induction of the interferon-stimulated genes, the addition of MG132 to cells undergoing infection in the presence of ICP0 should result in an increase in the abundance of interferon-stimulated gene products.

Where indicated, HEL cells were treated with 5 μ M MG132 for 30 min. prior to being mock-infected or infected with d106 or d109. MG132 treatment was continued until RNA or protein was harvested at the indicated time postinfection. The addition of MG132 resulted in elevated levels of ISG54 mRNA in d106-infected cells (Figure 17A), despite the presence of ICP0 (Figure 17B). ISG 54 was not induced in the MG132-treated mock-infected cells. As before, d109-infected cells showed induction of ISG54, which was slightly lower in the MG132-treated cells (Figure 17A). MG132 treatment also resulted in slightly decreased GFP expression from the human cytomegalovirus

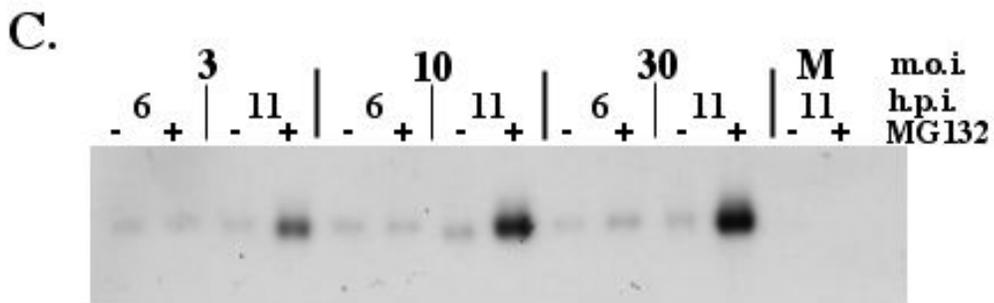
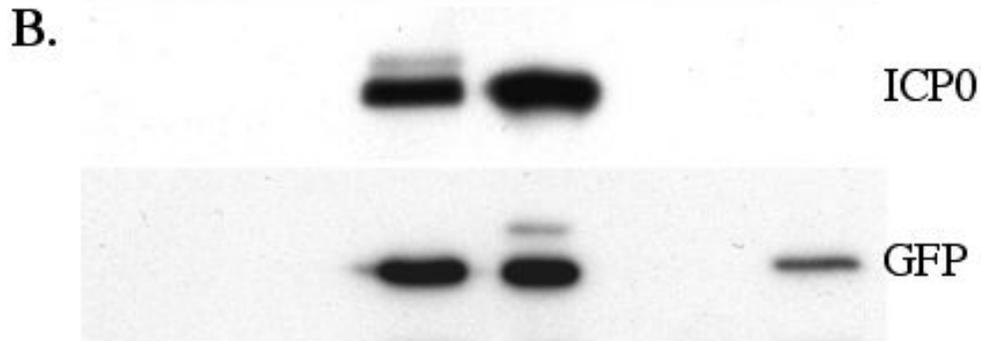
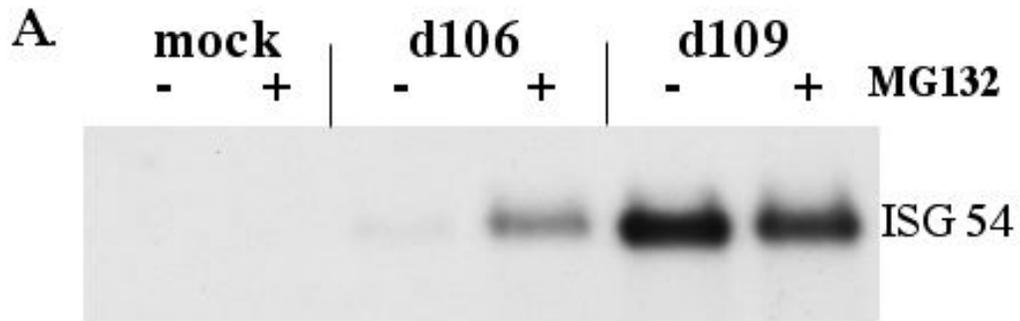
promoter in the d106 genome, but stimulated expression in d109-infected cells (Figure 17B). Interestingly, ICP6 (UL39) expression, which was dramatically lower in MG132-treated d106-infected cells, was also stimulated in d109-infected cells by the addition of MG132 (Figure 17D).

The effect of virus input on the levels of ISG54 accumulation in MG132-treated d106-infected cells was also examined. ISG54 was induced in MG132-treated d106-infected cells as a function of MOI (Figure 17C). Untreated d106-infected cells showed a very low level of ISG54 RNA at both 6 and 11 h. At the 11- but not the 6- h time point, the induction was greatly increased in MG132-treated d106-infected cells. This suggests that the amount of ISG54 RNA at early times postinfection may result from the activation of IRF-3 by TBK-1 that occurs prior to the accumulation of sufficient ICP0 to inhibit further induction.

Lastly, microarray experiments were conducted to obtain a more general view of the effects of MG132 on the levels of interferon-stimulated genes in d109- and d106-infected cells (Figure 17D). RNA from MG132-treated d106-infected cells was compared to RNA from untreated d106-infected cells on the same array. The same type of comparison was conducted for d109- and mock-infected cells. Shown are the data for the interferon-stimulated genes induced fivefold or greater in d109-infected cells relative to uninfected cells. The arrays also contained DNA encoding parts of the genes for GFP and ICP6 (UL39), which are abundantly expressed in d106-infected cells and poorly expressed in d109-infected cells (202). Four genes, including ISG54, were induced by a factor of threefold or greater in MG132-treated d106-infected cells relative to untreated d106-infected cells. MG132 did not affect the expression of these genes in

uninfected cells. Therefore, these gene were induced as a function of infection in the presence of ICP0 under inhibition of the proteasome.

