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Manipulation of the Intracellular Redox Environment by HSV-1

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Manipulation of the Intracellular Redox Environment by HSV-1

Brandon S. Albright, PhD

University of Connecticut, 2015

This thesis describes three separate but related projects exploring how herpes simplex virus 1 (HSV-1) induces and copes with a pro-oxidant environment during infection. We found that the UL6 portal ring, the conduit responsible for the uptake and release of viral DNA, required disulfide bonds for ring formation and stability. Mutagenesis studies revealed that these disulfide bonds might be mediated by cysteines 166 and 254. We next examined the protein UL32 as a potential virally encoded oxidoreductase. UL32 contains three C-X-X-C motifs reminiscent of proteins that regulate disulfide bond formation. Mutation of two of these C-X-X-C motifs abrogated protein function. We used a thiol-alkylation assay to show that viral capsid proteins, including UL6, had altered disulfide bond profiles in the absence of UL32, suggesting that UL32 influences disulfide bond formation in capsid proteins during assembly and maturation. We then confirmed that HSV induces oxidation using a redox-sensitive GFP probe. We also found that treating HSV-infected cells with the antioxidant glutathione (GSH) inhibited viral growth. This growth defect was likely due to delayed viral protein expression and replication compartment formation. We further investigated the role of ROS generating NADPH-oxidases (NOXs), which are important

for the growth of several viruses. Using a pan-NOX inhibitor, we found similar defects in viral growth, protein expression and replication compartment formation as observed with GSH treatment, suggesting that NOX-derived ROS might be important for HSV infection. Collectively, we propose that HSV has evolved to use and manipulate the intracellular redox-balance for its own benefit.

Manipulation of the Intracellular Redox Environment by HSV-1

Brandon Scott Albright

B.S., Central Connecticut State University, 2009

A Dissertation

Submitted in Partial Fulfillment of the

Requirement for the Degree of

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University of Connecticut

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Brandon Scott Albright

2015

APPROVAL PAGE

Doctor of Philosophy Dissertation

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This Thesis is Dedicated to:
My Father, John Brian Albright
My Mother, Dolores Lynn Sniffen

Thank you!

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Chapter 1

Introduction

General overview

Herpes simplex virus 1 (HSV-1) is one of many enveloped, double stranded DNA (dsDNA) viruses that constitute the *Herpesviridae* family, which include some of the most ancient and ubiquitous viruses in nature. Herpesviruses have been recognized since ancient times and were named by Hippocrates based on the “creeping” morphology of the lesions caused by the herpes simplex virus 2 (HSV-2). Beginning in the early 1900’s, advances in laboratory and virological techniques allowed intensive studies into the herpesviruses, and to this day over 200 have been isolated and characterized, with the numbers likely to increase with the availability of better sequencing technologies. Although herpesviruses have been recognized and studied for centuries, there are still a surprising number of questions that remain.

Nine herpesviruses are known to cause disease in humans (Table 1-1) and the disease presentation can range from minor lesions, as in the oral fever blisters observed in HSV-1, to very serious manifestations, such as lymphomas (EBV) and sarcomas (KSHV). It is currently estimated that over 90% of adults are infected with at least one herpesvirus, with many people carrying up to five (Wald and Corey 2007, Yamanishi, Mori et al. 2013). The long co-evolutionary history of these viruses with humans has likely resulted in attenuated pathogenicity; however, they still cause significant disease, especially in immunocompromised individuals.

Formal name	Synonym	Sub family	Predominant Disease association
Human Herpesvirus-1	Herpes simplex virus-1	α	Oral fever blisters
Human Herpesvirus-2	Herpes simplex virus-2	α	Genital warts
Human Herpesvirus-3	Varicella-zoster virus	α	Chickenpox, Shingles
Human Herpesvirus-4	Epstein-Barr virus	γ	Infectious mononucleosis, Burkitt's Lymphoma
Human Herpesvirus-5	Cytomegalovirus	β	Congenital infections
Human Herpesvirus-6A	Human Herpesvirus-6A	β	Unclear
Human Herpesvirus-6B	Human Herpesvirus-6B	β	Roseola
Human Herpesvirus-7	Human Herpesvirus 7	β	Roseola, febrile respiratory disease
Human Herpesvirus-8	Kaposi's sarcoma-associated Herpesvirus	γ	Kaposi's Sarcoma

Table 1-1 The Human Herpesviruses

Furthermore, it has been shown that infection with herpesviruses can contribute to increased infection rates of other serious pathogens, such as HIV, HPV and KSHV (Lusso, De Maria et al. 1991, Corey, Wald et al. 2004, Smith 2004, Freeman, Weiss et al. 2006, Abu-Raddad, Magaret et al. 2008, Qin, Feng et al. 2011).

Although significant diversity exists among herpesviruses, several morphological features are shared within this family of viruses, including a dsDNA genome protected by an icosahedral proteinaceous capsid, a layer of tegument proteins and a glycoprotein-rich lipid envelope. Several aspects of the viral life cycle are also shared by all herpesviruses. A well recognized trait of the herpesviruses is their ability to establish lifelong infections in which they exist in a latent, or dormant, state with periodic episodes of reactivation. Herpesviral DNA synthesis occurs in the nucleus during lytic infection. Upon induction of latency, viral genomes circularize and acquire chromatin (Roizman, Knipe et al. 2013). A final property shared by herpesviruses is that they carry genes that code for their own replication machinery and protein modifying enzymes, such as kinases. (Pellett and Roizman 2013)

In addition to the common characteristics shared by all herpesviruses, there are distinguishing characteristics in viral life style that have resulted in their classification into three subfamilies: the alpha, the beta and the gammaherpesviruses. Viruses within these subfamilies are defined according to their host range, tissue tropism and resulting disease. Alphaherpesviruses (HSV-1, HSV-2 and VZV) are the most widely studied and are characterized by short, destructive lifecycles and a variable host range (Pellett and Roizman 2013). These viruses establish latency in neuronal ganglia and, upon reactivation, generate

infectious virions that are released from nerve endings. This results in lytic infection of the surrounding cells in the tissue, including fibroblasts and epithelial cells, leading to tissue destruction.

The betaherpesviruses (CMV, Human herpesvirus 6A, Human herpesvirus 6B and Human herpesvirus 7) are recognized by restricted host range and their long reproductive cycle. Infection with betherpesviruses results in the enlargement of the host cell. There are two genera within the human betaherpesvirus subfamily which are distinguished by tissue tropism. CMV, a member of the *Cytomegalovirus* genus, can establish latency in myeloid progenitor cells (Reeves and Sinclair 2008) and possibly other cell types, but this is still a controversial and poorly understood area of investigation. Several cell types are susceptible to CMV infection, including endothelial, epithelial, myeloid cells and, to a lesser extent, fibroblasts. Fibroblasts are typically used to culture and study CMV, and the lab-adapted strains have been shown to accumulate mutations that make them grow more efficiently in this cell type (Dargan, Douglas et al. 2010). The other human betaherpesviruses, HHV-6A, 6B and 7, are members of the *Roseola* genus, and they typically infect lymphocytes. HHV-6A and 6B were designated as two separate viruses in 2012 due in part to differences in their abilities to replicate in specific subtypes of differentiated T-cells (Yamanishi, Mori et al. 2013). HHV-7 establishes latency in activated CD4⁺ T-cells; whereas, the reservoirs of HHV-6A and HHV-6B are still being studied (Mori and Yamanishi 2007).

The human gammaherpesviruses include EBV and KSHV which preferentially infect B- and T- cells, respectively, although these viruses can infect epithelial cells.

EBV can establish latency in both epithelial and B-cells and exhibits a unique characteristic of existing in one of three latency states within B-cells. The state of latency is determined by the viral proteins being expressed which, in turn, influence the growth properties of the B-cell (Amon and Farrell 2005) , including transformation of the B-cells (Yates, Warren et al. 1985). KSHV, the most recently identified herpesvirus, is the causative agent of Kaposi's sarcoma and is associated with several lymphoproliferative diseases, such as primary effusion lymphoma and multicentric Castleman's disease. It is believed that latency drives transformation and hyperproliferation; however, unlike EBV, latent KSHV cannot transform cells in culture. Another interesting phenomenon is that cells harboring a latent KSHV genome will lose that genome after several passages in cell culture. Thus, it has been proposed that persistent infection relies on a combination of latent and lytic infection (Damanian and Cesarman 2013).

Of all the herpesviruses, HSV has proven to be one of the most important tools for virologists over the past century. Studies on HSV have led to important observations that have not only advanced our understanding of the biology of enveloped, DNA viruses, but have also been integral in uncovering virus-host interactions involved in disease. HSV, like all viruses, needs to usurp cellular metabolic processes for its own proliferation. During this process it has to combat cellular antiviral mechanisms, the host immune response and keep the cell alive long enough to maximize virus production. Conversely, the cells and the organism have developed many strategies to limit virus replication and spread. This dynamic has led to a molecular arms race, where both the virus and host have been forced to

adapt to each other throughout evolutionary time. Thus, viruses have served an important role in shaping cellular biology. The study of viruses not only allows us to understand how to develop therapeutics to specific microbes but gives us important insight to cellular processes that may have evolved to be antiviral but now serve other, essential functions. This thesis explores how HSV influences and navigates one of these processes - the cellular redox system. Here, we provide evidence that HSV-1 induces cellular oxidation, which may have initially involved as an antiviral response for its own benefit. We also provide examples of strategies the virus has evolved to survive in this altered cellular milieu.

