Regulation of stress associated protein kinase signaling pathways and apoptosis by Herpes Simplex Virus Type 1 infected cell protein 27

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Abstract

Infected cell protein 27 (ICP27) is an immediate early (IE) herpes simplex virus type 1 (HSV-1) regulatory protein. During the course of a HSV-1 infection, ICP27 has been implicated in a variety of functions important for viral replication including host shut-off, viral gene expression, stress activated protein kinase (SAPK) activation, and apoptosis inhibition. However, many of these studies have relied on infections with viral deletion mutants to make these observations, but have failed to separate the direct and indirect effects of ICP27 on viral replication and cellular function.

To examine the direct functions of ICP27, we engineered stable HeLa cell lines that inducibly express ICP27. We showed that ICP27 expression is sufficient to activate p38 signaling to a level similar to that which is observed during wild type HSV-1 infection. Interestingly, ICP27 expression is only able to induce a modest level of JNK signaling, compared to the high levels observed during WT HSV-1 infection. Using chemical inhibitors, we show that the ICP27-mediated activation of p38 signaling triggers apoptosis in the inducible cell lines demonstrating that ICP27 is a pro-apoptotic factor. However, it is likely that additional downstream ICP27-dependent viral factors inhibit apoptosis during HSV-1 infection.

In an effort to better understand the regulation of apoptosis ICP27, we wanted to determine the effects of ICP27 expression on NF-κB activation. During HSV-1 infection ICP27-mediated JNK signaling is required for the activation of NF-κB, which is required for the inhibition of apoptosis. Using our

inducible cell lines, we found that ICP27 expression alone is unable to activate NF-κB, suggesting that at least one additional ICP27-dependent viral factor is required. We demonstrate that the HSV-1 protein UL37 is expressed in an ICP27/JNK signaling-dependent manner and is capable of activating NF-κB. Furthermore, expression of UL37 in an ICP27-independent fashion is able to delay apoptosis induced by infection with an ICP27 null mutant.

Finally, we wanted to characterize the effects of ICP27-mediated p38 signaling on normal cellular function. Using the inducible cell lines, we demonstrate that ICP27 and subsequent p38 signaling are capable of stabilizing the ARE-containing IEX-1 transcript in the absence of infection, by manipulating cellular pathways regulating mRNA decay. During the course of these studies, we also observed that ICP27 expression activates elements of the cellular integrated stress response and the endoplasmic reticulum stress pathways leading to eIF2α phosphorylation and IRE1 activation, respectively. The cellular response to ER stress induced by ICP27 could provide a mechanism for induction of SAPK signaling. These combined results indicate that ICP27 functions as an important regulator of cell fate during HSV-1 infection.

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Chapter 1

Background and Literature Review

Herpesviruses.

Herpesviruses are a large group of pathogens that can infect a diverse array of animals ranging from invertebrate mollusks all the way to humans. They are comprised of double-stranded enveloped viruses with large DNA genomes ranging from 120 to 230 kbp (119). It is important to note that there is a distinct subset of genes that are conserved amongst all herpesviruses that carry out key steps in viral replication. These conserved genes encode proteins that are responsible for regulatory functions, nucleic acid metabolism (e.g., thymidine kinase), DNA synthesis (e.g., DNA polymerase), and protein processing.

Another defining characteristic of *Herpesviridae* is that they cause infections with two distinct phases. Initially, herpesviruses replicate lytically in a broad range of cell types leading to acute disease (119, 129). During lytic infection gene expression is characterized by a tightly regulated temporal cascade of gene expression that occurs in three distinct phases: immediate-early (IE), delayed early (DE), and late (L). After the initial lytic infection, herpesviruses will establish long-term latent infections in specific cell type where the virus remains throughout the life of the host. Latent infection is characterized by minimal expression of a few genes required to maintain latency. Spontaneous reactivation of latent infections can occur, leading to lytic infection and/or virus shedding (reviewed in (129)).

Herpesviruses all have the same basic virion structure ranging in size from 120 to 300 nm. The viral particle is composed of a 100 nm icosahedral capsid that contains the linear viral genome. The capsid is surrounded by a tegument layer, which is an amorphous-appearing region similar to the matrix of RNA viruses (104). The tegument has been shown to have a loosely defined structure containing at least 20 viral proteins. The tegument proteins have a variety of functions that stimulate viral replication and control host cell function prior to viral gene expression. Some key tegument proteins of herpes simplex virus 1 (HSV-1) are virion protein (VP) 16 (pUL48), which is the major transcriptional transactivator of IE genes, and virion host shut-off (vhs) protein (pUL41), which reduces host protein synthesis by nonspecifically degrading RNA (145). The tegument can also contain viral mRNA (148) and cellular proteins, e.g. heat-shock proteins (72). The tegument is surrounded by a host-derived lipid envelope that is studded with viral glycoproteins. These glycoproteins mediate immune evasion, cell attachment, and membrane penetration.

The members of the family *Herpesviridae* are separated into three distinct subfamilies, *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*, based on their site of latency, replication kinetics, and genome sequence (119, 129). Alphaherpesviruses have short life cycles and relatively broad host ranges. Human alphaherpesviruses include herpes simplex type 1 (HSV-1) and type 2 (HSV-2) and Varicella-Zoster virus (VZV), which are classified as (human herpes virus (HHV) 1-3 respectively. Alphaherpesviruses initiate acute infections in epithelial cells where they quickly replicate. Due to their neurotropic nature,

they then establish latent infection in sensory neurons that enervate the location of the primary acute infection. Since alphaherpesviruses replicate quickly, they are used as a model for studying not only herpesvirus replication but also host cell biology as well.

Betaherpesviruses have significantly longer reproductive cycles and more restricted host ranges than alphaherpesviruses. The subfamily *Betaherpesvirinae* contains multiple ubiquitous human pathogens such as human cytomegalovirus (HCVM/HHV-5), HHV-6 (causative agent of exanthem subitum/roseola) and HHV-7 (causative agent of roseola infantum). These viruses are characterized by their ability to form enlarged infected cells known as cytomegalia. Betaherpesviruses establish latent infections in secretory glands, lymphoreticular cells, and the kidneys. While these viruses are all associated with relatively mild childhood disease they can lead to serious complications during reactivation or in immunocompromised patients. HCMV is a major cause of congenital birth defects and graft rejection, while HHV-6/7 can lead to mononucleosis or meningoencephalitis.

Members of the subfamily *Gammaherpesvirinae* have a slow replication cycle like betaherpesviruses with a very restricted host range. Gammaherpesviruses are lymphotropic viruses that have specificity for either B- or T-lymphocytes. Members of this family have been shown to transform lymphocytes *in vitro* and have been associated with oncogenesis *in vivo*. Two major human pathogens, Epstein-Barr virus (EBV/HHV-4) and Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) are gammaherpesviruses. While both

EBV and KSHV most often lead to relatively asymptomatic infections, EBV is associated with Burkitt's lymphoma and Hodgkin's disease, and KSHV is associated with Kaposi's sarcoma.

Herpesviruses can cause a variety of diseases in humans ranging from simple sores on the skin or mucosal surfaces to encephalitis (119). By adulthood the average person in the developed world is infected by six out of the eight human herpesviruses (129). Most herpesviral infections occur as part of normal childhood exposure and result in only minor symptoms, except for chicken pox (VZV). This is due in part to control of the infection by host's immune system as well as to the long-term evolution of herpesviruses, driven by host pathogen interactions, towards the establishment of latent infections. However in individuals with compromised immune systems (e.g., infants, the elderly, HIV infected individuals, and immunosuppressed transplant recipients) either acute or reactivating latent infections can spread, leading to severe disease.

Herpes Simplex Virus Type-1.

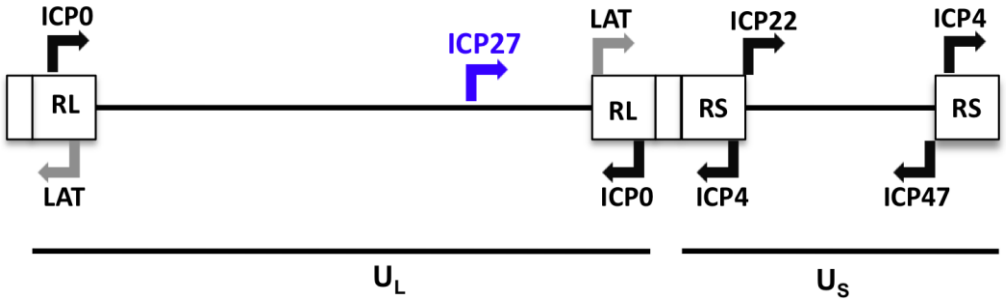
HSV-1 is an alphaherpesvirus that has been extensively studied to gain a better understanding of herpesvirus replication. In addition to being a model for herpesviral infection, HSV-1 has also been a useful tool for studying the regulation of eukaryotic promoters (97), transcriptional activators (133, 171, 172), homologous recombination, and DNA damage repair. HSV-1 has a large 152-kb genome that encodes more than 80 viral proteins. The genome is relatively GC rich (68%). The HSV-1 genome consists of two distinct domains dubbed the

unique long (U_L) and unique short (U_S) regions that are flanked by inverted repeats (Fig. 1.1). Each of these domains includes a unique sequence specific for a subset of viral genes identified as either U_L or U_S respectively. Genes within these regions are largely intronless and many are encoded in overlapping fashion. This can result in messages for multiple viral proteins sharing coding sequences or genes encoded in antisense orientation on complementary strands. The unique arrangement of the genome's terminal repeats allows for both recombination and genome segment inversion to occur during viral replication.

Herpes simplex virus has a long history as a human pathogen with descriptions of herpes-like symptoms dating to some of the earliest human civilizations (129). Today it is a widespread human pathogen with greater than 90% of some adult populations testing as seropositive. Classically, HSV-1 is associated with mild orolabial infections resulting in mild gingivostomatitis or cold sores. Recently, however, HSV-1 has become an emerging cause of genital herpesvirus infections as well. These initial acute infections are easily limited by the immune system. In the majority of cases, following the initial stage of the acute infection, HSV-1 almost invariably establishes latency in the sensory ganglia that enervates the site of the primary infection. The virus then remains latent for extended periods and sporadically reactivates over the life of the host. HSV-1 can lead to more severe infections depending upon the site of primary infection and the immune status of the host. Such more serious infections include keratitis of the eye, encephalitis, and neonatal herpes.

Fig. 1.1. Diagram of the HSV-1 genome. A representation of the HSV-1 genome indicating the approximate locations of the IE genes, the LAT gene, and the major regions of the genome [U_S, U_L, and repeats (R)].

Fig. 1.1



HSV-1 Replication.

The replication cycle of HSV-1 has been extensively studied (reviewed in (129)). HSV-1 infection is initiated by virion attachment to the cell. Binding is mediated by envelope glycoproteins, glycoprotein C (gC) and glycoprotein B (gB), binding to heparan sulfate moieties on the surface of the target host cell. Following initial attachment, the viral glycoprotein D (gD) binds to one of its coreceptors referred to as Hve (for herpesvirus entry). Currently there are three classes of these receptors: a member of the tumor necrosis factor (TNF) receptor family (HveA or herpes virus entry mediator (HVEM)), nectins (HveB and HveC), and a specific form of 3-O-sulfated heparan sulfate (3-OS HS) (26, 81, 106, 151, 177). After gD has bound to its coreceptor, it undergoes a conformational change allowing it to interact with gB and the gH/gL heterodimer (39, 89, 143). This complex of viral glycoproteins then mediates membrane fusion via a pH-independent mechanism.

Membrane fusion allows the tegument proteins and viral capsid to be released directly into the cytoplasm. Some tegument proteins remain in the cytoplasm where they quickly begin to disrupt normal cell functions (e.g., vhs and translation, or US11 and the adaptive immune response). Other tegument proteins (e.g., VP16), along with the capsid, traffic to the nucleus along microtubules. The genome is extruded into the nucleus once the capsid has reached the nuclear pore [(158), reviewed in (129)]. Once inside the nucleus, the HSV-1 genome is believed to circularize. However, based on recent studies by Jackson and DeLuca, genome circularization may not be required for

productive lytic infection (68), but instead may be a key step for the establishment of latency.

Once the genome has reached the nucleus, transcription of viral genes is mediated by host RNA polymerase II (Pol II) (129). A defining feature of HSV-1 infection is a tightly regulated temporal cascade of viral gene transcription starting with immediate early genes (IE), progressing to delayed early genes (DE), and ending with the late genes (L) (129). The expression of the IE genes begins almost immediately upon infection and is independent of viral protein synthesis. IE gene expression is transactivated by tegument protein VP16 binding host transcription factors Octamer transcription factor 1 (Oct-1) and Host Cell Factor 1 (HCF1). The complex of VP16/Oct-1/HCF1 acts as a strong transcriptional activator specific for the TAATGARAT motifs in promoters of IE genes. Once bound to the promoter regions, the complex recruits Pol II and co-activators (59, 129, 182) leading to robust IE gene transcription. Following translation most of the IE gene products are imported into the nucleus where they activate the expression of DE and L genes.

The DE stage starts at approximately 3 hpi and reaches its peak at 5-7 hpi. Genes in this kinetic class require the expression of IE gene products to mediate their expression. DE gene products are primarily responsible for replication of the viral genome, and thus, most DE genes are involved in processes such as nucleotide synthesis (e.g., thymidine kinase (TK)) and DNA replication [e.g., infected cell protein 8 (ICP8)]. Upon translation, these viral

proteins traffic to the nucleus and mediate genome replication and the transition to late gene expression.

L genes have been categorized into two different subsets based on their dependence on DNA replication for expression. True-L genes, e.g., gC, require viral genome replication to be expressed whereas leaky -L genes, e.g., VP5, can be partially expressed in the absence of viral DNA synthesis (129). These are not two clearly defined kinetic classes, but are instead expressed as a continuum. L genes encode the structural proteins required for virion assembly, i.e., capsid proteins, tegument proteins, and glycoproteins.

Virion assembly occurs at distinct sites throughout the cell, which late in infection has been converted into a viral factory. In the nucleus, genome replication occurs through a unique rolling circle mechanism producing long concatemeric strands of viral DNA (reviewed in (129)). These strands are cleaved by a viral nuclease and loaded into preformed capsids (12). Genome-containing capsids egress the nucleus via a process of envelopement/de-envelopement across the nuclear membrane and are released as naked nucleocapsids into the cytoplasm. Once in the cytoplasm, the capsids associate with tegument proteins before being transported to the Golgi. Nucleocapsids associated with tegument proteins then bud into the Golgi compartment at regions of the membrane enriched with viral glycoproteins. This budding process leads to the envelopment of the virion core. Completed virions can then be released by the secretory pathway, cell-to-cell transfer, or virally induced lysis of the infected cell.

As mentioned above, HSV-1 establishes latency in sensory neuronal cells. Many of the early stages of infection, i.e. attachment, penetration, and transport to the nucleus, occur in an identical fashion to lytic infection. The viral genome is maintained in its episomal form inside the nucleus associated with nucleosomes. Unlike lytic infection, however, IE gene expression is repressed leading to an arrest of viral replication. In fact, few if any viral transcripts are produced in latently infected cells. The virally encoded latency-associated transcript (LAT) is the only highly expressed viral RNAs in infected cells, and LAT is closely associated with the establishment of latency (LAT function is reviewed in (14)). The role that LAT plays is controversial and is the focus of an extensive body of research. Interestingly, although the transcript is readily detectable, no LAT protein has ever been definitively identified. It has been suggested that LAT expression actively represses the transcription of viral lytic genes. Two potential models to explain this repression focus on the LAT transcript either limiting ICP0 expression via an antisense mechanism or interacting directly with the viral genome leading to broader repression. The LAT transcript has also been shown to play an important role in viral reactivation from latency. Following productive reactivation, virion particles traffic down axons and spread back to the initial site of acute infection.

Immediate Early Proteins.

As mentioned above, IE gene products are associated with the regulation of viral gene expression. There are five IE proteins: ICP0, ICP4, ICP22, ICP27,

and ICP47. The first four function as transactivators/regulators of viral gene expression. ICP47, however, functions as an inhibitor of the adaptive immune response. The focus of this study is ICP27, which is required for viral replication and activates the expression of a subset of ICP27 dependent genes (116, 174), and will be addressed in detail later.

ICP0 is a 775 amino acid protein that is required for growth at low MOI (multiplicity of infection) and in some cell types (37). It is one of the few intron containing HSV-1 genes. ICP0 is initially localized to the nucleus, but as the infection progresses it gradually moves into the cytoplasm in an ICP27 dependent manner (150, 192-194). ICP0 functions as a promiscuous transcriptional transactivator capable of stimulating all classes (IE, DE, and L genes) of viral genes as well as heterologous promoters (reviewed in (129)). ICP0 has also been shown to be able to dissociate class II histone deacetylases (HDACS) from their target genes, which could be an additional mechanism of ICP0 transactivation (91, 122, 190). Additionally, ICP0 functions as an E3 ubiquitin ligase that is responsible for targeting proteins for proteasomal degradation (175). ICP0 expression has also been closely associated with reactivation from latency (52, 86). The role of ICP0 in reactivation from latency, however, remains contentious as recent findings have indicated ICP0 is not required for reactivation (169).

ICP4 is a 1,298 amino acid protein that has variable localization during viral infection, similar to ICP0 (reviewed in (129)). ICP4 acts as the major transcriptional activator of HSV-1 gene expression (116). Not surprisingly,

mutants lacking the ICP4 gene are unable to replicate in non-complementing cell lines. While the mechanism of ICP4 mediated transactivation is poorly understood, it has two key properties that are required for this function: it binds DNA and interacts with host transcription factors. The strength of ICP4's binding to a particular DNA site appears to play a role in gene expression. It binds tightly to several promoters, including its own, where it functions as a repressor by interacting with sites that overlap transcription initiation sites (reviewed in (129)). Conversely, ICP4 binds to multiple low affinity sites throughout the genome where it transactivates viral gene expression through an unknown mechanism. ICP4 binds to multiple host transcription factors responsible for transcription initiation, e.g., TATA-binding protein and TFIIB. It is assumed that ICP4 is required to recruit these host transcription factors to viral genes and to stimulate transcription.

ICP22 is a 68 kDa IE phosphoprotein that is dispensable for growth in many cell lines. However, ICP22 mutants display significant growth defects and attenuation in mouse models of HSV-1 infection (reviewed in (129)). ICP22 expression is also required for viral growth and the efficient expression of several true-L genes in certain cell lines. ICP22 along with viral UL13 protein kinase have been shown to alter the phosphorylation and levels of RNA Pol II in infected cells (40, 41). ICP22 and UL13 have also been shown to trigger the degradation of cellular cyclins (2). Recently, ICP22 has been linked to the recruitment of topoisomerases to promote L gene expression (3).

Unlike the other four IE genes, ICP47 does not regulate the expression of either viral or cellular genes. It is a small (~9 kDa) immunomodulatory protein that inhibits the adaptive immune response. While the other IE proteins traffic back to the nucleus, ICP47 remains in the cytoplasm after translation. There it binds to the transporter associated with antigen processing (TAP) blocking the translocation of cytoplasmic antigenic peptides into the endoplasmic reticulum (ER). By blocking the TAP transporter, ICP47 inhibits MHC I peptide loading/presentation of viral antigens to cytotoxic T lymphocytes.

ICP27.

Structure and Function.

The final IE protein, ICP27, is the focus of this thesis. ICP27 is a 63 kDa phosphoprotein encoded by the UL54 gene. It is required for HSV-1 replication. Furthermore, all herpesviruses express an ICP27 homologue, underscoring the critical nature of ICP27 and its homologues in herpesvirus replication. ICP27 is a multifunctional viral regulatory protein that has been shown to affect both viral and cellular gene expression. It is required for efficient expression of a subset of DE and L genes (95, 125, 174). Previous research has demonstrated that ICP27 can alter both the transcription of viral genes (69) and the translation of their mRNAs (32). Additionally, ICP27 has been shown to bind RNA (101) and shuttle between the cytoplasm and nucleus (102, 121, 140, 160). These activities are consistent with data that indicate it serves as an mRNA export factor for intronless viral transcripts (22, 23, 79). Furthermore, ICP27 inhibits normal

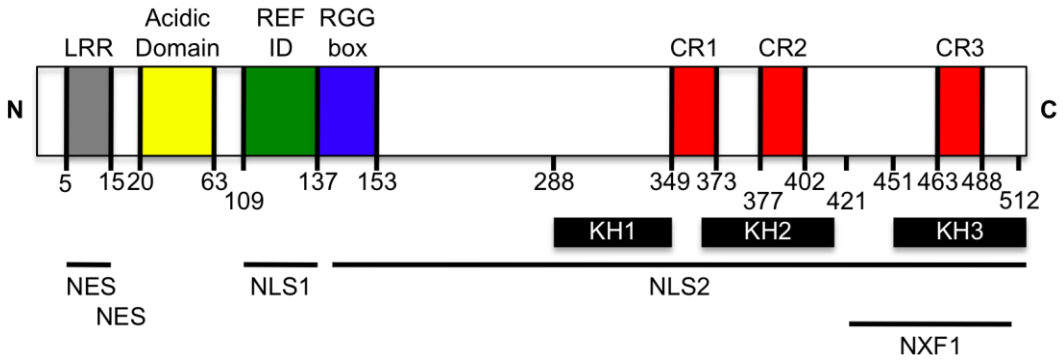
cellular gene expression by inhibiting mRNA splicing as well as the transcription of some host genes (54, 55, 140, 164). ICP27 expression can also lead to increased expression of some cellular mRNAs due to stabilization of transcripts containing AU-rich elements (17, 27).

While the three-dimensional structure of ICP27 is unknown, several key functional domains have been identified throughout the protein (Fig. 1.2). The N-terminal portion of the protein appears to be made up of several unique functional domains. There is a leucine-rich nuclear export signal (NES) (aa 5-17) required for shuttling (136). An acidic domain that spans aas 12-63 has been implicated in the ability of HSV-1 to trigger p38 and JNK signaling (27, 56). The N-terminal portion of the protein also contains a strong nuclear localization signal (NLS) (aa 109-137), while the C-terminal portion has a large weak NLS (100). The N-terminal half has also been ascribed to play a key role in RNA export and shuttling, based on its interaction with the cellular RNA export factor (REF) (aa 138-171) and an RNA interaction domain known as arginine- and glycine-rich RGG box (102).

The C-terminal half of ICP27 consists of a conserved sequence present in all herpesvirus ICP27 homologues. The three highly conserved regions CR1, CR2, and CR3 are the defining feature of ICP27 homologues (reviewed in (96)). The importance of this conserved sequence is underscored by the observation that mutations in the C-terminus of ICP27 have significant impacts in viral replication (95, 125, 128, 161). Large regions of the C- terminal half of ICP27 have been associated with ICP27's ability to activate (amino acids 262-512) or

Fig. 1.2. ICP27 functional regions. Diagram of HSV-1 ICP27 and its functional regions. NES: leucine-rich nuclear export signal; AR: acidic region; REF ID: REF interacting domain; RGG box: arginine/glycine-rich region; CR1, CR2, CR3: conserved regions 1-3; NXF1: NXF1 interacting domain; KH1, KH2, KH3: hnRNP K homology regions; NLS1: strong nuclear localization signal; NLS2: weak nuclear localization signal.

Fig. 1.2


Acidic
REF
RGG
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Domain
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box
CR1
CR2
CR3
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5
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109
137
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373
377
402
421
451
463
488
512
KH1
KH2
KH3
NES
NES
NLS1
NLS2
NXF1

repress (amino acids 413-512) expression of viral genes (99). More recent studies have shown N-terminal regions also are required for the transactivation of viral gene expression (127). The C-terminal portion of ICP27 is also required for its interaction with both itself and a nuclear RNA export factor NXF1 (22). Finally, three KH-like domains have been identified throughout the C-terminal half of ICP27. These KH domains are loosely homologous to cellular hnRNP K RNA binding regions and appear to mediate L gene enhancement and nuclear shuttling though unknown mechanisms

ICP27-mediated regulation of viral gene expression.

Initial studies with temperature sensitive and viral deletion mutants showed that ICP27 was required for lytic infection (132). Mutants lacking ICP27 expressed elevated levels of IE gene products while displaying severely reduced levels of L gene products. ICP27 deletion mutants also display significantly reduced genome replication (95) and are unable to lead to the normal shut-off of translation of cellular gene products (54, 163).

Further studies with temperature sensitive mutants as well as IE deletion mutants have shown that ICP27 is responsible for transactivating the expression of several DE genes including UL5, UL8, UL9, UL30, UL42,and UL52 (125, 157, 174). The DE genes transactivated by ICP27 are required for DNA replication (125, 157, 174). Mutants lacking the DE genes transactivated by ICP27 or ICP27 itself display at least a 5-fold reduction in viral DNA synthesis (174). In addition to stimulating the expression of a subset of DE genes, ICP27 also enhances L

gene expression. The ability of ICP27 to stimulate synthesis of viral DNA, outlined above, plays a key role in stimulating the expression of L genes that have weak promoter elements (95, 132, 174). Portions of both the N- and C-terminus are critical for efficient DNA synthesis (125, 127). However, there is evidence that ICP27 plays an additional role in the activation of L gene expression. Targeted deletion mutants of the C-terminal domain of ICP27 have been shown to significantly impact the expression of L gene expression without significantly affecting DNA replication (125, 126). Furthermore, deletion of amino acids 103-178 from ICP27's amino terminus also significantly impacts the expression of L genes (61). Recent work by our lab has shown that ICP27 can transactivate the expression of L genes by a post-transcriptional mechanism ((120) and Sedlackova, in press).

The mechanisms behind the activation of DE and L gene expression are still poorly characterized. Recent research using ICP27 deletion mutants has shown that ICP27 can have several effects on target gene transcripts including altered patterns of mRNA splicing, enhancement of polyadenylation of pre-mRNA, and stabilization of mRNA normally subject to rapid degradation. As mentioned above, most viral genes are intronless. HSV-1 exploits this during infection by repressing host mRNA splicing, and ICP27 is required for this effect (18, 25, 33, 157). ICP27 expression has also been shown to alter the splicing of the viral gC transcript during infection (149). Additionally, ICP27 has been shown to interact with and alter the localization of several essential members of the cellular splicing complex including SRPK1 (SR protein kinase 1), p32, and

SAP145 (spliceosome-associated protein 145) (18, 25, 33, 63, 146). However, the significance of these interactions on splicing inhibition is currently unknown.

ICP27 has also been shown to stabilize the expression of several unstable mRNAs. Studies in the early 1990's clearly demonstrated that ICP27 was able to stabilize β-interferon mRNA expressed from transfected plasmids (25, 157). This message is normally subject to rapid turnover due to an adenosine-uracil rich element (ARE) in its 3' untranslated region (UTR) that targets the message for degradation. Many rapid response genes, e.g. cytokines, carry these elements to ensure rapid mRNA turnover to allow the cell to quickly respond to environmental cues while tightly controlling the duration of the response. More recently, Hsu et al. showed that another AU-rich cellular transcript IEX-1 is also stabilized during HSV-1 infection (63). A paper by Corcoran et al. showed the stabilization of IEX-1 transcript required the acidic domain of ICP27 as well as p38 MAPK signaling (27). The mechanism behind the stabilization of these mRNAs is unknown but could be due to changes in the recruitment or availability of cellular factors mediating mRNA stability. In addition to altering cellular mRNA stability, ICP27 expression can also stabilize viral mRNA. Work from our lab suggests that ICP27 prevents the rapid degradation of the gC message ((120) and Sedlackova, in press).

ICP27 has also been shown to bind to both cellular and viral mRNAs in the cytoplasm and nucleus (137, 161). The ability of ICP27 to bind intronless mRNA via its RGG box (102, 136) as well as its ability to shuttle between the nucleus and cytoplasm (101, 121, 136, 160) has led to a model wherein ICP27

functions as an mRNA export factor. This model is further strengthened by the observation that ICP27 interacts with two components of the cellular mRNA export pathway, REF and NXF1 (22, 73, 79, 87). While no specific ICP27-binding sites on viral mRNAs have been identified, ICP27 was shown to selectively bind to several viral mRNAs by a yeast-three-hybrid screening assay (159). This same study also verified the importance of the RGG box and to a lesser degree the KH3 domain of ICP27 in this interaction.

In addition to its ability to post-transcriptionally regulate gene expression, ICP27 has also been reported to have effects on transcription. Research by Jean et al. found that ICP27 is required for the transcription of two viral L genes, UL47 and gC (69). As mentioned above, however, findings by Perkins et al. suggest that, at least in the case of gC, ICP27 does not enhance transcription but rather stabilizes gC mRNA (120). Additionally, ICP27's ability to bind to both ICP4 and Pol II have led some to suggest that ICP27 plays a role in transcription (117, 191). In the case of ICP4, the interaction with ICP27 may be more important for proper localization of ICP4 and its incorporation into the virion late in infection (150). Finally, ICP27 localizes to replication compartments that are the sites of L gene transcription in the nuclei (28, 168, 191). However, the significance of this observation is unknown.

ICP27 is also known to stimulate translation. The C-terminal portion of ICP27 has been shown to enhance the translation of reporter constructs *in vivo* (85). The same study found that ICP27 sediments with the polyribosomal fraction of infected cells indicating that ICP27 is associated with active

translation. ICP27 has also been show to stimulate the translation of HSV-1 ICP4 and VP16 proteins (32, 155). Furthermore, ICP27 has been shown to interact with the cellular translation initiation factors poly(A) binding protein (PABP), eukaryotic initiation factor (eIF) 3, and eIF4G (38). However, the significance of these interactions remains unknown.

ICP27 mediated stress associated protein kinase (SAPK) signaling

In addition to transactivating DE and L gene expression, ICP27 has also been linked to the stimulation of SAPK signaling. SAPKs p38 and c-Jun N-terminal kinase (JNK) are mitogen activated protein kinases (MAPK). MAPKs play diverse roles as regulators of normal cellular functions. In addition to p38 and JNK, ERK1, ERK2, and ERK5 belong to this group of serine/threonine kinases. The SAPKs, p38 and JNK, are responsible for controlling cellular responses to environmental stress (e.g., UV irradiation, osmotic shock, and oxidative stress), cellular damage (e.g., DNA and RNA damage), and cytokine signaling (1, 6, 65, 130, 170). Internal and external signaling from these diverse sources leads to the activation of sensor kinases, which trigger signaling cascades. Signaling cascades begin with the phosphorylation and activation of MAPK kinase kinases (MAPKKKs). MAPKKKs then phosphorylate the MAPK kinases (MKKs) that are responsible for phosphorylating MAPKs, which in the case of p38 and JNK are primarily MKK3/MKK6 and MKK4/MKK7, respectively. In addition to this classic kinase cascade of activation, p38 can also be autophosphorylated by interacting with TAB1 (43).

Once activated, p38 and JNK kinases then mediate a variety of cellular responses through stimulation of transcription, post-transcriptional modifications, and translational regulation. Following activation, SAPK signaling has been shown to play fundamental roles in a variety of cell functions, including apoptosis, oncogenesis, cell division, differentiation, immune activation, and inflammation [reviewed in (131) and (170)]. Interestingly ICP27 has been associated with the regulation of several of these functions.

Initial work by Zachos et al. showed that the SAPKs JNK and p38 were activated during HSV-1 infection (185). The importance of SAPK signaling was further underscored by a study that showed small molecule inhibitors of either JNK or p38 lowered viral yield by 85-91% (76, 98). During infection by wild type HSV-1, p38 and JNK signaling are activated at 3 hours post infection and reach maximal levels by 8 hpi. Signaling levels remain elevated until late times post infection falling just before virally mediated cell death. Little is known about the mechanism behind the signaling activation except that it occurs downstream of the master signaling regulator Ras (98). Recently, a study that used multiple IE deletion mutants was able to show that, in the context of a viral infection, ICP27 is required for the activation of both the JNK and p38 signaling pathways (56). The same paper also identified amino acids 12-63 of ICP27 as playing a critical role in stimulating SAPK signaling. In a follow-up study, this same group found that ICP27 mediated JNK signaling is required for the activation of NF-κB during infection (57). While the mutant screening was extensive, it was unclear from this study whether the observed JNK activation was due to ICP27 directly or

indirectly through a downstream ICP27 dependent viral effectors. Additionally, at about the same time, other studies were published that showed that HSV-1 is able to prevent infected cells from undergoing apoptosis. One paper linked ICP4 and ICP27 to the stabilization of Bcl-2 after p38 activation (186), while another showed that activation of NF-κB is required for preventing apoptosis (47). It is important to note that NF-κB activation can be one of the downstream effects of SAPK signaling (56, 98). The IKK-IκB-p65 pathway has also been shown to play a critical role in the efficient replication of HSV-1 (50). The combined results of these studies give credence to the idea that SAPK signaling and inhibition of apoptosis are linked in HSV-1 infected cells. The pleiotropic nature of the signaling pathways makes them attractive candidate for mediators of some of ICP27's diverse functions.

Regulation of apoptosis during HSV-1 infection.

Apoptosis is a tightly controlled process of programmed cell death that commonly occurs in all multicellular organisms (reviewed in (112)). Controlled cell death is advantageous the organism since it allows the removal of damaged or unnecessary cells without triggering the inflammatory response that follows necrotic cell death. Apoptosis is characterized by a series of biochemical changes that ultimately results in morphological changes. During apoptosis the nuclei of cells first condense and then become fragmented due to chromatin condensation and DNA degradation or laddering. In the cytoplasm, organelles remain largely intact, but cease functioning as normal. Mitochondrial membrane

potential is lost, resulting in the release of cytochromes and respiratory arrest. Cellular apoptotic proteases called caspases are activated and cleave target cellular proteins. The cellular cytoskeleton is also degraded resulting in changes in cellular morphology and the disruption of normal protein trafficking. The cellular membrane also undergoes a series of morphological changes. Phospholipids, e.g. phosphatidylserine, flip from the inner layer to the outer layer, identifying the cell as a target for phagocytosis by macrophages. The membrane also becomes ruffled in appearance due to membrane blebbing. Finally, these membrane blebs bud from the cells forming apoptotic bodies that are easily phagocytosed and cleared by the organism's immune system.

Apoptosis can be triggered by either external or internal cellular signals, but either pathway leads to the same ultimate fate. The extrinsic apoptotic pathway is mediated by the binding of cellular death receptors, TNF-R (tumor necrosis factor receptor) or Fas, to their ligands, TNFα or Fas ligand. Signaling through these death receptors then leads to the activation of procaspase 8 though their cognate death domain proteins, TRADD or FADD, respectively. Following cleavage and activation, caspase 8 cleaves various effector procaspases, e.g., procaspase 3. These effector caspases then mediate apoptosis by degrading key cellular proteins, e.g. PARP, actin, and DFF-45. The mitochondrion serves as the master regulator of the intrinsic pathway activation. Internal cellular signals such as DNA damage, oxidative stress, or viral infection can either directly or indirectly impair mitochondrial function resulting in the release of cytochromes. Free cytoplasmic cytochrome C then complexes with

Apaf-1 (Apoptotic peptidase activating factor 1). This activated complex, known as the apoptosome, then leads to the cleavage and activation of procaspase 9. Activated caspase 9 then functions in a manner similar to caspase 8 by activating effectors caspases, which mediate apoptosis. Finally, apoptosis can also be triggered indirectly by any one of several signaling kinase pathways. These signaling pathways, which include MAPKs, mediate the cellular response to various extracellular and intracellular signals. These signaling pathways then can trigger or inhibit the activation of apoptosis by stabilizing/destabilizing proteins via post-translational modification or changes in the cellular transcriptional program.

Apoptosis serves along the frontline of the innate immune response during viral infection. Viral infection can directly lead to changes in normal cellular functions leading to apoptosis of infected cells. Additionally, translational arrest triggered by the interferon response can lead to growth arrest and apoptosis of uninfected cells near infected cells. Therefore, most viral pathogens have developed a variety of mechanisms to evade or inhibit the cellular apoptotic response. Herpesviruses are no exception to this rule and block apoptosis through a variety of different mechanisms. KSHV encodes a homologue of the cellular FLICE protein, vFLIP, which blocks the activation of caspases 3, 8, and 9 (13). EBV encodes small viral RNAs called EBERs (110, 111) and a viral protein EBNA 1 (77) that inhibit apoptosis in latently infected cells. Finally, HCMV expresses vICA, an inhibitor of caspase 8 (152), and vMIA, an inhibitor of the proapoptotic factor BAX (46).