A number of d109-induced genes were not induced in MG132-treated d106-infected cells. Interestingly, the abundance of a number of these RNAs was reduced in MG132-treated d109-infected cells relative to that in untreated d109-infected cells. It is possible that the global effects of inhibiting the proteasome may result in the altered abundance of gene-specific transcription factors that function in the expression of specific interferon-stimulated genes.



D.

Gene_ID	d106+/d106	d109+/d109	d109/mock	mock+/mock
EST, similar to interferon-induced protein 56	9.25	0.53	7.17	1.04
interferon-induced protein 56	5.22	0.61	26.95	0.99
interferon-induced protein 54	4.32	0.62	9.92	0.92
small inducible cytokine subfamily B (Cys-X-Cys), member 10	3.52	0.67	10.99	1.09
guanosine monophosphate reductase	2.14	0.97	6.33	1.44
interferon-induced protein 35	1.33	0.94	13.55	0.81
proteasome (prosome, macropain) subunit, beta type, 9	1.33	2.42	14.19	0.80
myxovirus (influenza) resistance 1, homolog of murine	1.14	0.21	28.06	2.09
interferon-stimulated transcription factor 3, gamma (48kD)	1.03	0.24	8.06	1.53
interferon, alpha-inducible protein (clone IFI-6-16)	0.99	0.74	7.55	1.60
interferon-stimulated protein, 15 kDa	0.95	0.12	373.43	1.16
interleukin 6 (interferon, beta 2)	0.44	1.42	5.33	0.97
viral UL39 ribonucleotide reductase large subunit	0.02	7.52	1.05	0.51
GFP	0.43	20.71	0.74	1.14

Figure 17. Effect of Proteasome-inhibition on interferon-stimulated gene expression in virus-infected cells.

A. Effect of MG132 treatment on abundance of ISG54 in d106- and d109- infected cells was examined by Northern blot analysis. Cells were treated as indicated with 5 μ M MG132 from 30 min. prior to infection until RNA isolation. At 11 hours post infection, total RNA was isolated from mock-infected cells and cells infected with the indicated virus at an MOI of 30. B. Effect of MG132 treatment on ICP0 and GFP protein expression in d106- and d109- infected cells was examined by Western blot analysis. Cells were treated as in A. Total protein was isolated from mock-infected cells and cells infected with the indicated virus at 11 hours post infection. C. Effect of MG132 treatment on the abundance of ISG54 in cells infected with d106 at various MOI. Cells were treated as indicated with 5 μ M MG132 from 30 min. prior to infection until RNA isolation. Total RNA was isolated from mock-infected cells and cells infected with the indicated MOI of d106 at 6 and 11 hours post infection (h.p.i.). Northern blot analysis was performed, probing for ISG54 message. D. Microarray analysis was performed on the RNA from A. as described in Materials and Methods. Shown are the induction ratios for comparisons of MG132-treated and untreated cells that have been either mock, d109, or d106 infected. Also shown is a comparison of d109 (MOI = 30) and mock-infected cells.

5.4.6 Expression of interferon-stimulated genes during non-productive infection does not require HSV nuclear entry

The specific viral component responsible for induction of interferon-stimulated genes by IRF-3 has not been determined. It is known that viral entry is required (163), though viral replication is not needed as demonstrated by the ISG expression induced by UV-inactivated HSV, HSV infection in the presence of cycloheximide, or replication-

incompetent viruses deficient for IE gene expression (60, 163, 169) (Table 1 & Figure 3). However, all of these viruses still mediate viral DNA entry into the nucleus, which the cell may have the ability to detect. Therefore, we sought to determine whether ISG induction requires viral DNA nuclear entry.

The HSV temperature-sensitive mutant tsB7, described and characterized by Batterson et al. (13), expresses all viral gene products, including ICP0, and replicates normally at the permissive temperature 34°C. At the non-permissive temperature 39.5°C, the virus enters the cell normally, but fails to eject its DNA into the nucleus via the nuclear pore complex and thus does not express any viral genes nor undergo replication (13). Thus, we can use this virus to examine the ISG induction pathway in the absence of viral DNA. Total RNA from 24 hour tsB7 infections at 34°C and 39.5°C were compared by northern blot analysis along with RNA from mock-infected cells, d109-, d106-, and wild-type HFEM strain infections (Figure 18). At 34°C d109 infection resulted in the induction of ISG54 expression. At 39.5°C however, both tsB7 and d109 induced ISG54 expression. While a small amount of ISG54 expression was induced in d106 infection at 34°C but not 39.5°C, only at 39.5°C was ISG54 expression detectable in tsB7 infection. At neither temperature was ISG54 induction detectable in the wild-type HFEM strain. While d109 at 39.5°C induced ISG54 to a greater degree than tsB7, the induction in tsB7 is robust enough to conclude that HSV DNA entry into the nucleus is not required for ISG induction.

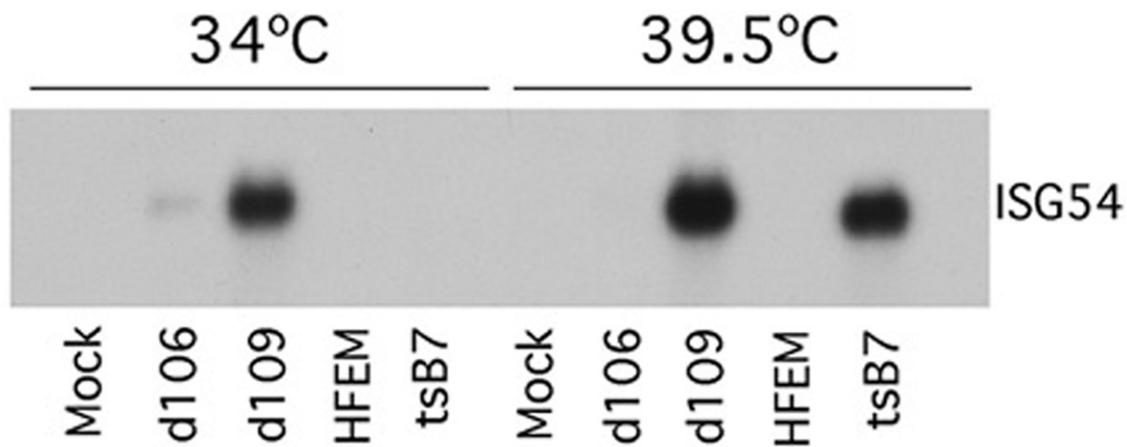


Figure 18. HSV-1 DNA entry into nucleus is not required for ISG induction.