The HSV virion

The mature HSV virion measures ~200nm and, like all herpesviruses, contains a DNA-filled capsid, a layer of tegument and a host-derived lipid envelope. The virion protects the viral DNA from the hostile extracellular environment and serves as the vehicle to deliver the genome to a susceptible host cell. The isolated virion carries ~30 viral proteins as well as several host proteins. The viral proteins were originally named VP, meaning “virion polypeptide”, which is a term that is still used for some of the proteins (ie. VP5) with the number corresponding to the relative banding position of virion proteins on an SDS-PAGE gel. Some viral proteins are called ICP for “Infected Cell Protein”, and these were characterized by the banding patterns of whole cell lysates from infected cells. Another commonly used naming strategy, and the one most widely used now, is to name the protein based on the

location of its open reading frame within the unique long or short arms of the HSV genome (ie. UL32 or US1).

The genome

The 152 kilobase pair genome is packaged as a linear duplex molecule and codes for ~90 known gene products. The genome is divided into two covalently linked segments that are termed the unique short (U_S) and the unique long (U_L). In 1988, McGeoch et al mapped the open reading frames within the U_L segment, numbering them sequentially from left to right (McGeoch, Dalrymple et al. 1988). The unique segments are bracketed by inverted repeat regions such that the genome arrangement is ab-U_L-b'a'a'c'-U_S-ca (Fig. 1-1) where the terminal a sequences are in the same orientation and the a' sequences within the junction are inverted (Wadsworth, Jacob et al. 1975, Wagner and Summers 1978). Sequences within the terminal repeats are essential for the encapsidation, or packaging, of the viral DNA (Jenkins and Roizman 1986). The *cis*- acting elements within the a sequence required for packaging are discussed in more detail below.

A unique feature of the genome is the presence of nicks and gaps in DNA isolated from virions. When virion DNA is transfected into cells, the nicks and gaps elicit a DNA damage response by activating the sensing kinase DNA-PK which must be overcome by the virus to establish infection, as shown in recent work from our lab (Smith, Reuven et al. 2014). It is somewhat surprising that the virus would evolve to have elements, such as a nicked and gapped genome, that would make it easily detected by the host. The role of the nicks and gaps in the genome is unclear, but it

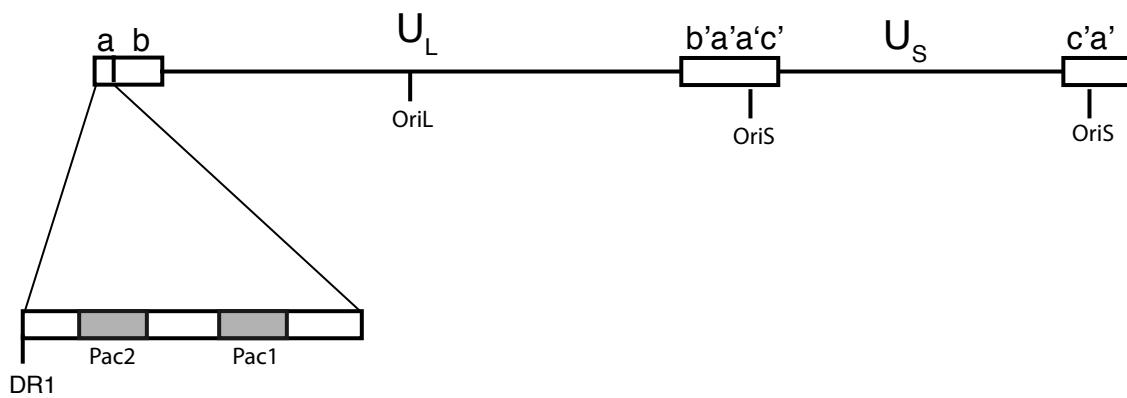


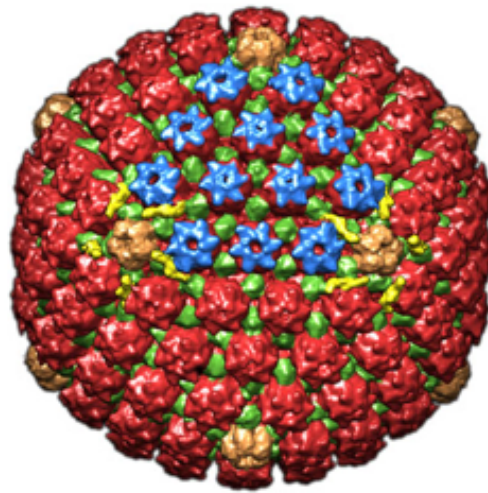
Figure 1-1 Diagram of the HSV-1 genome. The 152 kbp genomes contains two unique segments, U_L and U_S , that are flanked by reat regions. The genome has three origins of replication that are recognized by the origin binding protein, UL9. Pac1 and Pac2 sites are contained within the terminal a sequences and are essential for the cleavage and packaging of viral DNA.

is possible that they play a role in viral DNA replication. Alternatively the nicks and gaps may facilitate viral DNA packaging. Our lab is currently investigating these possibilities.

The HSV capsid

The HSV-1 capsid is an icosahedral proteinaceous shell that is mainly composed of the major capsid protein VP5 and the minor capsid proteins VP19c, VP23 and VP26 (Fig 1-2). VP5 monomers form capsomers with hexameric and pentameric geometries that ultimately form the capsid shell made predominantly of hexons, with pentons at eleven of the twelve capsid vertices. The capsomeres are held together by VP19c and VP23 which exist in a 1:2 stoichiometry to form a complex called the triplex (Newcomb, Trus et al. 1993). The smallest minor capsid protein is VP26, and this protein associates exclusively with hexonal VP5 in a 1:1 ratio (Wingfield, Stahl et al. 1997). VP26, unlike the other capsid proteins, isn't essential for growth in cell culture; however, work in a mouse model shows that VP26 is important for efficient infection *in vivo*, especially within the trigeminal ganglia (Desai, DeLuca et al. 1998). Another feature of the outer capsid is a ring made from twelve monomers of the UL6 protein which occupies one of the capsid vertices and is presumed to form a portal responsible for the uptake and the release of the viral DNA (Newcomb, Juhas et al. 2001, Trus, Cheng et al. 2004, Cardone, Winkler et al. 2007, Chang, Schmid et al. 2007).

During infection, the capsid forms prior to DNA packaging. This capsid, called



(J. Conway and F. Homa, 2011, *Alphaherpesviruses*)

Figure 1-2 The anatomy of the HSV-1 capsid. The HSV-1 capsid is predominantly made up of the major capsid protein VP5 (red). The minor capsid proteins VP19c and VP23 form a complex (green and yellow) and hold the VP5 hexons and pentons together. VP26 (blue) associates with the distal tips of VP5 and may play a role in intracellular trafficking.

the procapsid, requires an internal scaffold comprised of the UL26 and the co-terminally expressed UL26.5 gene products. Procapsids contain 10-fold more UL26.5 than UL26 (Homa and Brown 1997) (Table 1-2). Both UL26 and 26.5 share the last 329 amino acids, however, full-length UL26 contains a viral protease (VP24) at the N-terminus which is responsible for the cleavage and release of the internal scaffold during genome packaging (described below). The scaffold interacts with VP5 and UL6 and is an important determinant in the formation and the size of the capsid (Newcomb, Thomsen et al. 2003). In 1994, several groups were able to assemble capsids *in vitro* from proteins individually expressed in insect cells infected with recombinant baculovirus individual capsid proteins (Newcomb, Homa et al. 1994, Tatman, Preston et al. 1994, Thomsen, Roof et al. 1994). Many studies have subsequently used this technology to uncover the minimum requirements for capsid formation, as well as to identify many important interactions that make this process possible (Newcomb, Homa et al. 1994, Tatman, Preston et al. 1994, Thomsen, Roof et al. 1994, Homa and Brown 1997, Newcomb, Homa et al. 2001, Newcomb, Thomsen et al. 2003).

Although the composition of the capsid has been well-defined, it is still unknown exactly how it is formed *in vivo*. Another mystery is how the UL6 portal is incorporated at only one vertex. It has been shown that the UL6 portal is incorporated into the capsid at an early step during capsid assembly, and thus is believed to be a nucleating factor (Newcomb, Homa et al. 2005). This would suggest that the UL6 rings forms prior to the rest of the capsid, leading to more questions as to how the portal itself is formed. Our lab has found that a leucine zipper region

within the UL6 protein is important for self-interaction and portal ring formation (Nellisery, Szczepaniak et al. 2007). In this thesis (Chapter 2), we show that disulfide bond formation is another essential binding interaction for portal ring formation and stability (Albright, Nellisery et al. 2011).