Viral modulation of the cellular apoptotic response plays a critical role in the replication of HSV. HSV infection leads to changes in cellular morphology that resemble many of the alterations observed during apoptosis. Following infection, cells undergo cytopathic effects (CPE) that include alterations to the nucleolus, cellular chromatin condensation, cell fusion, and rounding. Normal productive WT HSV infections result in virally mediated lysis/necrosis. This virally mediated lysis, however, may be due to a careful balance of apoptotic signals and viral replication. In many ways HSV infection can be viewed as a race between viral replication and apoptosis. Studies by Koyama and Adachi originally showed that infection by HSV-1 in the absence of viral protein expression triggers apoptosis (80). This initial observation led to a model where viral infection triggers apoptosis, but viral factors then act as brakes allowing HSV-1 replication to win the race. Since that initial observation, significant research has gone into identifying HSV-1 associated activators and inhibitors of apoptosis.

While it is well established that HSV-1 infection triggers apoptosis, few proapoptotic viral factors have been definitively identified. Aubert et al., using a time course of cyclohexamide treatment (CHX), concluded that apoptosis is triggered at early times following HSV infection (9). ICP0 has long been implicated in the activation of apoptosis following infection (20, 134). Recent work by Sanfilippo and Blaho has shown that the ICP0 mRNA is sufficient for triggering apoptosis. (141). Work by Hagglund et al. has also shown that US1.5

and UL13 are able to trigger apoptosis (51). It is unknown if there are other viral proapoptotic factors.

There is an extensive list of antiapoptotic factors encoded by HSV-1 that have been identified, but the mechanism by which most of these factors work is poorly understood. Interestingly, one of them, ICP22, is highly related to US1.5 in amino acid sequence, but has been shown to reduce apoptosis (9). ICP4 null mutants result in nonproductive infections that trigger apoptosis, implicating ICP4 in the inhibition of apoptosis. Based on observations by Goodkin et al. that gene expression from 3-6 hpi is required to inhibit apoptosis, it is assumed that this is an indirect effect due to ICP4 being required for the expression of DE/L gene products (11, 47). Consistent with this idea, several DE and L gene products have been implicated in the inhibition of apoptosis. The viral kinase US3 protein has been shown to inhibit virally induced caspase 3 cleavage by blocking the JNK signaling pathway (51, 107). The HSV-1 LAT gene has also been shown to block apoptosis induced by caspase 8 and caspase 9 in latently infected cells. Viral glycoproteins have been implicated in the regulation of apoptosis via receptor binding. HSV gJ has been shown to inhibit apoptosis in T cells (70, 71). Glycoprotein D has also been shown to inhibit apoptosis by binding to its receptor HVEM and triggering the activation of NF-κB (147). Activation of NF-κB is a recurrent theme in the inhibition of apoptosis during HSV-1 infection. Several lines of evidence have shown that NF-κB activation is required for the inhibition of apoptosis (47, 147). Additionally, Bcl-2, a target for NF-κB mediated

transcription, plays an important role in inhibiting apoptosis during viral infection (10, 42, 186).

ICP27 is required for the prevention of apoptosis during infection (8). The exact mechanism behind this remains unclear. Several papers have proposed that this inhibition is due to ICP27's ability to transactivate DE and L gene expression (11, 47). However, there are several other possible explanations for ICP27-mediated inhibition of apoptosis. For example, ICP27 expression stabilizes the cellular IEX-1 transcript, which can act as either a pro- or anti-apoptotic factor (27). Alternatively, SAPK signaling activated by ICP27 during viral infection is required for NF-κB activation, which has been associated with inhibition of apoptosis (57). Furthermore, there are also several lines of evidence that SAPK signaling can directly play an anti-apoptotic role as well (31, 180, 188). Thus additional research is needed to clarify the role of ICP27 in the regulation of apoptosis in HSV-1 infected cells.

Thesis statement

Our laboratory is interested in understanding the role of the IE regulatory protein ICP27 in the regulation of viral gene expression during lytic HSV-1 infection. The fact that ICP27 has no known cellular homologues and is highly conserved among all herpesviruses makes it an ideal candidate for antiviral therapy. Determining the effects of ICP27 in the absence of viral infection on host cell function, particularly SAPK signaling, is critical to understanding HSV-1 replication as there is evidence that ICP27 mediated regulation of cellular SAPK signaling pathways has drastic and widespread effects on both host cell functions

and viral replication. Identifying the direct effect of ICP27 expression on these pathways and understanding the downstream effectors of the signaling event will provide valuable insight into how HSV-1 modulates apoptosis during infection. Furthermore, SAPK signaling could play a critical role in ICP27's ability to transactivate the expression of viral gene products. There were three mains goals of the study presented here. First, we wanted to identify the effects of ICP27 expression on normal cellular functions including SAPK signaling. Second, we sought to determine the downstream effects of this signaling on host cell function and viral replication. Third, we hoped to identify the mechanism by which ICP27 induces SAPK signaling. In pursuing these goals, we found evidence that ICP27 functions as an important regulator of both pro- and anti-apoptotic signaling during viral infection. Furthermore, we show that ICP27-mediated SAPK signaling is required for the efficient expression of multiple DE and L gene products. Finally, we have identified a potential ICP27-mediated trigger for the SAPK signaling event.

Chapter 2

Materials and Methods

Cells.

HeLa cells (human cervical epithelial cells) were obtained from American Type Culture Collection (ATCC). HeLa Tet-On (HTO) cells were obtained from Clontech. HTO cells stably express a modified tetracycline transactivator from *E. coli* fused to the HSV-1 VP16 activation domain, which induces expression of genes controlled by the tetracycline operator in the presence of doxicycline (49). Vero cells (African green monkey kidney cells) were obtained from ATCC. V27 (125) cells are Vero cells that contain a stably transfected copy of the HSV-1 ICP27 gene. The ICP27 gene is not constitutively expressed by V27 cells, but is induced by HSV-1 infection. HTO and Vero cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented to contain 10% and 5% heat-inactivated fetal bovine serum (FBS), respectively, 50 U of penicillin/ml, and 50 µg of streptomycin/ml. The medium for the V27 cells was the same as that for Vero cells except that it also contained 300 µg/ml G418. The construction of the HTO-H, HTO-L, TE12, and TE51 cell lines is described below. They were maintained in DMEM supplemented to contain 10% tetracycline-free FBS, 50 U of penicillin/ml, 50 µg of streptomycin/ml, 400 µg /ml G418, and 100 µg/ml hygromycin B.

Plasmids.

The pTE27 plasmid was created by cloning the ICP27 gene from pBH27 (124) into the pTRE-Tight vector purchased from Clontech. Briefly, pBH27 was cleaved at an Age I restriction site 75 bp downstream of the ICP27 gene transcriptional start site and at the Hind III restriction site. The Age I-generated DNA ends were blunted using the Klenow fragment of *E. coli* DNA polymerase. The ICP27 gene fragment was ligated into the multiple cloning site of the pTRE-Tight plasmid using the Pvu II (blunted as above) and Hind III restriction sites. This created an ICP27 gene under the control of the minimal cytomegalovirus IE gene promoter and seven repeats of a modified tetracycline operator site.

The UL37 expression construct, pXL-37v5, has been previously described (90). It was generously supplied by David Knipe.

The TTP expression construct, pCMV.TTPh.tag has been previously described (84). It was a generous gift from Perry Blackshear, and kindly provided by Paul Bohjanen.

Stable cell line generation.

TE12 and TE51 are HeLa cell lines that conditionally express ICP27 via a doxicycline-regulated system (48, 49). HTO-L is a HeLa cell line that conditionally expresses luciferase using the same system. HTO cells were co-transfected with either pTE27 and the linear hygromycin selection marker (BD Biosciences) to generate the TE12 and TE51 cell lines, or pTRE-Tight-Luc (BD Biosciences) and the linear hygromycin selection marker (BD Biosciences) to

generate the HTO-L cell line. Additionally, the selection marker alone was transfected in to create the HTO-H cell line. Transfections were performed using Lipofectamine-2000 (Invitrogen) at a 20:1 ratio (w/w) of plasmid to linear selection marker following the manufacturer's recommended protocol. HTO-H cells were isolated as a pooled population of hygromycin B-resistant cells, while clones of potential luciferase and ICP27 cell lines were selected in the presence of hygromycin B (200 µg/ml, BD Biosciences). Drug-resistant colonies were isolated using cloning cylinders and screened for the inducible expression of luciferase or functional ICP27 using immunoblots, plaque assays, and immunofluorescence.

Hela-H37 is a HeLa cell line that stably expresses the HSV-1 UL37 protein. It was made by stably transfecting HeLa cells with the pXL-37v5 plasmid. To do this, HeLa cells were co-transfected with either pXL-37v5 and the linear hygromycin selection marker (BD Biosciences) to generate the HeLa-H37 cell line, or the selection marker alone to create the HeLa-H control cell line. Transfections were performed using Lipofectamine-2000 (Invitrogen) at a 20:1 ratio (w/w) of plasmid to linear selection marker following the manufacturer's recommended protocol. HeLa-H cells were isolated as a pooled population of hygromycin B-resistant cells, while clones of UL37 expressing cell lines were selected in the presence of hygromycin B (600 µg/ml, BD Biosciences). Drug-resistant colonies were isolated using cloning cylinders through two successive rounds of cloning and selected for uniform expression of UL37 using immunoblots and immunofluorescence.

Viruses and infections.

The wild-type (WT) strain of HSV-1 used in these studies was KOS1.1 (64). Viral ICP4 (d120) (30) and ICP27 (d27-1) (125) deletion mutants have been described. Additional ICP27 N-terminal deletion mutants were used: d1-2, a mutant lacking amino acids 12-63 (127); d2-3, a mutant lacking amino acids 64-108 (11); d3-4, a mutant lacking amino acids 109-138 (100); d4-5, a mutant lacking amino acids 139-153 (100); d5-6, a mutant lacking amino acids 154-173 (102); and d6-7, a mutant lacking amino acids 174-200 (11).

Infections were carried out at a multiplicity of infection of 10 or 1, as indicated, in phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% glucose and 0.1% heat-inactivated newborn calf serum. Viral adsorption was carried out for 1 h at 37°C, at which time the viral inoculum was replaced. The viral inoculum was replaced with either 199 medium containing 2% heat-inactivated newborn calf serum, 50 U of penicillin/ml, and 50 µg of streptomycin/ml, or spent media from duplicate wells as indicated. Following media replacement, all infections were incubated at 37°C.

Viral plaque assays were performed using the same media as for viral infections except that 1% heat-inactivated normal pooled human serum (MP Biomedicals) was added. Plaque assay mixtures were incubated at 37°C for 4 days to allow plaques to develop. Viral yield experiments used monolayers infected at an MOI of 1 that were treated at 2 hpi with an acid-glycine wash (pH 3.0) to reduce background due to viral particles that had not entered cells by this

time (21). The infections were stopped after 24 h and virus was harvested by the addition of 5 ml of sterile milk to each flask, followed by three cycles of freeze-thawing at –80 ºC. The amount of infectious virus was determined by plaque assay of the infected cell lysate on complementing V27 cells. Confluent monolayers were infected with 1-ml aliquots of 100-fold and higher dilutions to test for plaque formation. Therefore, the limit of detection of viral replication for each infection was 1 x 10^2 PFU.

Cell growth assays.

To measure effects on cell growth over time due to ICP27 expression, 30,000 HTO-H, TE12, and TE51 cells were plated in 12.5 cm^2 tissue culture flasks. Half of the flasks were immediately treated with 2 µg/ml of doxicycline. At each time point, six flasks per cell line were trypsinized (3 induced and 3 uninduced). The cells were resuspended in DMEM and counted using a hemacytometer.

Activator and inhibitor treatment.

Sorbitol was used to induce SAPK signaling by treating cells with a final concentration of 0.5 M sorbitol (Invitrogen) for 30 min (15). Apoptosis was induced by treating cells with staurosporine (ICN Biomedicals) dissolved in DMSO at final concentration of 1 µM for 1 and 3 h. Pharmacological inhibitors of SAPK signaling SB203580 (p38 inhibitor; Promega) and SP600125 (JNK

inhibitor; Biosource) were added 30 min prior to infection or sorbitol induction at final concentrations of 5 μM and 1 μM, respectively.

Immunoblots.

Confluent monolayers of cells were harvested for all immunoblots. Analysis of ICP27 protein expression by immunoblotting was carried out as described previously (43). Cell lysates were prepared first by washing cells with cold phosphate-buffered saline (PBS) supplemented with TLCK and PMSF. Cells were then lysed directly in PSB buffer (40 mM Tris pH 6.8, 1% SDS, 10% glycerol, 2% beta-mercaptoethanol, and 0.01% bromophenol blue). The lysate was then boiled 5 min and frozen overnight at –80 ºC. For SDS-PAGE, the samples were boiled again for 5 min and loaded onto 10% acrylamide gels. Proteins were transferred to nitrocellulose membranes and probed for SAPKs and ICP27. The antibody for ICP27, H1119 (mouse monoclonal used at a dilution of 1:5,400), was purchased from the Rumbaugh-Goodwin Institute for Cancer Research (Plantation, FL). Cellular p38 and phospho-p38 were detected using rabbit polyclonal antibodies 9212 and 9211, respectively (Cell Signaling Danvers, MA), at a dilution of 1:1000. Polyclonal rabbit antibodies against cellular JNK1 (44-690G) and phospho-JNK (44-682G) were purchased from Biosource (Camarillo, CA) and used at dilutions of 1:10,000 and 1:1000, respectively. The rabbit polyclonal antibody used to detect IκB (sc-203) was from Santa Cruz and used at a 1:200 dilution. The anti-UL37 rabbit serum was a generous gift from Frank Jenkins (University of Pittsburgh) and used at a 1:200

dilution. For ICP8, a 1:1000 dilution of mouse monoclonal antibody H1115 was used (Rumbaugh-Goodwin Institute). For gD, a 1:3,000 dilution of mouse monoclonal antibody H1103 was used (Rumbaugh-Goodwin Institute). For VP5, a 1:1000 dilution of a mouse monoclonal antibody (ab6508; Abcam) was used. The antibody used to detect eIF2α phosphorylation was a phospho-eIF2α (S51)-specific rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) used at a 1:1000 dilution. PARP cleavage was detected using mouse monoclonal anti-PARP C2-10 from R & D Systems (Minneapolis, MN) at a 1:500 dilution. The cellular early endosomal antigen (EEA1) was detected using a mouse monoclonal antibody (BD Biosciences) diluted 1:2,500. ß-actin was detected using a mouse monoclonal antibody (ab6276; Abcam) at a 1:10,000 dilution. The secondary antibodies used for immunoblot detection were horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G and horseradish peroxidase goat anti-rabbit, purchased from Jackson Immuno-Research (West Grove, PA), and were both diluted 1:5,000. Secondary antibodies were detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham).

Indirect immunofluorescence.

For infection and induction experiments to examine ICP27 expression and JNK signaling, cells were fixed in 3.7% formaldehyde in PBS for 10 min followed by acetone permeabilization for 2 min (123). Cells used for caspase-3 immunofluorescent experiments were fixed and permeablized in methanol at –20

ºC for 10 min. For fluorescent staining, coverslips were incubated at 37°C for 1 h with various primary antibodies. The primary antibodies were mouse monoclonal anti-ICP27 (H1119) at a 1:1000 dilution, rabbit polyclonal anti-ICP27 Clu38 (a kind gift from Saul Silverstein, Columbia University) at a dilution of 1:800, rabbit polyclonal anti-JNK1 at a 1:1000 dilution (Biosource 44-690G), rabbit polyclonal anti-phospho-JNK at a 1:1000 dilution (Biosource 44-682G), goat polyclonal anti-caspase-3 p11 (K19) at a 1:100 dilution (Santa Cruz sc-1224), rabbit polyclonal anti-NF-κB p65 (C-20) a 1:100 dilution (Santa Cruz sc-372), and goat polyclonal anti-luciferase at a 1:500 dilution (Promega G7451). After primary incubation, cells underwent secondary staining for 1 h at 37°C. For ICP27 immunofluoresence alone, a 1:1000 dilution of Cy3-conjugated goat anti-mouse immunoglobulin G was used. For ICP27 and JNK co-staining in HTO-H, TE12 and TE51 cell lines, a combination of mouse and rabbit primary antibodies was used; secondary staining was done with a 1:1,000 dilution of Cy3-conjugated goat anti-rabbit immunoglobulin G and a 1:200 dilution of Cy2-conjugated goat anti-mouse immunoglobulin G. Luciferase expression and JNK phosphorylation in HTO-L cells were visualized by using a combination of goat and rabbit primary antibodies respectively. The HTO-L cells were then costained with Cy2-conjugated donkey anti-rabbit immunoglobulin G diluted 1:200 and a 1:1000 dilution of Cy3-conjugated donkey anti-goat immunoglobulin G. When cells were costained for ICP27 and caspase-3, secondary staining was done sequentially starting with a 1:1000 dilution of Cy3-conjugated donkey anti-goat immunoglobulin G antibody followed by a 1:200 dilution of Cy2-conjugated goat

anti-mouse immunoglobulin G antibody. When cells were costained for NF-κB p65 and ICP27, Cy3-conjugated goat anti-rabbit immunoglobulin G at a 1:1,000 dilution and Cy2-conjugated goat anti-mouse immunoglobulin G at a 1:200 dilution were used. All secondary antibodies were purchased from Jackson ImmunoResearch. Nuclei of all cells were co-stained using Hoechst stain at a 1:1000 dilution. Cells were observed with an Olympus BX 60 fluorescent microscope linked to a video camera. Images were taken using Scion-CG7 frame-grabber and saved as TIFF files. Images were adjusted for brightness, contrast, and size using Adobe Photoshop CS2.

Flow cytometry analysis.

Approximately 25% confluent monolayers of HTO-L, TE12, and TE51 cells were left uninduced or induced with 2 μg/ml doxicycline for 72 hours. Cells were then trypsinized and pelleted. The level of apoptosis in the cells was assayed using APOAF Annexin V-FITC Apoptosis Detection Kit (Sigma). The stained cells were then analyzed using a FACScaliber flow cytometer (BD Biosciences), and the collected data were analyzed using Flow Jo version 8.7 (Tree Star) software. Samples were gated using forward scatter and side scatter to exclude any cell debris. Apoptotic cells were identified as cells that were Annexin V FITC positive.

Cell adherence.

To measure the effects UL37 expression on cell viability following viral infection with KOS 1.1 and d27-1, HeLa-H and HeLa-H37 cells were plated in 6 well trays. Cells were then either mock-infected or infected with KOS 1.1 or d27-1. At 16 hpi, viable adherent cells were fixed with methanol and stained with Accustain Geimsa stain (Sigma). Images were captured using a digital camera system from Fotodyne Inc.. The area of viable/adherent cells was measured using ImageJ.

Analysis of TTP localization and stress granule formation.

HTO-L, TE12, and TE51 cells were grown to 75% confluence on glass coverslips. Cells were transfected with either salmon sperm DNA or pCMV.TTPh.tag using Lipofectamine-2000 (Invitrogen) following the manufacturer's recommended protocol. After four hours, media was replaced and doxicycline (2µg/ml) was added to induce protein expression. Stress granule formation was induced in some cells 2.5 hours prior to fixation using 1 mM sodium arsenite (Sigma) for 30 min and then allowed to recover for 2 h in normal media. Twelve hours post doxicycline-induction, cells were fixed with 2% formaldehyde for 10 min followed by permabilization in PHEM buffer (60 mM PIPES, 25mM Hepes, 10mM EDTA, 2 mM $MgCl_2$, (pH 6.9)) with 0.1% Triton X-100. Coverslips were blocked in PBSTT (PBS with 0.5% Tween-20 and 0.1% Triton X-100) with 0.5% BSA and 1% horse serum for 30 min. Coverslips were incubated with goat anti-T-cell internal antigen 1 (TIA-1)-related protein (TIAR) antibody (sc-1750; Santa Cruz Biotechnology, Inc.), rabbit anti-TTP (ab36558;

Abcam), and mouse anti-ICP27 (H1119) and washed with PBS. Bound antibodies were detected using sequential staining followed by blocking with PBS with 5% goat serum using Cy3-conjugated donkey anti-goat, Cy2-conjugated donkey anti-rabbit, and Cy5-conjugated goat anti-mouse secondary antibodies. All secondary antibodies were purchased from Jackson ImmunoResearch. Nuclei of all cells were co-stained using Hoechst stain at a 1:1000 dilution. Cells were observed and images taken using an Olympus IX70 confocal microscope with PixCell II LCM. Images were adjusted for brightness, contrast, and size using Adobe Photoshop CS2.

RNA preparation.

For preparation of total RNA, RNA was harvested from 10-cm^2 cell culture dishes for Northern blot analysis or 6 well trays for RT-PCR analysis. HeLa, HTO-L, and TE12 cells were mock infected, infected, or induced with doxicycline (2 µg/ml) as indicated. For Northern blot analysis of IEX-1 messages stability, parallel cultures were treated with actinomycin D (source/amount) to arrest RNA synthesis for 1 additional hour prior to harvest. RNA was prepared using Trizol reagent (Invitrogen) following the manufacturer's guidelines. The RNA was treated with RNase-free DNase (Roche Diagnostics Corp) to remove DNA contaminates. The RNA was then purified and precipitated using a phenol-chloroform extraction followed by ethanol precipitation.

IEX-1 Northern blots.

For Northern blotting, 5 µg of each sample were denatured with Glyoxal Loading Dye (Ambion) for 1 hour at 50˚C. Diluted samples were loaded into a 1.2% agarose gel prepared with 1X BPTE, pH 6.5 (10 mM PIPES, 30 mM Bis-Tris, 1mM EDTA (pH 8.0)). RNA was transferred to GeneScreen Plus membranes (Perkin-Elmer). Membranes were UV-crosslinked using a Strata-linker (Stratagene). Following crosslinking, membranes wee pre-hybridized in 10 ml of preheated (60˚C) ULTRAhyb solution (Ambion) for 30 minutes. Membranes were hybridized overnight at 42˚C with ^{32}P-labeled DNA probes in ULTRAhyb solution and washed according to manufacturer's instructions. Probes specific for IEX-1 were generated with a random primer labeling kit (Invitrogen) using the 710-bp EcoR1-XhoI fragment from the previously described IEX-1 cDNA IMAGE clone (63).

RT-PCR measurement of XBP-1 splicing.

Reverse transcription-PCRs (RT-PCRs) were done using the OneStep RT-PCR kit (Qiagen). The forward 5'-AACTCCAGCTAGAAAATCAGC-3' and reverse 5'-CCATGGGAAGATGTTCTGGG-3' primers used have been previously described (58). PCR was done for 35 cycles of melting (94 ˚C, 30 s), annealing (55 ˚C, 30 s), and extension (72 ˚C, 1 min) with a final 7 min extension. PCR products representing the unspliced 241 bp and spliced 215 bp fragments were detected by running the PCR products on a 2% agarose gel and staining with ethidium bromide.

Chapter 3

Herpes Simplex Virus Type 1 ICP27 Induces p38 Mitogen-activated Protein Kinase Signaling and Apoptosis in HeLa Cells

Introduction

ICP27 is an essential multifunctional viral regulatory protein that has been shown to affect both viral and cellular gene expression. Several studies have shown that ICP27 is also involved in activating p38 and JNK MAPK signaling pathways during HSV-1 infection. Both of these MAPKs are known as stress-activated protein kinases (SAPK)s due to their involvement in controlling cellular responses to various types of stress. Initial studies showed p38 and JNK signaling pathways become activated during the initial stages of infection, as early as 3 hours post infection (hpi), with a peak of activation at 6-8 hpi (185). The activation of SAPK signaling pathways appears to be important for normal viral replication, since the presence of pharmacological inhibitors of p38 and JNK signaling leads to a significant drop in viral yields (76, 98). The mechanism behind the activation of the SAPK signaling pathways by HSV-1 has yet to be fully understood. It is known that the activation occurs downstream of Ras (98). The timing of the activation pointed towards an IE gene product as being responsible for initial induction since IE proteins are present at high levels at 3 hpi. Consistent with this, studies showed that ICP27 is required in the context of viral infection for activation of the SAPK pathways (27, 56, 57). The N-terminal end of the ICP27 protein, which plays a functional role in nuclear export, is required for SAPK activation (27, 56). Additionally, it was shown that ICP27-

mediated JNK signaling leads to the activation of NF-κB during viral infection (57). Interestingly, activation of NF-κB during HSV-1 infections has been associated with the inhibition of apoptosis, which is activated by very early events in the viral infection (47). ICP27 is also required during viral infection to prevent activation of apoptosis associated with p38-mediated destabilization of Bcl-2 (186).

It is currently unclear whether the requirement for ICP27 in SAPK activation during viral infection is a direct effect of the protein or an indirect downstream effect dependent on other viral factors, which are themselves ICP27-dependent. In order to look at the effects of ICP27 alone on SAPK signaling and other host cell functions, we sought to develop a system allowed inducible expression of ICP27 in uninfected cells, and in the absence of transient transfection or viral transduction, both of which could potentially activate SAPK's. For this we utilized a doxicycline-inducible promoter system in HeLa cells to express ICP27. Using this system, we demonstrate that ICP27 expression is sufficient to induce p38 signaling to levels similar to those seen during wild-type HSV-1 infection. Furthermore, we show that ICP27-induced p38 signaling inhibits normal cellular growth and leads to apoptosis. In contrast, ICP27 expression alone appears to be insufficient for the full activation of JNK signaling. Therefore, it appears that ICP27 activates the p38 and JNK signaling pathways by two distinct mechanisms.

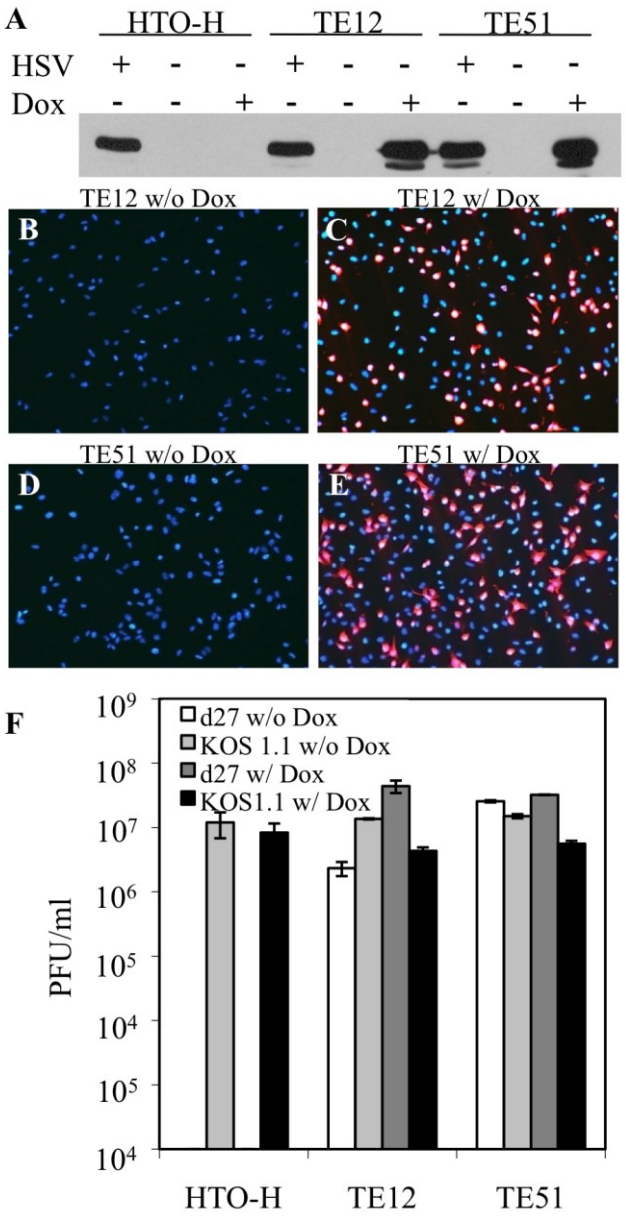
Results

Isolation and initial characterization of cell lines that inducibly express ICP27.

Most prior attempts to study the effects of ICP27 on host cell function have been conducted in the context of viral infection. Such studies have identified a host of potential functions that affect cellular gene expression and physiology. However, analyses in infected cells do not clearly separate the primary functions of ICP27 from secondary effects which are mediated by viral gene products whose expression is ICP27-dependent. Therefore, in an effort to identify the primary effects of ICP27 on cell function, we sought to engineer human cell lines that express ICP27 in an inducible manner. For this purpose, we first engineered a doxicycline-regulatable ICP27 gene contained on a plasmid (pTE27). We then transfected G418-resistant HTO cells, which are HeLa cells stably expressing the Tet-on transactivator (49), with pTE27 and a linear hygromycin-resistance gene. Sixty hygromycin- and G418-resistant clones were selected and screened for ICP27 expression both in the absence and presence of doxicycline (2 μg/ml for 24 h). The level of ICP27 expression was then compared to that in virally infected cells and uninduced cells using immunoblots of cell lysates. Hygromycin-resistant HTO cells, HTO-H, were included as a control. Two clones, TE12 and TE51, showed no detectable background expression of ICP27 and high levels of inducible ICP27 that were equal to or exceeded the expression levels seen during HSV-1 infection (Fig. 3.1A).

Fig. 3.1. TE12 and TE51 cell lines are able to inducibly express functional ICP27. (A) Immunoblot assay comparing HTO-H, TE12, and TE51 ICP27 expression levels. Cells were infected with WT HSV-1 at an MOI of 5 and harvested at 8 hpi. For induction samples, cells were untreated or treated with 2 μg/ml doxicycline for 24 h. (B-E) Immunofluorescent analysis of ICP27 expression in the TE12 (B, C) and TE51 cell lines (D, E) in the presence (C, E) or absence of doxicycline (B, D) (24 h post treatment). The red signal indicates ICP27 expression, and the blue signal is due to Hoechst staining of nuclei. (F) Single-cycle yield assays. Replicate cultures of HTO-H, TE12, and TE51 cells were infected in duplicate at an MOI of 1 with WT HSV-1 or an ICP27 null mutant d27-1 in the presence or absence of doxicycline. After 24 h, the infections were terminated by freezing. Virus was released from the cells by three cycles of freeze-thawing, and the total infectious virus in the lysate was determining by a plaque assay on V27 cells.

Fig. 3.1


A
HTO-H
TE12
TE51
HSV
Dox
TE12 w/o Dox
TE12 w/ Dox
B
C
TE51 w/o Dox
TE51 w/ Dox
D
E
F
d27 w/o Dox
KOS 1.1 w/o Dox
d27 w/ Dox
KOS1.1 w/ Dox
PFU/ml
HTO-H
TE12
TE51

To determine if these cell lines show uniform expression of ICP27 after induction we used immunofluorescent microcopy. This analysis indicated that there was a very low level of ICP27 expression in uninduced cells on a per cell basis (Fig. 3.1B, D) as well as a high percentage of cell expressing ICP27 after induction (Fig. 3.1C, E). Both TE12 and TE51 cell lines showed less than 1% of cells that were ICP27 positive in the absence of doxicycline. After 24 h of induction, approximately 50% of the cells were ICP27 positive, a result that was consistently seen over several experiments (data not shown). Sub-cloning of the two lines did not result in an increased percentage of cells expressing ICP27 following induction. Moreover, following infection with the HSV-1 ICP27 deletion mutant d27-1, nearly 100% of TE12 and TE51 cells expressed ICP27 (data not shown). This indicates that the vast majority of TE12 and TE51 cells carry the ICP27 gene which can be activated by infection (36, 60), but that only approximately 50% of them express it to detectable levels following induction. At present, we do not understand why a sub-population of cells is refractory to induction.

In order to ensure that the ICP27 expressed by TE12 and TE51 was still functional, we infected them with d27-1 in the presence and absence of doxicycline to determine if the ICP27 expressed by the cells is able to complement the ICP27 defect of d27-1. Both cell lines were able to restore growth to d27-1 to a level similar to the WT virus KOS1.1 (Fig. 3.1F). It is important to note that infection with HSV-1 has been shown to induce the expression of stably-transfected genes, including genes controlled by tetracycline

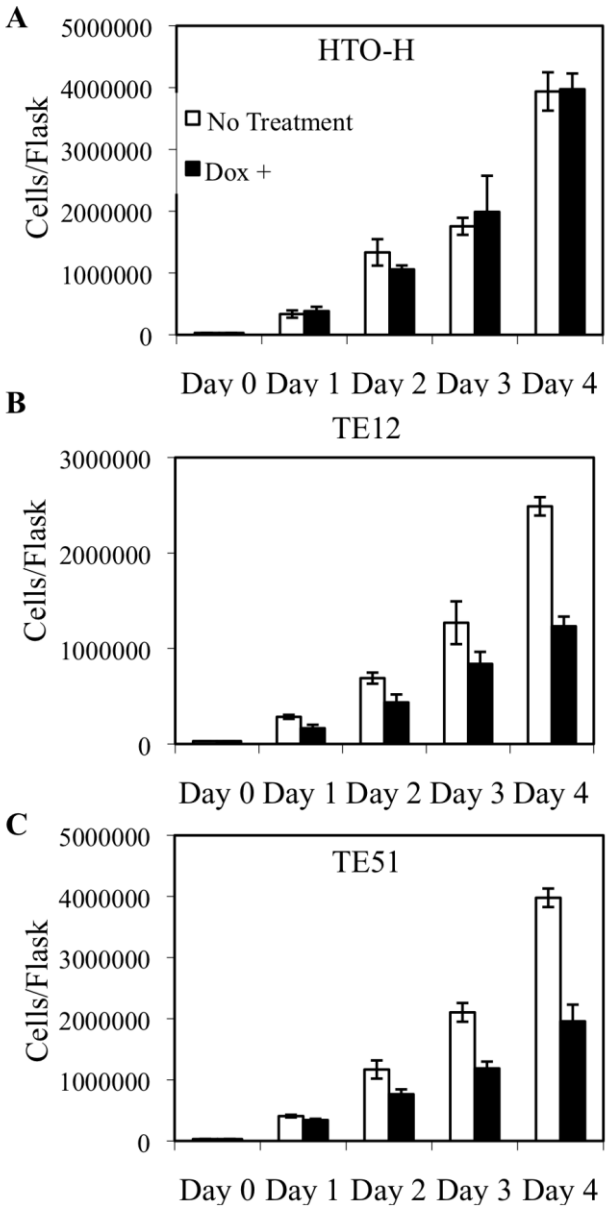
regulatable elements (36, 60), likely accounting for the high level of viral growth observed in TE12 and TE51 cells in both the absence and presence of doxicycline. Consistent with this, and as stated above, nearly 100% of TE12 and TE51 cells expressed ICP27 following infection with d27-1 (data not shown).

After ensuring that the both inducible cell lines expressed functional ICP27, we next sought to characterize the expression kinetics of ICP27 following doxicycline induction. By immunoblot analysis and immunofluorescent microscopy, we found that ICP27 expression was detectable by 8 hr post-induction and was highly expressed at a constant level from 12 h to 24 h post induction (data not shown). It was observed that at early timepoints (6-8 h) ICP27 initially localizes to the nucleus. However, over time (12-24 h), the localization of ICP27 became increasingly cytoplasmic. After 24 h, expression was slowly lost in the cell population over the course of several days until approximately only 5% of the cells remained ICP27 positive at 4 days post induction as assayed by immunofluorescent microcopy (data not shown). This loss of expression was typically associated with cytopathology in the cultures, suggesting that ICP27 expression was toxic to cells.