HEL cells were infected with indicated virus at an MOI of 30 and incubated at either 34°C or 39.5°C. Total RNA was isolated at 24 hours post infection and Northern blot analysis was performed using a probe for interferon-stimulated gene 54. At both temperatures, the virus d109 induced ISG54 production while d106, which produces ICP0, did not. tsB7, a temperature-sensitive HSV-1 HFEM strain mutant, did not induce ISG54 expression at the permissive temperature. At the non-permissive temperature, at which tsB7 does not eject its DNA into the nucleus and viral gene expression does not occur, tsB7 induced ISG54 expression.

5.5 DISCUSSION

d109 induced interferon-stimulated genes very similar to other non-IE-expressing mutants, including *in1312*, used by Nicholl et al., and KM110, used by Mossman et al. (163, 169). Interferon-stimulated gene expression increased with increasing MOI, abruptly becoming more abundant when the MOI exceeded 10 PFU/cell. Interestingly,

the plaquing of wild-type virus on d109-infected cells was only significantly inhibited when the MOI of d109 used to establish the antiviral state exceeded 10 PFU/cell. The quantitative differences between this and previous studies (163, 169) may simply be a function of different PFU/particle ratios. Perhaps this cellular response functions to attenuate robust acute infections in vivo and has less influence on low-level and/or latent infections. Alternatively, the cellular response to infection with respect to interferon-stimulated gene induction may differ from cell type to cell type (188).

In previous work, no HSV-1 mutants expressing ICP4 or ICP0 were found to significantly induce the expression of interferon-stimulated genes unless inactivated by UV or treated with cycloheximide (163, 169). From Mossman et al. and previous microarray data from our lab, it is clear that an ICP4-defective virus d120, cannot induce interferon-stimulated genes (60, 163) (Table 1). From our previous array data it was clear that ICP4⁻ mutants expressing ICP0, regardless of other IE genes not expressed, also did not significantly induce interferon-stimulated genes; the only mutant besides d109 found to robustly induce interferon-stimulated genes was d104, which does not express ICP0 but does express ICP22 and ICP47 (Table 1) (60). Consistent with these results, an adenovirus expressing very low levels of ICP0 also inhibited the induction of interferon-stimulated genes by d109. Together, these results suggest that the expression of ICP0 can inhibit the induction of the interferon-stimulated gene by the HSV virion, most likely through the inhibition of TBK-1-mediated phosphorylation of the transactivating protein IRF-3.

Many of ICP0's actions promoting viral replication are mediated through the proteasome, including the degradation of CENP A & C, PML, DNA-PK, and SP-100 (71,

72, 146, 179, 180). However, apart from DNA-PK, none of the proteins in the interferon-stimulated gene induction pathway – including TBK-1, IRF-3, IRF-7, TLR3, and CBP, have been found to be degraded during HSV-1 infection (Figure 4 & Figure 9) and Lin et al. (142). Proteasome-inhibitors have also been found to affect the localization of ICP0 during infection, inhibiting nuclear to cytoplasmic translocation when administered early in infection (76) and causing cytoplasmic to nuclear translocation when administered after DNA synthesis (147). In the present study, proteasome inhibition restored interferon-stimulated gene expression, even in the presence of ICP0. Although ICP0's ability to inhibit the induction of interferon-stimulated genes requires functional proteasomes, the actual responsibility of the proteasome in the ISG inhibition mechanism is still unclear; the proteasome may only be involved in mediating the localization of ICP0 or ICP0 may yet be found to proteasomally degrade a component of the IRF-3 activation pathway.

Previous studies using uv-inactivated wild-type HSV or performing infections in the presence of cycloheximide have shown that DNA replication and viral gene expression are not required for ISG induction (163, 169). Further, the d109 virus used in this study to induce interferon-stimulated genes is not only replication-incompetent but does not express viral genes. However, UV-inactivated HSV-1 has intact DNA, viral DNA is transcribed during cycloheximide treatment, and in some cells d109 spontaneously expresses the GFP gene – thus it can be inferred that DNA entry into the nucleus is intact in these infection conditions. HSV DNA entry into the nucleus is mediated by the UL36 gene product VP 1/2 included in the virion tegument (54). The tsB7 virus has a temperature-sensitive lesion in the coding region of the UL36 gene. Its

phenotype at the non-permissive temperature is the accumulation of DNA-filled capsids at the nuclear pore complex. At the restrictive temperature, the tsB7 virus does not eject its DNA into the cell nucleus; when brought to the permissive temperature, the DNA is immediately ejected and viral gene expression occurs (13). As tsB7 infection at the non-permissive temperature results in the induction of interferon-stimulated genes, viral DNA entry into the nucleus is not the stimulus for ISG induction. The components of the HSV-1 virion and tegument are presumably not affected by any of the conditions that allow interferon-stimulated gene expressions to occur during HSV infection. As viral entry is known to be required for interferon-stimulated gene induction, and now DNA entry into the nucleus is found *not* to be required, it can be hypothesized that the cellular antiviral defense mechanisms are induced by some component(s) of the HSV tegument or virion or an action thereof between virus entry and DNA nuclear entry.