The envelope and tegument

The viral envelope consists of a lipid bilayer and at least twelve viral glycoproteins are embedded within the host-derived lipid bilayer. Since the glycoproteins gB, gD, gH and gL are the only ones required for growth in cell culture, and consequently the most widely studied, these will be the only ones noted here (Reske, Pollara et al. 2007). When exiting the nucleus, the virus buds through the inner nuclear membrane where it obtains an initial envelope. This envelope fuses with the outer nuclear membrane, depositing the naked capsid into the cytoplasm. The virus then acquires a golgi-derived envelope, which remains part of the virion until the subsequent round of infection. This is supported by the observation that the composition of the lipids within the viral envelope is consistent with the lipids found in golgi-derived membrane (van Genderen, Brandimarti et al. 1994).

The tegument is the layer between the capsid and the envelope and comprises ~20 proteins that are involved in every aspect of the viral lifecycle - from physical viral processes, such as capsid transport, to inhibiting cellular antiviral activities. Most of the tegument is deposited into the cytoplasm along with the viral capsid upon fusion of the viral envelope with the host membrane. Some tegument proteins associate with the capsid, such as UL36 and UL37, and are required for

targeted transport to the nucleus and subsequent release of the genome through the nuclear pore (Batterson, Furlong et al. 1983, Ojala, Sodeik et al. 2000, Copeland, Newcomb et al. 2009). Other proteins set the stage for infection. For instance the virally encoded ubiquitin ligase, ICP0, may inhibit the innate antiviral response through degradation of key cellular proteins while the transactivators ICP4 and VP16 initiate viral gene expression (Roizman, Knipe et al. 2013). Although there are many other important tegument proteins, they will not be discussed here and are reviewed elsewhere (Kelly, Fraefel et al. 2009).

The HSV lifecycle

Entry

The initial recognition of host cells is mediated by gB and gC, which initially bind to heparin sulfate proteoglycans, followed by attachment of gD to the herpesvirus mediated entry receptor (HVEM), Nectin-1, or 3-O-Sulfated heparin sulfate (3-OS HS) (Spear, Eisenberg et al. 2000, Roizman, Knipe et al. 2013). Once the virus attaches, the virus can enter by fusion with the plasma membrane or through an endocytic pathway, which is primarily determined by cell-type (Qie, Marcellino et al. 1999, Milne, Nicola et al. 2005, Nicola, Hou et al. 2005). Although entry mechanisms vary, they all require the formation of a four protein complex mediated by the interactions of gD with the class III fusion protein, gB, and the gH/gL complex. The gB/gH/gL complex is likely responsible for the actual fusion event, however this process is still unclear (Eisenberg, Atanasiu et al. 2012).

Following entry, dynein motors associate with the minor capsid protein VP26 and possibly the tegument proteins UL36 and UL37 to transport the capsid to the nucleus (Sodeik, Ebersold et al. 1997, Douglas, Diefenbach et al. 2004, Wolfstein, Nagel et al. 2006) where it docks with the nuclear pore complex, followed by release of the genome, most likely through the UL6 portal, into the nucleus (Copeland, Newcomb et al. 2009). How this event is coordinated is still a mystery. We have shown that disulfide bonds are important for the formation and stability of the UL6 ring, and it is possible that these interactions are disrupted upon entering the reducing environment of the cytoplasm, thus serving as a molecular trigger for genome release (Albright, Nellissery et al. 2011). Once the genome is inside the nucleus, the host cell and the virus will partake in a molecular tug of war that will result in either the onset of a lytic infection or the establishment of latency.

Viral gene expression

During lytic infection, viral gene expression occurs in a stepwise, orderly manner. Immediate early (IE) genes are the first kinetic class and are expressed soon after the genome enters the nucleus, a process independent of *de-novo* protein synthesis. The five IE genes are ICP0, ICP4, ICP22, ICP27 and ICP47. ICP0 is an E3 ubiquitin ligase that aids in gene expression mostly by counteracting the cellular antiviral response. ICP4 is the major viral transcription factor and activates the transcription of most of the viral genes (Wagner, Bayer et al. 2013). It also represses the expression of a number of viral genes, including itself (DeLuca 2011). Thus, ICP4 is required for nearly every step of infection. ICP27 is a multifunctional protein

involved with recruiting RNA pol II to sites of gene transcription, inhibiting cellular mRNA splicing, and transport of viral mRNAs (Roizman, Knipe et al. 2013). ICP27 has been shown to work in coordination with ICP4 and ICP0 to activate or repress viral genes (Sekulovich, Leary et al. 1988, Rice and Knipe 1990). Thus, ICP27 works on many levels to shift the cellular machinery in favor of viral gene expression. ICP22 is the least well understood IE gene product and is required for efficient infection in animal models; however, it is not required in all cell types. ICP22 may play a role in regulating gene transcription and this is due to its influence on the phosphorylation status on the C-terminal domain of RNA pol II (Rice and Fraser 2006). An interesting property of ICP22 is its ability to reorganize the molecular chaperone Hsc70 into nuclear inclusions. These inclusions, called VICE domains (Virus-Induced Chaperone-Enriched) contain several chaperones as well as components of the cellular protein quality control machinery, such as the 20S proteasome, and ubiquitinated and misfolded proteins (Burch and Weller 2004, Livingston, DeLuca et al. 2008, Livingston, Ifrim et al. 2009, Bastian, Livingston et al. 2010, Weller 2010). The role of VICE domains is unclear, although our lab is intrigued by the possibility that these inclusions might serve to sequester the molecular chaperones, especially Hsc70, away from possible antiviral functions. The fifth IE gene, ICP47 modulates the immune response by inhibiting antigen presentation (Fruh, Ahn et al. 1995, Hill, Jugovic et al. 1995).

The early genes are the next wave and these code for a number of proteins. Most notably, all of the replication proteins are expressed at this time. Late gene expression follows and this class is further divided into genes that express with

early-late or true-late kinetics. Early-late proteins can be detected in the absence of viral DNA replication, however, their expression levels increase at the onset of replication. True-late genes, on the other hand, require viral DNA replication; proteins are classified as true-late if their expression cannot be detected during infection with a DNA replication-defective mutant or during treatment with a viral polymerase inhibitor, such as phosphonoacetic acid (PAA). An example of this is shown in Chapter 3, where we confirm on the protein level that the DNA packaging protein, UL32, is indeed a true-late gene (Albright, Kosinski et al. 2015). The structural genes are mostly expressed in the late class and are discussed elsewhere in this thesis.

Viral DNA replication

HSV viral DNA replication requires seven virally encoded proteins and occurs in the nucleus of the cell within globular domains called replication compartments. The ssDNA binding protein, ICP8, plays a critical role in the formation of replication compartments which begin as small foci, called prereplicative sites, and coalesce to fill up the nucleus as the infection progresses (Roizman, Knipe et al. 2013). Prereplicative sites contain ICP8, the origin-binding protein UL9, and the three subunit helicase/primase complex. It is believed that UL9 and ICP8 mediate the distortion of the DNA at any one of three origins of replication that are present in the genome (labeled OriS and OriL, Fig. 1-1). The model predicts that this distortion would recruit the three subunit helicase/primase (H/P) complex which would in turn

recruit the viral polymerase (UL30) and its processivity factor (UL42) (Weller and Coen 2012).

The end product of DNA synthesis is a longer than unit-length concatemer and there are a few models for how this is formed. As opposed to the long held belief that the HSV genome circularizes and that concatemers are formed through a rolling-circle replication mechanism, we and others have observed that recombination of the viral DNA might play a significant role in concatemer formation. Some lines of evidence include: 1) replicating viral genomes are complex, branched and don't enter a pulsed-field gel, 2) the virus encodes two proteins (ICP8 and UL12) that may work as a two-subunit recombinase, 3) recombination is a frequent event between two co-infecting genomes and 4) HSV-1 activates some recombination pathways while disabling others (Bataille and Epstein 1994, Severini, Morgan et al. 1994, Zhang, Efsthathiou et al. 1994, Martinez, Sarisky et al. 1996, Severini, Scraba et al. 1996, Schumacher, Mohni et al. 2012).