To explore the possible effects of ICP27 on cell growth and viability, equal numbers of HTO-H, TE12, and TE51 cells were plated and the total number of cells was followed for four days in the presence or absence of doxicycline induction. Doxicycline had no discernable effect on the growth of control HTO-H cells (Fig. 3.2 A). However, doxicycline treatment of both TE12 and TE51 cells led to a significant reduction in cell number that was clearly evident by day 2 and

Fig. 3.2. Doxicyline-induced expression of ICP27 reduces cellular growth. Equal numbers (3×10^4) of HTO-H (A), TE12 (B), and TE51 (C) cells were plated in triplicate in the presence or absence of doxicycline (2 µg/ml). Cells were harvested at 1, 2, 3, and 4 days post plating and counted using a hemacytometer.

Fig. 3.2


A
HTO-H
No Treatment
Dox +
Cells/Flask
5000000
4000000
3000000
2000000
1000000
0
Day 0 Day 1 Day 2 Day 3 Day 4
B
TE12
Cells/Flask
3000000
2000000
1000000
0
Day 0 Day 1 Day 2 Day 3 Day 4
C
TE51
Cells/Flask
5000000
4000000
3000000
2000000
1000000
0
Day 0 Day 1 Day 2 Day 3 Day 4

dramatic by day 4 (Fig. 3.2 B, C).

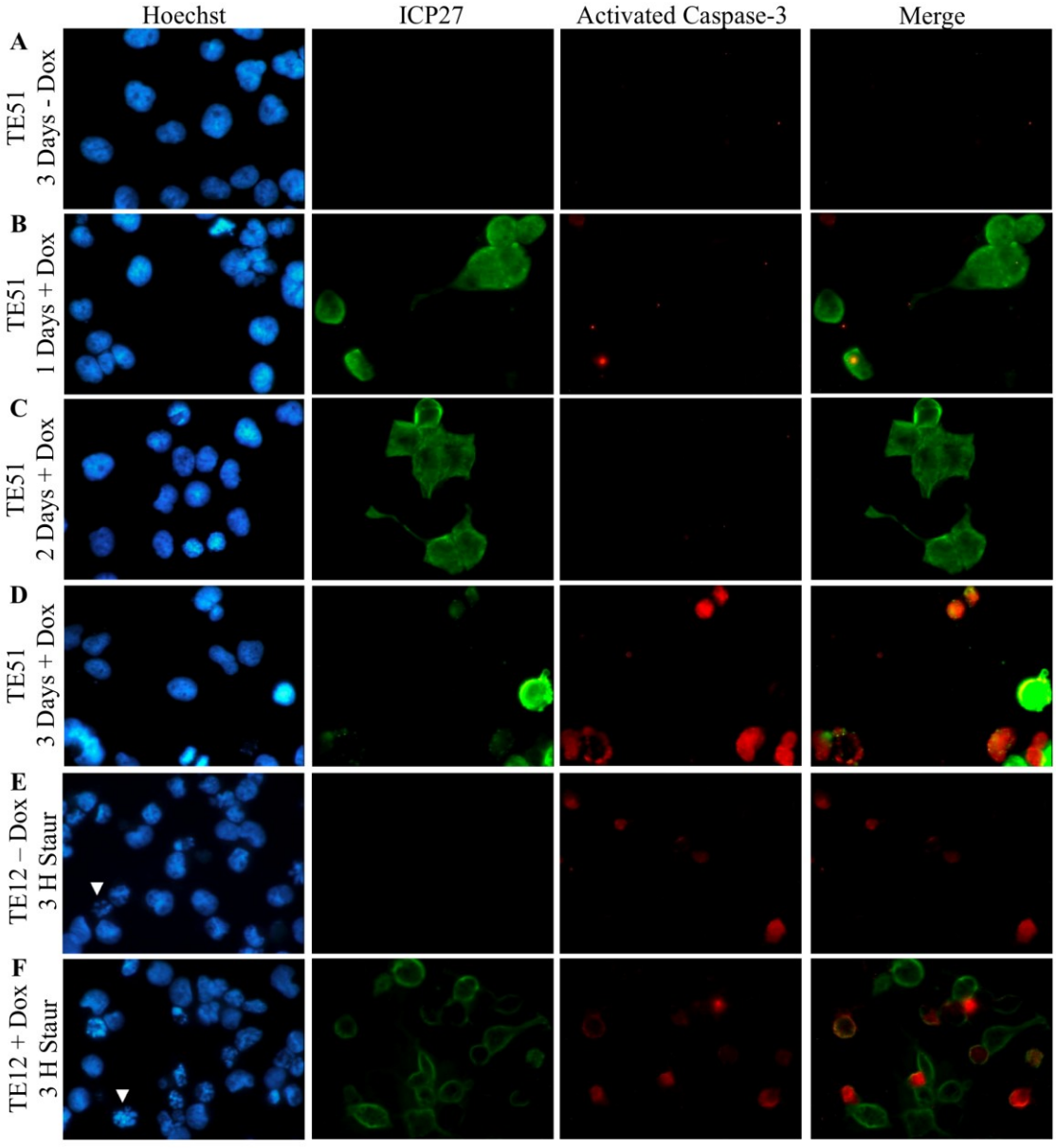
ICP27 expression activates apoptosis.

The apparent growth inhibition seen above, coupled with the cytopathic effects observed, suggested that ICP27 might be inducing apoptosis in the TE12 and TE51 cells. In order to determine if this was the case, we used immunofluorescent microscopy to monitor caspase-3 activation over time following the induction of ICP27 expression. Little to no activated caspase-3 was seen in control HTO-H or HTO-L cells at any time following addition of doxicycline indicating that neither doxicycline treatment nor induced protein expression is sufficient to trigger apoptosis (data not shown). Additionally, little activation was observed in uninduced TE51 cells (Fig. 3.3 A), or in the TE51 cells during the first 2 days of doxicycline induction (Fig. 3.3 B and C). However, after three days of induced expression of ICP27, caspase-3 activation indicative of apoptosis was observed, predominantly in cells expressing ICP27 (Fig. 3.3 D). Similar results were observed in TE12 cells (data not shown).

As an independent measure of apoptosis, we used flow cytometry to measure the number of Annexin V-positive cells following induction of either luciferase expression in HTO-L cells or ICP27 expression in TE51 cells. While there was no change in the percentage of apoptotic cells following three days of luciferase expression when compared to uninduced HTO-L cells, there was a significant increase in the number of Annexin V-positive cells following three days of ICP27 expression (Fig. 3.4 A). Interestingly, the approximately 12%

Fig. 3.3. ICP27 expression activates apoptosis. (A-D) Induction of apoptosis by ICP27. Immunofluorescence analysis of caspase-3 activation in cells expressing ICP27. TE51 cells were incubated in the absence (A) or presence (B-D) of doxicycline (2 μg/ml). Cells were fixed and processed at 1 (B), 2 (C), or 3 (A, D) days post induction using antibodies specific for activated caspase-3 and ICP27. (E,F) Effect of ICP27 on staurosporine-induced apoptosis. Untreated TE12 cells (E) or TE12 cells following 24 h of doxicycline-induced ICP27 expression (F) were treated with staurosporine (1μM). Cells were fixed and processed 3 hours post staurosporine treatment using antibodies specific for activated caspase-3 and ICP27. Representative figures are shown.

Fig. 3.3

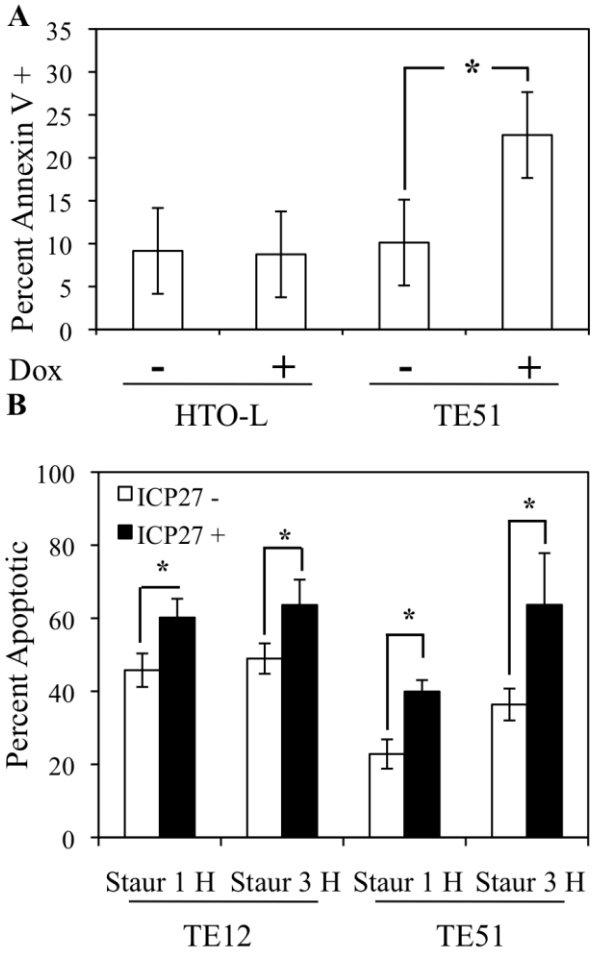

Hoechst
ICP27
Activated Caspase-3
Merge
A
TE51
3 Days - Dox
B
TE51
1 Days + Dox
C
TE51
2 Days + Dox
D
TE51
3 Days + Dox
E
TE12 – Dox
3 H Staur
F
TE12 + Dox
3 H Staur

increase in apoptotic cells observed in cells expressing ICP27 correlates to the relative ICP27 expression percentage following three days of expression (data not shown). These results, coupled with the caspase-3 studies, demonstrate that ICP27 expression in HeLa cells leads to apoptosis, likely explaining the observed defect in cellular growth that is associated with ICP27 expression in the TE12 and TE51 cells.

The above results were somewhat surprising because, as mentioned in the Introduction, prior research has indicated that ICP27 inhibits apoptosis during viral infection (8). Therefore, we asked whether ICP27 could inhibit staurosporine-induced apoptosis in TE12 and TE51 cells following 24 h of doxicycline-induced expression. Staurosporine treatment induced apoptosis in uninduced cells at 1 and 3 h post treatment, as has been previously shown (data not shown and Fig. 3.3 E). ICP27 had no obvious inhibitory effect on the induction of apoptosis since ICP27-expressing cells did not appear to be protected from apoptosis (data not shown and Fig. 3.3 F). In fact, caspase-3 activation and chromatin condensation at these time points appeared to preferentially occur in cells expressing ICP27, suggesting that ICP27 sensitizes cells to staurosporine-induced apoptosis. To examine this further, we analyzed the level of apoptosis of ICP27-negative versus ICP27-positive staurosporine treated cells by counting results of three separate experiments. Cells were identified as apoptotic based on staining for activated caspase-3 or chromatin condensation (as shown by white triangles in Fig. 3.3 E, F). As shown in Fig. 3.4B, there is a significant increase in the percent of apoptotic cells in cells

Fig. 3.4. Quantitation of ICP27-induced apoptosis. (A) Flow cytometric analysis of Annexin V staining in HTO-L and TE51 cells. Cells were left untreated or treated with doxicycline (2 μg/ml) for 3 days to induce either luciferase or ICP27 expression as indicated. Cells were stained using Annexin V conjugated to FITC and counted using a FACSCalibur flow cytometer. The data were analyzed using Flow Jo software. (* $p < 0.05$) (B) Quantitation of apoptosis in staurosporine-treated cells. TE12 and TE51 cells were induced with doxicycline for 24 h, then treated with 1 μM staurosporine. Cells were fixed 1 or 3 h after staurosporine addition and stained for ICP27 and activated Caspase-3. Cells were classified as apoptotic based on presence of activated Caspase-3 or chromatin condensation. The level of apoptosis in ICP27-negative cells (white bars) versus ICP27-positive cells (black bars) is shown. Greater than 1000 cells were counted per time point per cell line. (* $p < 0.05$)

Fig. 3.4


A
Percent Annexin V +
35
30
25
20
15
10
5
0
*
Dox
-
+
-
+
HTO-L
TE51
B
Percent Apoptotic
100
80
60
40
20
0
ICP27 -
ICP27 +
*
*
*
*
Staur 1 H
Staur 3 H
Staur 1 H
Staur 3 H
TE12
TE51

expressing ICP27 compared to non-expressing cells at both 1 and 3 h post-staurosporine treatment. These results combined with those above, demonstrate that in the absence of infection, ICP27 has a pro-apoptotic effect in HeLa cells.

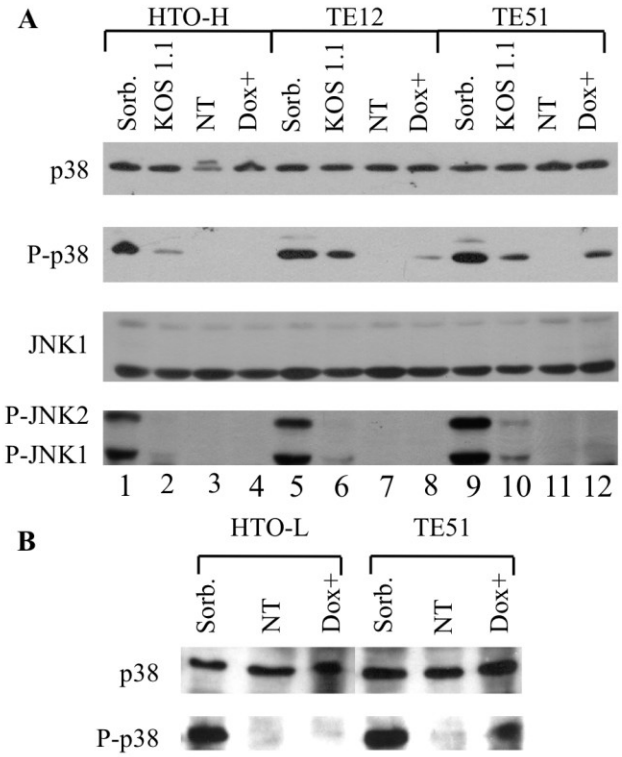
ICP27-mediated activation of SAPKs.

We speculated that the activation of apoptosis in cells expressing ICP27 could be due to ICP27-mediated SAPK signaling, since both p38 and JNK signaling have been shown to be pro-apoptotic in some cases (170). To see whether ICP27 activates SAPK signaling pathways in TE12 and TE51 cells, lysates of control or induced cells were probed for both total and phosphorylated p38 and JNK. The results of the experiment showed that both sorbitol, an inducer of SAPK signaling through osmotic stress, and viral infection (MOI 10 at 8 hpi) led to the phosphorylation of both p38 and JNK in HTO-H, TE12 and TE51 cells (Fig. 3.5 A lanes, 1, 5, and 9 and 2, 6, and 10 respectively), although in general sorbitol appeared to be a more effective activator at the time points examined. No major changes in levels of total JNK1 or p38 were seen under any of the tested conditions. As expected, untreated cells (Fig. 3.5 A, lanes 3, 7, and 11) showed little to no SAPK signaling, nor did HTO-H control cells treated with doxicycline (Fig. 3.5 A, lane 4). However, TE12 and TE51 cells that had been treated with doxicycline for 24 h showed a clear increase in p38 phosphorylation that was comparable to that seen in virally infected cells (Fig. 3.5 A, lanes 8 and 12). In contrast, JNK phosphorylation was not observed in TE12 and TE51 cells following either 12 h or 24 h of ICP27 induction (data not shown and Fig. 3.5 A,

Fig. 3.5. ICP27 activates p38 signaling in the absence of viral infection.

(A) Immunoblot analysis of SAPK signaling activation in HTO-H, TE12, and TE51 cells following 30 min of sorbitol treatment, WT HSV-1 infection (MOI 10, 8 hpi), control cells left untreated (NT), or doxicycline induction (2 μg/ml for 24 h) as indicated. (B) Immunoblot analysis of p38 phosphorylation in HTO-L and TE51 cells following 30 minute of sorbitol treatment, control cells left untreated (NT), or doxicycline induction (2 μg/ml for 24 h) as indicated. Protein extracts were analyzed by immunoblotting for p38, phosphorylated p38, JNK1, and phosphorylated JNK1/2.

Fig. 3.5


A
HTO-H
TE12
TE51
Sorb.
KOS 1.1
NT
Dox+
p38
P-p38
JNK1
P-JNK2
P-JNK1
1 2 3 4 5 6 7 8 9 10 11 12
B
HTO-L
TE51
Sorb.
NT
Dox+
p38
P-p38

lanes 8 and 12). In an effort to ensure that the activation of p38 was due to the biochemical effects of ICP27 expression and not simply due to the over expression of an induced protein, we compared p38 phosphorylation in induced HTO-L and TE51 cells. While both cell lines showed strong p38 phosphorylation following sorbitol treatment, only TE51 cells showed p38 phosphorylation after doxicycline induction (Fig. 3.5 B). These results show that ICP27 expression in HeLa cells is sufficient for the phosphorylation of p38 MAPK.

The observation that ICP27 expression alone activates p38 signaling but is apparently insufficient to activate JNK signaling is interesting in light of the results of Hargett et al. (56, 57), who showed that ICP27 is required for JNK activation during infection. However, it is possible that the JNK immunoblotting assay is not sensitive enough to detect low levels of JNK phosphorylation in a subset of the total cell population. Therefore, to further investigate the effect of ICP27 expression on JNK activation, we used immunofluorescent microscopy to look at JNK phosphorylation in cells expressing ICP27. Little or no phospho-JNK was seen in untreated TE12 and TE51 cells or in control HTO-H cells in the presence of doxicycline (Fig. 3.6 A and data not shown). Consistent with the immunoblot results, sorbitol induction and WT HSV-1 infection led to intense nuclear staining for phospho-JNK, indicative of JNK phosphorylation and translocation to the nucleus (Fig. 3.6 B and C). Interestingly, some phosphorylated JNK was seen in TE12 and TE51 cells that had been treated with doxicycline, and its presence correlated with ICP27 expression (Fig 3.6 D and data not shown). However, the overall level of staining was lower than in

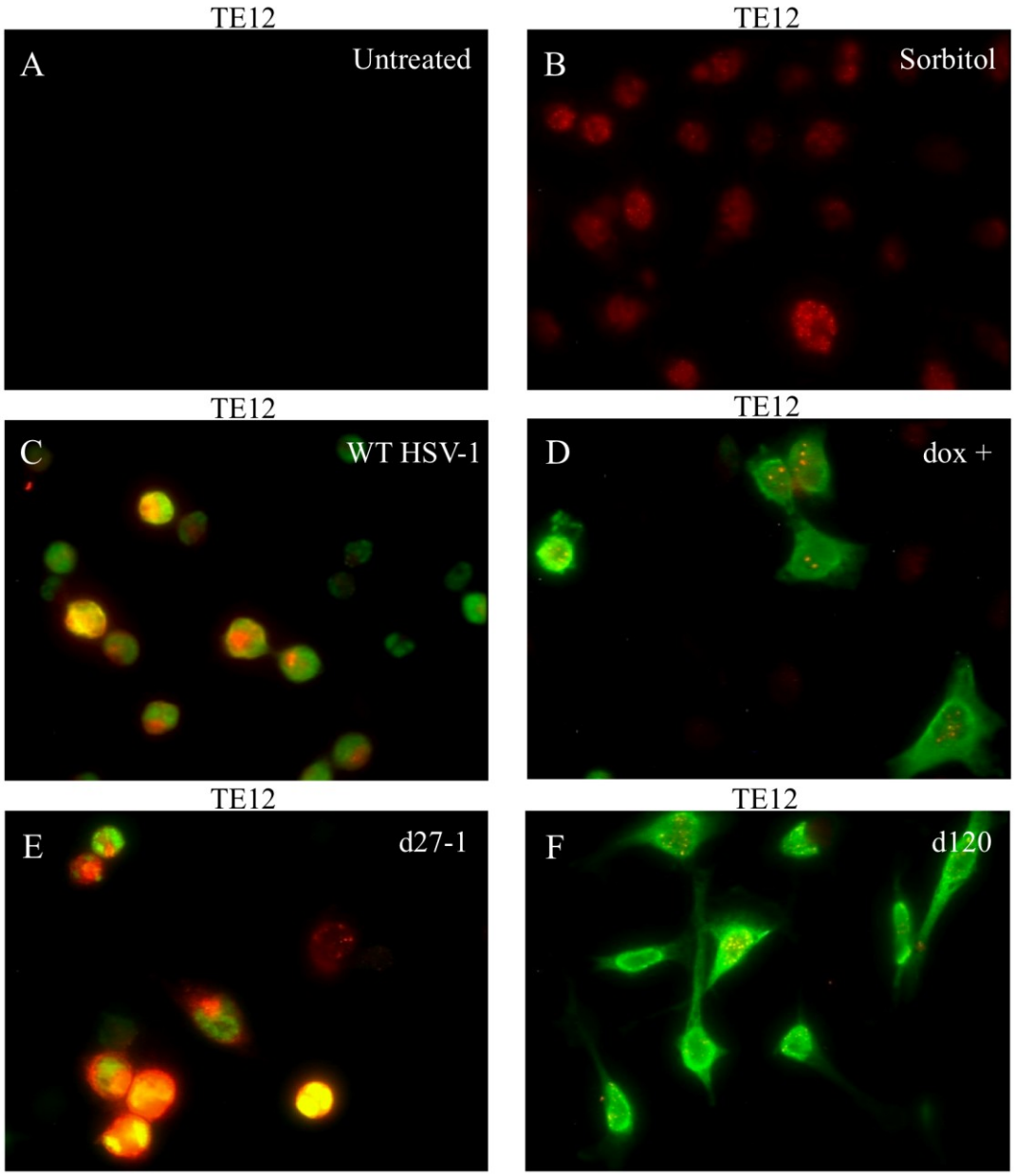
sorbitol-treated or infected cells and the staining pattern showed nuclear speckles of phosphorylated-JNK instead of the intense diffuse staining pattern. Immunofluorescent staining with an anti-PML antibody indicated that these speckles did not correspond to ND10 domains (data not shown). Thus, it appears that ICP27 expression alone is sufficient to induce some JNK phosphorylation, but the levels are reduced compared to that seen during viral infection. This suggests that additional HSV-1 factors may be required for the full phosphorylation observed during infection.

To further investigate whether factors other than ICP27 contribute to JNK phosphorylation during infection, we infected HTO-H, TE12 and TE51 cells with KOS 1.1, d27-1, and d120 (an ICP4 deletion mutant) and compared phosphor-JNK levels at 8 hpi using immunofluorescent microscopy. As expected, HTO-H cells infected with d27-1 virus show little if any JNK phosphorylation (data not shown), consistent with prior results that indicated ICP27 is required for JNK activation (56, 57). In contrast, high levels of nuclear phosphorylated JNK were seen in both KOS and d27-1-infected TE51 cells (data not shown and Fig. 3.6 E). Cells infected with d120 virus, however, which lacks the ICP4 gene required for DE and L gene expression, displayed less intense nuclear phosphorylated-JNK staining (Fig. 3.6 F). Interestingly, the d120-infected cells showed a speckling pattern similar to that seen in cells expressing ICP27 (Fig. 3.6 D). Similar results were seen in TE51 cells (data not shown). These results suggest that, in addition to ICP27, at least one other viral factor (ICP4 or a DE/L gene product) is

Fig. 3.6. ICP27 expression leads to phosphorylation of JNK.

Immunofluorescence analysis of JNK activation in cells expressing ICP27. (A) TE12 cells were left untreated or (B) treated with sorbitol for 30 min, (C) infected for 8 h with WT HSV-1 (MOI 10), (D) treated with doxicycline (2μg/ml for 24 h), TE12 cells were also (E) infected for 8 h with d27-1, or (F) infected for 8 h with d120. Infections were performed at an MOI of 10. Following treatment, cells were fixed and processed using antibodies specific for phosphorylated JNK1/2 (red signal) and ICP27 (green signal).

Fig. 3.6


TE12
A
Untreated
TE12
B
Sorbitol
TE12
C
WT HSV-1
TE12
D
dox +
TE12
E
d27-1
TE12
F
d120

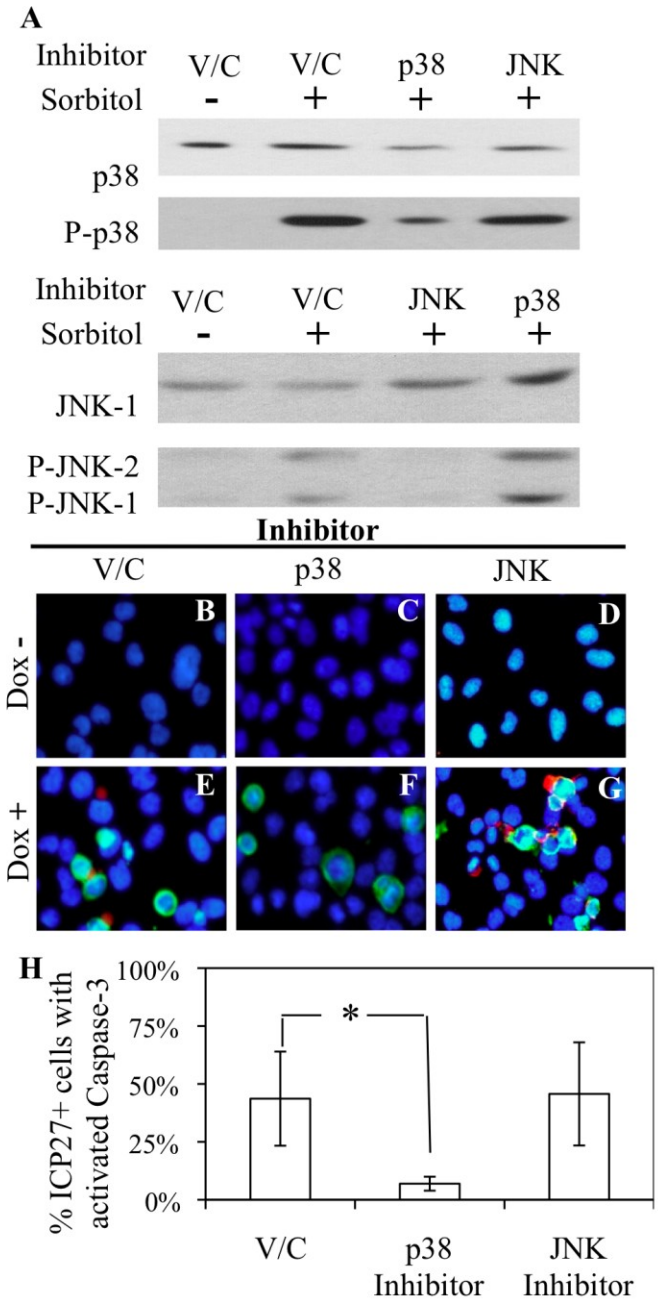
required for full induction of JNK phosphorylation. This experiment further strengthens the conclusion that ICP27 is only partially sufficient for the activation of JNK signaling during infection.

ICP27-mediated activation of p38 signaling leads to apoptosis.

As mentioned above, previous research has associated HSV-1 induced JNK signaling with NF-κB activation during infection (57). Additionally, other work has shown the importance of NF-κB activation in preventing apoptosis during infection (32). Therefore, we hypothesized that in TE12 and TE51 cells, ICP27-mediated activation of p38 signaling in the absence of strong JNK activation results in apoptosis. To test this, we conducted a series of experiments using pharmacological inhibitors of p38 and JNK signaling. First, we verified the activity of the inhibitors, SB203580 and SP600125 (p38 and JNK inhibitors, respectively), by adding them to cells 30 minutes prior to sorbitol-induced stress. Cells were then lysed after 30 min of sorbitol exposure and SAPK activation was examined by immunoblot analysis. Fig. 7A shows significant and specific reductions in SAPK activation, although the p38 inhibition did not completely prevent phosphorylation of p38. We next asked whether treatment with JNK and p38 inhibitors could suppress the apoptosis induced by ICP27 expression. We induced expression of ICP27 in the TE12 and TE51 cell lines in the presence or absence of SB203580 and SP600125 for three days. The cells were then fixed and stained for activated caspase-3 and examined using immunofluorescent microscopy. In uninduced TE51 cells, where no ICP27

Fig. 3.7. Treatment with p38 inhibitors prevents ICP27 induced apoptosis. (A) Immunoblot analysis of SAPK phosphorylation in HTO-H cells. Cells were either left untreated or treated with sorbitol for 30 min. Cells were then treated with vehicle control (DMSO), p38 inhibitor (SB203580 at 5 μM), or JNK inhibitor (SP600125 at 1μM) for 30 min as indicated. Following treatment, protein extracts were collected and analyzed by immunoblotting for p38, phosphorylated p38, JNK1, and phosphorylated JNK1/2. (B-G) Immunofluorescence analysis of caspase-3 activation in cells expressing ICP27 treated with inhibitors. Uninduced TE51 cells were treated with DMSO (B), p38 inhibitor (C), and JNK inhibitor (D). TE51 cells induced with doxicycline (2 μg/ml) were treated with DMSO (E), p38 inhibitor (F), and JNK inhibitor (G). Cells were fixed and processed 3 h post treatment using antibodies specific for activated caspase-3 (red-Cy3) and ICP27 (green-Cy2). (H) Enumeration of the percent of ICP27-positive cells staining positive for activated caspase-3 following treatment with vehicle control, p38 inhibitor, and JNK inhibitor (>1200 cells counted for each condition) (* $p < 0.05$).

Fig. 3.7

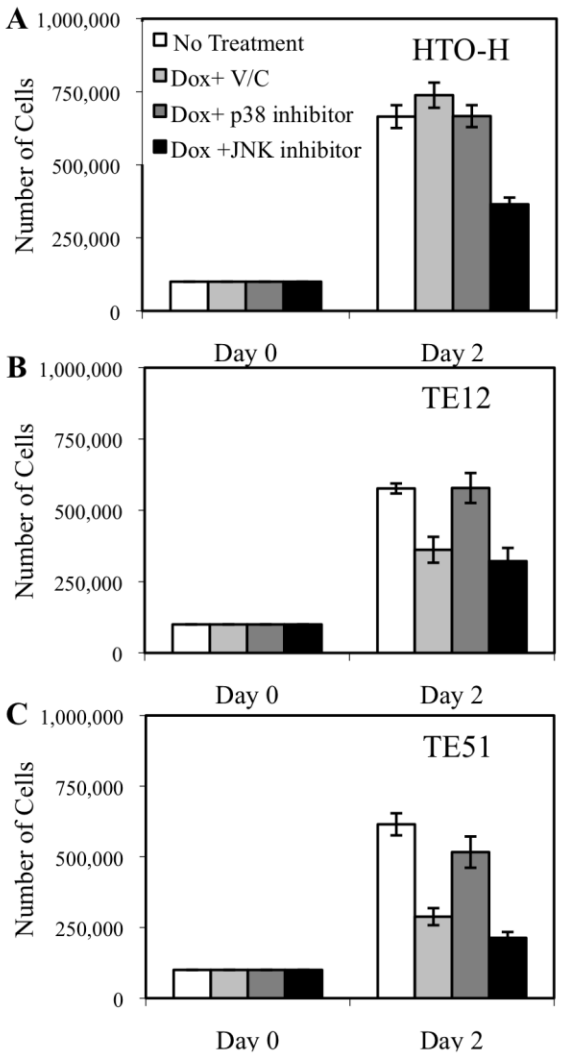

A
Inhibitor
Sorbitol
V/C
V/C
p38
JNK
-
+
+
+
p38
P-p38
Inhibitor
Sorbitol
V/C
V/C
JNK
p38
-
+
+
+
JNK-1
P-JNK-2
P-JNK-1
Inhibitor
V/C
p38
JNK
Dox -
Dox +
B
C
D
E
F
G
H
% ICP27+ cells with activated Caspase-3
100%
75%
50%
25%
0%
*
V/C
p38 Inhibitor
JNK Inhibitor

was expressed, vehicle control treatment with DMSO as well as p38 and JNK inhibitor treatment led to little or no activation of caspase-3 (Fig. 3.7 B-D). As expected, induction of ICP27 by doxicycline led to significant apoptosis in the vehicle control-treated cells (Fig. 3.7 E). Treatment of induced cells with JNK inhibitor also had little or no effect on caspase-3 activation (Fig. 3.7 G, H). Cells treated with p38 inhibitor, however, showed a significant reduction in apoptosis based on caspase-3 activation and nuclear morphology (Fig. 3.7 F, H). Similar overall results were observed in TE12 cells (data not shown). Thus, p38 activation appears to be necessary for ICP27-induced apoptosis in HeLa cells.

The observation that p38 inhibitor treatment prevents apoptosis induced by ICP27 led us to ask if p38 inhibition could also restore normal growth to TE12 and TE51 cells after induction of ICP27 expression. To examine this, we conducted a cell growth assay in the presence and absence of SAPK inhibitors. Equal numbers of HTO-H, TE12, and TE51 cells were plated, and inhibitors were added to the media prior to the induction of ICP27 expression. Cells were counted two days post induction and compared to uninduced and induced cells that had not been treated with inhibitors. We noted that the JNK inhibitor had a negative effect on all cell lines including HTO-H cells, consistent with data that indicate JNK signaling is required for HeLa cell proliferation (62). While treatment of HTO-H cells with p38 inhibitor had little or no effect on cellular growth (Fig. 3.8 A), it almost fully restored growth to TE12 and TE51 cells following two days of induced expression (Fig. 3.8 B, C). These data suggest that ICP27-mediated p38 activation leads to apoptosis and is responsible for the

Fig. 3.8. Cell growth following SAPK inhibitor treatment. Equal numbers of HTO-H (A), TE12 (B), and TE51 (C) cells were plated in triplicate in the presence or absence of doxicycline (2 μg/ml). Cells were treated upon induction with vector control (DMSO), p38 inhibitor (SB203580 at 5 μM), or JNK inhibitor (SP600125 at 1μM). Cells were harvested and counted 2 days post induction/treatment using a hemacytometer.

Fig. 3.8


A
1,000,000
750,000
500,000
250,000
0
Number of Cells
No Treatment
Dox+ V/C
Dox+ p38 inhibitor
Dox +JNK inhibitor
HTO-H
Day 0
Day 2
B
1,000,000
750,000
500,000
250,000
0
Number of Cells
TE12
Day 0
Day 2
C
1,000,000
750,000
500,000
250,000
0
Number of Cells
TE51
Day 0
Day 2

defect in cellular growth that we observe in TE12 and TE51 cells following ICP27 induction.

Discussion

ICP27 expression activates p38 signaling.

Previous reports using viral deletion mutants have shown that ICP27 expression is required during HSV-1 infection for p38 activation (27, 56, 57). The study presented in this paper, however, is the first to show that ICP27 expression alone is sufficient for the activation of p38 signaling. The importance of p38 activation during infection has been shown by previous research which demonstrated that inhibition of p38 signaling leads to a significant defect in HSV-1 replication (76). Signaling through p38 could potentially have a host of effects on viral replication and cellular function. Research from Corcoran et al. has shown that HSV-1-mediated p38 signaling is required for stabilization of certain cellular transcripts which have AU-rich instability elements in their 3' UTRs (27). Additionally, p38 signaling can lead to global upregulation of translation by activating MNK 1/2 (43, 178). In fact, Walsh and Mohr have shown that HSV-1-mediated activation of p38 leads to MNK1 activation and the stimulation of viral protein synthesis (176). Activated p38 can also translocate to the nucleus where it is able to activate a range of transcription factors leading to increased cytokine expression, apoptosis, cell growth, or differentiation (170). Interestingly, a recent study has shown that p38 signaling during HSV-1 infection of T cells is required for IL-10 synthesis, which could help lead to the induction of immunological tolerance (153).

The mechanism behind ICP27-mediated activation of p38 remains to be characterized. Previous studies have shown that the activation of p38 and JNK in HSV-1 infected cells appears to occur downstream of Ras (98). Additional studies have identified regions of ICP27 that are required for p38 activation (27, 56), but little is known about the method of activation. The differential activation of JNK and p38 observed in this paper argues against a shared mechanism of signaling activation. Therefore it is not likely that the activation is occurring through cellular receptors for oxidative stress, UV exposure, or surface receptors for cytokine signaling, e.g. Fas, since these signal receptors activate both pathways, as occurs following sorbitol treatment (164). This could point towards a potentially novel mechanism for p38 activation by ICP27. It is possible that the known effects of ICP27 on cellular gene expression could play a role, including its reported ability to inhibit pre-mRNA splicing (18, 27, 54, 55, 140, 164). Further research, however, will be required to identify the mechanism involved. The inducible cell lines described here could be useful tools in such studies.