Cellular interferon responses and antiviral pathways vary greatly between cell types and inducers and use multiple signaling pathways. In addition, the actions of many interferon-stimulated genes remain under-characterized, and the counter-acting viral mechanisms are difficult to characterize. Two of the most studied antiviral pathways, involving the interferon-stimulated genes double-stranded RNA-dependent protein kinase and the 2'5'-oligoadenylate synthetase, are both inhibited by HSV-1 (33, 97, 184, 219). It is clear that HSV can also inhibit the induction of interferon-stimulated genes by the virion, and it has been postulated that multiple HSV gene products may be sufficient for this activity (163, 169). Our studies very strongly suggest that one such gene product is ICP0. Therefore, ICP0 expressed very early in infection may

simultaneously stimulate viral gene expression and contribute to the inhibition of interferon-stimulated gene induction, thus promoting lytic infection.

6.0 SUMMARY AND GENERAL DISCUSSION

This study has examined the induction and inhibition of the IRF-3 antiviral pathway during herpes simplex virus-1 infection. Using HSV-1 mutants distinguished genetically only in the expression of one viral gene product, the IE gene ICP0, we examined the activation of the interferon-regulatory factor 3 (IRF-3) pathway and the production of a protective antiviral response as a function of ICP0. These results have implications in understanding the early events in HSV-1 infection that allow the virus to replicate while the cell attempts to initiate an antiviral state.

6.1 IRF-3 ACTIVATION

The activation of IRF-3 by RNA viruses comprises hyperphosphorylation on multiple serine sites by at least 3, perhaps 4 different kinases (79, 120, 209, 215), dimerization, translocation to the nucleus, retention by association with the transactivating factors CBP/p300, and binding on interferon-stimulated regulatory elements (ISRE) of multiple interferon-stimulated genes (ISG) (Figure 2) (102, 141, 213, 242, 243, 250). HSV-1 infection in the absence of gene expression (e.g. UV-inactivated or cycloheximide-treated) has been shown to induce ISG's (163, 169). Further, induction of ISG56 and a

protective antiviral response by UV-inactivated HSV-1 requires IRF-3 (41). While Preston et al. showed binding of IRF-3 to the ISRE of ISG15 by electro-mobility shift assay (188) neither hyperphosphorylation nor nuclear translocation and retention have been observed during HSV-1 infection. Collins et al found that infection with UV-inactivated HSV-1 did not induce detectable IRF-3 nuclear localization, and additional IRF-3 phosphorylation was also not observed (41). Collins et al hypothesize that the induction of ISGs and an antiviral response by HSV-1 infection does not involve additional IRF-3 phosphorylation nor detectable nuclear IRF-3, rather IRF-3 hyperphosphorylation and robust nuclear localization were hallmarks of vigorous RNA viral replication and production of dsRNA (41).

In contrast to the hypothesis put forth by Collins et al., in our study, HSV infection in the absence of viral gene expression, represented by infection with the d109 HSV-1 mutant virus that does not express any viral genes, was found to induce IRF-3 phosphorylation as well as readily detectable IRF-3 nuclear localization. This activation of IRF-3 was functional in that a variety of ISG's were induced by d109, and a protective antiviral state was established in d109-infected cells. From our study, it is clear that nuclear localization of IRF-3 does not require viral replication as d109 is not capable of replication, and d109 only expresses GFP in a small number of cells, so cellular gene expression is most likely not a factor in induction. While it's not clear why UV-inactivated virus failed to induce detectable nuclear IRF-3 (41), the detection of phosphorylated IRF-3 due to d109 infection required two-dimensional electrophoretic analyses.

In both our study and two-dimensional analysis of IRF-3 performed by Sarkar et al (209), constitutive IRF-3 protein exists in four isoforms. Two of the isoforms represent phosphorylation as they disappeared after treatment with alkaline phosphatase (209). d109 infection resulted in the appearance of two additional more positive isoforms, reflecting phosphorylation of the protein. Sarkar et al. found that upon dsRNA treatment in the presence of an inhibitor of phosphatidylinositol-3 kinase (PI3K), only one additional isoform was detected and ISG56 expression was not observed (209). The additional isoform, however, was identified as phosphorylation at the serine 396, which is the only site that has been found to be required for IRF-3 activity (141).

While the Sarkar et al study demonstrated the requirement of PI3K activity for IRF-3 activation and ISG expression by dsRNA, the induction was shown to proceed through the toll-like receptor-3 (TLR3). TLR3 has been shown to be dispensable in induction of IRF-3 by viral infection (61) and the requirement for PI3K in ISG induction varies between viruses (41). As phosphorylation of serine 396 on IRF-3 has been shown to be required for nuclear localization and ISG activation (141), from the IRF-3 translocation data in our study along with the kinetics of ISG expression, it may be possible that the first additional isoform visible in our two-dimensional gel electrophoresis represents IRF-3 phosphorylation at serine 396. As other IRF-3 phosphorylation has been found to be mediated by DNA-PK in the nucleus of the cell (120), we speculate that the last isoform to appear, visible by 18 hours post d109 infection, but not d106 infection, represents DNA-PK phosphorylation. This hypothesis is substantiated by the finding that two-dimensional gel analysis of IRF-3 phosphorylation revealed one additional isoform of IRF-3 in DNA-PK expressing cells

(MO59K) than in the MO59 J (DNA-PK⁻) cells. However, regardless of the identity of the serine residue represented by the 5th isoform of IRF-3, d109 infection clearly results in the phosphorylation of IRF-3 at multiple residues, translocation from the cytoplasm to the nucleus and nuclear retention. From the microarray and Northern blot analysis of d109 infection, the induction of IRF-3 phosphorylation and nuclear retention leads to interferon-stimulated gene (ISG) induction and establishment of a protective antiviral response.