DNA packaging

One of the last steps of the viral lifecycle before egress from the nucleus is the packaging of viral DNA into the capsid, which, like almost all intranuclear viral events, occurs within replication compartments. This multistep process involves a preformed procapsid acquiring a full-length genome with the concurrent proteolysis and expulsion of the internal scaffold by the viral protease, VP24 (Figure 1-3) (Brown, McVoy et al. 2002, Baines and Weller 2005, Conway and Homa 2011). The steps of this process are unclear and will likely remain so in the absence of an *in*

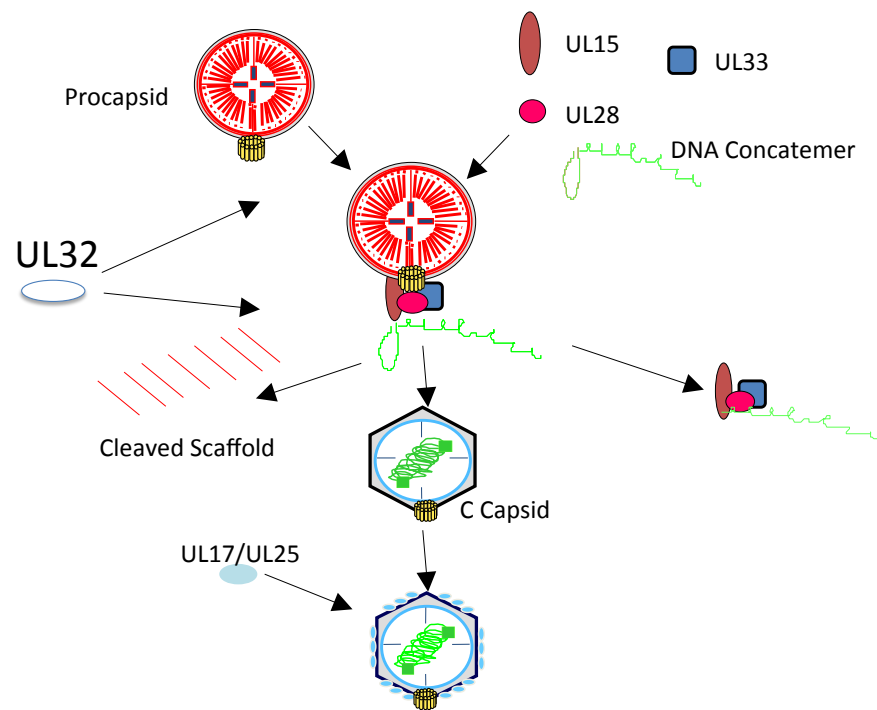


Figure 1-3 The proposed model for HSV-1 DNA packaging. During viral genome packaging, UL15, UL28 and UL33 form a terminase complex that binds viral DNA and docks with the viral portal (yellow). DNA is driven into the capsid, most likely through the portal in an ATP-driven process. At this time, the internal scaffold is cleaved and expelled from the capsid, resulting in angularization of the capsid. The accessory proteins UL17 and UL25 bind to the capsid to aid in retention of the viral genome within the capsid.

vitro packaging system. Capsids can have one of three fates during this stage, becoming A-, B- or C-capsids. The separate capsid types are distinguished based on where they band on a sucrose gradient. A-capsids have the lowest mass and migrate toward the top of the gradient. These capsids are devoid of viral DNA and the internal scaffold, suggestive of capsids that were able to begin the packaging reaction but unable to retain the genome. B-capsids migrate in the middle due to the presence of the internal scaffold and are presumed to be dead-end products that never initiated packaging. C-capsids migrate the farthest down a gradient and have successfully packaged a full-length genome.

Seven essential proteins form the components required for the formation of C-capsids: the UL6 portal protein, a terminase complex containing UL15, UL28 and UL33, UL25 and UL15 which form a capsid-vertex specific complex (CVSC) and the putative chaperone protein UL32. The terminase complex recognizes viral DNA, docks with the portal protein and drives the DNA into the capsid in an ATP-dependent manner (Yu and Weller 1998, Yu and Weller 1998, Dasgupta and Wilson 1999, Adelman, Salmon et al. 2001, Beard, Taus et al. 2002, Yang, Homa et al. 2007, Higgs, Preston et al. 2008). The CVSC associates with the pentons and may play a role in the retention of the viral DNA after packaging. Mutants lacking UL25 can still cleave viral DNA, however there is an accumulation of A-capsids, suggesting that DNA had been packaged but could not be retained within the capsid (McNab, Desai et al. 1998). It is interesting to note that although UL25 and UL17 interact to form the CVSC, the UL17-null virus does not cleave viral DNA and these mutants produce B-capsids (Salmon, Cunningham et al. 1998). UL32 is the only

packaging protein that has not been found associated with the capsid. Its function in DNA encapsidation has been a mystery, however our lab previously described UL32 as a chaperone responsible for the proper localization of the major capsid protein VP5 into replication compartments (Lamberti and Weller 1998). Work presented in this thesis describes a novel role for UL32. In Chapter 3, we present evidence that UL32 influences disulfide bond formation within several viral proteins, including the protease, VP24, and UL6 (Albright, Kosinski et al. 2015). Although we have observed that disulfide bonds are important for the formation and stability of the portal, these exciting results suggest that disulfide bonds may also be important for other essential processes, such as the activation of VP24.

DNA packaging relies on the presence of motifs within the a sequence of the genome that serve as substrates for recognition and cleavage by the terminase. For instance, UL28 has been shown to bind specifically to the *Pac 1* site within direct repeat elements at the UL terminus (DR1, Fig. 1-1) (Adelman, Salmon et al. 2001). Thus, packaging would begin at this end of the genome and stop when the terminase recognizes a *Pac 2* site in the proper orientation, which would be at the US terminus (Vlazny, Kwong et al. 1982). This mechanism ensures that one complete copy of the genome is inserted into the capsid. During packaging, the scaffold is expelled and the capsid angularizes. When packaged, the DNA is compacted to an almost liquid-crystalline state with internal pressure of the capsid reaching up to ~ 18 atmospheres (Bauer, Huffman et al. 2013). This internal pressure most likely serves a role in the ejection of the viral genome during a subsequent round of infection.

Egress

Packaged capsids face several barriers before final release into the extracellular environment. They have to exit the nucleus, transverse the cytoplasm and finally bud from the plasma membrane, all while acquiring an envelope and a layer of tegument. Exit from the nucleus is a difficult task due to the presence of the nuclear lamina, which is a meshwork of many proteins and serves as the internal framework of the inner nuclear membrane (INM). Two viral proteins, UL31 and UL34, form a heterodimer called the nuclear egress complex (NEC) which is essential for the initial budding event through the INM (Reynolds, Ryckman et al. 2001). This event is also dependent on the viral kinase UL13 and US3 and the cellular atypical PKCs which phosphorylate nuclear lamins, thus disrupting this barrier (Leach and Roller 2010). The primary envelopment is followed by de-envelopment via fusion with the outer nuclear membrane which was shown to be dependent on gB and gH/gL, the same glycoproteins needed for the entry of the virus (Connolly, Jackson et al. 2011). The naked capsid goes through a secondary envelopment, possibly through the *trans-Golgi* network, where it acquires its tegument as well as a double-membrane envelope such that it is essentially an enveloped virus within a vesicle (Turcotte, Letellier et al. 2005, Sugimoto, Uema et al. 2008). This outer membrane fuses with the plasma membrane, releasing the enveloped virion into the extracellular environment.

HSV-1 manipulates cellular stress pathways

HSV-1 infection induces a robust cellular response. Throughout evolutionary time, the genes of the virus and host have been shaped to counteract each other. The virus may have picked up genes that were originally useless, but conferred a selective advantage somewhere along the timeline. Conversely, cellular pathways may have originally evolved to be antiviral but provided an advantage thus being fixed in the host. For instance, upon entry into the nucleus, the nicked and gapped viral genome encounters restrictive factors, such as PML bodies that localize to, and silence, the incoming genome. The virus has evolved a mechanism (ICP0 – mediated degradation) to disrupt this response. These measures and countermeasures are pretty obvious; a restrictive factor evolves, and the virus gets around it.

HSV hijacks the host-cell protein quality control machinery (PQC), including a number of heat shock proteins (HSP) and the proteasome system (Burch and Weller 2004, Livingston, DeLuca et al. 2008, Livingston, Ifrim et al. 2009, Bastian, Livingston et al. 2010, Weller 2010). We have found that individual components of the PQC are important for infection. For instance, treatment with the Hsp90 inhibitor, geldanamycin, prevents the localization of the viral polymerase to the nucleus and thus inhibits growth (Burch and Weller 2005). Also, viral growth is somewhat attenuated (< 1 log) when infecting cells transiently express a dominant/negative Hsc70 (Li, Johnson et al. 2008). However, the dependence on these heat shock proteins for efficient viral infection does not explain how and why they are sequestered in VICE domains. VICE domains are localized on the periphery

of replication compartments. Does the virus use these sites for assembling or remodeling viral protein complexes, or is the virus actively degrading proteins? If so, what are these proteins and why? Alternatively, could sequestration prevent PQC proteins from executing yet unknown antiviral functions?

It is known that the transcription factor responsible for upregulating cellular chaperones (heat shock factor 1, HSF-1) is modified during infection, and preventing its phosphorylation is detrimental to the virus (Livingston, et al, unpublished). We have been interested in how and why it gets modified, as well as what the exact modifications are. There are a few possibilities that could explain this modification: 1) viral kinases could possibly phosphorylate HSF-1, 2) viral infection could prevent phosphatases from dephosphorylating HSF-1 shifting the predominant species to the active form or 3) the intracellular environment could be altered in a way that would activate HSF-1. These aren't mutually exclusive and it is possible that a combination of factors could elicit a stress response. Thus, it seems that this stress-response pathway plays a vital role in HSV infection and deserves further investigation. In the next few sections, and later in the thesis, we will discuss how HSV does in fact alter the cellular milieu and how these changes might be important.