ICP27 expression does not fully activate JNK signaling.

In our experiments, we observed some activation of the JNK pathway by ICP27 based on the levels of phospho-JNK. However, it did not appear to be as robust as the activation seen during HSV-1 infection. Interestingly the phosphorylated JNK induced by ICP27 accumulated in faint nuclear speckles, a pattern that is quite distinct from the robust nuclear staining seen during normal HSV-1 infection. The speckles are reminiscent of the activated JNK speckles

that have been reported during VZV infection (187). Our findings are significant since prior reports have shown that during infection ICP27 is required for robust JNK activation (56, 57). Together, the data suggest that an additional DE or L gene is required to fully activate JNK signaling. The incomplete activation of JNK we describe is consistent with several previous observations. Most recently, Hargett et al. showed that during infection, ICP27 expression initiated NF-κB activation and ICP4 expression further increased it (69). In the same study, evidence was provided that activation of NF-κB is due in part to ICP27-mediated activation of JNK signaling. Furthermore, Goodkin et al. have previously shown that a DE or leaky-late gene expressed between 3 and 6 hours post infection is required for NF-κB activation (47). Finally, research has shown that the regions of ICP27 that are responsible for nuclear export and RNA binding are required for JNK activation, which suggests that ICP27's role may be at least in part to transactivate the expression of a key viral gene product (27, 56). These previous findings along with the results of this study strongly argue that there are one or more ICP27-dependent DE or L genes that together with ICP27 lead to full JNK activation during infection. Further research is needed to identify the relevant viral gene or genes responsible for full activation.

ICP27-mediated activation of p38 signaling leads to apoptosis.

Our studies indicate that in HeLa cells ICP27-mediated activation of p38 signals leads to activation of the cellular apoptotic response. This finding not only explains the loss of expression observed in our cell lines over extended

periods of ICP27 expression, but also may help explain why ICP27 genes introduced into cells by stable transfections are generally silenced (S. Rice, unpublished). The findings of this study also shed light on the complex regulation of apoptosis by HSV-1 factors. Research has shown that HSV-1 blocks apoptosis and alters p38 signaling during infection (186). Sanfilippo et al. showed that IE gene expression is required for apoptosis induction (142) and further work by these investigators implicated the ICP0 mRNA (141). In this work, we found that expression of ICP27 can induce apoptosis, as well as sensitize cells to staurosporine-induced apoptosis. Thus, it appears that ICP27 is also a pro-apoptotic factor. This is in contrast to previous reports which have shown that ICP27 prevents apoptosis during HSV-1 infection (8). However, previous work was carried out in the context of infection and thus could not differentiate between direct and indirect effects of ICP27. Our finding that ICP27 expression is not sufficient for inhibition of induced apoptosis agrees with work by Aubert et al. that showed ICP27 expressed from transiently transfected plasmids is unable to block staurosporine induced apoptosis (10).

In our HeLa cell system, ICP27 expression is induced after 8 h of doxicycline treatment, but the resulting ICP27-dependent cytopathology and apoptosis are not evident for 2-3 d. These somewhat slow kinetics presumably reflect both the need for ICP27 to build up to critical levels and the specific mechanism, currently unknown, by which it stimulates p38 signaling and apoptosis. Regarding the induction of apoptosis, it is possible that effects on Bcl-2 are involved, as Zachos et al. have shown that activation of p38 signaling leads

to destabilization of Bcl-2 resulting in apoptosis during infection with ICP27 mutant viruses (186). Additionally, work by Galvan et al. showed that over expression of Bcl-2 is able to prevent apoptosis associated with the expression of IE proteins (42). Thus, in our system, expression of ICP27 activates p38, which without additional viral factors could lead to a loss of Bcl-2, resulting in sensitization to or induction of apoptosis. Obviously, further work is required to delineate the pathway by which ICP27 leads to apoptosis.

NF-κB activation has been shown to play a critical role in inhibiting cellular apoptotic pathways during HSV-1 infection (47). Interestingly, NF-κB activation during infection has been associated with ICP27-mediated JNK signaling (57), which our work suggests involves the expression of DE or L gene(s) along with ICP27. This fits well with findings from Aubert et al., who showed that DE and L gene expression are required for the prevention of apoptosis (11). Thus, it seems likely that during HSV-1 infection, ICP27 directly induces pro-apoptotic signaling early during infection, along with other IE factors such as the ICP0 mRNA (141). However, this induction is later suppressed by the effects of one or more ICP27-dependent DE or L gene products.

Based on our work and previous studies, we propose the following model to explain the relationship between ICP27-mediated SAPK signaling and the regulation of apoptosis during HSV-1 infection. First, ICP27 directly leads to activation of p38 through an as yet uncharacterized interaction with cellular signaling components. The p38 signaling triggers the host cell apoptotic response through an as yet unknown mechanism. Second, ICP27, along with

ICP4, mediates the expression of one or more DE or L gene products that are responsible for the activation of robust JNK signaling. The activation of JNK signaling subsequently leads to NF-κB activation. Finally, NF-κB activation leads to the expression of host cell anti-apoptotic factors that serve to block host cell death. Further work is certainly needed to test the various aspects of this model. A central feature of this model is that ICP27, through its direct and indirect effects on SAPK signaling pathways, is a key regulator of cell fate during HSV-1 infection.

Chapter 4

HSV-1 ICP27 mediated JNK signaling is required for efficient viral gene expression, NF-κB activation, and apoptosis inhibition

Introduction

Previously, we demonstrated that HSV-1 ICP27, in the absence of a viral infection, leads to a modest activation of the c-Jun N-terminal kinase (JNK) signaling pathway, but that at least one additional viral factor is required to phosphorylate JNK to the high levels observed during infection (44). Additionally, we showed that ICP27 fully activates p38 signaling in the absence of viral infection, leading to apoptosis (44). Prior research by others has indicated that NF-κB activation inhibits apoptosis during HSV-1 infection (47) and that JNK signaling is required for this activation to occur (57). This led us to hypothesize NF-κB activation/apoptosis inhibition requires ICP27 acting along with at least one other as yet unidentified viral factor.

In this work, we sought to determine if ICP27-induced JNK signaling is sufficient for the activation of NF-κB during infection, and if not, to identify the additional viral factor(s) that is(are) required for NF-κB activation and the associated inhibition of apoptosis. We found that expression of ICP27, in the absence of viral infection, is insufficient to activate NF-κB. Moreover, we identified an ICP27-dependent L gene product, UL37, that is capable of activating NF-κB and delaying the onset of apoptosis during infection with an ICP27 null mutant. Our results suggest that during viral infection, UL37 acts as

one of the ICP27-dependent anti-apoptotic factors that inhibit apoptosis triggered by HSV-1 infection.

Results

ICP27 expression alone does not activate NF-κB.

We previously showed that ICP27 expression in the absence of a viral infection strongly activates p38 signaling, and induces a modest level of JNK signaling (44). Our findings using an ICP4 null mutant indicated that at least one additional viral factor is required to fully activate JNK signaling to the high levels observed during HSV-1 infection. Prior research by Hargett et al. has shown that ICP27-induced JNK signaling is required for NF-κB activation during infection (57). Together these studies suggest that either ICP4 and/or a DE or L gene product(s) is required along with ICP27 for robust JNK signaling and subsequent activation of NF-κB.

The only prior report of NF-κB activation following HSV- infection in HeLa cells is from a study by Gimble et al. that showed HSV-1 activates transcription of the HIV LTR via NF-κB (45). Therefore, in order to show that NF-κB is activated in HeLa cells during infection, we used immunofluorescent microscopy to determine if the p65 subunit of NF-κB translocates to the nucleus following HSV-1 infection. In uninduced, mock-infected HTO-L or TE12 cells, which are HeLa cells that have a doxicycline-inducible luciferase or ICP27 gene respectively, NF-κB p65 is located primarily in the cytoplasm (data not shown and Fig. 4.1 D). In contrast, in HTO-L and TE12 cells infected with WT HSV-1, the NF-κB p65 subunit was localized to the nucleus in almost every infected cell (Fig. 4.1 A and E). However, the nuclear translocation of p65 subunit did not occur in HTO-L cells infected with an ICP27 null mutant, d27-1 (Fig. 4.1 B), consistent with the

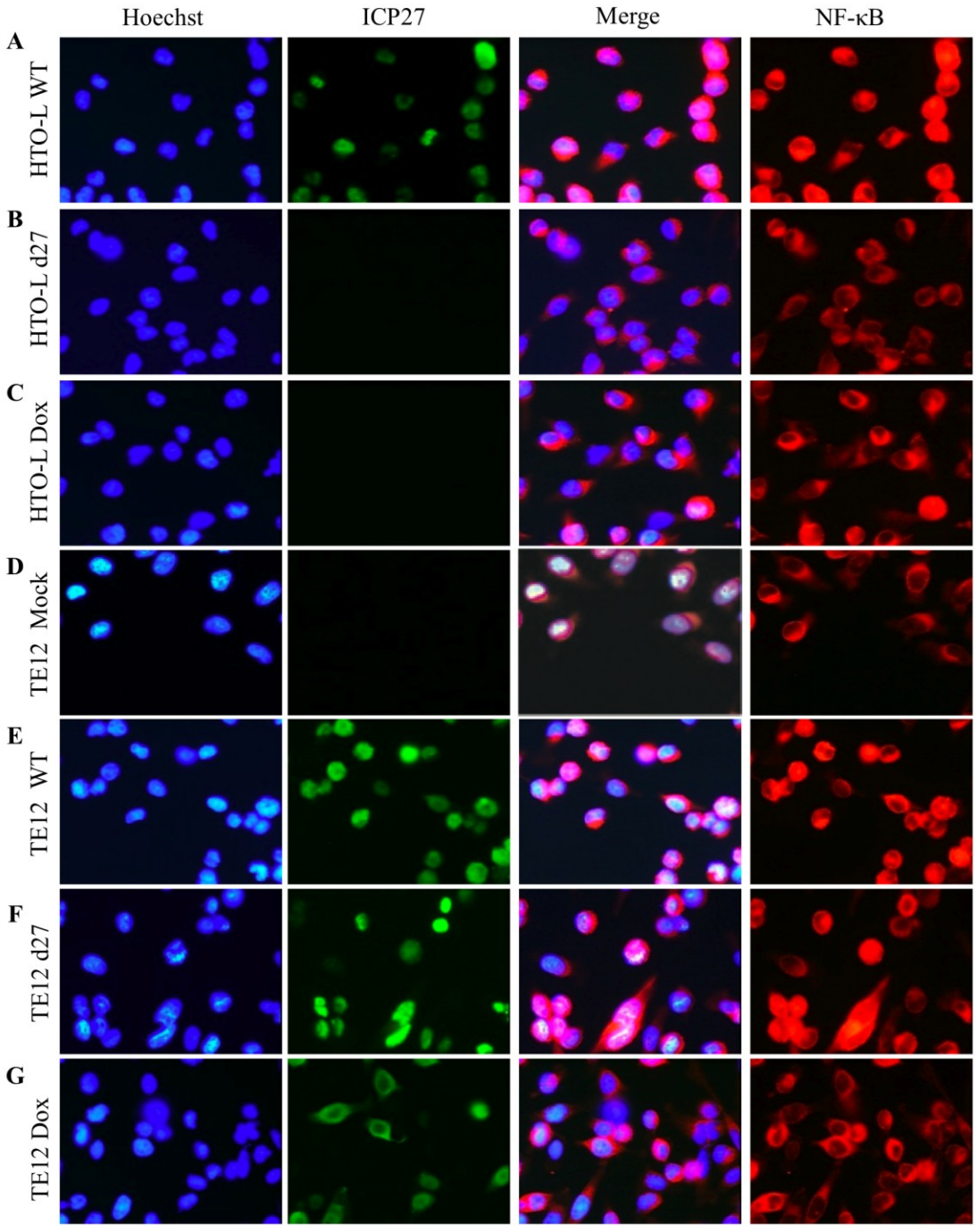
previous findings that showed ICP27 expression is required for activation of NF-κB during HSV-1 infection (57). As expected, in the TE12 cells, the NF-κB activation defect of d27-1 is complemented by the stably transfected ICP27 gene, resulting in a restoration of nuclear translocation of the p65 subunit (Fig. 4.1 F). These results demonstrate that ICP27 expressed in our inducible cell lines is capable of activating NF-κB during viral infection.

Based on our previous observation that ICP27 expression in the absence of viral infection is pro-apoptotic, and that an additional viral factor(s) besides ICP27 is required for the robust activation of JNK, we suspected that ICP27 expression alone is insufficient for NF-κB activation. To test this, we induced ICP27 expression in TE12 cells and observed the localization of the p65 subunit. Little to no nuclear translocation of the p65 subunit was observed in either the control HTO-L cells or TE12 cells following doxicycline induction (Fig. 4.1 C and G). To quantitate this, immunofluorecently stained cells were counted and no significant difference was observed between the localization of the NF-κB p65 subunit in the TE12 cell line compared to HTO-L cells following doxicycline treatment (Table 4.1). These results strongly suggest that ICP27 expression is insufficient to induce NF-κB activation. Thus, similar to our previous observation on JNK signaling, the activation of NF- κB during viral infection, while being ICP27 dependent, is likely to require one or more additional viral factor(s).

JNK signaling is a critical factor in NF-κB activation.

Fig. 4.1. Induced expression of ICP27 does not lead to nuclear translocation of the NF-κB p65 subunit. Immunofluorescence analysis of cellular localization of the NF-κB p65 subunit in cells following mock, WT, or d27-1 infection or doxicycline treatment. HTO-L cells were infected with KOS 1.1 (A) or d27-1 (B) at an MOI of 5 and fixed at 8 hpi or treated with doxicycline (2 μg/ml) (C) and fixed 24 hour later. TE12 cells were mock infected (D), infected with KOS 1.1 (E) or d27-1 (F) at an MOI of 5 and fixed at 8 hpi or treated with doxicycline (2 μg/ml) (G) and fixed 24 hour later. Cells were stained using antibodies specific for the NF-κB p65 subunit (red) and ICP27 (green). Nuclei were stained using Hoechst dye (blue). Representative figures are shown.

Fig. 4.1


Hoechst
ICP27
Merge
NF-κB
A
HTO-L WT
B
HTO-L d27
C
HTO-L Dox
D
TE12 Mock
E
TE12 WT
F
TE12 d27
G
TE12 Dox

After observing that ICP27 expression alone does not lead to the translocation of the NF-κB p65 subunit, we wanted to ensure that JNK signaling is required for the activation of NF-κB during HSV-1 infection of HeLa cells. SAPK signaling pathways are highly sensitive and can lead to multiple cellular responses based on cell type, signal source, and activation of additional signaling pathways (15, 65, 130, 180). To assess the importance of JNK signaling in the activation of NF-κB in HeLa cells, we infected HeLa cells with KOS1.1 in the absence or presence of the JNK inhibitor SP600125 and used immunofluorescent microscopy to observe the cellular localization of the NF-κB p65 subunit at 8 hpi. While high levels of NF-κB were seen in the nucleus during infection of untreated cells (Fig. 4.2 A), the NF-κB p65 subunit was almost exclusively cytoplasmic during infection of treated cells (Fig. 4.2 B). In fact, the localization of the NF-κB p65 subunit is nearly indistinguishable from that observed in mock infected cells (Fig. 4.2 C). Thus, the nuclear translocation of the NF-κB p65 subunit following infection is dependent on JNK signaling.

To confirm the above data and further quantitate the effects of JNK signaling on NF-κB activation, we assessed the consequences of JNK inhibition on the levels of IκBα, the inhibitor of NF-κB. In order for NF-κB to become activated, IκBα is first phosphorylated by cellular kinases. Following phosphorylation, IκBα disassociates from NF-κB and is targeted for rapid degradation by the proteosome. HeLa cells were either mock-infected or infected with KOS 1.1 at an MOI of 5 in the presence of increasing levels of SP600125

Table 4.1. Quantitation of nuclear translocation of NF-κB p65 subunit.

Enumeration of the percent of ICP27-negative and ICP27-positive HTO-L and TE12 cells with nuclear staining for the NF-κB p65 subunit following mock, WT, or d27-1 infection or treatment with doxicycline (>100 cells counted for each condition)

Table 4.1

HTO-L

	ICP27 Negative Cells % Nuclear	ICP27 Positive Cells % Nuclear
Mock	8.3	0
WT	0	95.6
d27-1	8.9	0
Dox	11.0	0

TE12

	ICP27 Negative Cells % Nuclear	ICP27 Positive Cells % Nuclear
Mock	9	0
WT	0	72.0
d27-1	0	60.0
Dox	17.9	13.8

(0, 5, and 20 μM), and proteins were harvested at 8 hpi. The samples were analyzed by immunoblot analysis for both phosphorylated-JNK and IκBα (57) (Fig. 4.2 D). ImageJ analysis was used to quantitate the blots from three separate infections. Sample protein levels were normalized to the loading control β-actin. The results are presented in Fig. 4.2 E as percentages of IκBα relative to mock infection and P-JNK1 relative to WT infection.

The immunoblots show that as expected JNK inhibition had little or no effect on IκBα levels in mock-infected cells (Fig. 4.2 D lanes 1-3). During infection, however, there was an inverse relationship between the level of JNK phosphorylation and the amount of IκBα remaining (Fig. 4.2 D lanes 4-6), suggesting that JNK signaling is required for much of the NF-κB activation. Treatment with the lowest level of inhibitor resulted in a significant reduction in JNK phosphorylation, and modest increases in the level of IκBα. Furthermore, the highest dose of inhibitor led to undetectable levels of JNK phosphorylation in 2 out of 3 samples. Interestingly, samples treated with the highest dose of inhibitor (Fig 4 D lane 6) still underwent a significant loss of IκBα when compared to mock infected samples (lanes 1-3) (Fig. 4E). This suggests that during HSV-1 infection some loss of IκBα might occur independently of JNK signaling. Additionally, in this experiment, we analyzed cells infected with the ICP27 null mutant d27-1 and the ICP4 null mutant d120. As expected, cells infected with the d120 virus show a significant induction of JNK phosphorylation (Fig. 4.2 D lane 8), whereas those infected with d27-1 did not (lane 7). However, both d27-1 (lane 7) and d120 (lane 8) infections displayed a significant loss of IκBα

compared to mock infected cells. While the loss of IκBα was not nearly as large as that observed during WT infection (lane 4), the levels of IκBα were similar to that observed during WT infection following high dose JNK inhibitor treatment (lane 6). These combined results suggest that the ICP27-mediated JNK signaling is required for NF-κB activation.

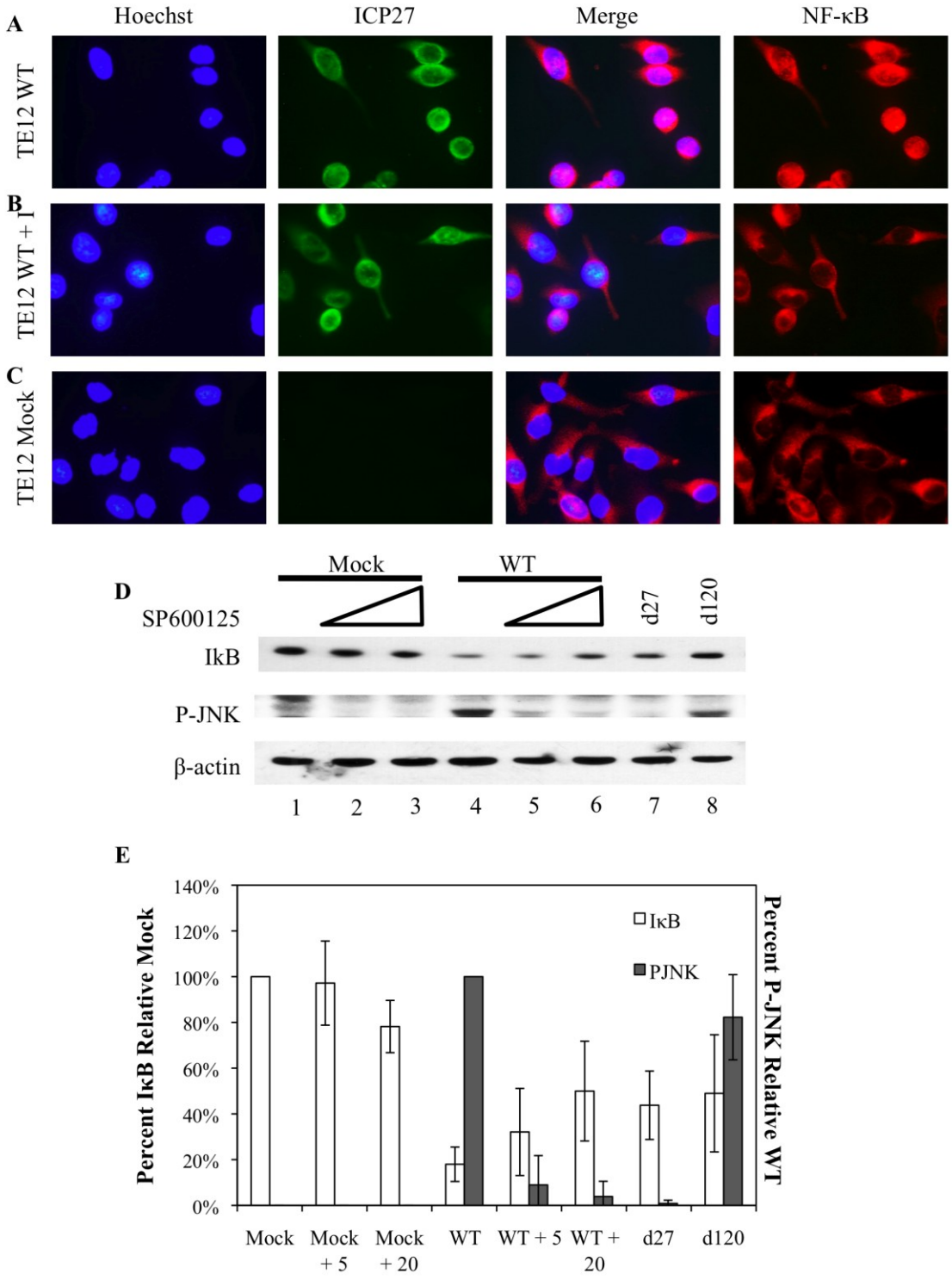
These findings confirm the previous results from Hargett et al. which showed that ICP27 expression and the resulting JNK signaling are required for activation of NF-κB during HSV-1 infection (57). However, it appears likely that there is a pathway independent of JNK signaling that contributes to the loss of IκBα following HSV-1 infection. This was unexpected since previous findings have shown that, in CV-1 cells, JNK inhibitor treatment completely prevents the loss of IκBα during HSV-1 infection (57). However, the combined observations that ICP27-mediated JNK signaling is required but not sufficient for NF-κB activation fits well with a model where DE and/or L gene expression is required for the inhibition of apoptosis in infected cells. Several lines of evidence have shown that in addition to NF-κB activation by viral infection, the expression of DE or L gene products at 3-6 hpi is required for the inhibition of apoptosis (9, 11, 47).

JNK phosphorylation is required for the efficient expression of DE and L viral proteins.

Our results suggest that ICP27-induced JNK signaling as well as at least one additional viral factor are required for NF-κB activation during HSV-1 infection. Additionally, previous observations by others have found that JNK

Fig. 4.2. JNK signaling is required for activation of NF-κB in infected HeLa cells. Immunofluorescence analysis of cellular localization of the NF-κB p65 subunit in HeLa cells treated with JNK inhibitor (SP600125 at 5 μM) following mock (A), WT (B), or d27-1 (C) infection at an MOI of 5. Cells were fixed and processed at 8 hpi. Cells were stained using antibodies specific for the NF-κB p65 subunit (red) and ICP27 (green). Nuclei were stained using Hoechst dye (blue). Representative figures are shown. (D) Immunoblot analysis of IκB levels and JNK phosphorylation in HeLa cells. Cells were either mock infected (lanes 1-3) or infected with KOS1.1 (MOI 5) (lanes 4-6) in the presence of increasing concentrations of SP600125 (0, 5, 20 μM) as indicated. Cells were also infected at an MOI of 5 with ICP27 deletion mutant d27-1 (lane 7) and ICP4 deletion mutant d120 (lane 8). Protein extracts were collected at 8 hpi and analyzed by immunoblotting for IκB, phosphorylated JNK1/2, and β-actin. (E) Quantitation of three sets of immunoblot analyses of IκB and phosphorylated JNK1 levels using NIH ImageJ analysis. Levels of IκB are expressed as percent remaining relative to mock-infected samples. Levels of P-JNK1 are expressed as percent P-JNK1 relative to WT infected cells.

Fig. 4.2


A
Hoechst
ICP27
Merge
NF-κB
TE12 WT
B
TE12 WT + I
C
TE12 Mock
D
Mock
WT
d27
d120
SP600125
IkB
P-JNK
β-actin
1 2 3 4 5 6 7 8
E
Percent IκB Relative Mock
Percent P-JNK Relative WT
140%
120%
100%
80%
60%
40%
20%
0%
IκB
PJNK
Mock
Mock + 5
Mock + 20
WT
WT + 5
WT + 20
d27
d120

inhibitor treatment significantly reduces viral yield following wild type HSV-1 infection (98). Furthermore, as discussed above, DE and L genes are required for inhibiting apoptosis. Therefore, we hypothesized that JNK signaling plays a role in inducing DE and L gene expression, and that the resulting DE and L gene products are responsible for activation of NF-κB and the inhibition of apoptosis. In order to test this hypothesis, HeLa cells were infected with KOS 1.1 in the presence of increasing doses of SP600125 (0, 5, and 20 μM). For comparison, cells were also infected with d27-1 or d120. Proteins from all infections were harvested at 8 hpi. Viral protein expression was characterized by immunobloting for DE (ICP8) and L (gD, VP5, and UL37) gene products.

Consistent with our hypothesis, inhibitor treatment led to a dose responsive decrease in the expression of all tested viral proteins compared to untreated wild type infection (Fig. 4.3 A, lane 1-3). As expected, expression of ICP8, gD, VP5, and UL37 is severely limited following d120 infection (Fig. 4.3 A, lane 5), since ICP4 is the major transactivator of DE and L gene expression. However, somewhat surprisingly, VP5 and UL37 were detected in the d120 infection. It is possible that this is due to detection of virion-associated proteins from the initial infection, since UL37 (tegument protein) and VP5 (capsid protein) are structural virion components. It was also somewhat surprising that the ICP27 null mutant showed reduced expression of all proteins tested (Fig. 4.3 A lane 4), including ICP8, which in Vero cells is not ICP27-dependent. This suggests that in HeLa cells ICP27 may play an even greater role in viral gene expression than it does in Vero cells.

In an effort to quantitate the reduction in viral protein expression, the above experiment was performed in triplicate and the resulting immunoblots were used to quantitate protein expression levels using ImageJ analysis. The results are shown in Fig. 4.3 B. Interestingly, all inhibitor treatments or deletion mutants resulted in a significant reduction in the expression of all viral proteins tested. Furthermore, high dose inhibitor treatment led to a reduction in viral protein expression that is similar to that observed during d27-1 infection. Therefore, it appears that JNK signaling is required for the efficient expression of viral DE and L gene products in HeLa cells.

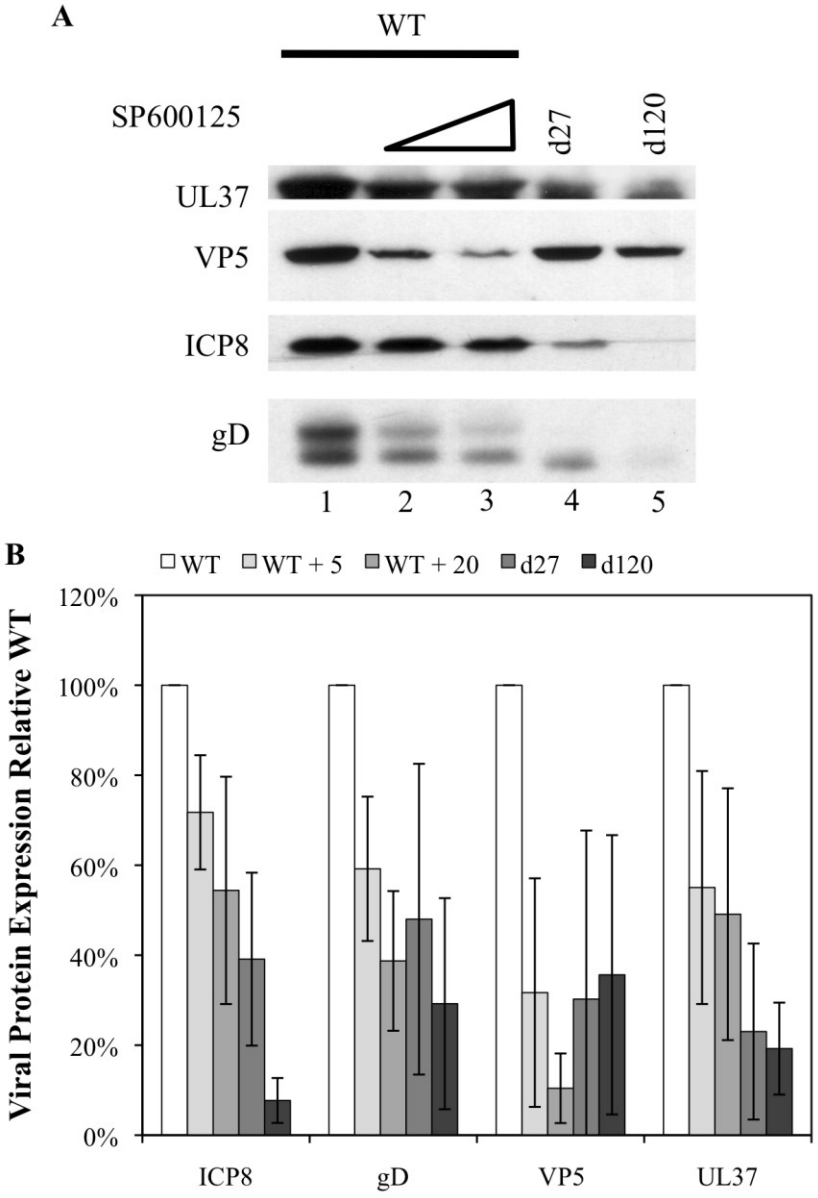
ICP27 mutants with defects in JNK signaling exhibit reduced UL37 expression.

Interestingly, the tegument protein UL37, encoded by a viral late gene, has recently been shown to activate NF-κB during infection (90, 147). UL37 triggers NF-κB activation in the cytoplasm through TRAF6 (90). The dependence of UL37 expression on JNK signaling, its ability to activate NF-κB, and the fact that it is an essential viral protein led us to explore the role of UL37 as a potential downstream factor of ICP27 in mediating apoptosis inhibition.

To determine whether UL37 expression is dependent on ICP27, and to study the possible relationship between UL37 and SAPK signaling, we used a panel of ICP27 N-terminal deletion mutants that have been previously shown to have a variety of defects in viral replication including reductions in SAPK

Fig. 4.3. JNK inhibitor treatment leads to reduced DE/L gene expression in HSV-1 infected cells. (A) Immunoblot analysis of UL37, VP5, ICP8, and gD expression. HeLa cells were infected with KOS1.1 (MOI 5) (lanes 1-3) in the presence of increasing concentrations of JNK inhibitor SP600125 (0, 5, 20 μM) as indicated. Cells were also infected at an MOI of 5 with ICP27 deletion mutant d27-1 (lane 4) and ICP4 deletion mutant d120 (lane 5). Protein extracts were collected at 8 hpi and analyzed by immunoblotting for UL37, VP5, ICP8, and gD. (B) Quantitation of three sets of immunoblot analysis for UL37, VP5, ICP8, and gD expression using NIH ImageJ analysis. Protein levels are expressed as percentage relative to untreated WT infected cells.

Fig. 4.3


A
WT
SP600125
d27
d120
UL37
VP5
ICP8
gD
1
2
3
4
5
B
WT
WT + 5
WT + 20
d27
d120
Viral Protein Expression Relative WT
120%
100%
80%
60%
40%
20%
0%
ICP8
gD
VP5
UL37

signaling (Table 4.2). Previous studies have shown that d1-2 is highly deficient in inducing both JNK and p38 signaling (27, 56, 57). However, the same studies produced conflicting data about d2-3, d3-4, and d4-5 and their ability to activate SAPK signaling during viral infection. Moreover, the ability of the mutants to induce JNK signaling in HeLa cells has never been tested. Therefore, we either mock-infected HeLa cells or infected them with KOS1.1, d1-2, d2-3, d3-4, d4-5, d5-6, or d6-7 at a MOI of 5. Proteins were harvested at 8 hpi and used for immunoblot analysis. We found that d1-2 was severely attenuated for JNK phosphorylation (Fig. 4.4 A, lane 3), whereas, d3-4 and d4-5 showed a slight reduction in JNK phosphorylation compared to wild type infection (Fig. 4.4 A lanes 5 and 6). The remaining viral mutants (Fig. 4.4 A lanes 4, 7, and 8) displayed levels of JNK phosphorylation that were comparable to those observed during WT infection (lane 1).

We also determined the level of IκB in the infected cells. Infection with the d1-2 mutant led to little or no loss of IκB following infection. Conversely, the mutants that lead to moderate or high levels of JNK phosphorylation display a reduction of IκB comparable to what is observed during wild type infection. Thus, it appears that amino acids 12-63 of ICP27 play a critical role in mediating JNK signaling in HeLa cells and that this ICP27-induced JNK signaling is required for the full NF-κB activation observed during WT HSV-1 infection.

We next wanted to determine if UL37 expression was reduced in the ICP27 mutants that displayed reduced JNK signaling compared to WT HSV-1.

Table 4.2. ICP27 N-terminal mutants differ in their ability to induce SAPK signaling. The results of two previous studies looking at SAPK signaling activation following infection by a series of N-terminal deletion mutants in either CV-1 or HeLa cells.