Recent studies have identified proteins upstream of TBK-1 in the IRF-3 activation scheme. TICAM-1 and NAP1 are found to mediate dsRNA-dependent induction of TBK-1 through the toll-like receptor 3 (TLR3), with a required role for PI3K (177, 209, 210, 247). LPS-mediated induction through TLR4 requires the additional protein TICAM-2 (177); as yet, neither PI3K nor NAP1 involvement has been reported. The Rho GTPase RAC1 and its effector p21-activated kinase (PAK1) also activate IRF-3 upstream of TBK-1 (59), and have been implicated in both TLR2- (9) and TLR3- (59, 113) mediated signaling. To respond to RNA viral infection, IRF-3 requires the RNA helicase RIG-1, which acts to detect viral ribonucleoproteins (249). None of these effectors have been shown to be involved in the activation cascade of IRF-3 during DNA virus infection. The induction of IRF-3 by HSV-1 in the absence of gene expression makes TLR3 an unlikely TBK-1 activator as dsRNA is most likely not present. HSV-1 manipulates cellular ribonucleoproteins through ICP27 rather than producing its own, giving RIG-1 nothing to detect. Noyce et al (170) hypothesize that PAK1 is involved in cytoskeleton modulation and may respond to cytoskeletal rearrangements induced by pseudorabies virus and HHV-8. Dominant-negative PAK1 did not preclude ISG

expression induced by an RNA virus (170), however Noyce et al did not report investigating the effect of dominant-negative PAK1 on HSV-1 induced ISG expression.

HSV-1 mutants deficient for viral entry, but able to attach to cells have been shown to be unable to induce ISG expression (163). We used a temperature-sensitive HSV-1 mutant, which at the permissive temperature expresses all viral gene products, including ICP0, replicates, and produces mature virions. At the non-permissive temperature, the mutant cannot eject viral DNA into the nucleus, and thus cannot express viral genes or go through a productive viral life cycle. This mutant demonstrated that nuclear viral DNA was not required for induction of interferon-stimulated genes. Thus, as entry is required – eliminating viral glycoproteins as inducing agents, and viral DNA is not required – eliminating the detection of viral DNA as the inducer, it can be hypothesized that a component of the virion – a virion, tegument, or capsid protein, is responsible for the activation of the IRF-3 pathway in non-productive HSV-1 infection.

Of the tegument proteins, many perturb the intracellular environment upon virion entry. The virion protein 22 (VP22) has been shown to interact with actin microfilaments (63) and reorganize the cytoskeletal network of microtubules (62). It also has been shown to exhibit cell to cell spread in transfected cells (63). A recent study suggested that microtubules serve as transport tracks for toll-like receptor (TLR) vesicles (235); however this study did not examine the ability of any TLR to detect changes in the microtubule network. Another study, however, found that inhibition of microtubules resulted in an impaired response to activation by LPS through the TLR2 receptor (128). While neither study examined the induction of ISG's as a result of microtubule disruption, the possibility of PAK1 detection of VP22 cytoskeletal and microtubule

perturbations is intriguing. Other tegument proteins include two kinases, UL13 and US3; UL13 is known to phosphorylate the cellular translation factor EF-1delta (125) and can mimic the function of the cellular protein kinase cdc2 (also known as cdk1) (123), which can phosphorylate many proteins (105, 148, 175). The functions of many of the other tegument proteins are unknown, but one or more of them may perturb the cell in such a way as to activate an antiviral response.

6.2 IRF-3 INHIBITION BY ICP0

Previously, we showed that the replication incompetent HSV-1 mutant, d106, a mutant deficient in all IE expression but ICP0, did not induce the expression of interferon-stimulated genes (60) (Table 1 & Figure 3). In this study, we demonstrate that ICP0 can induce a change in localization of the IRF-3 activating kinase TBK-1. The mis-localization itself is not alone indicative of inhibition, however, combined with the decrease in IRF-3 phosphorylated isoforms during infection with d106 and the observation that IRF-3 does not accumulate in the nucleus (a function requiring IRF-3 phosphorylation), the mis-localization of TBK-1 takes on a functional significance. Interestingly, we showed that all of the actions of ICP0, as well as the inhibition of interferon-stimulated gene expression, require the function of the proteasome. ICP0 has been shown to have intrinsic E3 ubiquitin ligase function associated with its RING finger domain (22). The requirement of the RING finger domain for ISG inhibition (142) along with the proteasome-dependence of ICP0's inhibition of ISG expression suggest that the mechanism by which ICP0 inhibits the IRF-3 antiviral pathway is to mediate the

degradation of an essential protein of the pathway. Yet neither our studies nor Lin et al detected degradation of either TBK-1 or IRF-3 (142). CBP (the protein essential for nuclear IRF-3 retention) was also not degraded during HSV-1 infection (142). No studies have reported the protein state of the upstream TBK-1 activators TICAM-1 & 2, NAP1, RIG-1, PAK1, or RAC1 in response to infection with an ICP0 expressing virus.