Viruses and ROS

The relationship between ROS and microbial infection is a topic of intense investigation. The severity of disease caused by many infections is directly correlated with ROS levels due to direct damage of macromolecules as well as ROS-induced inflammation (Lin, Liu et al. 2000, Imai, Kuba et al. 2008, Seet, Lee et

al. 2009, Ibeh and Emeka-Nwabunnia 2012, Ivanov, Bartosch et al. 2013, Lee, Lai et al. 2013, Paracha, Fatima et al. 2013, Olaghier, Peri et al. 2014, Soundravally, Hoti et al. 2014). Although this response would be presumed to clear pathogens, a remarkable number of viruses are not only able to tolerate, but to thrive in a pro-oxidant environment. Recent work suggests that 1) virus production is more efficient in the presence of oxidants (Paiva and Bozza 2014) and 2) these viruses are sensitive to antioxidant treatment (references listed below). Here I will highlight a few of the viruses in which the induction and role of ROS is well studied. This is not a comprehensive review; however, extensive reviews on this topic can be found elsewhere (Fraternali, Paoletti et al. 2009, Campagna and Rivas 2010, Bottero, Chakraborty et al. 2013, Paiva and Bozza 2014). Before I discuss the viruses, it is important to understand what ROS are.

Reactive oxygen species

Reactive oxygen species (ROS) are the partially reduced metabolites of molecular oxygen and are known for their ability to cause damage to most cellular macromolecules. These species include peroxides (i.e. H_2O_2), superoxide anions (O_2^-) and hydroxyl radicals (OH^\cdot). Although ROS are generally thought to be deleterious, they are being recognized as essential signaling molecules involved in regulating cellular processes, such as cell proliferation, cell death and gene transcription (Sauer, Wartenberg et al. 2001, Valencia and Moran 2004, Collins, Chouchani et al. 2012). They also serve as an important host defense against

microbial pathogens; phagocytic cells generate oxidative bursts for pathogen clearance and stimulate the inflammatory response (Iles and Forman 2002). Thus ROS are necessary but must be kept in check.

Not all ROS exhibit the same reactivities, and the three species that will be discussed follow the hierarchy $\text{H}_2\text{O}_2 < \text{O}_2^- < \text{OH}^\cdot$. H_2O_2 is the most stable ROS and is membrane permeable, making it of significant interest as a potential signaling molecule. By itself, H_2O_2 is a poor oxidant, but can react with Fe through Fenton chemistry to form the extremely reactive OH^\cdot radical, which is the suggested mechanism for why H_2O_2 is toxic (Luo, Han et al. 1994). The predominant cellular buffer against ROS is the small tripeptide glutathione (GSH) that exists in cells at an estimated 1-10 mM concentration. An important characteristic of GSH is the presence of a thiol group on the molecule, which can serve as an antioxidant by directly reacting with ROS. GSH can also form adducts with free thiols on other proteins to protect them from being oxidized (Lushchak 2012). The properties of the thiol groups, as well as the high concentration, make GSH the most important antioxidant defense mechanism for the cell. The cell has many other mechanisms to maintain homeostatic levels of ROS comprised of other antioxidant molecules (vitamin C, vitamin E, etc.) and enzymes (catalase, superoxide dismutase (SOD), etc.).

Endogenous Sources of ROS

It has been difficult to study ROS *in vivo* due to a lack of specific probes. For instance, most studies use redox-sensitive fluorescent dyes, but these can have

different selectivities to different reactive oxygen species or, even worse, react with other cellular components, such as cytochrome c, which can influence the interpretation of the results (Wardman 2007). The design of better molecular tools to study these volatile systems *in vitro* and *in vivo* has led to a rapid expansion in the field of redox biology. Although it is important to understand how ROS influence cellular processes, it is equally important to understand where these ROS come from. There are many exogenous sources of ROS, from xenobiotics to ultra-violet radiation, however we will limit our discussion to the endogenous species.

Mitochondrial sources of ROS

During cellular respiration, a series of transmembrane enzymes in the inner mitochondrial membrane participate in an electron transfer relay known as the “electron transport chain” (Fig 1-4). Each relay provides the energy to actively pump protons into the intermembrane space, resulting in an electrochemical gradient. The protons re-enter the inner membrane through the ATP-synthase enzyme (complex V), thus acting as the driving force for ATP synthesis. This event, known as oxidative phosphorylation, is essential for providing energy for the cell, but it can also generate ROS in the process. O₂ serves as the final electron acceptor at the end of the chain (complex IV) where it gets converted to water. Premature leakage of electrons from complexes I and III in the chain can result in partial, or incomplete, reduction of O₂ leading to the formation of O₂^{•-}. Unlike other mitochondrial ROS generators, complex III releases O₂^{•-} into both the matrix and the intermembrane space and thus is the most likely source of mitochondrial ROS that leaks into the

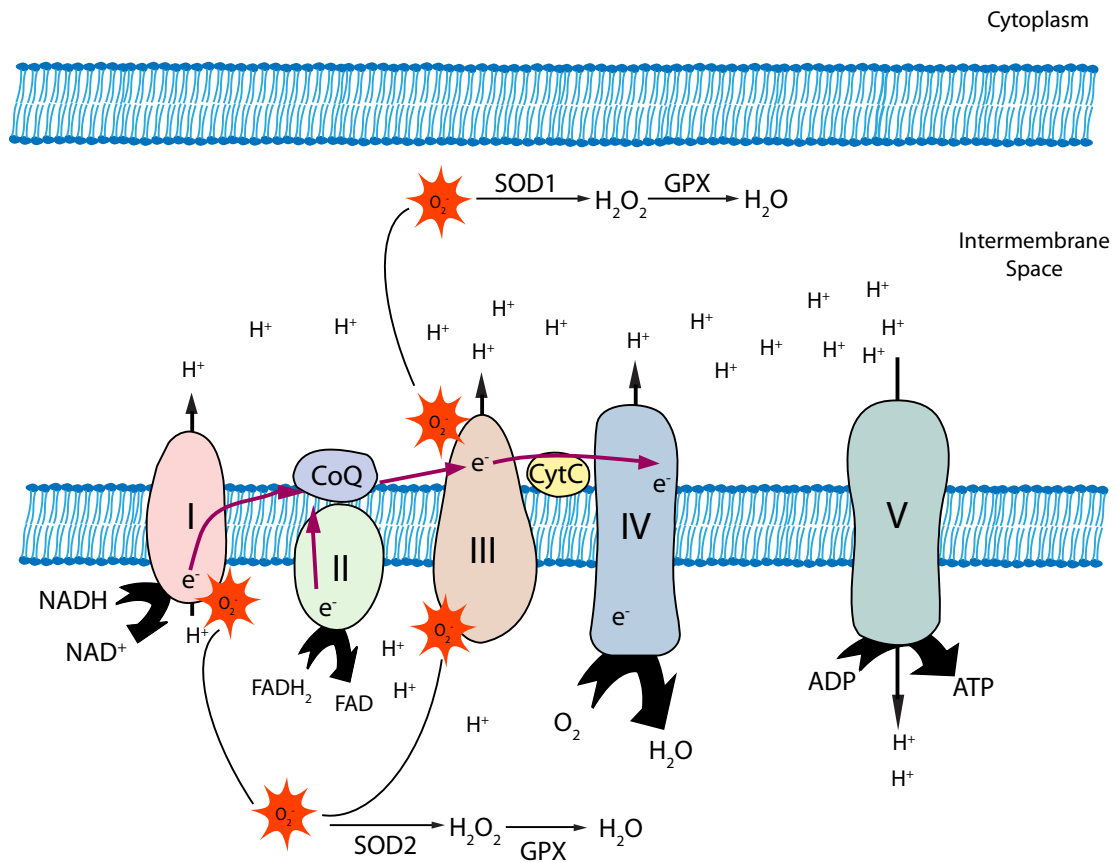


Fig.1-4 Model of mitochondrial ROS generation. During cellular respiration, electrons are transferred from NADH and FADH₂ to complexes I and II in the electron transport chain. Oxygen (O₂) serves as the final electron acceptor and is ultimately reduced to form water at complex IV. Mitochondrial ROS are generated from the leakage of electrons to form superoxide at complex I and complex III. Superoxide is produced within the matrix at complex I; whereas, at complex III superoxide is released towards both the matrix and the intermembrane space. Once generated, superoxide is dismutated to hydrogen peroxide by superoxide dismutase 1 (SOD1) or SOD2. This is followed by reduction to water by glutathione peroxidase (GPX). Both superoxide and hydrogen peroxide are produced in this process.

cytoplasm (Brand 2010). These ROS are easily handled by the many antioxidant systems in the mitochondria. It was initially thought that this accidental ROS generation was one of the major contributors to intracellular ROS, however, this idea is falling out of favor as the studies that show this were either done *in vitro* with purified mitochondria or with drugs that may skew the results (Brand 2010, Sena and Chandel 2012). Thus, there is still no real evidence that mitochondria contribute substantially to the intracellular ROS pool *in vivo* under normal physiological conditions. Even under pathological conditions where mitochondrial dysfunction increases cellular ROS, the observed ROS is generated by another set of enzymes called the NADPH-oxidases (NOXs) which are being recognized as possibly the biggest contributors of intracellular ROS (Bedard and Krause 2007, Jiang, Zhang et al. 2011, Bauer, Huffman et al. 2013).