Table 4.2

Viral Mutant	Residues Deleted	p38 signaling		JNK Signaling	
		CV-1 [a]	HeLa [b]	CV-1 [a]	HeLa [b]
d1-2	12-63	-	-	-	N/D
d2-3	64-108	+	+/-	+	N/D
d3-4	109-138	+	-	+	N/D
d4-5	139-153	+/-	-	+	N/D
d5-6	153-173	+	+	+	N/D
d6-7	174-200	+	+	+	N/D

a Data from Hargett et al. (56, 57)
b Data from Corcoran et al. (27)
N/D= not determined

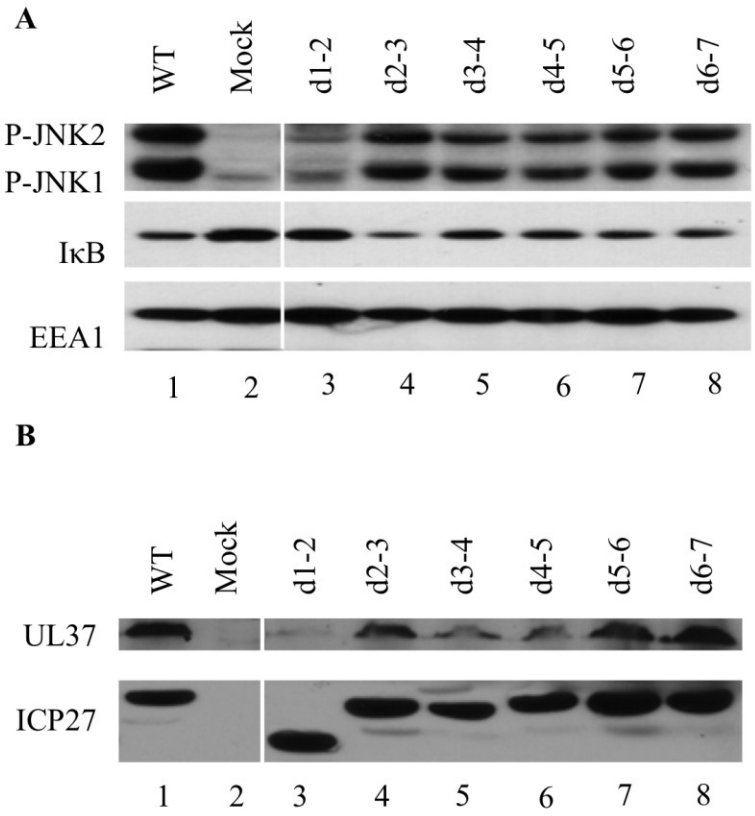
Using the lysates from mock, KOS1.1, d1-2, d2-3, d3-4, d4-5, d5-6, or d6-7 infections described above, we used immunoblot analysis to measure the expression of UL37. The level of ICP27 accumulation was similar in all samples indicating that cells are infected at roughly equivalent levels and that any changes in UL37 expression are unlikely to be attributable to different levels of ICP27 (Fig. 4.4 B lanes1 and 3-8). Interestingly, UL37 expression closely correlated with the level of JNK signaling observed during deletion mutant infections. In ICP27 deletion mutants d2-3, d5-6, and d6-7 (lanes 4, 7, and 8), which have similar levels of JNK phosphorylation and IκB compared to KOS1.1 (lane 1), UL37 was expressed at levels similar to that seen in the WT infection. Mutants d3-4 and d4-5 (lanes 5 and 6) exhibit moderate levels of JNK-phosphorylation and express decreased amounts of UL37. Finally, the d1-2 mutant, which leads to little or no JNK phosphorylation or NF-κB activation, expresses barely detectable levels of UL37. Therefore, based on the combined results of this and the inhibitor study, we conclude that expression of UL37 is both ICP27- and JNK signaling-dependent. Furthermore, amino acid residues 12-63 of ICP27 are required for JNK signaling, efficient expression of UL37, and activation of NF-κB.

ICP27-independent UL37 expression delays apoptosis during infection with an ICP27 null mutant.

The above observations show that ICP27 expression and subsequent JNK phosphorylation are required for the efficient expression of the UL37 protein.

Fig. 4.4. ICP27 N-terminal mutants show reduction in JNK signaling and UL37 expression. (A) Immunoblot analysis of IκB levels and JNK phosphorylation in HeLa cells following infection with a panel of N-terminal deletion mutants. HeLa cells were infected with KOS1.1 (MOI 5) (lane 1) or mock infected (lane 2). Cells were also infected at an MOI of 5 with ICP27 deletion mutants d1-2, d2-3, d3-4, d4-5, d5-6, or d6-7 (lanes 3-8). Protein extracts were collected at 8 hpi and analyzed by immunoblotting for levels of IκB and phosphorylated JNK. EEA1 served as a loading control. (B) Immunoblot analysis of UL37 and ICP27 expression in HeLa cells following infection with a panel of N-terminal deletion mutants. Lysates harvested from the infection in (A) were analyzed by immunoblotting for UL37 and ICP27.

Fig. 4.4


A
WT
Mock
d1-2
d2-3
d3-4
d4-5
d5-6
d6-7
P-JNK2
P-JNK1
IκB
EEA1
1 2 3 4 5 6 7 8
B
WT
Mock
d1-2
d2-3
d3-4
d4-5
d5-6
d6-7
UL37
ICP27
1 2 3 4 5 6 7 8

Furthermore, data from Liu et al. indicates that UL37 is capable of activating NF-κB (90), and activation of NF-κB has been shown to be critical for the inhibition of apoptosis during HSV-1 infection. However, there are no reports to date of UL37 functioning as a viral anti-apoptotic factor. This led us to hypothesize that UL37 could be a key ICP27-dependent anti-apoptotic factor during HSV-1 infection. In order to test this, we developed a HeLa cell line derivative that constitutively expresses UL37. To engineer this cell line, we transfected HeLa cells with an UL37 expression plasmid, pXL-37V5 (a generous gift from David Knipe), along with a linear hygromycin selection marker. Following two rounds of clonal selection, a cell line was isolated (HeLa-H37) that constitutively expresses UL37 in most cells (Fig. 4.5 A). Work by Liu et al. demonstrated that transient expression of UL37 results in an activation of NF-κB up to 48 hours post transfection (90). Given the time required to generate a stable cell line, however, we were unsure if NF-κB would be activated in the HeLa-H37 cells. We found that the HeLa-H37 cell line had a modest increase in nuclear translocation of NF-κB compared to HeLa-H cells (Fig. 4.5 B and C, respectively). Thus, it appears that the UL37 protein in HeLa-H37 cells is able to activate NF-κB, although not likely to the levels observed during wild type infection. This could be due to the fact that additional viral gene products are required for full NF-κB activation observed during wild type HSV-1 infection (90) or that sustained activation of NF-κB is detrimental to cell viability.

Having found that the UL37 gene in HeLa-H37 cells is both expressed in a uniform fashion and has retained its ability to activate NF-κB, we next used these

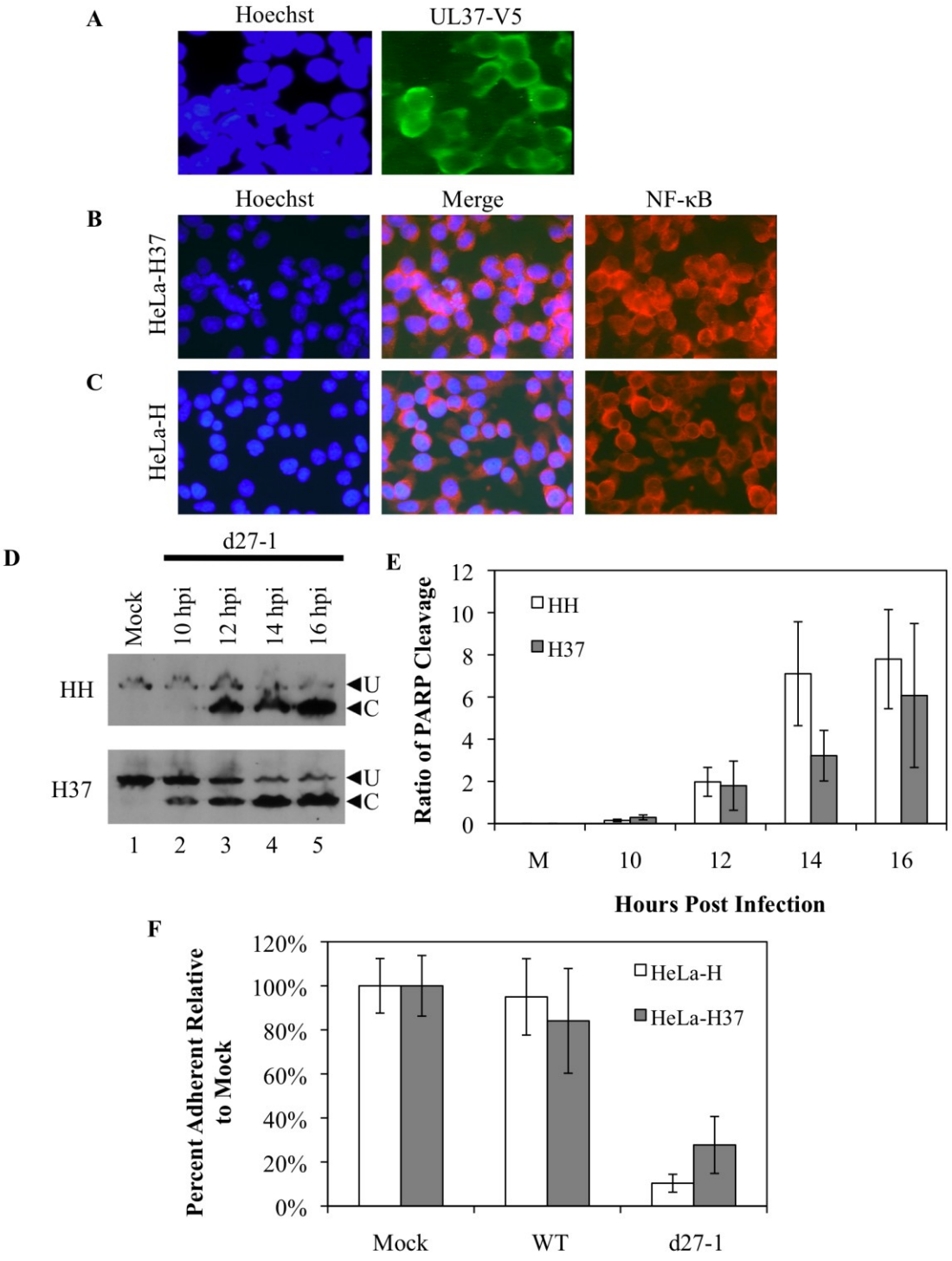
cells to explore the possible role of UL37 in NF-κB activation and apoptosis inhibition during HSV-1 infection. It has been shown that HeLa cells undergo apoptosis at approximately 14 hpi following infection with an ICP27 null mutant (8). We hypothesized that this is due, at least in part, to the expression of UL37 in WT but not d27-1 infected cells. Our HeLa-H37 cells allowed us to test this by allowing ICP27-independent expression of UL37 during a d27-1 infection. Therefore, we mock-infected HeLa-H or HeLa-H37 cells or infected them with d27-1 or KOS1.1. We then used immunoblot analysis to measure the ratio of uncleaved to cleaved Poly (ADP-ribose) polymerase (PARP) (Fig. 4.5 D). PARP is cleaved by activated caspases during apoptosis; therefore, a greater fraction of PARP will be cleaved in cells undergoing apoptosis. This experiment was completed in triplicate, results were quantitated using ImageJ, and the results graphed as the ratio of cleaved to uncleaved PARP (Fig. 4.5 E).

As expected, no PARP cleavage was observed in mock-infected cells (Fig. 5D lanes 1). Furthermore, little or no PARP cleavage was observed in cells infected with KOS 1.1 since HSV-1 infection blocks apoptosis (data not shown). In the d27-1 infection, at 10 hpi, only slight amounts of cleaved PARP were present in both HeLa-H and HeLa-H37 cells (Fig. 4.5 D lanes 2 and 7 and Fig. 4.5 E). In HeLa-H cells, PARP cleavage became evident at 10 hpi (Fig. 4.5 D lane 3) and increased to significantly higher levels at 14 hpi (lane 4) with little further change by 16 hpi (lane 5) (Fig. 4.5 E). Similar to what is observed in HeLa-H cells, PARP cleavage in d27-1 infected HeLa-H37 cells first became evident at 10 hpi (Fig. 4.5 D lane 7 and Fig. 4.5 E). However, the kinetics of the

process appeared to differ, as the d27-1 infected HeLa-37 cells showed significantly less PARP cleavage at 14 hpi compared to HeLa-H cells (lane 8 and Fig. 4.5 E). Both cell lines appeared to have extensive PARP cleavage by 16 hpi indicating high levels of apoptosis. These findings correlate well with the observation that a significantly higher percentage of d27-1 infected H37 cells remained adherent at the later time points of infection (Fig. 4.5 F). Together, these results indicate that ICP27-independent expression of UL37 is able delay apoptosis induced by infection with an ICP27 null mutant. Thus, UL37 is an important anti-apoptotic factor in infected HeLa cells. It is important to note that apoptosis still occurs in the presence of UL37. This suggests that other ICP27-dependent anti-apoptotic factors exist.

Fig. 4.5. ICP27-independent expression of UL37 activates NF-κB and partially inhibits apoptosis induced by an ICP27 null mutant. (A) Immunofluorescence analysis of UL37 expression in HeLa-H37 cells. Cells were fixed and stained using an antibody specific UL37 (green) and nuclei were stained using Hoechst dye (blue). (B and C) Immunofluorescence analysis of cellular localization of the NF-κB p65 subunit in HeLa-H37 and HeLa-H cells. Cells were fixed and processed using antibodies specific for the NF-κB p65 subunit (red) and nuclei were stained using Hoechst dye (blue). Representative figures are shown. (D) Immunoblot analysis of PARP cleavage following d27-1 infection. HeLa-H (HH) and HeLa-H37 (H37) cells were either mock infected or infected with ICP27 null mutant d27-1 (MOI=5). Protein extracts were collected at 10, 12, 14, and 16 hpi and analyzed by immunoblotting levels of uncleaved (U) and cleaved (C) PARP. (E) Quantitation of three sets of immunoblot analysis for PARP cleavage using NIH ImageJ analysis. Cleavage is expressed as a ratio of cleaved PARP relative to uncleaved PARP (higher numbers indicate increased cleavage). (F) Quantitation of cell adherence following d27-1 infection. Triplicate wells of 6-well trays of HeLa cells were mock infected or infected with WT KOS1.1 or d27-1 at a MOI of 5. Adherent cells were fixed and stained at 16 hpi using methanol and Geimsa. Images of the plates were captured using a digital camera and the area of adherent cells was quantitated using NIH ImageJ analysis.

Fig. 4.5


A
Hoechst
UL37-V5
B
Hoechst
Merge
NF-κB
HeLa-H37
C
HeLa-H
D
d27-1
Mock
10 hpi
12 hpi
14 hpi
16 hpi
HH
H37
U
C
1 2 3 4 5
E
Ratio of PARP Cleavage
HH
H37
M 10 12 14 16
Hours Post Infection
F
Percent Adherent Relative to Mock
HeLa-H
HeLa-H37
Mock
WT
d27-1

Discussion

ICP27-induced JNK signaling is insufficient to activate NF-κB in the absence of a viral infection.

The importance of NF-κB activation during HSV-1 infection was first noted by Patel et al. in 1998 when they found that infection leads to nuclear translocation of NF-κB at 6 hpi (118). Furthermore, this study showed that NF-κB activation is required for efficient viral replication. Later studies went on to show that the reduced levels of viral replication in the absence of NF-κB activation was due to host cell apoptosis (47). The mechanism behind the NF-κB dependent inhibition of apoptosis remains unclear. NF-κB activation is known to have a variety of effects on normal cellular function including antiviral responses, growth stimulation, and both pro- and anti-apoptotic roles depending upon the context of the signaling event. Additionally, the growth of several viruses is known to be stimulated by NF-κB activation. The LTR of HIV has a NF-κB responsive promoter sequence that helps to reactivate viral gene expression in activated T cells (45). NF-κB activation has also been shown to play a key role in the replication of several herpesviruses. During latent EBV infection, LMP1 activates NF-κB via TRAF signaling (105). During HCMV infection, NF-κB is first activated during virion binding by a glycoprotein/receptor interaction, and then later activated by another mechanism requiring viral gene expression (135, 183, 184).

Similar to the situation for HCMV, binding of HSV-1 envelope protein gD to its cognate receptor (HVEM) has been shown to mediate an initial activation of NF-κB during HSV-1 infection (147). Following this initial activation, several

other HSV-1 specific interactions have been suggested to activate NF-κB. Previous reports have shown that ICP27 expression is required during HSV-1 infection for SAPK signaling (27, 44, 56, 57), and Hargett et al. showed that ICP27-induced JNK signaling is critical for the activation of NF-κB (57). Experiments involving cyclohexamide treatment of cells at varying times post infection have suggested that DE or L gene products are the actual mediators of NF-κB activation (11). Based on these findings, two non-mutually exclusive models have been proposed for ICP27-dependent activation of NF-κB. The first posits that ICP27-induced SAPK signaling directly activates NF-κB via downstream effector kinases. The second hypothesizes that ICP27 and the resulting SAPK signaling induce the expression of DE and L gene products that mediate NF-κB activation. This study is the first to show that ICP27 expression, while required for NF-κB activation, is insufficient alone to activate of NF-κB. This finding supports the second model, in which ICP27 promotes the expression of DE and L gene products that activate NF-κB.

ICP27-mediated JNK signaling is required for the efficient expression of DE and L genes.

As mentioned above, previous work has suggested that JNK signaling is required for the activation of NF-κB during HSV-1 infection. Much of this research was performed using either chemical inhibitors of JNK signaling or ICP27 deletion mutants that are deficient for the activation of JNK signaling, e.g., d1-2 (56). Research has shown that treatment with the JNK inhibitor SP600125

significantly reduces the efficiency of viral replication (98), but no studies have been performed to investigate the mechanism behind the defect in viral growth. Similarly, mutant d1-2 has an attenuated growth phenotype resulting in a 1-2 log decrease compared to WT KOS1.1 in Vero cells, and has been shown to display increased levels of apoptosis (11, 127), but the nature of its growth defect is not well understood. Here we report for the first time that JNK inhibitor treatment leads to reduced expression of several DE and L gene products in infected HeLa cells. This is unlikely to be due to the effects of apoptosis, as we have shown that PARP cleavage is barely detectable in cell infected with d27-1 at 10 hpi. Furthermore, no significant differences in cellular morphology, adherence, or number are observed between untreated or treated cells at this time. Thus, we suggest that, at least in HeLa cells, JNK signaling plays an important role in HSV-1 gene expression.

The mechanism behind the stimulation of viral gene expression by JNK signaling is currently unclear. Changes in viral gene expression could be due to a variety of mechanisms including changes in transcription, post-transcriptional regulation, translation, or reduced genome replication. It is possible that viral gene expression is directly stimulated by transcription factors downstream of JNK, e.g. c-Jun, ATF-2, or p53. Therefore, it would be valuable to measure effects of inhibitor treatment on the levels of affected transcripts using either Northern blot analysis or quantitative RT-PCR. If we found that transcript levels were reduced following inhibitor treatment, we could then determine if this was due to changes in the rate of transcription by using nuclear run-on assays.

However, we are unaware of any reports of HSV-1 gene promoters having binding sites for or being responsive to these transcription factors. Furthermore, activation of many of these transcription factors is usually considered to be pro-apoptotic, which does not fit well with the observation that JNK signaling is anti-apoptotic during infection.

In addition to directly stimulating expression of viral genes, JNK signaling could also lead to the expression of cellular genes that result in the increased expression of viral proteins. Several targets of JNK signaling have been shown to play a role in HSV-1 replication. The transcription factor heat shock factor 1 (HSF1) is stimulated via JNK signaling and leads to the expression of cellular heat shock proteins, e.g. Hsp70 and Hsp90, which have been shown to play an important role in HSV replication (7, 19, 114, 167). Additionally, increased expression of heat shock proteins during HCMV infection has been shown to increase the translational capacity of cells (67). JNK-mediated c-Jun/AP-1 has also been shown to promote cell cycle progression and to function as an antipoptotic signal both *in vivo* and *in vitro* (reviewed in (170)). The latter effect seems unlikely given the observation that JNK signaling alone has no protective effect. However, it is possible that cell cycle progression could be favorable for HSV-1 replication since G1/S phase cyclin-dependent kinases have been shown to stimulate viral gene expression (144). Further experiments, however, are required to fully elucidate the effects of JNK signaling on viral and cellular gene expression.

Interestingly, infection with the d1-2 mutant, which is severely attenuated for both JNK and p38 signaling, demonstrated a defect in UL37 gene expression that was similar to that seen in inhibitor-treated cells. In Vero cells, d1-2 exhibits a growth phenotype similar to that which is observed during WT HSV-1 infection following JNK inhibitor treatment (98, 127). Unpublished data from our lab suggests that d1-2 growth is more attenuated in HeLa and Hep2 cells than in Vero cells, suggesting that SAPK signaling could play an even more significant role in HSV-1 replication in these cell types. Therefore it will be important to further explore the effects of JNK signaling in other cell types.

The work with d1-2 points towards amino acid residues 12-63 of ICP27 playing a key role in the activation of JNK signaling and the subsequent efficient expression of viral proteins. This region of the ICP27 protein has previously been shown to be critical for nuclear export of ICP27, and mutants lacking this region are impaired in their ability to shuttle between the nucleus and cytoplasm (136). Thus, we speculate that ICP27 is either actively shuttling an unknown component from the nucleus leading to SAPK activation, or that ICP27 has a unique function in the cytoplasm that leads to activation. Further studies are required to test this idea and elucidate the mechanism involved.

UL37 is an ICP27-dependent anti-apoptotic factor during viral infection.

Our data show that both ICP27 expression and JNK signaling are required for the efficient expression of UL37, which has been previously shown to activate NF-κB during viral replication (90). As NF-κB expression has been linked to the

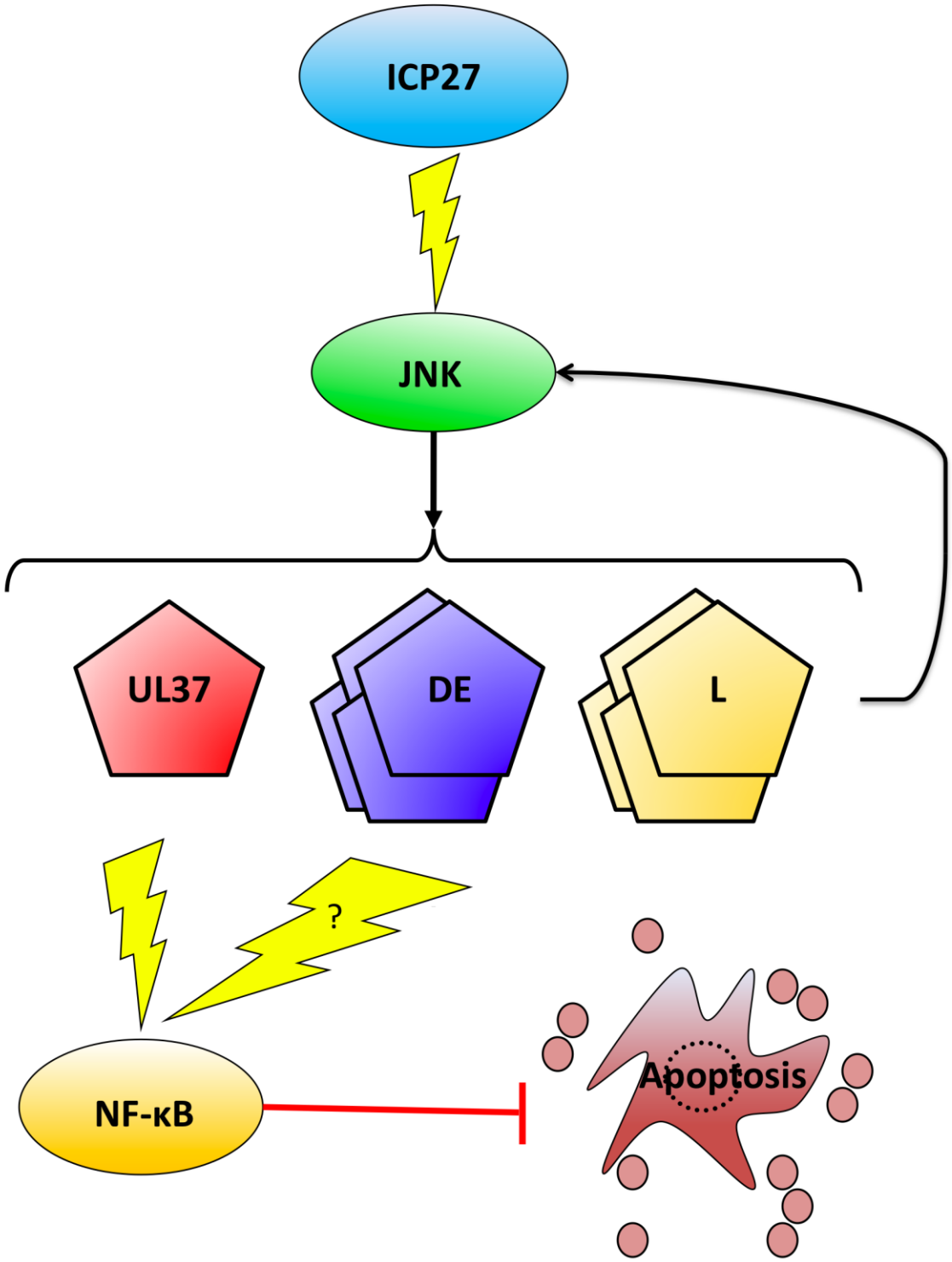
suppression of apoptosis, it is possible that induction of UL37 by ICP27 is partially responsible for ICP27's ability to inhibit apoptosis. Here we provide evidence for this by showing that ICP27-independent expression of UL37 can delay the apoptotic response in cells infected with an ICP27 null mutant. While this inhibition is only temporary, it is significant and suggests that UL37 plays a role in blocking apoptosis. It is perhaps unsurprising that UL37 expression alone is insufficient to completely block apoptosis since previous research has shown that it is only partly responsible for the activation of NF-κB during WT infection (90). Additionally, HSV-1-mediated inhibition of apoptosis can be viewed as a transient effect used by the virus to gain enough time to replicate before lysing the host cell. Our UL37-expressing cell line is still unable to complement the additional defects in viral growth associated with the ICP27 null virus, and thus it is expected that d27-1 infection would eventually lead to apoptosis.

Based on this work and previous research, we propose the following model to explain the relationship between ICP27 function, JNK signaling, NF-κB activation, and apoptosis during viral infection in HeLa cells (Fig. 4.6). First, ICP27 alone activates JNK during infection through an unknown mechanism. Second, this initial activation of the JNK signaling pathway enhances the expression of multiple DE and L gene products. Third, one or more of these DE and L gene products further stimulate JNK signaling, as suggested in our previous research. Fourth, JNK-mediated expression of a subset of the viral gene products, e.g., UL37, leads to the activation of NF-κB. Finally, the activation of NF-κB leads to the inhibition of apoptosis via its effects on host cell

factors. This scheme is consistent with the concept that ICP27 functions as a regulator of host cell fate during viral infection.

Fig. 4.6. Model for ICP27/JNK signaling dependent DE/L gene expression and inhibition of apoptosis.

Fig. 4.6



Chapter 5

ICP27 stabilizes ARE-containing transcripts and appears to induce ER stress

Introduction

Following HSV-1 infection, host gene expression is repressed due to the combined function of ICP27 and virion host shutoff protein (vhs). ICP27 and vhs function by inhibiting the normal synthesis of cellular mRNA and triggering mRNA degradation, respectively (54, 55, 82, 83, 154). Interestingly, certain cellular transcripts seem immune to the host shutoff effect and are instead enhanced in expression following HSV-1 infection. One class of transcripts with increased expression is a group of messages containing AU-rich elements (AREs) in their 3' UTRs. These messages are usually subject to rapid degradation and turnover in cells to ensure that the expression of the proteins they encode is tightly regulated. Messages with AREs encode a wide variety of proteins associated with cellular stress responses and inflammation and must be tightly controlled for the health of the organism.

Previous work by another group has suggested that HSV-1 vhs is responsible for the stabilization of ARE-containing messages (34, 35). However, other studies have linked ICP27 to ARE message stabilization (25, 27, 33, 63). In a recent paper from James Smiley's lab, it was demonstrated that amino acids 12-63 of ICP27 are critical for the stabilization of messages containing AREs (27). Additionally, using a chemical inhibitor of p38, they showed that p38 signaling was required for the stabilization of the ARE-containing cellular mRNA

that encodes the IEX-1 protein (27). This finding fits well with what is known of the normal cellular regulatory mechanism of mRNA decay. Under normal conditions, cytokine and stress-mediated signaling through the p38 pathway leads to the stabilization of mRNAs with AREs. This stabilization is mediated by the MK2 effector kinase that is phosphorylated and activated by p38. Once activated, MK2 carries out a variety of functions in the cytoplasm, allowing the cell to rapidly respond to stress stimuli. The targets of MK2 signaling include protein components of the cytoplasmic complexes responsible for the regulation of ARE mRNA turnover, e.g., the RNA-binding protein tristetraproline (TTP) (16, 165). MK2-mediated phosphorylation leads to the exclusion of TTP and similar proteins from the RNA degradation complexes (165). While the phosphorylated proteins are still able to bind AREs, they are unable to trigger degradation of these mRNAs (165).

Based on our prior observation that ICP27 expression alone is sufficient for p38 signaling as well as the work of the Smiley group, we hypothesized that ICP27 expression alone is sufficient to stabilize mRNAs with AREs. The inducible cell lines detailed in Chapter 3 provides an excellent system to test this hypothesis. Using these inducible cell lines, we demonstrate that ICP27 and subsequent p38 signaling are capable of mediating ARE message stabilization in the absence of infection. Furthermore, we provide evidence that ICP27-mediated p38 signaling leads to the phosphorylation of TTP and its subsequent sequestration from sites of RNA degradation. Interestingly, over the course of these studies we also observed that ICP27 expression activates elements of the

cellular integrated stress response pathway, eIF2α and PERK. Activation of the ER stress response by ICP27 could provide a mechanism by which it induces SAPK signaling.

Results

ICP27 expression and subsequent p38 signaling are sufficient to induce ARE message stabilization.

Most prior work addressing the effect of ICP27 on p38 signaling has been conducted in the context of HSV-1 infection. These studies have suggested that both ICP27 and p38 signaling play a role in the stabilization of mRNAs containing AREs. The mechanism behind this stabilization, however, remains unknown. Cellular pathways of ARE mRNA stabilization are well understood, but there are only a few reports of stabilization of these messages during viral infections. Therefore, it remains unclear if there are additional viral mediators of ARE message stabilization or if ICP27 activated p38 signaling is sufficient for message stabilization. In order to study the effects of ICP27 in the absence of viral infection, we used the ICP27 inducible cells lines TE12 and TE51, described in Chapter 3, to determine if ICP27 is sufficient to lead to either increased levels or stabilization of the ARE-containing message encoding IEX-1. To measure changes in IEX-1 message level/stability, replicate cultures of both HTO-L (control cells inducibly expressing luciferase) and TE12 cells were left untreated, infected with KOS 1.1 for 6 hours at an MOI 5, or treated with doxicycline for either 12 or 24 hours to induce protein expression. Following infection or treatment, one culture from each treatment condition or infection was harvested and total cellular RNA was collected. To measure the stability of messages, the remaining cultures were treated with actinomycin D, an inhibitor of RNA synthesis, for one hour and then total cellular RNA was harvested. We used

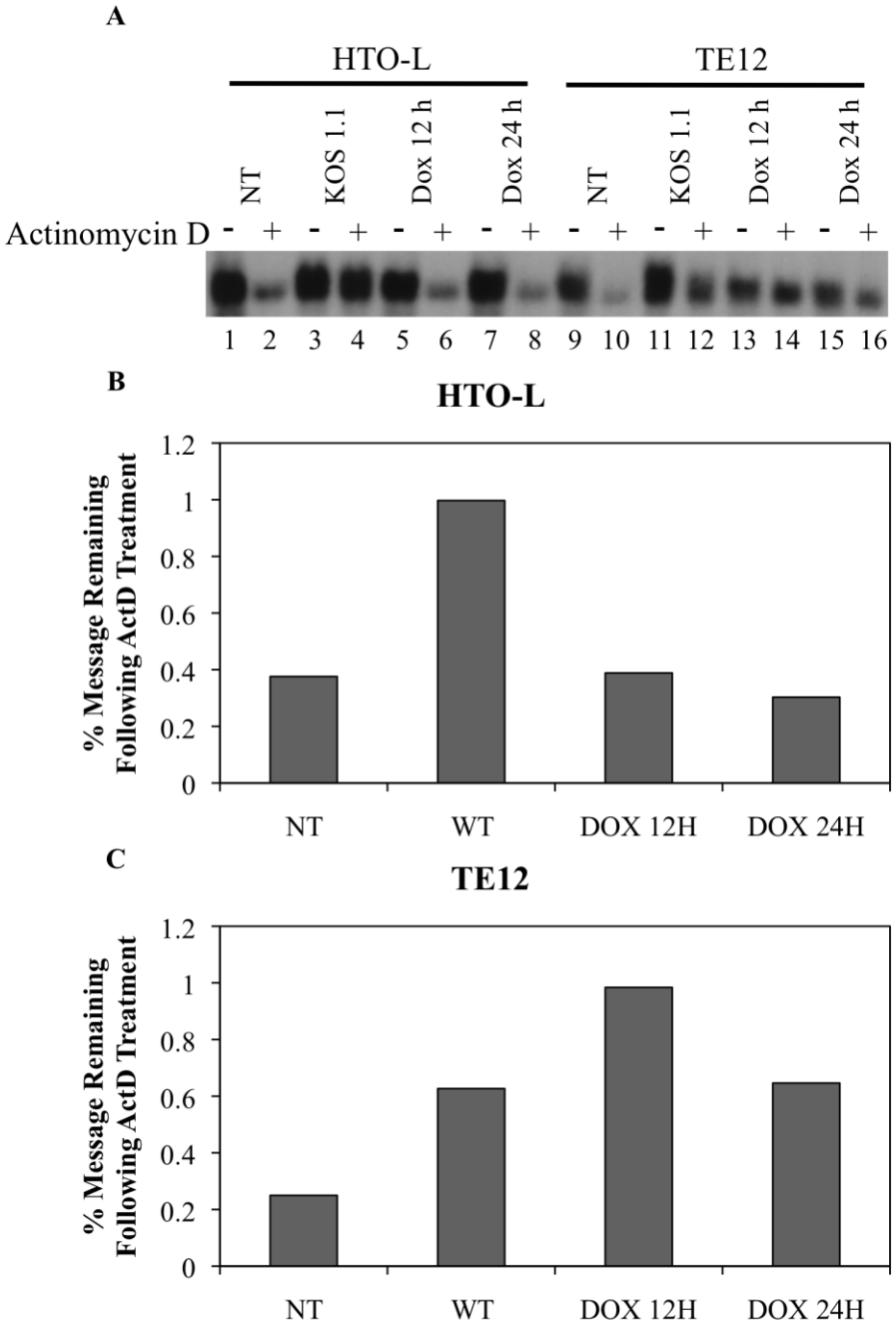
Northern blot analysis to measure the levels of the ARE-containing IEX-1 message both before and after actD treatment (Fig. 5.1 A). No significant increase in IEX-1 message levels was observed in cells following induced expression of either luciferase (lane 1 compared to 5 and 7) or ICP27 (lane 9 compared to 13 and 15). Additionally, viral infection seemed to lead to modest increase in the levels of IEX-1 mRNA (lanes 3 and 11).

The IEX-1 mRNA is subject to rapid degradation under normal conditions. Consistent with this, in untreated cells significantly less message was observed following actD treatment (lanes 2 and 10). As has been previously shown, viral infection led to a significant stabilization of the IEX-1 transcript (lanes 4 and 12). As expected, expression of luciferase failed to stabilize IEX-1 mRNA following actD treatment as only small amounts of transcript were detectable in induced HTO-L cells (lands 6 and 8). In contrast, following induced expression of ICP27, large amounts of IEX-1 transcript are still detectable (lanes 14 and 16), suggesting their ICP27 is capable of stabilizing IEX-1 mRNA.

We quantified the percent of IEX-1 message remaining following actD treatment relative to untreated cells using ImageJ analysis. While viral infection of HTO-L cells resulted in a dramatic increase in message stability, little or no difference is detectable in the stability of the IEX-1 transcript in doxicycline-treated HTO-L cells (Fig. 5.1 B). However, doxicycline induction of TE12 cells led to levels of message stabilization greater than or equal to what was observed following viral infection (Fig. 5.1 C). This leads us to conclude that expression of ICP27 is sufficient for the stabilization of the ARE-containing IEX-1 transcript.

Fig. 5.1. ICP27 expression leads to IEX-1 message stabilization. (A) Northern blot analysis of IEX-1 message stability in infected and induced cells. HTO-L (lanes 1-8) and TE12 (lanes 9-16) cells were plated in duplicate and then left untreated, infected with KOS1.1 (MOI 5 for 8 h), or treated with doxicyline (2 μg/ml) for 12 or 24 h. Total RNA was harvested from one replicate while the other was treated with actinomycin D for one hour to halt RNA synthesis. Total RNA from actinomycin D treated cells was then harvested. Northern blot analysis was performed using probes specific for IEX-1 mRNA. IEX-1 message stability in HTO-L (B) and TE12 (C) cells was measured using NIH ImageJ to quantitate IEX-1 message levels before and after actinomycin D treatment. Message stability is shown as the percent message remaining following actinomycin D treatment.