Recently, in the context of RNA viral infection, the association of TBK-1 with the cytoplasmic chaperone/heat shock protein Hsp90 was found to be required for TBK-1-mediated phosphorylation of IRF-3 (248). Yang et al found that Hsp90 formed a stable cytoplasmic complex with TBK-1 *in vitro* and *in vivo*, independent of TBK-1 activation (248). An inhibitor of Hsp90, geldanamycin, was found previously to inhibit dsRNA- or virus-induced phosphorylation of IRF-3 (113). Yang et al found that geldanamycin was sufficient to inhibit the interaction between Hsp90 and TBK-1 and resulted in the proteasome-dependent degradation of TBK-1 (248). Interestingly, Hsp90 is also involved in HSV-1 replication; geldanamycin treatment results in viral DNA polymerase mis-localization and proteasome-mediated degradation, leading Burch and Weller to identify the viral DNA polymerase as a client protein of Hsp90 (25). In this same study, Hsp90 was found to localize to the virus-induced chaperone-enriched (VICE) domains with Hsp70/Hsc70, Hsp40, and components of the proteasome (25). At early times of infection, ICP0 has been identified as essential to the formation of the VICE domains (26). The ICP0-induced formation of VICE domains coupled with the inclusion of Hsp90 in these domains may be the mechanism for the mis-localization of TBK-1 in wild-type and d106-infected cells. While TBK-1 proteasome-degradation does not appear to occur during infection with either HSV-1 virus, the uncoupling of TBK-1 from Hsp90

during wild-type and d106 infection may result in TBK-1 sequestration in the Golgi apparatus or uncharacterized cytoplasmic aggregates, respectively.

TBK-1 localization during wild-type infection differs from that observed during d106 infection. In wild-type infection TBK-1 is distinctly reorganized to the perinuclear region occupied by the Golgi apparatus, however d106 infection only induces cytoplasmic aggregation of TBK-1. Proteasome-inhibition abrogates the perinuclear TBK-1 reorganization and cytoplasmic ICP0 localization in wild-type infection. In d106 infection in the presence of proteasome-inhibition, TBK-1 aggregation still occurs, albeit to a lesser degree, and cytoplasmic ICP0 is slightly detectable. The localization of ICP0 during wild-type infection has been found to be controlled partly by its posttranslational modification by viral and cellular kinases (4), and examination of these factors may explain the discrepancy between d106 ICP0 localization and that in wild-type infection.

Davido et al made mutations of putative phosphorylation sites in three regions of ICP0, two in the transactivation domain downstream of the RING finger domain and the third in the nuclear localization signal (46). Only the third set of mutations resulted in differential ICP0 cellular localization from wild-type infection, these mutations include putative phosphorylation sites for casein kinase II (CKII) and cyclin-dependent kinase (cdk-1) (46). The cellular localization of SV40 T-antigen is regulated by phosphorylation of the nuclear localization signal; phosphorylation by CKII induces nuclear localization (116, 196) while phosphorylation by cdk-1 inhibits transport into the nucleus (115). As the phosphorylation state of ICP0 can determine its ability to translocate from the nucleus, it can be theorized that regulation similar to that of SV40 may be occurring.

The UL13 protein kinase has been found to phosphorylate the same site on EF1-delta as cdk-1 (123, 125). Recently, UL13 was shown to affect the nuclear localization of two HSV-1 proteins, UL34 and UL31, either by direct phosphorylation or indirectly by phosphorylation of Us3 kinase (121). While the localization of both proteins was nuclear regardless of UL13, they were isolated in punctate patterns in the absence of UL13. Our data showed that in the absence of UL13 protein kinase, ICP0 is found exclusively in the nucleus in many cells. While ICP0 translocation to the cytoplasm does occur, it is greatly delayed when compared to wild-type infection. We can theorize that, as with cdk-1 phosphorylation of SV40 T-antigen, phosphorylation of ICP0 at the putative cdk-1 site by UL13 would result in ICP0 translocation to the cytoplasm.

Paradoxically however, the localization of ICP0 also affects its phosphorylation. If ICP0 is not localized to the right subcellular space, it may not be phosphorylated by a kinase confined to that area of the cell (46). The post-translational modification of ICP0 is complex and involves interplay between multiple kinases. The absence of one kinase may prevent ICP0's localization to an area where it is phosphorylated by another kinase. Both the UL13 kinase and the US3 kinase are included in the tegument of wild-type HSV-1. However, the expression of a truncated form of the HSV-1 Us3 kinase occurs in cells infected by a virus lacking the IE gene ICP22 expression (185). The d106 virus is grown in cells complemented for ICP0, ICP4, and ICP27, but not ICP22. Therefore we could expect normal UL13 kinase, but not normal US3 kinase to be present in the d106 tegument. The small amount of cytoplasmic ICP0 observed in d106 infection versus wild-type infection may be a result of failure of ICP0-UL13 co-localization. Hypothetically, If ICP0 is phosphorylated by the Us3 kinase, the decreased

amount of functional Us3 kinase may contribute to the mostly nuclear localization of ICP0 seen in the IE mutant d106. The decreased amount of cytoplasmic ICP0 may explain the incomplete mis-localization of TBK-1 observed during d106 infection.

The involvement and requirements of the proteasome in the subcellular localization of ICP0, especially in the case of replication incompetent viruses, is unclear. Inhibition of the proteasome in wild-type HSV-1 infection prevents ICP0 nuclear to cytoplasmic translocation when administered early in infection (76) and induces cytoplasmic to nuclear translocation when given later in infection (147). Lopez et al hypothesize that the translocation of ICP0 to the cytoplasm is due to an HSV-1 protein expressed late in infection (147). However, the localization of ICP0 during infection with a replication-incompetent virus, d120, deleted for ICP4, was found to be cytoplasmic very early in infection (147). While seemingly contradictory to what we observe during d106 infection, this localization may be due to the over-expression of ICP22 found in d120 infection (52) and its effect on cellular kinases, such as US3. Lopez et al found about 1/3 of cells exhibit cytoplasmic ICP0 even in the presence of a proteasome-inhibitor (147) suggesting post-translational modification of ICP0 is incomplete when early genes are not expressed, allowing some ICP0 to localize independently of the proteasome. The mostly nuclear ICP0 localization in d106 infection may also be due to incomplete phosphorylation of ICP0, and the partial inhibition of ICP0 cytoplasmic localization in the presence of proteasome-inhibitors supports this. During d106 infection, the inability of proteasome-inhibition to completely disrupt the mis-localization of TBK-1 suggests that proteasome-inhibition is more likely affecting ICP0's nuclear egress rather than degrading a mediator of the TBK-1 activation cascade.