NADPH – oxidases and ROS

The NOXs are a family of conserved transmembrane proteins that are the major source of intentional ROS generation. There are seven NOXs that have been identified and they all display unique tissue and cellular distribution. NOX2, the first NOX identified and the most well studied, is expressed in macrophages and NK cells and produces a robust oxidative burst essential for pathogen clearance (Bedard and Krause 2007). The other NOXs (1,3-5 and Duox 1,2) are less well studied and their roles under normal physiological conditions aren't known. Unlike mitochondria, NOXs can provide an immediate oxidative burst that can be shut off making it a more tightly regulated redox signaling system, possibly as a stress response (Jiang,

Zhang et al. 2011). Another possibility is that these enzymes provide a respiratory burst, as in NOX2, to protect the cells from pathogens. This might not be the case with NOX4 which, unlike the other NOXs, generates H₂O₂ instead of O₂⁻ (Martyn, Frederick et al. 2006), suggesting that it may serve a unique signaling role.

Influenza and ROS

The relationship between influenza viruses and ROS has long been recognized. It was originally observed that ROS contribute to the pathogenesis of the virus (Akaike, Ando et al. 1990, Hennet, Peterhans et al. 1992, Peterhans 1997), and subsequent studies showed that antioxidant treatment attenuates viral growth in cell culture and animal models (Cai, Chen et al. 2003, Nencioni, Iuvara et al. 2003). Defective viral growth in cell culture seems in part due to improper maturation of the HA glycoprotein (Nencioni, Iuvara et al. 2003, Sgarbanti, Nencioni et al. 2011). The reasons for the attenuated growth in mouse models aren't entirely clear, but it is apparent that ROS are important for the growth of the virus. This is further supported by the observation that influenza growth is restricted in NOX2 knockout mice, or mice treated with the NOX2 inhibitor, apocynin (Vlahos, Stambas et al. 2011) suggesting that NOX2-induced ROS is required for efficient infection in animals. Surprisingly, a recent study shows that NOX4 and not NOX2 is important for influenza infection in lung epithelial cells (Amatore, Sgarbanti et al. 2015). The authors show that abrogating NOX4 activity, through shRNA knockdown or by using chemical inhibitors, prevents influenza-induced ROS and reduces viral growth. Furthermore, blocking NOX-4 mediated ROS generation results in inhibition of

MAPK signaling and accumulation of the viral ribonucleoprotein (vRNP) in the nucleus. This is especially interesting as a new study confirmed that caspase activation results in enlargement of the nuclear pores which is required for exit of the vRNPs from the nucleus (Muhlbauer, Dzieciolowski et al. 2015). Thus the work by Amatore, et al, suggests that NOX4-induced ROS might be responsible for caspase activation through MAPK signaling. It will be interesting to see if NOX4 is essential for influenza infection in mice.

HIV and ROS

Early studies on human immunodeficiency virus (HIV) revealed that oxidative stress (OS) played some role in disease. The first evidence was the accumulation of lipid peroxidation markers and depletion of the antioxidant GSH in serum samples of patients (Sonnerborg, Carlin et al. 1988, Droge, Eck et al. 1994, Favier, Sappey et al. 1994, Perl and Banki 2000). Although these markers of OS were seen even in asymptomatic patients (Buhl, Jaffe et al. 1989), it became apparent that OS directly correlated with the progression of the disease (Baruchel and Wainberg 1992, Droge, Eck et al. 1994, Perl and Banki 2000). The observation that ROS induce viral gene transcription by activating the transcription factor NF- κ B, which could be prevented by antioxidant treatment, provided clues to how OS was involved in the pathogenesis of HIV (Nabel and Baltimore 1987, Staal, Roederer et al. 1990). One way the virus induces OS is by downregulating key antioxidant enzymes (Masutani, Naito et al. 1992, Flores, Marecki et al. 1993). For instance, superoxide dismutase expression is downregulated by the viral protein, Tat (Flores, Marecki et al. 1993)

which reduces the cells ability to buffer against ROS. This is especially important since the HIV protease has been shown to cleave a protein responsible for the integrity of the mitochondrial membrane, Bcl-2 (Strack, Frey et al. 1996). Interestingly, a more recent study showed that Tat-induced ROS is mediated through NOX2 (Song, Ju et al. 2011). The viral proteins gp120 and VPR have also been shown to induce OS (Price, Ercal et al. 2005, Deshmane, Mukerjee et al. 2009). OS results in the constitutive release of TNF- α , which has been implicated in persistent activation of the immune response during HIV infection. This has been linked to the depletion of CD4⁺ cells which contributes to the onset of AIDS (Aukrust, Liabakk et al. 1995). The role of OS in this capacity is still murky; however, there is compelling evidence that OS is associated with HIV-induced neurodegeneration.

HSV-1 and ROS

The relationship between HSV-1 and ROS has been recognized since 1995 when it was observed that GSH levels were decreased in cells immediately after infection and that exogenous GSH treatment was deleterious for viral growth (Palamara, Perno et al. 1995). These studies were confirmed in rabbits (Nucci, Palamara et al. 2000) and mice (Vogel, Cinatl et al. 2005) using derivatives of GSH, suggesting that HSV requires a pro-oxidant state in cell culture and in live animal models. This is consistent with the accumulation of ROS in HSV infected cells, as detected with redox-sensitive fluorescent dyes (Kavouras, Prandovszky et al. 2007, Aubert, Chen et al. 2008, Schachtele, Hu et al. 2010, Hu, Sheng et al. 2011, Gonzalez-Dosal, Horan et al. 2012). Furthermore, protein carbonylation and lipid

peroxidation have been observed during infection, suggesting that the cells are undergoing severe OS (Kavouras, Prandovszky et al. 2007, Mathew, Bryant et al. 2010).

The role of OS during HSV infection is entirely unclear. Early work suggested that GSH blocks HSV growth at a late stage of the infection cycle and was likely due to a defect in expression or maturation of the viral glycoprotein, gB (Palamara, Perno et al. 1995). This has been the only mechanism proposed to explain why HSV needs a more oxidizing environment. In work presented in this thesis, I observed that there was a defect in viral protein expression, viral replication compartment formation and viral growth even at early times post infection in the presence of GSH (Chapter 4), suggesting that OS could play a role in infection much earlier than originally believed. Interestingly, there have been multiple observations that stress-related proteins, such as Jnk, p38 MAPK, ERK, NF- κ B and HSF-1, are phosphorylated (and presumably activated) during infection (Hargett, McLean et al. 2005, Schachtele, Hu et al. 2010, Livingston, et al unpublished, Hu, Sheng et al. 2011, Qiu, Chen et al. 2013). The immediate early protein ICP27 has been implicated in activating several of these pathways (Hargett, McLean et al. 2005, Hargett, Rice et al. 2006, Salaun, MacDonald et al. 2010), which is especially intriguing since ICP27 has been known to induce ROS (Kim, Choi et al. 2008). ICP27, as mentioned above, plays an important role in late gene expression and it is exciting to speculate that this regulatory role might involve some of these stress-induced transcription factors; similar to the role played by NF- κ B in HIV gene regulation (Nabel and Baltimore 1987, Staal, Roederer et al. 1990). Could ROS be a driver of late gene regulation

through these pathways? It is apparent that we have a long way to go before questions like this get answered. There is little doubt that the development of better technologies and the growing recognition of the importance of ROS will lead to dramatic advances in our understanding of many biological processes.

Objective of the study

The original focus of this work was to investigate the hypothesis that UL32 is a virally encoded chaperone involved in the regulation of disulfide bonds necessary for the assembly of encapsidation-competent virus. The original aims of this project were as follows:

Aim 1. Determine possible functions of UL32.

Aim 2. Investigate the hypothesis that UL32 is involved in the regulation of disulfide bond formation in the scaffold and other viral proteins.

Aim 3. Identify viral and cellular interacting partners for UL32.

In Chapter 3, we provide evidence that UL32 may in fact serve as a redox-sensitive chaperone responsible for proper disulfide bond formation within viral capsid proteins. This exciting result, as well as our observations that disulfide bond formation is an important bonding interaction for HSV-1 proteins (Chapter1,

(Albright, Nellissery et al. 2011, Szczepaniak, Nellissery et al. 2011)), led us to change course by investigating the role of reactive oxygen species and oxidative stress during viral infection.