Fig. 5.1


A
HTO-L
TE12
NT
KOS 1.1
Dox 12 h
Dox 24 h
NT
KOS 1.1
Dox 12 h
Dox 24 h
Actinomycin D - + - + - + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
HTO-L
% Message Remaining Following ActD Treatment
1.2
1
0.8
0.6
0.4
0.2
0
NT
WT
DOX 12H
DOX 24H
C
TE12
% Message Remaining Following ActD Treatment
1.2
1
0.8
0.6
0.4
0.2
0
NT
WT
DOX 12H
DOX 24H

ICP27 expression leads to the phosphorylation of TTP and its sequestration from sites of mRNA degradation.

After observing that ICP27 expression alone is sufficient to inhibit the degradation of the IEX-1 transcript, we next wanted to investigate whether p38 responsive pathways play a role in the stabilization of this ARE-containing transcript. TTP is one of the best-characterized proteins responsible for targeting ARE-containing messages for degradation. Under normal conditions, it is maintained primarily in the nucleus where it binds to AREs and targets them for degradation in the cytoplasm (16, 165). Following p38 signaling activation, TTP becomes phosphorylated by MK2 and translocates to the cytoplasm (16, 165). Additionally, the phosphorylation of TTP causes it to be associated with 14-3-3 proteins and excluded from RNA degradation complexes (165). We wished to use immunofluoresence to test the effects of ICP27 on TTP localization. However, since TTP is expressed at low levels in HeLa cells (29), we used transient transfection to express TTP to levels that would allow us to visualize it by immunofluoresence.

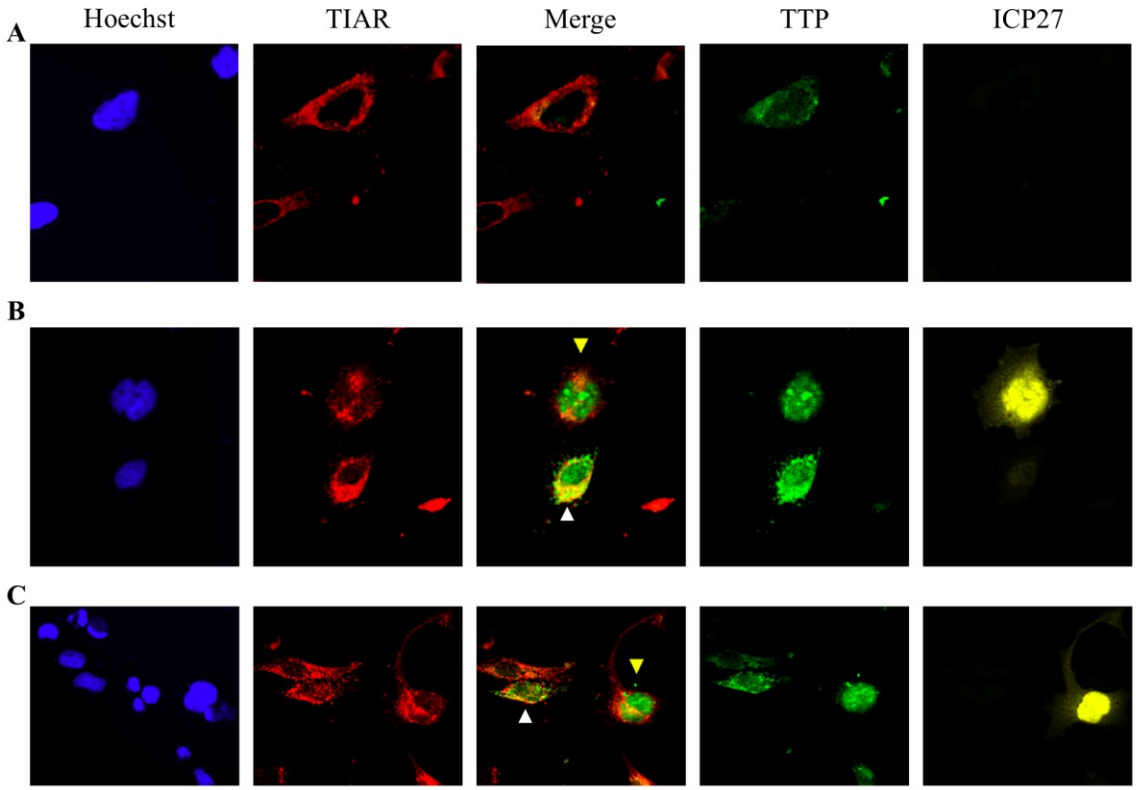
It is important to note that over expression of TTP can result in the formation of stress granules, which are cytoplasmic aggregates of stalled translational preinitiation complexes (reviewed in (4, 5)). These stress granules also contain proteins such as TTP that are responsible for the degradation of mRNA. However, once phosphorylated, TTP is excluded from stress granules. Therefore, we carried out an experiment to determine the localization of TTP with respect to stress granules in cells that had been induced to express ICP27. To

do this, HTO-L, TE12 and TE51 cells were treated with doxicycline and then either mock transfected or transfected with pCMV.TTPh.tag, a TTP expression plasmid, and allowed to recover for 4 hours. At 18 h post transfection, cells were fixed and stained for TTP, TIAR (a marker for stress granules), and ICP27. An additional control of sodium arsenite treatment was used as a positive control for stress granule formation. As expected, TTP transfection resulted in the formation of stress granule-like structures as indicated by the intense red speckles of TIAR in HTO-L, TE12, and TE51 cells (Fig. 5.2 A, B, C). In untransfected cells, low levels of TTP was found diffusely throughout the cell (data not shown). In induced HTO-L cells, TTP largely colocalized with TIAR in granular structures indicating that it likely remains unphosphorylated (Fig. 5.2 A). Furthermore, in TE12 and TE51 cells that are not expressing ICP27, TTP and TIAR remain colocalized suggesting that there is no change in the phosphorylation state of TTP (Fig. 5.2 B and C white arrows). However, in cells that are expressing ICP27, TTP was observed in a different pattern. It was localized throughout the cell (Fig. 5.2 B and C yellow arrows) with little or no intensely staining foci or colocalization with the TIAR rich stress granules. Thus, our results are consistent with a model in which ICP27-mediated p38 signaling causes TTP to be phosphorylated and excluded from sites of mRNA degradation.

ICP27 expression leads to stress granule formation and induction of the cellular integrated stress response.

Fig 5.2. ICP27 expression leads to sequestration of TTP from stress granules. Immunofluorescent analysis of TTP's localization with respect to stress granules in HTO-L (A), TE12 (B), and TE51 (C) cells. Cells were treated with doxicycline (2 μg/ml) and then transfected with pCMV.TTPh.tag. Cells were fixed and stained using indirect immunofluoresence for the TTP (green), TIAR (red), ICP27 (yellow), and nuclei were stained using Hoechst dye (blue).

Fig. 5.2



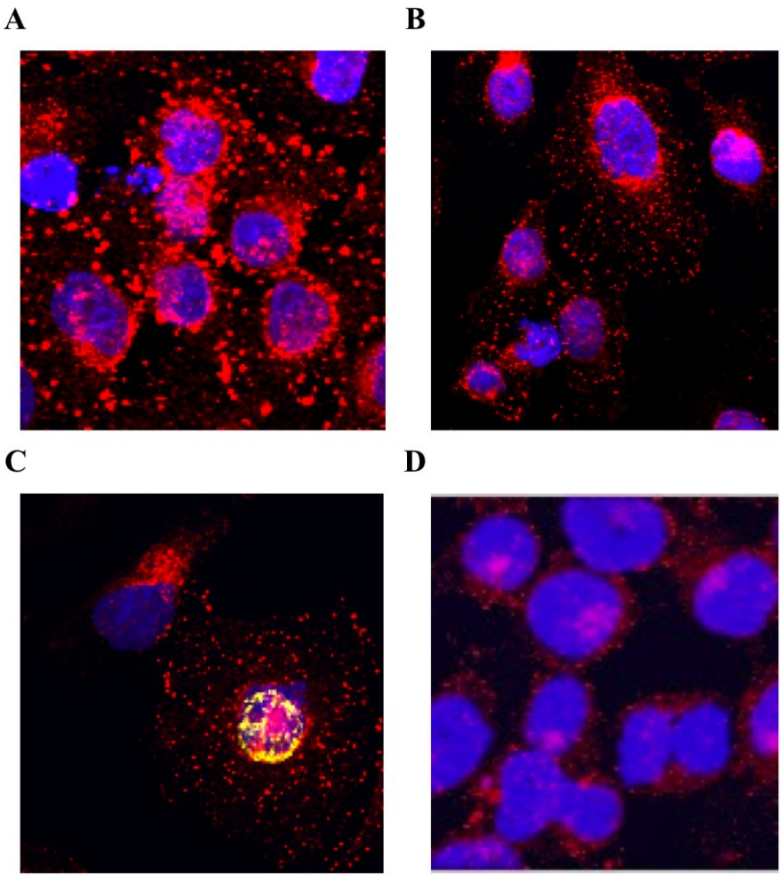
As expected, sodium arsenite treatment led to the formation of stress granules in the absence of TTP induction in HTO-L, TE12, and TE51 cells (Fig. 5.3 A, B, and data not shown, respectively). The induction of stress granule formation is indicated by the punctate staining pattern of TIAR is consistent with previous reports of stress granule formation (156, 165). Unexpectedly, similar punctate staining patterns were observed in TE12 cells expressing ICP27 (Fig. 5.3 C). Stress granule formation was also observed in TE51 cells expressing ICP27 (data not shown). Furthermore, this punctate staining pattern was only observed in cells expressing ICP27, indicating that the effect was associated with ICP27 expression and was not due to doxicycline treatment. Consistent with this, stress granule formation was not observed in HTO-L cells following doxicycline addition (Fig. 3D).

Stress granule formation in cells is normally associated with induction of the integrated stress response (ISR) pathway by a variety of cellular stresses. The formation of stress granules in cells expressing ICP27 was unexpected since there are no previous reports of ICP27 inducing the cellular integrated stress response pathway. Previous data have shown that accumulation of stalled translational complexes is often the result of translational inhibition due to eIF2α phosphorylation. Phosphorylation of eIF2α prevents the transfer of methionine-tRNA to the 40S subunit to form the 43S preinitiation complex, blocking a new round of translation. The block in translation leads to accumulation of stalled preinitiation complexes, which are bound by TIA-1 and TIAR and oligomerize to serve as the nucleus for stress granule formation.

Fig. 5.3. ICP27 expression induces stress granule formation.

Immunofluorescent analysis of stress granule formation in HTO-L (A) and TE12 (B) cells treated with sodium arsenite, or TE12 (C) and HTO-L (D) cells following 24 h of doxicycline treatment (2 μg/ml). Cells were fixed and stained using indirect immunofluorescence for the ICP27 (yellow), TIAR (red); and nuclei were stained using Hoechst dye (blue).

Fig. 5.3



In order to see whether ICP27-induced stress granules are linked to effects on eIF2α, we measured eIF2α phosphorylation following ICP27 induction. TE12 and HTO-L cells were treated with doxicycline for 0, 8, 16, and 24 h to induce the expression of ICP27 and luciferase, respectively. As a positive control, TE12 and HTO-L cells were also treated with thapsigargin to induce endoplasmic reticulum (ER) stress. KOS 1.1 infection (MOI 5 at 8 hpi) was used as a negative control for eIF2α phosphorylation, since HSV-1 encodes several proteins that block eIF2α phosphorylation (reviewed in (129)) and therefore this sample should have only minimal levels of phosphorylated eIF2α. Proteins were harvested and used for immunoblot analysis with antibodies specific for phosphorylated eIF2α (P- eIF2α) and early endosomal antigen 1 (EEA1) as a loading control. As expected, thapsigargin treatment led to a significant increase in the amount of phosphorylated eIF2α compared to untreated cells (Fig. 4 A and B, lane 1 vs. 2). Furthermore, viral infection led to a sizable reduction in eIF2α phosphorylation compared to untreated cells (Fig. 4 A and B, lane 3 vs. 2). As expected, little or no change in eIF2α phosphorylation was observed in HTO-L cells following doxicycline induction (Fig. 4A, lanes 4-6) compared to untreated cells (Fig. 4A, lane 2). However, ICP27 expression in TE12 cells appeared to result in elevated levels of eIF2α phosphorylation at 8, 16, and 24 h post induction, compared to untreated cells (Fig. 4B, lanes 4-6).

To measure the increase in the level of eIF2α phosphorylation caused by induced expression of ICP27, we quantitated the results of five independent

experiments. In all experiments, we treated HTO-L, TE12, and TE51 cells with thapsigargin as above for a positive control, left them untreated, or induced protein expression for 24 h with doxicycline. Cells were lysed and proteins were harvested for immunoblot analysis as above. Results were quantitated using ImageJ software. The level of eIF2α phosphorylation was normalized to EEA1 loading controls, and the results are presented in Figure 5.4 C as the level of P-eIF2α relative to untreated cells. As expected, luciferase expression in HTO-L cells resulted in little or no change in the level of eIF2α phosphorylation. In contrast, induced expression of ICP27 in TE12 cells led to over a two-fold increase in the level of phosphorylated eIF2α. In TE51 cells, a 50% increase was seen following doxicycline induction. The increase in eIF2α phosphorylation in induced TE12 and TE51 cells compared to untreated cells was significant with p values of 0.024 and 0.00024 respectively. Thus, we conclude that ICP27 expression in HeLa cells induces eIF2α phosphorylation and stress granule formation.

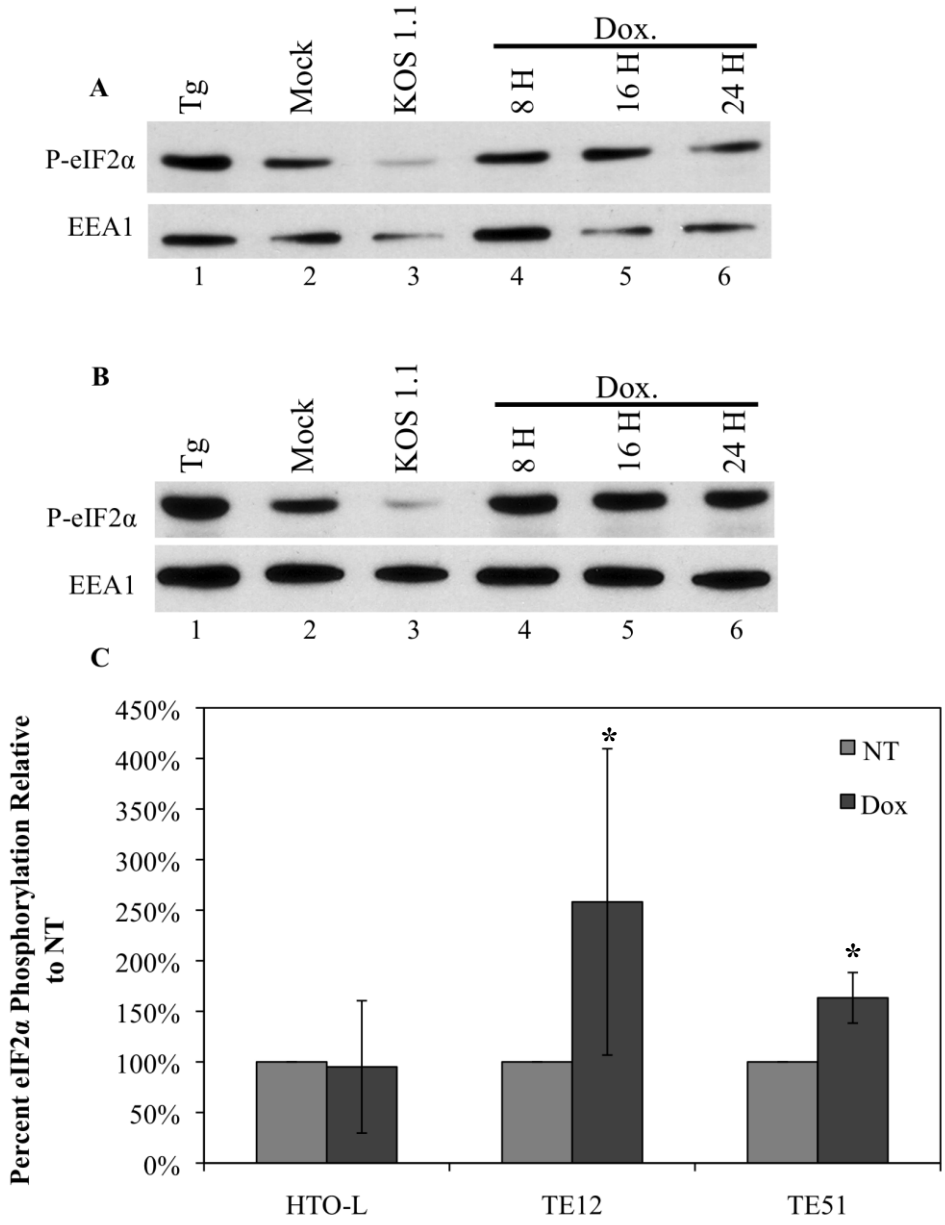
The ER stress response is triggered by ICP27 expression and at early times following HSV-1 infection.

Phosphorylation of eIF2α and the integrated stress response (ISR) pathway are regulated by a group of four cellular kinases that sense a variety of stress signals (reviewed in (179)). PKR (dsRNA-dependent protein kinase) is classically thought of as an antiviral sensor that when bound to dsRNA phosphorylates eIF2α through its kinase domain. PERK (PKR-like ER stress

sensor kinase) senses the accumulation of unfolded proteins in the ER (ER

Fig. 5.4. Expression of ICP27 leads to eIF2α phosphorylation. Immunoblot analysis of the levels of phosphorylated eIF2α in HTO-L (A) and TE12 (B) cells. Proteins were harvested from cells treated with thapsigargin (lane 1), mock infected (lane2), infected with KOS 1.1 (MOI 5 at 8 hpi) (lane 3), or treated with doxicycline (2 μg/ml) for 8 h, 16 h, or 24 h (lanes 4-6). Phosphorylation of eIF2α was measure using immunoblot analysis. EEA1 was used as a loading control. (C) The phosphorylated eIF2α over 5 experiments (24 h of doxicycline induction) was quantitated using ImageJ analysis and graphed as a percentage relative to untreated cells. (* $p < 0.05$, relative to NT)

Fig. 5.4


A
Tg
Mock
KOS 1.1
Dox.
8 H
16 H
24 H
P-eIF2α
EEA1
1
2
3
4
5
6
B
Tg
Mock
KOS 1.1
Dox.
8 H
16 H
24 H
P-eIF2α
EEA1
1
2
3
4
5
6
C
Percent eIF2α Phosphorylation Relative to NT
450%
400%
350%
300%
250%
200%
150%
100%
50%
0%
NT
Dox
*
*
HTO-L
TE12
TE51

overload). These unfolded proteins then induce dimerization and activation of PERK leading to eIF2α phosphorylation. GCN2 (general control non-derepressible-2 or eIF2a kinase 4) signaling is activated by amino acid starvation, which it senses via an accumulation of uncharged tRNAs. Finally, haem-regulated inhibitor (HRI) triggers eIF2α phosphorylation after sensing oxidative stress or heat shock. It is not obvious why ICP27 expression alone would trigger the activation of any of these responses. ICP27 mRNA is not known to have secondary structure and there should be no complementary RNA present to activate PKR. Furthermore, ICP27 is a largely nuclear protein that does not require ER processing for its translation so PERK should not be activated. Finally, the expression of ICP27 alone would not be expected to trigger amino acid starvation, heat shock, or oxidative stress.

One possibility is that ICP27 activates the ISR pathway via its effects on cellular gene expression. ICP27 is known to lead to message stabilization as discussed above, splicing inhibition, and intron retention (25, 44, 54, 75, 82, 83, 138, 139, 146, 149). It is conceivable that accumulation of atypical transcripts in the cytoplasm could cause cellular stress and potentially activate PKR. For example, one report showed that cytoplasmic accumulation of unspliced TNFα transcripts, which contains an ARE, leads to activation of PKR (115). However, HeLa cells do not express TNFα normally. ICP27 has also been shown to stimulate the translation of viral proteins and interact with cellular translational factors (32, 38, 85, 116, 138). Furthermore, p38 signaling can stimulate cellular translation by phosphorylating MNK1/2 (66, 93). If ICP27 expression stimulates

translation of cellular mRNA, this could potentially lead to ER overloading and PERK signaling.

In order to determine if ICP27 induces ER stress, we used XBP-1 mRNA splicing as a surrogate marker for PERK activation (reviewed in (53)). The XBP-1 transcript is normally present in the cytoplasm in its unspliced form. The accumulation of misfolded proteins in the ER lumen leads to the dimerization and activation of the endoribonuclease/kinase IRE1 as well as PERK. The activated IRE1 then processes the XBP-1 message, removing a small 26 bp intron allowing for the production of a transcription factor that induces transcription of stress-responsive genes (53). XBP-1 mRNA splicing can be used a marker for ER stress since IRE1 phosphorylation is difficult to detect directly (74, 94) and HSV-1 encodes a variety of proteins that make PERK activation difficult to detect (24, 108, 109).

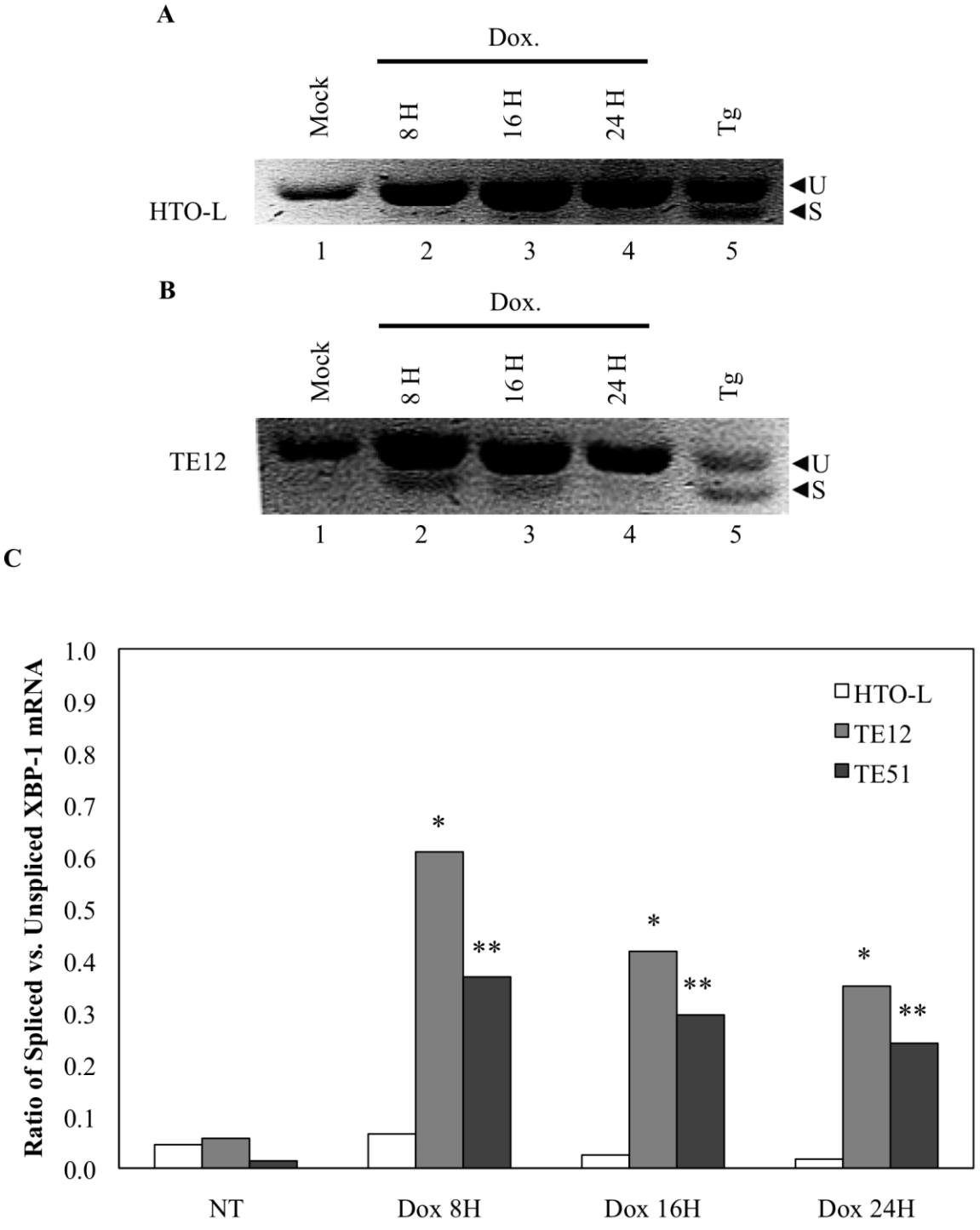
To see if XBP-1 mRNA splicing is induced by ICP27 expression in uninfected cells, total RNA was harvested from HTO-L, TE12, and TE51 cells at 0, 8, 16, and 24 h post doxicycline induction. Total RNA from thapsigargin treated cells was also collected as a positive control. RT-PCR analysis was carried out with primers spanning the intron and the splicing pattern was analyzed by agarose gel electrophoresis. As expected, at all times following doxicycline induction, HTO-L cells displayed only the slower migrating RT-PCR product that arises from the unspliced form of XBP-1 mRNA (Fig. 5.5 A, lanes 1-4). Thapsigargin-induced ER stress resulted in XBP-1 mRNA, as indicated by the presence the smaller faster migrating band (Fig. 5.5 A and B, lane 5).

Interestingly, expression of ICP27 in TE12 cells resulted in detectable splicing of XBP-1 mRNA. This splicing peaked at 8 h post induction and by 24 hours post induction was barely detectable (Fig. 5.5 B lanes 1-4). Similar results were observed in TE51 cells (data not shown). To ensure these observations were repeatable and to measure the splicing effect, this experiment was repeated three times and results were quantitated using ImageJ analysis. The results are shown in Figure 5.5 C. HTO-L cells showed little or no XBP-1 mRNA splicing at any time following induction. Conversely, both TE12 and TE51 cells showed high levels of XBP-1 mRNA splicing at 8 hour post induction, with lesser levels at the 16 and 24 hour time points. While splicing faded over time, it remained significantly elevated over the levels of XBP-1 mRNA splicing observed in both uninduced cells and HTO-L cell following induction. These results indicate that ICP27 expression alone is sufficient to induce XBP-1 mRNA splicing. This strongly suggests that ICP27 is inducing ER stress and activating IRE1 and likely PERK.

Having observed that ICP27 alone is able to induce splicing of the XBP-1 transcript, we wanted to determine whether XBP-1 mRNA splicing is triggered during HSV-1 infection. Previous research by Mulvey et al. has shown that in HSV-1 infected Vero and murine 3T3 cells, activation of PERK is blocked by the gB glycoprotein (108). This same study demonstrated that while the IRE1 activation and XBP-1 mRNA splicing are unaffected by this inhibition, XBP-1 mRNA splicing is not detectable at 7, 9, and 11 hpi (108). Based on our results that showed ICP27 alone is sufficient to lead to splicing of the XBP-1 transcript,

Fig. 5.5. ICP27 expression induces splicing of the XBP-1 transcript. RT-PCR analysis of XBP-1 splicing in HTO-L (A) and TE12 (B) cells. Cells were either left untreated (lane 1), treated with doxicycline (2 μg/ml) for 8 h, 16 h, or 24 h, or with thapsigargin for 30 min. Total RNA was harvested and used for RT-PCR with primers specific for XBP-1 mRNA. (C) Quantitation of XBP-1 mRNA splicing from three experiments using ImageJ analysis. XBP-1 splicing is expressed as the ratio of spliced versus unspliced XBP-1 mRNA. (* $p < 0.05$, ** $p < 0.005$, relative to NT)

Fig. 5.5

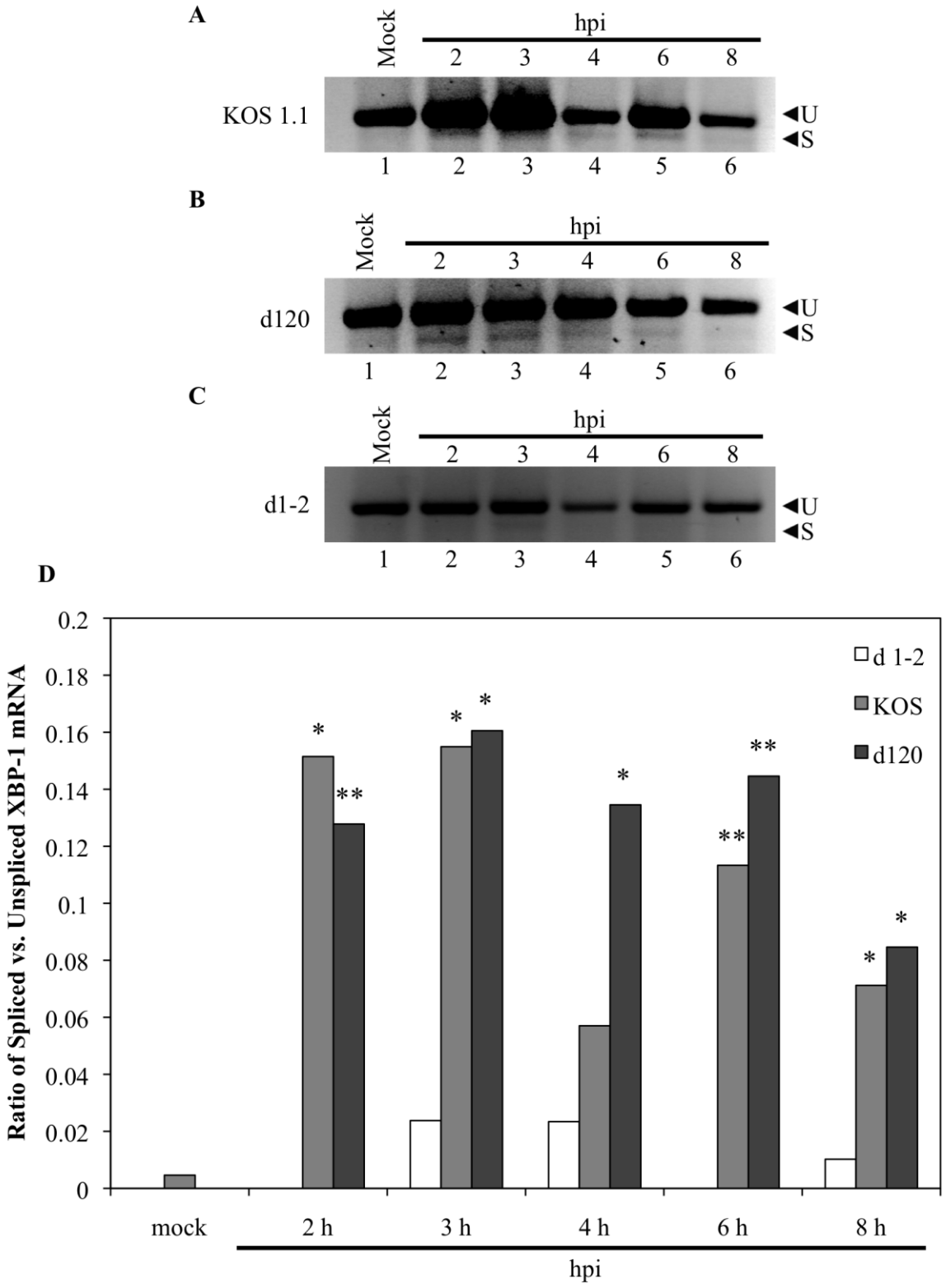

A
Dox.
Mock
8 H
16 H
24 H
Tg
HTO-L
U
S
1
2
3
4
5
B
Dox.
Mock
8 H
16 H
24 H
Tg
TE12
U
S
1
2
3
4
5
C
Ratio of Spliced vs. Unspliced XBP-1 mRNA
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
HTO-L
TE12
TE51
*
**
NT
Dox 8H
Dox 16H
Dox 24H

we hypothesized that ER stress and XBP-1 mRNA splicing is triggered at earlier timepoints during infection than those that were examined by Mulvey et al. To test this, we looked at XBP-1 mRNA splicing following WT HSV-1 infection. In an effort to determine if DE or L gene products are able to inhibit the ICP27-induced ER stress, we also examined XBP-1 mRNA splicing in the ICP4 mutant d120, which over expresses IE genes but is unable to express DE and L genes. We also examined XBP-1 mRNA splicing in the ICP27 deletion mutant d1-2, which lacks the acidic N-terminal region that has been shown to be required for efficient viral replication, shuttling, and SAPK signaling. This mutant was selected since activation of p38 signaling can stimulate translation directly via MNK1/2, and IRE1 has been shown to activate the SAPK signaling cascade via apoptotic signaling kinase 1 (ASK-1) (78). To carry out the experiment, we mock-infected or infected HeLa cells at a MOI of 5 and harvested total RNA at 2, 3, 4, 6, and 8 hpi. XBP-1 mRNA splicing was analyzed by RT-PCR followed by agarose gel electrophoresis. In WT-infected cells, we observed a modest level of XBP-1 mRNA splicing at early times post infection. This peaked at 2-3 hpi and then lessened (Fig. 5.6 A). In d120-infected cells, the duration of XBP-1 mRNA splicing appeared to be extended (Fig. 5.6 B). In contrast, infection with d1-2 resulted in no detectable splicing of XBP-1 transcript (Fig. 5.6 C). We went on to quantitate the level of XBP-1 mRNA splicing following viral infection, using data accumulated over 3 experiments. Figure 5.6 D shows that both KOS 1.1 and d120 infection lead to modest but significant levels of XBP-1 mRNA splicing, while d1-2 does not induce XBP-1 mRNA splicing. These results suggest that

ER stress is induced at early times following HSV-1 infection in an ICP27–dependent fashion, but appears to be counteracted at later times by DE/L gene products. Furthermore the acidic region of ICP27, spanning from amino acids 12-63, appears to be required for the activation of ER stress.

Fig. 5.6. XBP-1 splicing is induced early during HSV-1 infection of HeLa cells. RT-PCR analysis of XBP-1 splicing in HeLa cells following infection by KOS 1.1 (A), d120 (B), and d1-2 (C). Cells were mock infected (lane 1) or infected at an MOI of 5. Total RNA was harvested at 2, 3, 4, 6, and 8 hpi and used for RT-PCR with primers specific for XBP-1 mRNA. (D) Quantitation of XBP-1 mRNA splicing from three experiments using ImageJ analysis. XBP-1 splicing is expressed as the ratio of spliced versus unspliced XBP-1 mRNA. (* $p < 0.05$, ** $p < 0.005$, relative to mock)

Fig. 5.6


A
Mock
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6
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KOS 1.1
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3
4
5
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B
Mock
hpi
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3
4
6
8
d120
U
S
1
2
3
4
5
6
C
Mock
hpi
2
3
4
6
8
d1-2
U
S
1
2
3
4
5
6
D
Ratio of Spliced vs. Unspliced XBP-1 mRNA
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
d 1-2
KOS
d120
mock
2 h
3 h
4 h
6 h
8 h
hpi

Discussion

ICP27-mediated p38 signaling leads to the stabilization of an ARE-containing message.

Previous studies using viral deletion mutants have shown that during infection ICP27 can enhance the stability of certain transcripts prone to rapid degradation (25, 27, 33). Recently, this ICP27-dependent stabilization of mRNAs in infected cells has been shown to be dependent on p38 signaling (27), which we and others have shown requires ICP27 (44, 56, 57). The studies presented in this chapter are the first to show that ICP27 expression is sufficient to stabilize transcripts containing AREs, even in the absence of viral infection. Furthermore, we provide evidence that this stabilization is the result of p38 signaling-mediated changes to TTP, one of the cellular components normally responsible for the degradation of ARE-containing transcripts.