From the results presented in chapter 3, we hypothesized that the mis-localization of TBK-1 as a result of cytoplasmic ICP0 would correspond to an inhibition of IRF-3 phosphorylation as a function of ICP0. By 8 hours post d106 infection, we found that IRF-3 phosphorylation was induced; a fifth, more phosphorylated isoform of IRF-3 was detected by two-dimensional electrophoresis of d106-infected cells. Between 8 and 12 hours, the levels of this isoform decline, and by 18 hours post d106 infection, the fifth isoform is no longer detectable. While we hypothesize that the 5th isoform in d109 infection is due to phosphorylation on serine 396 mediated by TBK-1, the ISG expression seen in d106 early in infection suggests that the 5th isoform observed in d106 infection is also representative of serine 396. A sixth isoform is not observed during infection with d106, leading to the conclusion that DNA-PK does not phosphorylate IRF-3 during d106 infection. This suggests that the small amount of early phosphorylated IRF-3 observed in d106 infection may be less stable and is degraded by the proteasome. The early phosphorylation of IRF-3 in d106 correlates with the small amount of expression of interferon-stimulated genes observed at early time points during infection with d106. The absence of phosphorylated/activated IRF-3 later in infection correlates with the absence of interferon-stimulated gene expression seen later during d106 infection.

6.3 HYPOTHETICAL MODEL OF ICP0-MEDIATED INHIBITION OF ISG EXPRESSION

IRF-3 phosphorylation by TBK-1 results in nuclear translocation. Once in the nucleus, IRF-3 is phosphorylated by DNA-PK, which helps stabilize the active IRF-3 and prevent its proteasome-mediated degradation. However, ICP0 mediates the proteasome degradation of DNA-PK. Our model for the inhibition of ISG expression by ICP0 is thus (Figure 19): Upon infection, TBK-1 in association with Hsp90 (236) is activated and begins phosphorylation of IRF-3 (4 hours, Figure 10), followed by IRF-3 nuclear translocation. Upon viral gene expression, ICP0 mediates the degradation of DNA-PK in the nucleus (136, 180), which destabilizes the activated IRF-3 found in the nucleus. Concurrently, a small amount of ICP0 is present in the cytoplasm and begins to affect TBK-1 localization (Figure 5), possibly through the re-direction of the chaperone Hsp90. Very low levels of ICP0 are sufficient to inhibit ISG expression (Figure 16) and may be sufficient to inhibit a portion of TBK-1. As infection progresses, in the cytoplasm ICP0 continues the dissociation of TBK-1 from Hsp90 and directs Hsp90 to the newly formed VICE domains in the nucleus (25, 26). Without TBK-1 mediating further IRF-3 phosphorylation, the small amount of phosphorylated IRF-3, which has been translocating to the nucleus where it cannot undergo DNA-PK phosphorylation, is degraded by the proteasome (12 hours, Figure 10). While we see no change in overall levels of IRF-3 (Figure 9), the induced/degraded amount of IRF-3 may be too small to detect. ISG expression can only be detected very early in infection (Figure 17), because the small amount of activated nuclear IRF-3 is degraded later in infection. Thus, ICP0 inhibits the induction of the IRF-3 antiviral response pathway by targeting

both early activated IRF-3 through the degradation of the stabilizing kinase DNA-PK and preventing further IRF-3 activation through mis-localization of TBK-1.