Chapter 2

Disulfide Bond Formation in the HSV-1 UL6 Protein is Required for Portal Ring Formation and Genome Encapsidation

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1 – These authors contributed equally

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Abstract

The HSV-1 UL6 portal protein forms a 12-subunit ring structure at a unique capsid vertex which functions as a conduit for encapsidation of the viral genome. We have previously demonstrated that the leucine zipper region of UL6 is important for inter-subunit interactions and stable ring formation. We now demonstrate that inter-subunit disulfide bonds exist between monomeric subunits and contribute to portal ring

formation and/or stability. Inter-subunit disulfide bonds were detected in purified portal rings by SDS-PAGE under non-reducing conditions. Furthermore, treatment of purified portal rings with dithiothreitol (DTT) resulted in the disruption of the rings, suggesting that disulfide bonds confer stability to this complex structure. The UL6 protein contains nine cysteines that were individually mutated to alanine. Two of these mutants, C166A and C254A, failed to complement a UL6 null mutant in a transient complementation assay. Furthermore, viral mutants bearing the C166A and C254A mutations failed to produce infectious progeny and were unable to cleave or package viral DNA. In cells infected with C166A or C254A B-capsids were produced which contained some UL6 but at reduced levels compared to those seen in wild type capsids. In addition, C166A and C254A mutant proteins expressed in insect cells infected with recombinant baculovirus failed to form ring structures. Cysteines at positions 166 and 254 thus appear to be required for inter-subunit disulfide bond formation. Taken together, these results indicate that disulfide bond formation is required for portal ring formation and/or stability and for the production of procapsids that are capable of encapsidation.

Introduction

The products of Herpes simplex virus type 1 (HSV-1) DNA replication are head to tail concatemers which are resolved into monomeric genomic units and packaged into a preformed capsid shell in the nucleus of the infected cell (reviewed in (Brown, McVoy et al. 2002, Baines and Weller 2005, Conway and Homa 2011)). The HSV-1 capsid shell is composed of the major capsid protein (VP5), two triplex proteins (VP19C and VP23) and VP26. Minor capsid proteins

include UL6, UL15, UL17, UL25, UL28 and UL33. The process of cleavage and DNA packaging requires the six minor capsid proteins as well as UL32, which is not found associated with capsids (Lamberti and Weller 1998, Brown, McVoy et al. 2002, Baines and Weller 2005, Conway and Homa 2011).

HSV capsid formation and genome encapsidation are reminiscent of the double-stranded DNA bacteriophages in that a procapsid shell is preassembled around a scaffolding protein that is not present in the mature virion (Steven, Trus et al. 1997, Baker, Jiang et al. 2005, Steven, Heymann et al. 2005). Bacteriophage and herpesviruses share an important structural element, a dodecameric portal ring located at a unique capsid vertex (Newcomb, Juhas et al. 2001, Trus, Cheng et al. 2004, Cardone, Winkler et al. 2007, Chang, Schmid et al. 2007). During HSV genome encapsidation, the portal ring provides a docking site for the terminase, an ATP-driven molecular motor that facilitates the uptake of viral DNA (Yu and Weller 1998, Sheaffer, Newcomb et al. 2001, White, Stow et al. 2003, Yang, Homa et al. 2007). Terminase is responsible not only for viral DNA uptake but also for the specific cleavage of viral genomes such that a monomeric unit of viral DNA is packaged in each capsid (Koslowski, Shaver et al. 1997, Yu and Weller 1998, Yu and Weller 1998, Koslowski, Shaver et al. 1999, Adelman, Salmon et al. 2001, Beard, Taus et al. 2002, Przech, Yu et al. 2003, White, Stow et al. 2003, Yang and Baines 2006).

UL6 becomes incorporated into nascent HSV-1 capsids mediated by interaction with the UL26.5 major scaffold protein (Newcomb, Thomsen et al. 2003, Newcomb, Homa et al. 2005, Singer, Newcomb et al. 2005, Huffman, Newcomb et

al. 2008). Procapsids can assemble in the absence of UL6 via an interaction between UL26.5 and VP5 (Newcomb, Homa et al. 2001); however, when UL6 is present at initiation of assembly, UL6-containing capsids are formed, suggesting that the portal is incorporated at a very early step in assembly (Newcomb, Homa et al. 2005). These results may also suggest that capsid assembly is regulated such that capsids lacking UL6 do not assemble efficiently in infected cells. UL6 is known to self assemble into a dodecameric ring in lysates from insect cells infected with recombinant UL6-expressing baculovirus (Newcomb, Juhas et al. 2001). Interestingly, three UL6 mutant proteins, L429E L436E and D-LZ, bearing mutations in the leucine zipper region are unable to produce rings and form polymorphic aggregates instead (Nellisery, Szczepaniak et al. 2007). Moreover, these mutant viruses assemble B-capsids that are defective for virus growth, cleavage and packaging of the genomes. Thus, the ability to form a dodecameric portal ring appears to be essential for the formation of a procapsid that is competent for encapsidation.

In this paper we investigated another type of bonding interaction that contributes to ring formation and/or stability. UL6 portal rings from insect cells infected with recombinant baculovirus were disrupted when exposed to reducing agents. Although disulfide bonds have been reported previously between HSV-2 capsid proteins (Zweig, Heilman et al. 1979) and in HSV-1 scaffold proteins (Yang, Yang et al. 2000), this is the first report of disulfide linkages in the portal ring. Mutational analysis of UL6 identified cysteines 166 and 254 as essential for 1)

intermolecular disulfide bond formation; 2) formation and/or stability of portal rings and 3) production of procapsids that are capable of encapsidation.

Materials and Methods

Viruses, cells, antibodies and other reagents. The KOS strain of Herpes simplex virus 1 (HSV-1) was used as the wild type virus and as the parental strain for the generation of recombinant viruses C166A and C254A. The UL6 null virus, hr74, contains an insertion of the *E.coli* lacZ gene under control of the HSV-1 ICP6 promoter and was described previously (Lamberti and Weller 1996). African green monkey kidney fibroblast cells (Vero) were obtained from the ATCC and used to propagate the WT type virus. The UL6 complementing cell line, UL6-31 (Lamberti and Weller 1996, Nellissery, Szczepaniak et al. 2007), was used to propagate hr74, C166A and C254A recombinant viruses. Jay C. Brown (University of Virginia Health System) provided anti-UL6 monoclonal antibodies 1C9 and 4G9 (Burch and Weller 2004). *N*-Ethylmaleimide (NEM) was purchased from Sigma and dissolved in ethanol (0.5 M).

Recombinant baculoviruses and protein expression. Wild-type, C166A and C254A recombinant proteins were expressed in Sf9 insect cells and purified as described (Nellissery, Szczepaniak et al. 2007). Briefly, 3.2×10^8 insect cells were infected with recombinant baculoviruses a multiplicity of infection (MOI) of 5 plaque forming units (PFU) per cell incubated at 28°C for 48 h. Inclusion bodies containing UL6 were isolated and resuspended in 0.5 ml of arginine (1.0 M, pH 7.5), kept on ice for 15 min with occasional mixing and centrifuged at 30,000 x g in a Beckman TL-100 ultracentrifuge at 4°C for 15 min to remove insoluble UL6 as described in

(25). The supernatant containing solubilized UL6 protein was used in all subsequent experiments.

Purification of UL6 rings. UL6 rings were purified by sucrose gradient centrifugation. In short, the solubilized UL6 protein (0.25 ml) was loaded onto a 10–30% sucrose gradient containing 1.0 M arginine and spun at 48,000 rpm for 18 h at 4°C in a SW 55Ti rotor. Gradient fractions (250 µl each) were collected using a BIOCOMP Piston Gradient fraction collector, stored on ice and subjected to SDS-PAGE. UL6 was detected by silver staining as follows: the gel was fixed for 1 hour (two 30 min incubations) in a 30% ethanol and 10% acetic acid solution, washed twice in 20% ethanol (10 min each wash) followed by one 20 min wash with water. The gel was sensitized in 0.1% sodium thiosulfate for 1 min, briefly rinsed with water and incubated in cold 0.1% silver nitrate for 30 min at room temperature.. Silver nitrate solution was removed; the gel was washed twice in water (1 min each wash) and developed with 3% potassium bicarbonate and 0.05% formaldehyde. Once the protein bands were detected, staining was terminated by replacing the developing solution with 5% acetic acid solution for 10 min.

Electron Microscopy. Purified rings in 1 M arginine were either untreated (control) or treated with 100 mM DTT followed by the addition of 50 mM sodium bisulfite to react with the free –SH groups. For EM analysis, treated and untreated ring samples were adsorbed onto formvar – carbon coated grids, stained with uranyl acetate and visualized in a Philips CM10 transmission electron microscope operating at 60 kV (52,000X magnification). Wild type and mutant rings were also analyzed by EM as previously described (Nellisery, Szczepaniak et al. 2007).