Interestingly, few if any HSV-1 transcripts bear AREs in their 3' non-coding regions (27, 63). Therefore, ICP27's effects are presumably most relevant to cellular transcripts. Stabilization of transcripts with AREs could have a variety of effects on normal cellular gene expression during viral infection, which could have beneficial effects for HSV-1 replication or spread in the host. For example, many ARE-containing mRNAs encode proinflammatory cytokines which could be used to alter the immune response, leading to tissue damage and allowing for increased spread. Alternatively, inflammation could be a viral evolutionary adaptation to limit acute infections to short sporadic bursts. These bursts might ensure long term viral persistence, allowing viral spread over time while resulting

in minimal detrimental effect to the host. Furthermore, p38 signaling has also been shown to enhance the expression of the immunosuppressive cytokine IL-10 during HSV-1 infection *in vitro* and *in vivo* (153, 181). Interestingly, the IL-10 transcript has recently been shown to contain an ARE that is sensitive to TTP-directed decay (173).

Other herpesviruses have also been shown to stabilize mRNAs with AREs. KSHV has been shown to activate MK2 leading to the stabilization of messages containing AREs. Specifically, the KSHV Kaposin B protein binds and activates MK2, resulting in increased expression of cytokines required for the growth of KSHV in spindle cells. Pseudorabies virus, a porcine herpesvirus, leads to the stabilization of the ARE-containing cyclooxygenase-2 (COX-2) transcript. Expression of COX-2 is required for viral growth *in vitro.* Similarly, HSV-1 growth in specific cell types *in vivo* could be favored by the expression of certain cytokines. Many proto-oncogenes also carry ARE elements, e.g., c-myc, c-jun, and b-jun, and stabilization of these transcripts could stimulate cell growth in arrested cells allowing for enhanced viral replication.

Ultimately, the true effects of p38 signaling on HSV-1 replication are poorly understood. Research has shown that p38 signaling is required for efficient viral replication in Vero cells (76). However, p38 signaling does not appear to effect viral gene expression at the level of mRNA accumulation (76). Consistent with this, we have not observed defects in viral protein expression following p38 inhibitor treatment of infected HeLa cells (Gillis and Rice, unpublished data). Stabilization of cellular transcripts with AREs could explain

why HSV-1 has evolved the ability to induce p38 signaling. However, further research is needed to test this hypothesis.

ICP27 expression triggers the ISR.

Our studies have shown that ICP27 expression alone can trigger the ISR pathway leading to eIF2α phosphorylation and stress granule formation. Moreover, we have provided evidence that the induction of ER stress pathway is at least one mechanism involved in the activation of the ISR. This suggests that ICP27 induces an accumulation of misfolded proteins in the ER, leading to the dimerization and activation of PERK and IRE1. PERK signaling might then lead to eIF2α phosphorylation and translational arrest. IRE1 is responsible for activating the cellular unfolded protein response (UPR) to expand the capacity of the ER and allow the refolding of misfolded proteins (reviewed in (92)).

It is conceivable that ICP27 is also stimulating PKR, although we have not investigated this possibility. PKR has previously been associated with the activation of NF-κB and expression of cytokines during HSV-1 infection (103, 166). The ICP27 transcript alone, however, appears unlikely to cause PKR activation, which requires dsRNA. Additional research is necessary to explore if and how the ICP27 transcript or altered host transcripts induced by ICP27 can activate PKR.

ICP27 expression is not expected to lead to ER stress directly, since ICP27 is translated in the cytoplasm and does not enter the ER. This suggests that ICP27 might stimulate translation of cellular proteins leading to ER

overloading. Such a mechanism is feasible, since ICP27 has been shown to stimulate the translation of viral gene products (32, 85, 99, 138). Furthermore, ICP27 has been shown to interact with a variety of host translation initiation factors (38, 85). However, more work is required to understand the mechanism by which ICP27 induces ER stress.

An acidic region near the ICP27 N-terminus is required for activation of the ISR.

In an effort to determine it ICP27 induce ER stress during infection. we measured XBP-1 mRNA splicing following HSV-1 infection. We show that XBP-1 splicing can be detected following WT KOS1.1 infection. This is in contrast to a previous study that found no evidence of XBP-1 mRNA splicing during HSV-1 infection (108). The most likely explanation for this difference is that we have examined earlier time points (2-6 hpi) than those observed in the other study. We did find that by 8 hpi, XBP-1 mRNA splicing was greatly reduced. However, the difference could also be due to differences in cell type since the study by Mulvey et al. used murine 3T3 cells while we used HeLa cells. The reduction in XBP-1 mRNA splicing at late time points could be due to the expression of additional viral DE or L proteins that limit the ER stress response, reduced expression of IE genes at late times of infection, or resolution of the early ER stress. This last possibility is similar to a mechanism used by HCMV to promote expression of its structural proteins. Early during HCMV infection, viral proteins mediate a controlled ER stress response to trigger the cellular unfolded protein

response (UPR) (67). The UPR then stimulates the expression of cellular heat shock proteins and chaperones that expand the translational capacity of the ER to increase the efficiency of viral glycoprotein expression at later times.

It is important to note that the level of XBP-1 mRNA splicing was significantly lower in HSV-1 infected cells than in cells induced to express ICP27. This could be due to over expression of ICP27 in induced cells, leading to elevated levels of cellular translation compared to infection. Alternatively, there could be additional viral factors that limit the translation of cellular proteins during viral infection. For example, the tegument protein vhs is known to inhibit translation of both cellular and viral transcripts (34, 82, 83, 154).

Infection with the ICP4 null mutant d120 resulted in increased splicing of XBP-1 mRNA compared to KOS 1.1 infection. As this virus is unable to transition to the DE/L stages of infection, this result is consistent with the idea that DE and L proteins down-regulate the ER stress response. For example, expression of newly synthesized vhs could play a role in limiting the activation of ER stress through mechanisms discussed above. Furthermore, the over expression of IE gene products, including ICP27, by d120 could result in increased levels of ICP27-mediated ER stress.

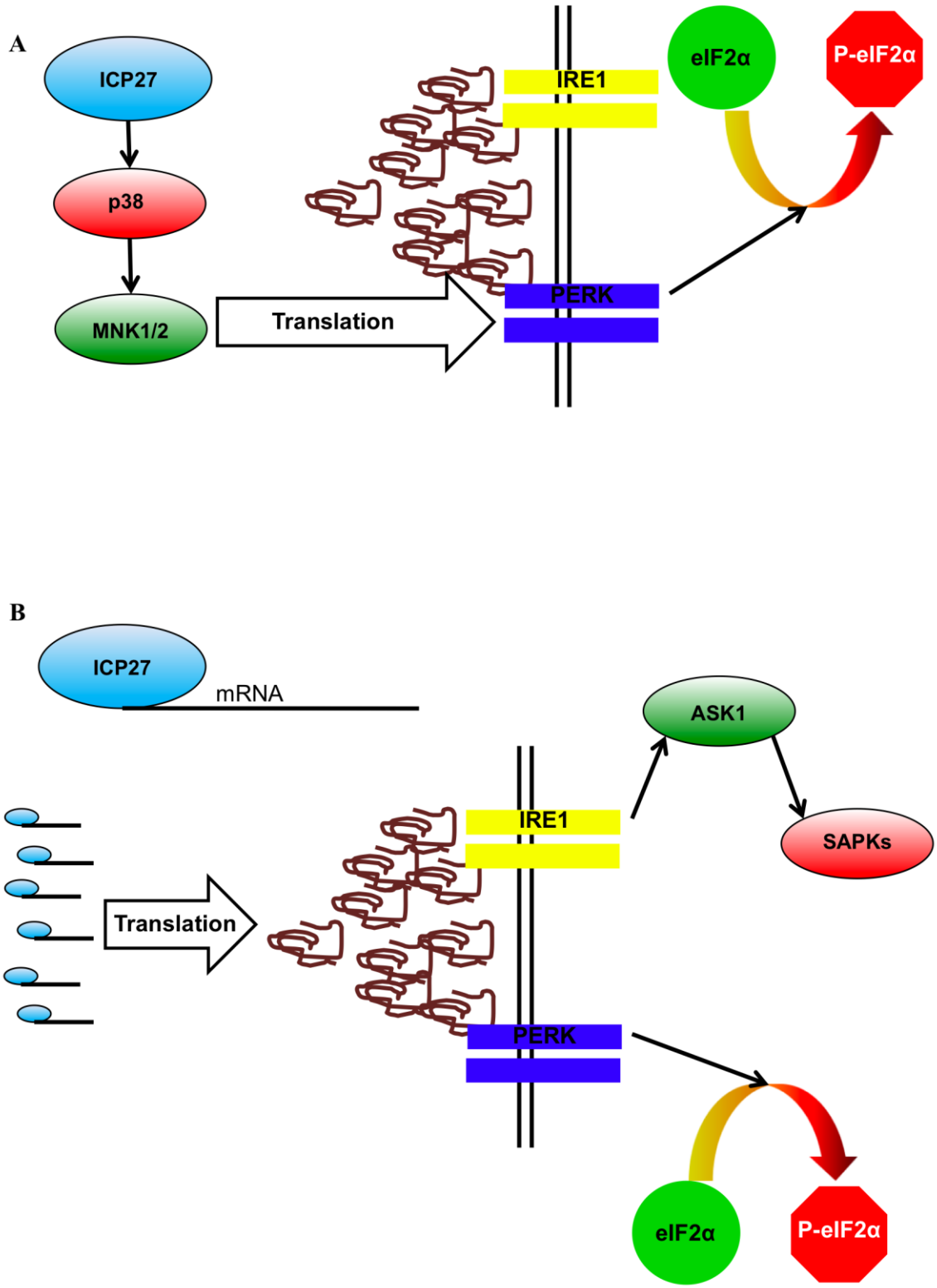
Finally, infection with the ICP27 mutant virus d1-2 potentially sheds light on possible mechanisms by which the ER stress response is induced by ICP27 and has potential implications for how ICP27 activates SAPK signaling. The 12-63 amino acid region of ICP27 has previously been shown to play an important role in the nucleocytoplsmic shuttling of ICP27 as well as its ability to induce

SAPK signaling pathways. Here, we provide evidence that this same domain is required for triggering ER stress and the subsequent activation of XBP-1 mRNA splicing. Based on our results, we suggest two models to explain how ICP27 expression leads to ER stress (Fig. 5.7). In one model (Fig. 5.7 A), ICP27 first triggers the activation of p38 and JNK signaling. As mentioned above, p38 signaling can stimulate translation by activating MNK1/2. Activated MNK1/2 phosphorylates eIF4E, leading to increased cellular translation (66, 93, 176) and the overloading of the ER. An alternative model is that ICP27 directly stimulates translation of cellular proteins through a combination of its mRNA export activity and its interactions with cellular translation factors (Fig. 5.7 B). That is, ICP27 might bind to viral and cellular mRNAs and shuttle them to the cytoplasm via interactions of its acidic region through Ref/TAP (22, 88, 101, 136, 160, 162). ICP27 then could stimulate the translation of the encoded proteins by recruiting translation initiation factors to the transcripts (38), ultimately resulting in ER stress. In this latter model, the ICP27-induced ER stress could then result in activation of SAPK signaling. Since IRE1 functions as both an endoribonuclease and a sensor kinase, upon dimerization it can lead to the phosphorylation and activation of apoptotic stimulating kinase 1 (ASK1) (78, 92). ASK1 has been shown to be capable of activating both the p38 and JNK signaling pathways via MKK6 (78). This ER stress-mediated SAPK signaling could not only trigger components of the UPR, but it could also activate apoptosis. This fits well with our previous observation that ICP27 expression in the absence of viral infection leads to apoptosis (Chapter 3). These combined results suggest that the

IRE1/ASK1 signaling pathway could play a key role in ICP27-mediated SAPK signaling.

Fig. 5.7. Two models for how ICP27 induces ER stress and SAPK signaling.

Fig. 5.7


A
ICP27
p38
MNK1/2
Translation
IRE1
PERK
eIF2α
P-eIF2α
B
ICP27
mRNA
Translation
IRE1
PERK
ASK1
SAPKs
eIF2α
P-eIF2α

Chapter 6

Major Findings and Future Directions

Introduction

At the time that the research in this thesis was begun, there had already been a significant body of work focusing on the functions of ICP27 during viral infection. Reports had implicated ICP27 in the shuttling of cellular and viral mRNA, stabilization of messages with AREs and weak poly-A signals, splicing inhibition, SAPK signaling, and inhibition of apoptosis (reviewed in (129, 138)). However, few of these reports had shown that ICP27 was directly responsible for these effects or had established the mechanisms behind them. Therefore, the focus of this research was to identify the direct effects of ICP27 on normal cellular functions. As an approach to doing this, we engineered human cell lines that inducibly express ICP27 in the absence of infection. Using this system, we discovered that ICP27 is able to stimulate the p38 and JNK signaling pathways in the absence of viral infection. These initial findings led us to further investigate the role of SAPK signaling pathways on viral replication. We found that SAPK signaling can have profound effects on both host cell function and viral replication. Additionally, we have potentially identified a mechanism for the activation of ICP27-mediated SAPK signaling during viral infection.

Major Findings

ICP27 activates SAPK signaling and induces apoptosis.

The research detailed in Chapter 3 shows that ICP27 expression in the absence of viral infection leads to the activation of both p38 and JNK signaling pathways. Although the p38 signaling is robust, the JNK signaling induced is significantly lower that that seen in HSV-1 infected cells. These observations were possible due to the creation of an inducible expression system that allowed us to study the effects of ICP27 on normal cellular functions in the absence of viral infection. Thus, we can conclude that ICP27 stimulates p38 signaling, and to a lesser degree, JNK signaling, during viral infection. This suggests that an additional viral factor(s) is/are likely to be required for the complete activation of JNK observed during viral infection. Our findings about SAPK signaling help to explain the multiple functions attributed to ICP27 during viral infection. For example, as we show in Chapter 5, ICP27-induced p38 signaling is responsible for the stabilization of cellular transcripts bearing AREs in their 3'-UTRs.

Using chemical inhibitors of SAPK signaling, we also found that ICP27-mediated p38 signaling leads to apoptosis in HeLa cells. This was surprising since there were several previous reports suggesting that ICP27 is anti-apoptotic factor during viral infection (8,11, 47). However, we failed to find any evidence that ICP27 alone has an anti-apoptotic effect. Thus, we conclude that, in the absence of viral infection, ICP27 is a pro-apoptotic factor. We speculate that ICP27 is also pro-apoptotic during viral infection, but that this effect is counteracted by additional ICP27-dependent anti-apoptotic viral factors.

JNK signaling is required for efficient expression of DE and L genes, activation of NF-κB, and inhibition of apoptosis.

NF-κB activation is critical for the prevention of apoptosis during HSV-1 infection (47). Previous reports have shown that both ICP27 and ICP27-mediated JNK signaling are required for the activation of NF-κB during viral infection (57). Since ICP27 expression in our inducible HeLa cell lines is sufficient to induce modest JNK signaling, we wanted to determine if expression of ICP27 is sufficient to active NF-κB. However, we found that ICP27 expression alone is not sufficient to induce activation of NF-κB, suggesting that additional viral factors are required. This observation fits well with studies from John Blaho's lab suggesting that DE or L gene products are required for the inhibition of apoptosis in human cells (9, 11, 112).

We hypothesized that ICP27-mediated JNK signaling is required for the expression of DE and L gene products capable of activating NF-κB and inhibiting apoptosis. Based on a recent report, one candidate gene product was the UL37 protein, as it is sufficient to activate NF-κB in transfected cells (90). Consistent with this, we found that chemical inhibition of JNK signaling leads to a significant reduction in the expression of UL37 during HSV-1 infection. Furthermore, UL37 expression is also significantly reduced in ICP27 mutants that are impaired in JNK signaling. To test whether UL37 is an important ICP27-dependent anti-apoptotic factor, we stably transfected the UL37 gene into HeLa cells to create a system in which UL37 is expressed in an ICP27-independent fashion. As expected based on our hypothesis, ICP27-independent expression of UL37

partially inhibits apoptosis during infection with an ICP27 deletion mutant. This identifies UL37 as an ICP27 dependent L protein that functions as an anti-apoptotic factor during viral infection. However, it is important to note that based on our research and the findings of others (90), it is likely that additional viral proteins also play a role in NF-κB activation and apoptosis inhibition during infection.

We found that in addition to impairing the expression of UL37 protein, chemical inhibition of JNK signaling also leads to significant reductions in the expression of all viral DE/L proteins tested. This is the first report that JNK signaling plays a role in the expression of viral proteins. Previous studies have shown that JNK signaling is critical for the efficient replication of HSV-1 in Vero cells (98), but did not investigate the mechanisms involved. Thus, in addition to being required for expression of anti-apoptotic viral proteins, JNK signaling may play a critical role in viral gene expression.

ICP27 expression leads to signs of ER stress and potentially triggers the cellular ISR.

In an effort to understand the downstream effects of p38 signaling, we examined the effects of ICP27 expression and subsequent p38 signaling on the stability of ARE-containing transcripts. We found that ICP27-mediated p38 signaling in the absence of viral infection leads to stabilization of the ARE-bearing IEX-1 transcript. Furthermore, we provided evidence that this stabilization is due to p38-mediated phosphorylation of key cellular proteins, e.g.,

TTP, that normally help mediate the degradation of ARE-containing messages, resulting in their exclusion from sites of mRNA degradation. Interestingly, ARE-containing transcripts are closely associated with regulating the immune response and responding to growth/death signals, and therefore require tight regulation. Thus, as speculated by others (153), ICP27-mediated p38 signaling could alter the host immune response to the advantage of the virus, or manipulate cellular growth so as to benefit viral replication *in vivo*.

Interestingly, during the course of this research, we noted that ICP27 induces stress granule formation in our HeLa cell system. Stress granule formation is indicative of translational arrest caused by the cellular ISR, which results in the phosphorylation of host translation factor eIF2α. Phosphorylation of eIF2α is the result of the activation of sensor kinases that sense internal stress signals. We confirmed that ICP27 expression led to the phosphorylation of eIF2α. Based on this, we hypothesized that ICP27 expression was triggering ER stress, resulting in PERK-mediated phosphorylation of eIF2α. This hypothesis was based on prior work by others that showed ICP27 associates with translation initiation factors and can stimulate the translation of viral genes during infection (32, 38, 85, 155). Due to the difficulty of detecting phosphorylated PERK during HSV-1 infection, we used IRE1-mediated splicing of XBP-1 mRNA as a surrogate marker of ER stress. We found the ICP27 expression is sufficient to induce splicing of XBP-1 mRNA, presumably by IRE1. Furthermore, XBP-1 mRNA splicing was also observed early during HSV-1 infection, indicating that ICP27 may trigger ER stress during viral infection. These finding suggest the ICP27

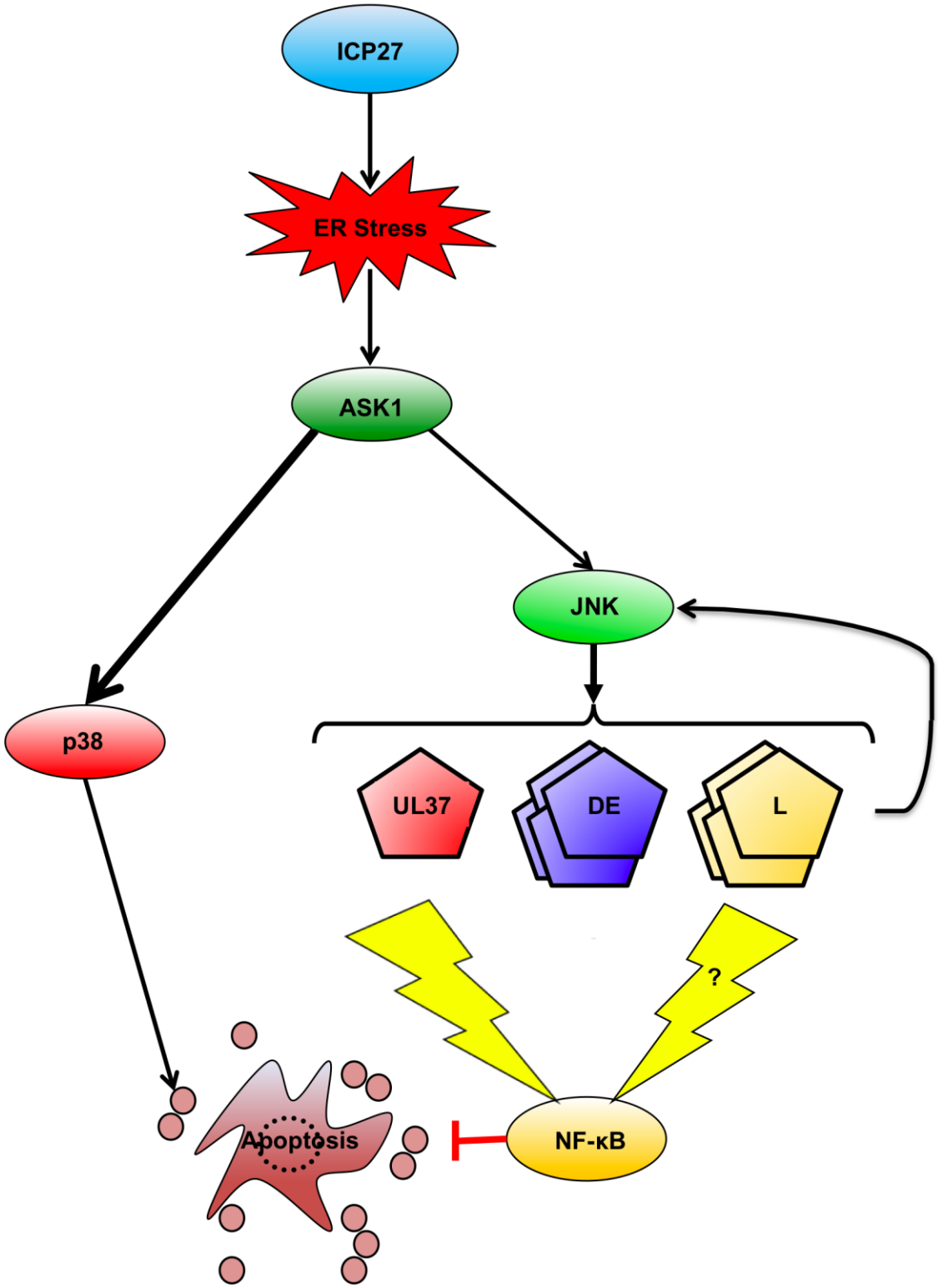
can trigger ER stress leading to activation of the ISR, but that additional viral factors subsequently block the response to allow for efficient viral replication.

The observation that IRE1 is likely activated by ICP27 expression also provides a potential mechanism to explain how ICP27 induces SAPK signaling. In addition to acting as an endoribonuclease, IRE1 functions as a sensor kinase and its activation leads to the phosphorylation of the host protein kinase ASK1 (78, 108). ASK1 is then capable of activating p38 and JNK signaling by phosphorylating MKK6 (78).

Ultimately, the results of this thesis lead us to a model to explain how ICP27's effects on SAPK signaling regulate viral gene expression and apoptotic fate (Fig. 6.1). In this model, ICP27 expression directly stimulates the translation of both cellular and viral transcripts during the earliest stages of HSV-1 infection. While the mechanism behind this translational stimulation remains unclear, amino acid residues 12-63 play an important role. The increased translation mediated by ICP27 then leads to ER stress, triggering the cellular integrated stress response. This benefits viral replication by triggering an UPR, expanding the translation capacity of the ER and allowing for efficient glycoprotein expression. Furthermore, ER stress leads to SAPK signaling which enhances the expression of viral DE and L genes. The negative aspects of the ISR (e.g., eIF2α phosphorylation, translational arrest, and apoptosis) are controlled by the expression of viral proteins that counteract these effects (e.g., US11, γ34.5, gB, and UL37) (24, 109, 129).

Fig. 6.1. Model of ICP27-mediated SAPK regulation of viral gene expression and apoptotic fate.

Fig. 6.1



Future Directions.

Determine the sequences of ICP27 that activate p38 and JNK signaling.

Several studies, including those presented in this thesis, have shown that the N-terminal portion of ICP27 plays a critical role in the activation of SAPKs (27, 56, 57). However many questions remain about the mechanism behind SAPK signaling as well as its importance. First, the published studies using ICP27 deletion mutants have yielded variable results and identified different regions of the N-terminal regions of ICP27 as being responsible for SAPK activation (27, 56, 57). Additionally, two prior studies disagreed on the importance of the C-terminal region for the activation of p38 signaling (27, 56, 57). The divergent results could be due to the fact that the studies were performed in different cell lines. Therefore, it would be interesting determine how cell type effects the activation of SAPK signaling following HSV-1 infection.

Further exploration of N- and C-terminal mutants generated by our lab and others could shed light on the mechanism of ICP27-mediated activation of SAPKs. Our work here has shown during viral infection of HeLa cells, ICP27 deletion mutant d1-2 is defective for JNK signaling, and mutants d3-4 and d4-5 display partially reduced JNK signaling. Additionally, C-terminal mutant M11 (R340L and D341E) (126) was also shown to be impaired for p38 signaling (27). It would be interesting to explore the effects of these ICP27 mutations on SAPK signaling in the absence of viral infection using the inducible expression system detailed in this thesis. This would allow for a clearer understanding of how these mutations directly affect signaling without the confounding effects of these

mutations on viral replication. Identification of the domains of ICP27 that are directly involved in SAPK signaling will allow for a better understanding of the mechanisms involved in the activation of this signaling.

Another important step forward in the understanding of the functions the N-terminal and C-terminal regions of ICP27 could come from determining the structure of the ICP27 protein using X-ray crystallography. Solving the protein structure will allow for a better understanding of how the N and C-terminal halves of ICP27 function to activate SAPK signaling. Furthermore, the structure of ICP27 will allow for prediction of how mutations could affect protein structure and function.

Determine if ICP27-mediated ER stress and ISR activation lead to SAPK signaling.

In addition to exploring the regions of ICP27 required for SAPK activation, it is critical to identify the cellular pathway(s) by which ICP27 triggers SAPK signaling. The results presented in this study are the first to suggest a potential mechanism for p38 and JNK activation. We have obtained evidence that ICP27 expression in the absence of viral infection leads to the activation of ISR pathways due to ER stress. Further studies exploring potential changes in translation and cellular signaling pathways associated with ER stress would provide valuable insight into the effects of ICP27 on normal cellular. Since ICP27 expression alone may be sufficient to induce ER stress, it would be interesting to determine what effects if any ICP27 has on cellular translation. Metabolic labeling

of cells using S^{35}-methionine would show if ICP27 expression is increasing the rates of cellular translation in a specific or global manner. Since ICP27 expression also leads to eIF2α phosphorylation, a time course of metabolic labeling would likely be needed to determine if ICP27 stimulates translation at early times post induction, while potentially inhibiting it at later times post induction. Additionally, cellular proteins whose expression is specifically affected by ICP27 could be potentially identified by proteomic analysis using isobaric tags for relative and absolute quantitation (iTRAQ) followed by tandem mass spectrometry. This system allows for analysis and identification of global changes in protein levels between multiple samples. Proteomic analysis using iTRAQ-based quantification has recently been used to identify changes in protein expression associated with HBV, HPV, and HIV infection (113, 189). Identification of cellular proteins whose synthesis is affected by ICP27 could provide valuable insights into how translation of mRNAs is affected by ICP27.

SAPK signaling could play a role in induction of ER stress or in the cellular response to it. Multiple approaches could be used to explore the relationship between ICP27 and SAPK signaling. First, induction of ER stress by ICP27 could be monitored via eIF2α phosphorylation and XBP-1 mRNA splicing in the absence or presence of the chemical inhibitors used in the studies detailed in this thesis. Use of inhibitors would show whether SAPK signaling activates ER stress, or if instead ER stress leads to the activation of SAPK signaling. Second, activation of IRE1 by ER stress has previously been shown to induce the phosphorylation and activation of ASK-1, leading to p38 and JNK signaling.

Antibodies against phosphorylated ASK-1 are commercially available and would potentially allow us to connect SAPK signaling to IRE1 activation. Third, ER stress and IRE1 activation could be monitored during infection with the ICP27 mutants that have defects in SAPK signaling. Finally, using siRNA knock down expression of either ASK1 or IRE1, the activation of SAPKs could be measured following induction/infection in cells unable to respond to ER stress.

Determine how JNK signaling stimulates HSV-1 gene expression.

Previous studies have shown that JNK signaling is beneficial for HSV-1 replication, and consistent with this, ICP27 mutants lacking the domain required for SAPK signal activation are deficient in replication. Unpublished data from our lab suggests that the region of ICP27 responsible for SAPK activation plays a significantly more important role in viral replication in HeLa cells than in Vero cells. Instead of the 1-2 log defect in viral replication seen in Vero cells, d1-2 displays a 3-4 log defect in growth in HeLa cells (J. Lengyel and S. Rice, unpublished observation). One potential explanation could be an increased dependence of HSV-1 protein expression on SAPK signaling in HeLa cells. Understanding the mechanism by which JNK signaling increases viral gene expression will provide valuable insight into the functions of ICP27 during viral replication. JNK signaling could affect gene expression at the transcriptional, post-transcriptional, or translational levels. To begin to determine which of these levels are affected, the steady state levels of viral gene mRNAs should be measured following wild-type HSV-1 infection, infection with inhibitor treatment,

and infection with SAPK deficient mutants. This could be done using Northern blots or quantitative RT-PCR. If the steady state levels of viral mRNAs are reduced, nuclear run-on assays can be used to determine if this is due to reduced transcription rates or to post-transcriptional message degradation (120, 149). Finally, if mRNA levels are unaffected, metabolic pulse-labeling followed by immunoprecipitation could be used to measure translation rates of specific viral proteins.

Inhibition of JNK signaling appeared to inhibit the expression of all of the viral proteins we examined. However, the mechanism behind this apparent requirement for JNK signaling and how widespread this effect is remains unknown. For these reasons, it would be interesting to examine the expression of a larger number of viral gene products. Most of the gene products examined in this study were L gene products that require genome replication to varying degrees for their efficient viral replication. Therefore, added focus should be placed on the inclusion of DE gene products that are both ICP27 dependent and independent. It would be best to measure the expression of additional viral genes by assaying both protein and mRNA levels as described above. Antibodies are currently available for detection of several DE and L viral gene products not examined in this study. Using these techniques, expression of viral proteins and mRNAs could be measured following wild-type infection, infection with inhibitor treatment, and infection with SAPK deficient mutants.

Identification of additional ICP27-dependent anti-apoptotic factors.

As discussed in Chapter 4, additional viral proteins besides UL37 are likely involved in the activation of NF-κB and the inhibition of apoptosis during HSV-1 infection. For example, glycoprotein D, which we have shown to be dependent on JNK signaling for its efficient expression, has recently been shown to activate NF-κB during viral infection (147). Furthermore, JNK signaling is required for activation of NF-κB during HSV-1 infection (57). Therefore, if we discover additional viral proteins whose expression is dependent on JNK signaling, it would be interesting to test whether any of these are involved in the activation of NF-κB or the inhibition of apoptosis during viral infection. To test this, we could ask if expression of the candidate protein(s) induces the nuclear localization of the NF-κB p65 subunit or causes IκB degradation. These assays, however, are not practical for screening a large number of viral genes due to technical limitations. Instead, as an initial screen, we could transiently transfect identified viral genes into cells expressing a reporter gene, e.g., β-gal or luciferase, under the control of a NF-κB responsive promoter allowing for a simple screening procedure. A similar approach was taken to identify UL37 as a protein capable of activating NF-κB (90). Although a large number of HSV-1 open reading frame were tested in that study, it is not clear if protein levels were measured to verify expression of candidate genes. This is a potentially important point, as we have shown that at least one ICP27 dependent viral gene product, gC, requires ICP27 for efficient protein expression following transfection (120, 149). For this reason, the inducible cell lines described in this thesis could be

used to create the reporter cell lines. Proteins identified as being capable of activating NF-κB by the reporter construct screening could then be confirmed by measuring the nuclear localization of the NF-κB p65 subunit and IκB degradation following protein expression. Finally, viral proteins capable of activating NF-κB could then analyzed for their ability to inhibit apoptosis during d27-1 infection as described in this thesis.

References

1. Adler, V., S. Y. Fuchs, J. Kim, A. Kraft, M. P. King, J. Pelling, and Z. Ronai. 1995. jun-NH2-terminal kinase activation mediated by UV-induced DNA lesions in melanoma and fibroblast cells. Cell Growth Differ 6:1437-46.
2. Advani, S. J., R. Brandimarti, R. R. Weichselbaum, and B. Roizman. 2000. The disappearance of cyclins A and B and the increase in activity of the G(2)/M-phase cellular kinase cdc2 in herpes simplex virus 1-infected cells require expression of the alpha22/U(S)1.5 and U(L)13 viral genes. J Virol 74:8-15.
3. Advani, S. J., R. R. Weichselbaum, and B. Roizman. 2003. Herpes simplex virus 1 activates cdc2 to recruit topoisomerase II alpha for post-DNA synthesis expression of late genes. Proc Natl Acad Sci U S A 100:4825-30.
4. Anderson, P., and N. Kedersha. 2002. Stressful initiations. J Cell Sci 115:3227-34.
5. Anderson, P., and N. Kedersha. 2002. Visibly stressed: the role of eIF2, TIA-1, and stress granules in protein translation. Cell Stress Chaperones 7:213-21.
6. Assefa, Z., M. Garmyn, R. Bouillon, W. Merlevede, J. R. Vandenheede, and P. Agostinis. 1997. Differential stimulation of ERK and JNK activities by ultraviolet B irradiation and epidermal growth factor in human keratinocytes. J Invest Dermatol 108:886-91.
7. Atanasiu, D., J. R. Kent, J. J. Gartner, and N. W. Fraser. 2006. The stable 2-kb LAT intron of herpes simplex stimulates the expression of heat shock proteins and protects cells from stress. Virology 350:26-33.
8. Aubert, M., and J. A. Blaho. 1999. The herpes simplex virus type 1 regulatory protein ICP27 is required for the prevention of apoptosis in infected human cells. J Virol 73:2803-13.
9. Aubert, M., J. O'Toole, and J. A. Blaho. 1999. Induction and prevention of apoptosis in human HEp-2 cells by herpes simplex virus type 1. J Virol 73:10359-70.
10. Aubert, M., L. E. Pomeranz, and J. A. Blaho. 2007. Herpes simplex virus blocks apoptosis by precluding mitochondrial cytochrome c release independent of caspase activation in infected human epithelial cells. Apoptosis 12:19-35.
11. Aubert, M., S. A. Rice, and J. A. Blaho. 2001. Accumulation of herpes simplex virus type 1 early and leaky-late proteins correlates with apoptosis prevention in infected human HEp-2 cells. J Virol 75:1013-30.
12. Baker, M. L., W. Jiang, F. J. Rixon, and W. Chiu. 2005. Common ancestry of herpesviruses and tailed DNA bacteriophages. J Virol 79:14967-70.

13. Belanger, C., A. Gravel, A. Tomoiu, M. E. Janelle, J. Gosselin, M. J. Tremblay, and L. Flamand. 2001. Human herpesvirus 8 viral FLICE-inhibitory protein inhibits Fas-mediated apoptosis through binding and prevention of procaspase-8 maturation. J Hum Virol 4:62-73.
14. Bloom, D. C. 2004. HSV LAT and neuronal survival. Int Rev Immunol 23:187-98.
15. Bogoyevitch, M. A., A. J. Ketterman, and P. H. Sugden. 1995. Cellular stresses differentially activate c-Jun N-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. J Biol Chem 270:29710-7.
16. Brook, M., C. R. Tchen, T. Santalucia, J. McIlrath, J. S. Arthur, J. Saklatvala, and A. R. Clark. 2006. Posttranslational regulation of tristetraprolin subcellular localization and protein stability by p38 mitogen-activated protein kinase and extracellular signal-regulated kinase pathways. Mol Cell Biol 26:2408-18.
17. Brown, C. R., M. S. Nakamura, J. D. Mosca, G. S. Hayward, S. E. Straus, and L. P. Perera. 1995. Herpes simplex virus trans-regulatory protein ICP27 stabilizes and binds to 3' ends of labile mRNA. J Virol 69:7187-95.
18. Bryant, H. E., S. E. Wadd, A. I. Lamond, S. J. Silverstein, and J. B. Clements. 2001. Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. J Virol 75:4376-85.
19. Burch, A. D., and S. K. Weller. 2005. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. J Virol 79:10740-9.
20. Cai, W., and P. A. Schaffer. 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. J Virol 66:2904-15.
21. Cai, W. Z., S. Person, C. DebRoy, and B. H. Gu. 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. J Mol Biol 201:575-88.
22. Chen, I. H., L. Li, L. Silva, and R. M. Sandri-Goldin. 2005. ICP27 recruits Aly/REF but not TAP/NXF1 to herpes simplex virus type 1 transcription sites although TAP/NXF1 is required for ICP27 export. J Virol 79:3949-61.
23. Chen, I. H., K. S. Sciabica, and R. M. Sandri-Goldin. 2002. ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway. J Virol 76:12877-89.
24. Cheng, G., Z. Feng, and B. He. 2005. Herpes simplex virus 1 infection activates the endoplasmic reticulum resident kinase PERK and mediates eIF-2alpha dephosphorylation by the gamma(1)34.5 protein. J Virol 79:1379-88.