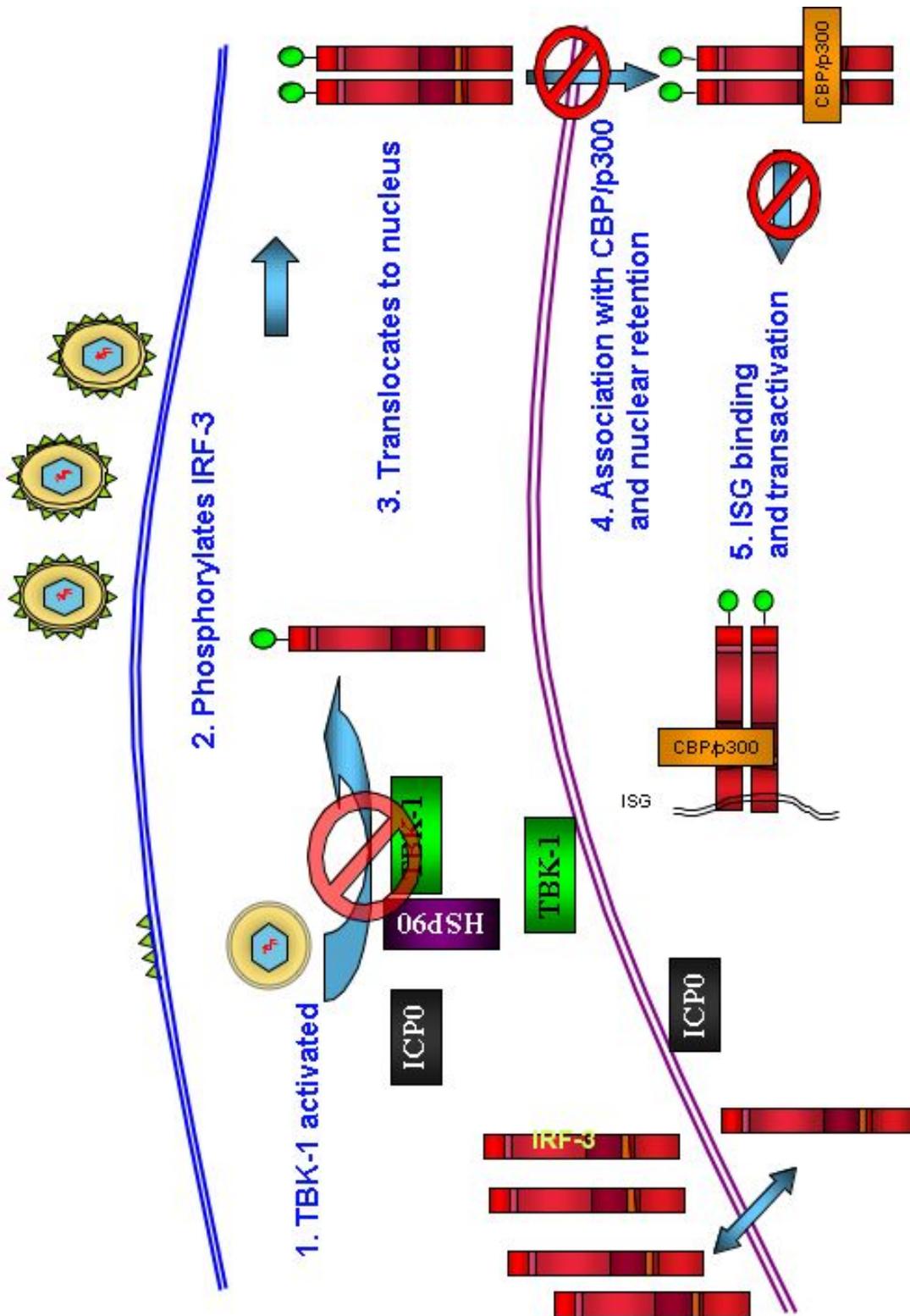


Figure 19. HSV-1 induced activation and ICP0-mediated inhibition of interferon-regulatory factor 3 (IRF-3).

To establish our proposed model, further experiments are required. First, the timing of ICP0's inhibition of the IRF-3 nuclear translocation needs to be examined more fully. Early in infection (<8 hours post infection), IRF-3 is phosphorylated during d106 infection and low levels of iSG are transactivated. However we never examined the changes in IRF-3 nuclear translocation at time points earlier than 12 hours. Through immunofluorescence staining of IRF-3, we would expect to see low levels of nuclear IRF-3 localization at time points earlier than 12 hours. To examine the role of HSP90 in the interaction of TBK-1 and IRF-3 we would first determine the localization of HSP90 as a function of ICP0. We would infect cells with d106, d109, and wt virus and using immunofluorescence we would stain for HSP90 & ICP0. If ICP0 affects the localization of HSP90 during d106 infection the immunofluorescence would give a change in HSP90 localization from diffusely cytoplasmic to nuclear, as is observed in wild-type infection (weller). We would expect this change to coincide with the timing of changes in TBK-1 localization already established during the studies presented here. As the interaction of TBK-1 and HSP90 were shown through immunoprecipitation studies (248) we would also perform similar studies to examine the interaction of HSP90/TBK-1/IRF-3 in HEL cells. Extracts of cells infected with d106, d109, and wild-type virus would be immunoprecipitated with antibodies to HSP90, TBK-1, or IRF-3 and Western blot performed for the two proteins not specifically immunoprecipitated. If TBK-1 and HSP90 interact we would expect the immunoprecipitates from each protein to include the other during mock- and d109-infection. The extracts from d109- infection would be expected to show less co-immunoprecipitation between HSP90 and IRF-3 than mock-infected, as activation of IRF-3 has been seen to result in less interaction with HSP90

(248). TBK-1 or HSP90 immunoprecipitates from d106 and wild-type infections would be expected to show little Western blot staining of HSP90 or TBK-1, respectively, indicating a change in interaction as a result of the presence of ICP0. Further, we would expect that at early time points in wild-type infection (< 4 hours post infection), the results of HSP90 and TBK-1 co-immunoprecipitation would be similar to that observed in wild-type infection, as at this time we observed ICP0 localization to be exclusively nuclear, and TBK-1 to exhibit normal localization. As a final test of TBK-1 and HSP90 interaction in our HSV-1 infection model, we would perform d106, d109, and wild-type infections under treatment with the HSP90 inhibitor geldanamycin. Geldanamycin has been shown to inhibit the interaction of TBK-1 and HSP90 as well as block the activation of IRF-3 (248). Performing immunofluorescence studies examining IRF-3 localization, we would expect to see reduced or abrogated amounts of nuclear IRF-3 during infection with d109 under geldanamycin treatment. Further we would expect to see reduced or abrogated levels of ISG expression during geldanamycin treated d109-infection as examined by Northern blot analysis. As geldanamycin would be expected to inhibit the activation of IRF-3, treatment during d106 and wild-type infection would not be expected to exhibit any difference in IRF-3 localization or ISG expression from untreated d106 and wild-type infection.

Despite needing further studies to completely establish the mechanism of action of ICP0 during infection, we have shown both the induction of the interferon-regulatory factor-3 pathway during HSV-1 infection, and inhibition of the IRF-3 pathway by the IE gene product ICP0. While we have seen ICP0-mediated mis-localization of the IRF-3 activating kinase, TBK-1, and have linked the mis-localization of TBK-1 with a functional

inhibition of IRF-3 phosphorylation by ICP0 at late time points in infection, inhibition of IRF-3 nuclear accumulation, and inhibition of interferon-stimulated gene expression. The mis-localization of TBK-1, the inhibited nuclear accumulation of IRF-3, and the inhibition of interferon-stimulated gene expression are all reversed by proteasome inhibition. We hypothesize that ICP0 does not degrade a mediator of the IRF-3 induction cascade by a proteasome-dependent mechanism, but rather the proteasome helps to mediate the cytoplasmic localization of ICP0 during infection.

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