25. Cheung, P., K. S. Ellison, R. Verity, and J. R. Smiley. 2000. Herpes simplex virus ICP27 induces cytoplasmic accumulation of unspliced polyadenylated alpha-globin pre-mRNA in infected HeLa cells. J Virol 74:2913-9.
26. Cocchi, F., L. Menotti, P. Dubreuil, M. Lopez, and G. Campadelli-Fiume. 2000. Cell-to-cell spread of wild-type herpes simplex virus type 1, but not of syncytial strains, is mediated by the immunoglobulin-like receptors that mediate virion entry, nectin1 (PRR1/HveC/HlgR) and nectin2 (PRR2/HveB). J Virol 74:3909-17.
27. Corcoran, J. A., W. L. Hsu, and J. R. Smiley. 2006. Herpes simplex virus ICP27 is required for virus-induced stabilization of the ARE-containing IEX-1 mRNA encoded by the human IER3 gene. J Virol 80:9720-9.
28. de Bruyn Kops, A., S. L. Uprichard, M. Chen, and D. M. Knipe. 1998. Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. Virology 252:162-78.
29. Dean, J. L., S. J. Sarsfield, E. Tsounakou, and J. Saklatvala. 2003. p38 Mitogen-activated protein kinase stabilizes mRNAs that contain cyclooxygenase-2 and tumor necrosis factor AU-rich elements by inhibiting deadenylation. J Biol Chem 278:39470-6.
30. DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer. 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J Virol 56:558-70.
31. Eferl, R., M. Sibilia, F. Hilberg, A. Fuchsbichler, I. Kufferath, B. Guertl, R. Zenz, E. F. Wagner, and K. Zatloukal. 1999. Functions of c-Jun in liver and heart development. J Cell Biol 145:1049-61.
32. Ellison, K. S., R. A. Maranchuk, K. L. Mottet, and J. R. Smiley. 2005. Control of VP16 translation by the herpes simplex virus type 1 immediate-early protein ICP27. J Virol 79:4120-31.
33. Ellison, K. S., S. A. Rice, R. Verity, and J. R. Smiley. 2000. Processing of alpha-globin and ICP0 mRNA in cells infected with herpes simplex virus type 1 ICP27 mutants. J Virol 74:7307-19.
34. Esclatine, A., B. Taddeo, L. Evans, and B. Roizman. 2004. The herpes simplex virus 1 UL41 gene-dependent destabilization of cellular RNAs is selective and may be sequence-specific. Proc Natl Acad Sci U S A 101:3603-8.
35. Esclatine, A., B. Taddeo, and B. Roizman. 2004. The UL41 protein of herpes simplex virus mediates selective stabilization or degradation of cellular mRNAs. Proc Natl Acad Sci U S A 101:18165-70.
36. Everett, R. D. 1985. Activation of cellular promoters during herpes virus infection of biochemically transformed cells. EMBO J 4:1973-80.
37. Everett, R. D. 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. J Gen Virol 70 (Pt 5):1185-202.

38. Fontaine-Rodriguez, E. C., T. J. Taylor, M. Olesky, and D. M. Knipe. 2004. Proteomics of herpes simplex virus infected cell protein 27: association with translation initiation factors. Virology 330:487-92.
39. Forrester, A., H. Farrell, G. Wilkinson, J. Kaye, N. Davis-Poynter, and T. Minson. 1992. Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. J Virol 66:341-8.
40. Fraser, K. A., and S. A. Rice. 2007. Herpes simplex virus immediate-early protein ICP22 triggers loss of serine 2-phosphorylated RNA polymerase II. J Virol 81:5091-101.
41. Fraser, K. A., and S. A. Rice. 2005. Herpes simplex virus type 1 infection leads to loss of serine-2 phosphorylation on the carboxyl-terminal domain of RNA polymerase II. J Virol 79:11323-34.
42. Galvan, V., R. Brandimarti, J. Munger, and B. Roizman. 2000. Bcl-2 blocks a caspase-dependent pathway of apoptosis activated by herpes simplex virus 1 infection in HEp-2 cells. J Virol 74:1931-8.
43. Ge, B., H. Gram, F. Di Padova, B. Huang, L. New, R. J. Ulevitch, Y. Luo, and J. Han. 2002. MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha. Science 295:1291-4.
44. Gillis, P. A., L. H. Okagaki, and S. A. Rice. 2009. Herpes simplex virus type 1 ICP27 induces p38 mitogen-activated protein kinase signaling and apoptosis in HeLa cells. J Virol 83:1767-77.
45. Gimble, J. M., E. Duh, J. M. Ostrove, H. E. Gendelman, E. E. Max, and A. B. Rabson. 1988. Activation of the human immunodeficiency virus long terminal repeat by herpes simplex virus type 1 is associated with induction of a nuclear factor that binds to the NF-kappa B/core enhancer sequence. J Virol 62:4104-12.
46. Goldmacher, V. S., L. M. Bartle, A. Skaletskaya, C. A. Dionne, N. L. Kedersha, C. A. Vater, J. W. Han, R. J. Lutz, S. Watanabe, E. D. Cahir McFarland, E. D. Kieff, E. S. Mocarski, and T. Chittenden. 1999. A cytomegalovirus-encoded mitochondria-localized inhibitor of apoptosis structurally unrelated to Bcl-2. Proc Natl Acad Sci U S A 96:12536-41.
47. Goodkin, M. L., A. T. Ting, and J. A. Blaho. 2003. NF-kappaB is required for apoptosis prevention during herpes simplex virus type 1 infection. J Virol 77:7261-80.
48. Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 89:5547-51.
49. Gossen, M., S. Freundlieb, G., Bender, G. Muller, W., Hillen, and H. Bujard. 1995. Transcriptional activation by tetracycline in mammalian cells. Science 268:1766-1769.
50. Gregory, D., D. Hargett, D. Holmes, E. Money, and S. L. Bachenheimer. 2004. Efficient replication by herpes simplex virus

type 1 involves activation of the IkappaB kinase-IkappaB-p65 pathway. J Virol 78:13582-90.

51. Hagglund, R., J. Munger, A. P. Poon, and B. Roizman. 2002. U(S)3 protein kinase of herpes simplex virus 1 blocks caspase 3 activation induced by the products of U(S)1.5 and U(L)13 genes and modulates expression of transduced U(S)1.5 open reading frame in a cell type-specific manner. J Virol 76:743-54.
52. Halford, W. P., and P. A. Schaffer. 2001. ICP0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. J Virol 75:3240-9.
53. Harding, H. P., M. Calfon, F. Urano, I. Novoa, and D. Ron. 2002. Transcriptional and translational control in the Mammalian unfolded protein response. Annu Rev Cell Dev Biol 18:575-99.
54. Hardwicke, M. A., and R. M. Sandri-Goldin. 1994. The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection. J Virol 68:4797-810.
55. Hardy, W. R., and R. M. Sandri-Goldin. 1994. Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. J Virol 68:7790-9.
56. Hargett, D., T. McLean, and S. L. Bachenheimer. 2005. Herpes simplex virus ICP27 activation of stress kinases JNK and p38. J Virol 79:8348-60.
57. Hargett, D., S. Rice, and S. L. Bachenheimer. 2006. Herpes simplex virus type 1 ICP27-dependent activation of NF-kappaB. J Virol 80:10565-78.
58. He, L., S. O. Kim, O. Kwon, S. J. Jeong, M. S. Kim, H. G. Lee, H. Osada, M. Jung, J. S. Ahn, and B. Y. Kim. 2009. ATM blocks tunicamycin-induced endoplasmic reticulum stress. FEBS Lett 583:903-8.
59. Herrera, F. J., and S. J. Triezenberg. 2004. VP16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. J Virol 78:9689-96.
60. Herrlinger, U., P. A. Pechan, A. H. Jacobs, C. Woiciechowski, N. G. Rainov, C. Fraefel, W. Paulus, and S. A. Reeves. 2000. HSV-1 infected cell proteins influence tetracycline-regulated transgene expression. J Gene Med 2:379-89.
61. Hibbard, M. K., and R. M. Sandri-Goldin. 1995. Arginine-rich regions succeeding the nuclear localization region of the herpes simplex virus type 1 regulatory protein ICP27 are required for efficient nuclear localization and late gene expression. J Virol 69:4656-67.
62. Holzberg, D., C. G. Knight, O. Dittrich-Breiholz, H. Schneider, A. Dorrie, E. Hoffmann, K. Resch, and M. Kracht. 2003. Disruption of the c-JUN-JNK complex by a cell-permeable peptide containing the c-JUN delta domain induces apoptosis and affects a distinct set of interleukin-1-induced inflammatory genes. J Biol Chem 278:40213-23.

63. Hsu, W. L., H. A. Saffran, and J. R. Smiley. 2005. Herpes simplex virus infection stabilizes cellular IEX-1 mRNA. J Virol 79:4090-8.
64. Hughes, R. G., Jr., and W. H. Munyon. 1975. Temperature-sensitive mutants of herpes simplex virus type 1 defective in lysis but not in transformation. J Virol 16:275-83.
65. Iordanov, M. S., D. Pribnow, J. L. Magun, T. H. Dinh, J. A. Pearson, and B. E. Magun. 1998. Ultraviolet radiation triggers the ribotoxic stress response in mammalian cells. J Biol Chem 273:15794-803.
66. Ishida, M., T. Ishida, H. Nakashima, N. Miho, K. Miyagawa, K. Chayama, T. Oshima, M. Kambe, and M. Yoshizumi. 2003. Mnk1 is required for angiotensin II-induced protein synthesis in vascular smooth muscle cells. Circ Res 93:1218-24.
67. Isler, J. A., A. H. Skalet, and J. C. Alwine. 2005. Human cytomegalovirus infection activates and regulates the unfolded protein response. J Virol 79:6890-9.
68. Jackson, S. A., and N. A. DeLuca. 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. Proc Natl Acad Sci U S A 100:7871-6.
69. Jean, S., K. M. LeVan, B. Song, M. Levine, and D. M. Knipe. 2001. Herpes simplex virus 1 ICP27 is required for transcription of two viral late (gamma 2) genes in infected cells. Virology 283:273-84.
70. Jerome, K. R., R. Fox, Z. Chen, P. Sarkar, and L. Corey. 2001. Inhibition of apoptosis by primary isolates of herpes simplex virus. Arch Virol 146:2219-25.
71. Jerome, K. R., R. Fox, Z. Chen, A. E. Sears, H. Lee, and L. Corey. 1999. Herpes simplex virus inhibits apoptosis through the action of two genes, Us5 and Us3. J Virol 73:8950-7.
72. Johannsen, E., M. Luftig, M. R. Chase, S. Weicksel, E. Cahir-McFarland, D. Illanes, D. Sarracino, and E. Kieff. 2004. Proteins of purified Epstein-Barr virus. Proc Natl Acad Sci U S A 101:16286-91.
73. Johnson, L. A., L. Li, and R. M. Sandri-Goldin. 2009. The cellular RNA export receptor TAP/NXF1 is required for ICP27-mediated export of herpes simplex virus 1 RNA, but the TREX complex adaptor protein Aly/REF appears to be dispensable. J Virol 83:6335-46.
74. Jordan, R., L. Wang, T. M. Graczyk, T. M. Block, and P. R. Romano. 2002. Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J Virol 76:9588-99.
75. Kang, W., R. Mukerjee, J. J. Gartner, A. G. Hatzigeorgiou, R. M. Sandri-Goldin, and N. W. Fraser. 2006. Characterization of a spliced exon product of herpes simplex type-1 latency-associated transcript in productively infected cells. Virology 356:106-14.
76. Karaca, G., D. Hargett, T. I. McLean, J. S. Aguilar, P. Ghazal, E. K. Wagner, and S. L. Bachenheimer. 2004. Inhibition of the stress-activated kinase, p38, does not affect the virus transcriptional program of herpes simplex virus type 1. Virology 329:142-56.

77. Kennedy, G., J. Komano, and B. Sugden. 2003. Epstein-Barr virus provides a survival factor to Burkitt's lymphomas. Proc Natl Acad Sci U S A 100:14269-74.
78. Kim, I., C. W. Shu, W. Xu, C. W. Shiau, D. Grant, S. Vasile, N. D. Cosford, and J. C. Reed. 2009. Chemical biology investigation of cell death pathways activated by endoplasmic reticulum stress reveals cytoprotective modulators of ASK1. J Biol Chem 284:1593-603.
79. Koffa, M. D., J. B. Clements, E. Izaurralde, S. Wadd, S. A. Wilson, I. W. Mattaj, and S. Kuersten. 2001. Herpes simplex virus ICP27 protein provides viral mRNAs with access to the cellular mRNA export pathway. EMBO J 20:5769-78.
80. Koyama, A. H., and A. Adachi. 1997. Induction of apoptosis by herpes simplex virus type 1. J Gen Virol 78 (Pt 11):2909-12.
81. Krummenacher, C., A. V. Nicola, J. C. Whitbeck, H. Lou, W. Hou, J. D. Lambris, R. J. Geraghty, P. G. Spear, G. H. Cohen, and R. J. Eisenberg. 1998. Herpes simplex virus glycoprotein D can bind to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. J Virol 72:7064-74.
82. Kwong, A. D., and N. Frenkel. 1989. The herpes simplex virus virion host shutoff function. J Virol 63:4834-9.
83. Kwong, A. D., J. A. Kruper, and N. Frenkel. 1988. Herpes simplex virus virion host shutoff function. J Virol 62:912-21.
84. Lai, W. S., E. Carballo, J. R. Strum, E. A. Kennington, R. S. Phillips, and P. J. Blackshear. 1999. Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol 19:4311-23.
85. Larralde, O., R. W. Smith, G. S. Wilkie, P. Malik, N. K. Gray, and J. B. Clements. 2006. Direct stimulation of translation by the multifunctional herpesvirus ICP27 protein. J Virol 80:1588-91.
86. Leib, D. A., D. M. Coen, C. L. Bogard, K. A. Hicks, D. R. Yager, D. M. Knipe, K. L. Tyler, and P. A. Schaffer. 1989. Immediate-early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J Virol 63:759-68.
87. Lengyel, J., C. Guy, V. Leong, S. Borge, and S. A. Rice. 2002. Mapping of functional regions in the amino-terminal portion of the herpes simplex virus ICP27 regulatory protein: importance of the leucine-rich nuclear export signal and RGG Box RNA-binding domain. J Virol 76:11866-79.
88. Lengyel, J., A. K. Strain, K. D. Perkins, and S. A. Rice. 2006. ICP27-dependent resistance of herpes simplex virus type 1 to leptomycin B is associated with enhanced nuclear localization of ICP4 and ICP0. Virology 352:368-79.
89. Ligas, M. W., and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by beta-

galactosidase sequences binds to but is unable to penetrate into cells. J Virol 62:1486-94.

90. Liu, X., K. Fitzgerald, E. Kurt-Jones, R. Finberg, and D. M. Knipe. 2008. Herpesvirus tegument protein activates NF-kappaB signaling through the TRAF6 adaptor protein. Proc Natl Acad Sci U S A 105:11335-9.
91. Lomonte, P., J. Thomas, P. Texier, C. Caron, S. Khochbin, and A. L. Epstein. 2004. Functional interaction between class II histone deacetylases and ICP0 of herpes simplex virus type 1. J Virol 78:6744-57.
92. Ma, Y., and L. M. Hendershot. 2004. The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 4:966-77.
93. Mahalingam, M., and J. A. Cooper. 2001. Phosphorylation of mammalian eIF4E by Mnk1 and Mnk2: tantalizing prospects for a role in translation. Prog Mol Subcell Biol 27:132-42.
94. Marciniak, S. J., C. Y. Yun, S. Oyadomari, I. Novoa, Y. Zhang, R. Jungreis, K. Nagata, H. P. Harding, and D. Ron. 2004. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. Genes Dev 18:3066-77.
95. McCarthy, A. M., L. McMahan, and P. A. Schaffer. 1989. Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J Virol 63:18-27.
96. McGeoch, D. J., F. J. Rixon, and A. J. Davison. 2006. Topics in herpesvirus genomics and evolution. Virus Res 117:90-104.
97. McKnight, S. L., E. R. Gavis, R. Kingsbury, and R. Axel. 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream control region. Cell 25:385-98.
98. McLean, T. I., and S. L. Bachenheimer. 1999. Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. J Virol 73:8415-26.
99. McMahan, L., and P. A. Schaffer. 1990. The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection. J Virol 64:3471-85.
100. Mears, W. E., V. Lam, and S. A. Rice. 1995. Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27. J Virol 69:935-47.
101. Mears, W. E., and S. A. Rice. 1998. The herpes simplex virus immediate-early protein ICP27 shuttles between nucleus and cytoplasm. Virology 242:128-37.
102. Mears, W. E., and S. A. Rice. 1996. The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines in vivo methylation. J Virol 70:7445-53.

103. Melchjorsen, J., F. S. Pedersen, S. C. Mogensen, and S. R. Paludan. 2002. Herpes simplex virus selectively induces expression of the CC chemokine RANTES/CCL5 in macrophages through a mechanism dependent on PKR and ICP0. J Virol 76:2780-8.

104. Mettenleiter, T. C., B. G. Klupp, and H. Granzow. 2006. Herpesvirus assembly: a tale of two membranes. Curr Opin Microbiol 9:423-9.

105. Miller, W. E., G. Mosialos, E. Kieff, and N. Raab-Traub. 1997. Epstein-Barr virus LMP1 induction of the epidermal growth factor receptor is mediated through a TRAF signaling pathway distinct from NF-kappaB activation. J Virol 71:586-94.

106. Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87:427-36.

107. Mori, I., F. Goshima, T. Koshizuka, N. Koide, T. Sugiyama, T. Yoshida, T. Yokochi, Y. Kimura, and Y. Nishiyama. 2003. The US3 protein kinase of herpes simplex virus attenuates the activation of the c-Jun N-terminal protein kinase signal transduction pathway in infected piriform cortex neurons of C57BL/6 mice. Neurosci Lett 351:201-5.

108. Mulvey, M., C. Arias, and I. Mohr. 2007. Maintenance of endoplasmic reticulum (ER) homeostasis in herpes simplex virus type 1-infected cells through the association of a viral glycoprotein with PERK, a cellular ER stress sensor. J Virol 81:3377-90.

109. Mulvey, M., C. Arias, and I. Mohr. 2006. Resistance of mRNA translation to acute endoplasmic reticulum stress-inducing agents in herpes simplex virus type 1-infected cells requires multiple virus-encoded functions. J Virol 80:7354-63.

110. Nanbo, A., K. Inoue, K. Adachi-Takasawa, and K. Takada. 2002. Epstein-Barr virus RNA confers resistance to interferon-alpha-induced apoptosis in Burkitt's lymphoma. EMBO J 21:954-65.

111. Nanbo, A., and K. Takada. 2002. The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis. Rev Med Virol 12:321-6.

112. Nguyen, M. L., and J. A. Blaho. 2007. Apoptosis during herpes simplex virus infection. Adv Virus Res 69:67-97.

113. Niu, D., J. Sui, J. Zhang, H. Feng, and W. N. Chen. 2009. iTRAQ-coupled 2-D LC-MS/MS analysis of protein profile associated with HBV-modulated DNA methylation. Proteomics 9:3856-68.

114. Nozawa, N., Y. Yamauchi, K. Ohtsuka, Y. Kawaguchi, and Y. Nishiyama. 2004. Formation of aggresome-like structures in herpes simplex virus type 2-infected cells and a potential role in virus assembly. Exp Cell Res 299:486-97.

115. Osman, F., N. Jarrous, Y. Ben-Asouli, and R. Kaempfer. 1999. A cis-acting element in the 3'-untranslated region of human TNF-alpha mRNA renders splicing dependent on the activation of protein kinase PKR. Genes Dev 13:3280-93.

116. Panagiotidis, C. A., E. K. Lium, and S. J. Silverstein. 1997. Physical and functional interactions between herpes simplex virus immediate-early proteins ICP4 and ICP27. J Virol 71:1547-57.
117. Papavassiliou, A. G., K. W. Wilcox, and S. J. Silverstein. 1991. The interaction of ICP4 with cell/infected-cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA. EMBO J 10:397-406.
118. Patel, A., J. Hanson, T. I. McLean, J. Olgiate, M. Hilton, W. E. Miller, and S. L. Bachenheimer. 1998. Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. Virology 247:212-22.
119. Pellet, P. E. a. B. R., B. 2007. The family *Herpesviridae*: a brief introduction, p. 2480-2500. *In* D. M. K. a. P. M. Howley (ed.), Fields Virology, 5th ed. Lippincott, Williams & Wilkins, Philadelphia, Pa.
120. Perkins, K. D., J. Gregonis, S. Borge, and S. A. Rice. 2003. Transactivation of a viral target gene by herpes simplex virus ICP27 is posttranscriptional and does not require the endogenous promoter or polyadenylation site. J Virol 77:9872-84.
121. Phelan, A., and J. B. Clements. 1997. Herpes simplex virus type 1 immediate early protein IE63 shuttles between nuclear compartments and the cytoplasm. J Gen Virol 78 (Pt 12):3327-31.
122. Poon, A. P., H. Gu, and B. Roizman. 2006. ICP0 and the US3 protein kinase of herpes simplex virus 1 independently block histone deacetylation to enable gene expression. Proc Natl Acad Sci U S A 103:9993-8.
123. Quinlan, M. P., L. B. Chen, and D. M. Knipe. 1984. The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell 36:857-68.
124. Rice, S. A., and D. M. Knipe. 1988. Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. J Virol 62:3814-23.
125. Rice, S. A., and D. M. Knipe. 1990. Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. J Virol 64:1704-15.
126. Rice, S. A., and V. Lam. 1994. Amino acid substitution mutations in the herpes simplex virus ICP27 protein define an essential gene regulation function. J Virol 68:823-33.
127. Rice, S. A., V. Lam, and D. M. Knipe. 1993. The acidic amino-terminal region of herpes simplex virus type 1 alpha protein ICP27 is required for an essential lytic function. J Virol 67:1778-87.
128. Rice, S. A., M. C. Long, V. Lam, and C. A. Spencer. 1994. RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. J Virol 68:988-1001.
129. Roizman, B., D. M. Knipe, and R.J. Whitley. 2007. Herpes simplex viruses, p. 2503-2602

In D. M. K. a. P. M. Howley (ed.), Fields Virology, 5th ed. Lippincott, Williams & Wilkins, Philadelphia, Pa.
130. Rosette, C., and M. Karin. 1996. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. Science 274:1194-7.
131. Roux, P. P., and J. Blenis. 2004. ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68:320-44.
132. Sacks, W. R., C. C. Greene, D. P. Aschman, and P. A. Schaffer. 1985. Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J Virol 55:796-805.
133. Sadowski, I., J. Ma, S. Triezenberg, and M. Ptashne. 1988. GAL4-VP16 is an unusually potent transcriptional activator. Nature 335:563-4.
134. Samaniego, L. A., L. Neiderhiser, and N. A. DeLuca. 1998. Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. J Virol 72:3307-20.
135. Sambucetti, L. C., J. M. Cherrington, G. W. Wilkinson, and E. S. Mocarski. 1989. NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. EMBO J 8:4251-8.
136. Sandri-Goldin, R. M. 1998. ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. Genes Dev 12:868-79.
137. Sandri-Goldin, R. M. 1998. Interactions between a herpes simplex virus regulatory protein and cellular mRNA processing pathways. Methods 16:95-104.
138. Sandri-Goldin, R. M. 2008. The many roles of the regulatory protein ICP27 during herpes simplex virus infection. Front Biosci 13:5241-56.
139. Sandri-Goldin, R. M., M. K. Hibbard, and M. A. Hardwicke. 1995. The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing. J Virol 69:6063-76.
140. Sandri-Goldin, R. M., and G. E. Mendoza. 1992. A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. Genes Dev 6:848-63.
141. Sanfilippo, C. M., and J. A. Blaho. 2006. ICP0 gene expression is a herpes simplex virus type 1 apoptotic trigger. J Virol 80:6810-21.
142. Sanfilippo, C. M., F. N. Chirimuuta, and J. A. Blaho. 2004. Herpes simplex virus type 1 immediate-early gene expression is required for the induction of apoptosis in human epithelial HEp-2 cells. J Virol 78:224-39.
143. Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B2) in virion infectivity. J Virol 29:1149-58.

144. Schang, L. M., J. Phillips, and P. A. Schaffer. 1998. Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription. J Virol 72:5626-37.
145. Schek, N., and S. L. Bachenheimer. 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. J Virol 55:601-10.
146. Sciabica, K. S., Q. J. Dai, and R. M. Sandri-Goldin. 2003. ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation. EMBO J 22:1608-19.
147. Sciortino, M. T., M. A. Medici, F. Marino-Merlo, D. Zaccaria, M. Giuffre-Cuculletto, A. Venuti, S. Grelli, P. Bramanti, and A. Mastino. 2008. Involvement of gD/HVEM interaction in NF-kB-dependent inhibition of apoptosis by HSV-1 gD. Biochem Pharmacol 76:1522-32.
148. Sciortino, M. T., B. Taddeo, A. P. Poon, A. Mastino, and B. Roizman. 2002. Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells for expression prior to viral infection. Proc Natl Acad Sci U S A 99:8318-23.
149. Sedlackova, L., K. D. Perkins, J. Lengyel, A. K. Strain, V. L. van Santen, and S. A. Rice. 2008. Herpes simplex virus type 1 ICP27 regulates expression of a variant, secreted form of glycoprotein C by an intron retention mechanism. J Virol 82:7443-55.
150. Sedlackova, L., and S. A. Rice. 2008. Herpes simplex virus type 1 immediate-early protein ICP27 is required for efficient incorporation of ICP0 and ICP4 into virions. J Virol 82:268-77.
151. Shukla, D., J. Liu, P. Blaiklock, N. W. Shworak, X. Bai, J. D. Esko, G. H. Cohen, R. J. Eisenberg, R. D. Rosenberg, and P. G. Spear. 1999. A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. Cell 99:13-22.
152. Skaletskaya, A., L. M. Bartle, T. Chittenden, A. L. McCormick, E. S. Mocarski, and V. S. Goldmacher. 2001. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc Natl Acad Sci U S A 98:7829-34.
153. Sloan, D. D., and K. R. Jerome. 2007. Herpes simplex virus remodels T-cell receptor signaling, resulting in p38-dependent selective synthesis of interleukin-10. J Virol 81:12504-14.
154. Smiley, J. R. 2004. Herpes simplex virus virion host shutoff protein: immune evasion mediated by a viral RNase? J Virol 78:1063-8.
155. Smith, I. L., M. A. Hardwicke, and R. M. Sandri-Goldin. 1992. Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. Virology 186:74-86.
156. Smith, J. A., S. C. Schmechel, A. Raghavan, M. Abelson, C. Reilly, M. G. Katze, R. J. Kaufman, P. R. Bohjanen, and L. A. Schiff. 2006. Reovirus induces and benefits from an integrated cellular stress response. J Virol 80:2019-33.

157. Smith, R. W., P. Malik, and J. B. Clements. 2005. The herpes simplex virus ICP27 protein: a multifunctional post-transcriptional regulator of gene expression. Biochem Soc Trans 33:499-501.
158. Sodeik, B., M. W. Ebersold, and A. Helenius. 1997. Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. J Cell Biol 136:1007-21.
159. Sokolowski, M., J. E. Scott, R. P. Heaney, A. H. Patel, and J. B. Clements. 2003. Identification of herpes simplex virus RNAs that interact specifically with regulatory protein ICP27 in vivo. J Biol Chem 278:33540-9.
160. Soliman, T. M., R. M. Sandri-Goldin, and S. J. Silverstein. 1997. Shuttling of the herpes simplex virus type 1 regulatory protein ICP27 between the nucleus and cytoplasm mediates the expression of late proteins. J Virol 71:9188-97.
161. Soliman, T. M., and S. J. Silverstein. 2000. Herpesvirus mRNAs are sorted for export via Crm1-dependent and -independent pathways. J Virol 74:2814-25.
162. Soliman, T. M., and S. J. Silverstein. 2000. Identification of an export control sequence and a requirement for the KH domains in ICP27 from herpes simplex virus type 1. J Virol 74:7600-9.
163. Song, B., K. C. Yeh, J. Liu, and D. M. Knipe. 2001. Herpes simplex virus gene products required for viral inhibition of expression of G1-phase functions. Virology 290:320-8.
164. Spencer, C. A., M. E. Dahmus, and S. A. Rice. 1997. Repression of host RNA polymerase II transcription by herpes simplex virus type 1. J Virol 71:2031-40.
165. Stoecklin, G., T. Stubbs, N. Kedersha, S. Wax, W. F. Rigby, T. K. Blackwell, and P. Anderson. 2004. MK2-induced tristetraprolin:14-3-3 complexes prevent stress granule association and ARE-mRNA decay. EMBO J 23:1313-24.
166. Taddeo, B., T. R. Luo, W. Zhang, and B. Roizman. 2003. Activation of NF-kappaB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis. Proc Natl Acad Sci U S A 100:12408-13.
167. Tanguy Le Gac, N., and P. E. Boehmer. 2002. Activation of the herpes simplex virus type-1 origin-binding protein (UL9) by heat shock proteins. J Biol Chem 277:5660-6.
168. Taylor, T. J., and D. M. Knipe. 2004. Proteomics of herpes simplex virus replication compartments: association of cellular DNA replication, repair, recombination, and chromatin remodeling proteins with ICP8. J Virol 78:5856-66.
169. Thompson, R. L., and N. M. Sawtell. 2006. Evidence that the herpes simplex virus type 1 ICP0 protein does not initiate reactivation from latency in vivo. J Virol 80:10919-30.
170. Tibbles, L. A., and J. R. Woodgett. 1999. The stress-activated protein kinase pathways. Cell Mol Life Sci 55:1230-54.

171. Triezenberg, S. J., R. C. Kingsbury, and S. L. McKnight. 1988. Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. Genes Dev 2:718-29.
172. Triezenberg, S. J., K. L. LaMarco, and S. L. McKnight. 1988. Evidence of DNA: protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes Dev 2:730-42.
173. Tudor, C., F. P. Marchese, E. Hitti, A. Aubareda, L. Rawlinson, M. Gaestel, P. J. Blackshear, A. R. Clark, J. Saklatvala, and J. L. Dean. 2009. The p38 MAPK pathway inhibits tristetraprolin-directed decay of interleukin-10 and pro-inflammatory mediator mRNAs in murine macrophages. FEBS Lett 583:1933-8.
174. Uprichard, S. L., and D. M. Knipe. 1996. Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes. J Virol 70:1969-80.
175. Van Sant, C., R. Hagglund, P. Lopez, and B. Roizman. 2001. The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin-conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity. Proc Natl Acad Sci U S A 98:8815-20.
176. Walsh, D., and I. Mohr. 2004. Phosphorylation of eIF4E by Mnk-1 enhances HSV-1 translation and replication in quiescent cells. Genes Dev 18:660-72.
177. Warner, M. S., R. J. Geraghty, W. M. Martinez, R. I. Montgomery, J. C. Whitbeck, R. Xu, R. J. Eisenberg, G. H. Cohen, and P. G. Spear. 1998. A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. Virology 246:179-89.
178. Waskiewicz, A. J., A. Flynn, C. G. Proud, and J. A. Cooper. 1997. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J 16:1909-20.
179. Wek, R. C., H. Y. Jiang, and T. G. Anthony. 2006. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 34:7-11.
180. Wisdom, R., R. S. Johnson, and C. Moore. 1999. c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms. EMBO J 18:188-97.
181. Yan, X. T., M. Zhuang, J. E. Oakes, and R. N. Lausch. 2001. Autocrine action of IL-10 suppresses proinflammatory mediators and inflammation in the HSV-1-infected cornea. J Leukoc Biol 69:149-57.
182. Yang, W. C., G. V. Devi-Rao, P. Ghazal, E. K. Wagner, and S. J. Triezenberg. 2002. General and specific alterations in programming of global viral gene expression during infection by VP16 activation-deficient mutants of herpes simplex virus type 1. J Virol 76:12758-74.
183. Yurochko, A. D., E. S. Hwang, L. Rasmussen, S. Keay, L. Pereira, and E. S. Huang. 1997. The human cytomegalovirus UL55 (gB) and UL75

(gH) glycoprotein ligands initiate the rapid activation of Sp1 and NF-kappaB during infection. J Virol 71:5051-9.

184. Yurochko, A. D., M. W. Mayo, E. E. Poma, A. S. Baldwin, Jr., and E. S. Huang. 1997. Induction of the transcription factor Sp1 during human cytomegalovirus infection mediates upregulation of the p65 and p105/p50 NF-kappaB promoters. J Virol 71:4638-48.
185. Zachos, G., B. Clements, and J. Conner. 1999. Herpes simplex virus type 1 infection stimulates p38/c-Jun N-terminal mitogen-activated protein kinase pathways and activates transcription factor AP-1. J Biol Chem 274:5097-103.
186. Zachos, G., M. Koffa, C. M. Preston, J. B. Clements, and J. Conner. 2001. Herpes simplex virus type 1 blocks the apoptotic host cell defense mechanisms that target Bcl-2 and manipulates activation of p38 mitogen-activated protein kinase to improve viral replication. J Virol 75:2710-28.
187. Zapata, H. J., M. Nakatsugawa, and J. F. Moffat. 2007. Varicella-zoster virus infection of human fibroblast cells activates the c-Jun N-terminal kinase pathway. J Virol 81:977-90.
188. Zarubin, T., and J. Han. 2005. Activation and signaling of the p38 MAP kinase pathway. Cell Res 15:11-8.
189. Zhang, J., D. Niu, J. Sui, C. B. Ching, and W. N. Chen. 2009. Protein profile in hepatitis B virus replicating rat primary hepatocytes and HepG2 cells by iTRAQ-coupled 2-D LC-MS/MS analysis: Insights on liver angiogenesis. Proteomics 9:2836-45.
190. Zhang, Y., and C. Jones. 2001. The bovine herpesvirus 1 immediate-early protein (bICP0) associates with histone deacetylase 1 to activate transcription. J Virol 75:9571-8.
191. Zhou, C., and D. M. Knipe. 2002. Association of herpes simplex virus type 1 ICP8 and ICP27 proteins with cellular RNA polymerase II holoenzyme. J Virol 76:5893-904.
192. Zhu, Z., W. Cai, and P. A. Schaffer. 1994. Cooperativity among herpes simplex virus type 1 immediate-early regulatory proteins: ICP4 and ICP27 affect the intracellular localization of ICP0. J Virol 68:3027-40.
193. Zhu, Z., N. A. DeLuca, and P. A. Schaffer. 1996. Overexpression of the herpes simplex virus type 1 immediate-early regulatory protein, ICP27, is responsible for the aberrant localization of ICP0 and mutant forms of ICP4 in ICP4 mutant virus-infected cells. J Virol 70:5346-56.
194. Zhu, Z., and P. A. Schaffer. 1995. Intracellular localization of the herpes simplex virus type 1 major transcriptional regulatory protein, ICP4, is affected by ICP27. J Virol 69:49-59.