Capsid isolation. Confluent monolayers of Vero cells in T225 tissue culture flasks were infected with KOS wild type or mutant virus at 37°C. At 18-20 h post infection the medium was discarded, the monolayers were washed with PBS and the cells scraped into 20 ml of PBS. Cells were pelleted at 200 x g for 15 min in a Beckman S4750 rotor, and the pellet containing $4-5 \times 10^7$ cells was resuspended in 5 ml of 20 mM Tris pH 7.6 buffer followed by addition of 5 ml of 2x lysis buffer (2% Triton, 20 mM Tris pH 7.6, 1 M NaCl, 4 mM EDTA). Cell lysates were incubated on ice for 30 min, treated with $MgCl_2$ and DNase at final concentrations of 20 mM and 0.1 mg/ml, respectively, for 15-20 min at 37°C and briefly sonicated in a cup horn sonicator (three 20 sec bursts at 50% power) to reduce viscosity. Insoluble material was removed by centrifugation at 10,000 x g for 15 min in an Beckman F6X100 rotor. The supernatants containing intracellular capsids were spun through a 1.5 ml cushion of 30% (wt/vol) sucrose in TNE buffer at 71,000 x g for 1 h in an SW41 rotor. Pellets containing capsids were resuspended in 700 μ l of TNE, briefly sonicated in a cup horn sonicator to break up clumps and layered over a 20-50% (wt/vol sucrose in TNE) continuous gradient. Gradients were centrifuged at 71,000 x g for 1h in an SW41 rotor and fractionated using a BIOCAMP Piston Gradient fraction collector. Collected fractions (250 μ l each) were stored at -80°C for further analysis by SDS-PAGE and Western blotting. For capsids isolated in the presence of NEM, cell monolayers were incubated at 37°C in DMEM containing 10 mM NEM for 10 min prior to harvesting. NEM at a final concentration of 10 mM was also added to all other buffers used during the process of capsid isolation. Total capsids were isolated as described above except that instead of sucrose gradient

centrifugation, capsids pelleted through a sucrose cushion were reconstituted in reducing or non-reducing SDS-PAGE loading buffer.

Infected cell lysates. Infected cell lysates were prepared as follows: Vero cells in a 100-mm-diameter tissue culture dish (1×10^7 cells) were infected with wild type or mutant virus stocks at an MOI of 10 PFU per cell at 37°C. Infected cells were incubated at 37°C in DMEM containing 10 mM NEM for 10 min prior to harvesting by scraping with a rubber spatula at 14 h post infection. Cells were collected by centrifugation at 1,000 rpm for 10 min and rinsed twice with PBS containing 10 mM NEM. Cell pellets were resuspended in 1 ml of reducing or non-reducing SDS-PAGE loading buffer as described below and subjected to SDS-PAGE.

SDS-PAGE under reducing and non-reducing conditions. Rings, capsids and cell extracts were prepared either in reducing SDS buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT, 5% (vol/vol) β -mercaptoethanol, 0.02% (wt/vol) bromophenol blue) or non-reducing SDS buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS, 20 mM NEM, 0.02% (wt/vol) bromophenol blue). Samples were heated at 95°C for 3 min, briefly sonicated and resolved on a Tris-Glycine gel cast according to instructions provided by Biorad. Gels were silver stained or subjected to Western blotting.

Western blot analysis. Proteins were electro-transferred to PVDF membranes, blocked with 5% fat free milk in TBST (150 mM NaCl, 20 mM Tris pH 7.5, and 0.1% Tween) and incubated with antibodies against VP5 or UL6. Monoclonal antibodies IC9 or 4G9 (anti-UL6) (Nellissery, Szczepaniak et al. 2007) were used at a dilution of 1:10,000 or 3E8 (anti-VP5) (Blewett, Black et al. 1996) at a 1:2,000.

Membranes were washed with TBST and incubated with secondary antibodies conjugated to either horseradish peroxidase or alkaline phosphatase, washed again with TBST and proteins were detected as described in the manufacturer's protocol.

Generation of cysteine substitution mutations. Substitution mutagenesis was carried out as described previously (Nellisery, Szczepaniak et al. 2007). Sequences of the oligonucleotides used for mutagenesis are available upon request. HSV-1 genomic DNA was used as the template in a two-step PCR-based mutagenesis protocol (Higuchi, Krummel et al. 1988). To generate the single and double cysteine substitutions the PCR product was gel purified and cloned into the pENTR vector of Gateway system (Invitrogen) and then moved to pDEST40 vector following manufacture's protocol. The 166A and 254A mutations were moved to Gateway pDEST10 for transfer into recombinant baculovirus genomes as described (Nellisery, Szczepaniak et al. 2007). All plasmids containing mutant versions of UL6 were subjected to DNA sequencing to confirm the presence of the desired mutations.

Marker transfer. Monolayers of the UL6-31 cell line (60mm dishes at 70% confluence) were transfected with 1 µg mutant plasmid DNA and 1 µg hr74 virus DNA using the Lipofectamine Plus reagent (Invitrogen Corporation). Transfection was done in serum-free Dulbecco's modified Eagle medium (DMEM) containing PLUS reagent (8 µl) and LIPOFECTAMINE reagent (12 µl) in a total volume of 250 µl. Transfected cells were incubated in DMEM containing 5% fetal bovine serum for 24 h, and harvested along with the culture supernatant. Viral stocks were titered

on the UL6-31 cell line and replated at a suitable dilution to yield approximately 100 plaques on a 10-cm dish. The dishes were overlaid with 2% methyl cellulose in DMEM, and recombinant plaques were identified by overlaying with methyl cellulose solution containing 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (50 µg/ml) over-night. The parental hr74 plaques stain blue, while the recombinants that contain the mutant UL6 gene and hence have lost the lacZ gene are colorless. Colorless plaques were selected, and each mutant was plaque purified three times as described (Nellissery, Szczepaniak et al. 2007). The presence of the desired UL6 mutant was confirmed by sequence analysis following PCR amplification of the region spanning the deletion.

Virus yield and plaque assays. Virus yields were determined by infecting Vero or UL6-31 cells at an MOI of 0.5 PFU/cell and samples were collected at 24 hours post infection. Serial dilutions were plated on the UL6-31 cell line, overlaid with methyl cellulose, incubated for 72 h, fixed in 4% formaldehyde, and stained with 1% crystal violet solution. Viral plaques were counted by eye.

Transient complementation assay. The transient complementation assay was carried out as described previously (Martinez, Shao et al. 1992). In brief, freshly trypsinized exponentially growing Vero cells were transfected with 1 µg of either empty plasmid, wild type or mutant plasmid. At 18 h post transfection, cells were superinfected with hr74 at an MOI of 3 PFU per cell. At 24 h post infection, progeny viruses were harvested and assayed on the UL6-31 cell line for total yield. The percent complementation was calculated by dividing the titer obtained for the

mutant plasmid with the titer of the WT plasmid and multiplying by 100. The background titers from the empty plasmid samples were subtracted.

Immunofluorescence microscopy. Vero cells grown on cover slips (70% confluent) were transfected with 1 μ g of plasmids bearing WT or mutant UL6 genes; at 16 hours post transfection the cover slips were washed with PBS and cells fixed with 4% paraformaldehyde, washed again with PBS, permeabilized with 0.5% Triton X 100, and washed again with PBS. The cover slips were then incubated in 5% goat serum as blocking solution and treated with a 1:200 dilution of the 4G9 monoclonal antibody for 1 hr. The cover slips were washed again in PBS, and incubated with the secondary antibodies conjugated to AlexaFluor 488 for 30 min. They were then washed and mounted on glass slides using glycerol-gelatin. The slides were examined using a Carl Zeiss Axiovert A410 confocal microscope and images collected. The images were exported to Photoshop to generate the final figures.

Results

Purified UL6 rings are sensitive to reducing reagents

The UL6 protein plays multiple roles during viral assembly and encapsidation, including ring formation, interaction with scaffold and VP5 to nucleate the assembly of the procapsid shell, docking of the terminase and acting as a conduit for the uptake of viral DNA into capsids (Patel and MacLean 1995, Lamberti and Weller 1996, Patel, Rixon et al. 1996, Yu and Weller 1998, Newcomb, Juhas et al. 2001, White, Stow et al. 2003, Trus, Cheng et al. 2004, Newcomb,

Homa et al. 2005, Cardone, Winkler et al. 2007, Chang, Schmid et al. 2007, Nellissery, Szczepaniak et al. 2007). It is also likely that the UL6 portal plays a role during subsequent infections. UL6 exists as a dodecameric ring and the forces that promote UL6-UL6 inter-subunit assembly are not well understood. In this paper we tested the hypothesis that disulfide bonds contribute to UL6 ring formation and proper UL6 function. First we wanted to determine if purified UL6 protein contains disulfide bonds. To test this, UL6 was expressed in insect cells infected with recombinant baculovirus expressing wild type protein as described previously (Nellissery, Szczepaniak et al. 2007). Portal rings were purified by sucrose gradient sedimentation as described in Materials and Methods. UL6-containing fractions were identified by SDS-PAGE followed by Western blot analysis using UL6 polyclonal antibody (data not shown). Purified UL6 rings were subjected to SDS-PAGE under reducing and non-reducing conditions (Fig. 2-1A). In the presence of DTT, the major UL6 band migrated at a position expected for a monomeric UL6 corresponding to a molecular weight of 74 kDa. On the other hand, under non-reducing conditions a slower migrating species was observed, with no visible monomer, indicating the presence of inter-subunit cross-links. The appearance of a slow-migrating, cross-linked species that can be reduced to the monomeric form suggests the presence of inter-subunit disulfide bonds.

UL6 expressed in insect cells infected with recombinant baculovirus forms rings that can be observed by electron microscopy (Newcomb, Juhas et al. 2001, Nellissery, Szczepaniak et al. 2007). To address whether disulfide bonds contribute to ring stability, UL6 rings were treated with DTT followed by the addition

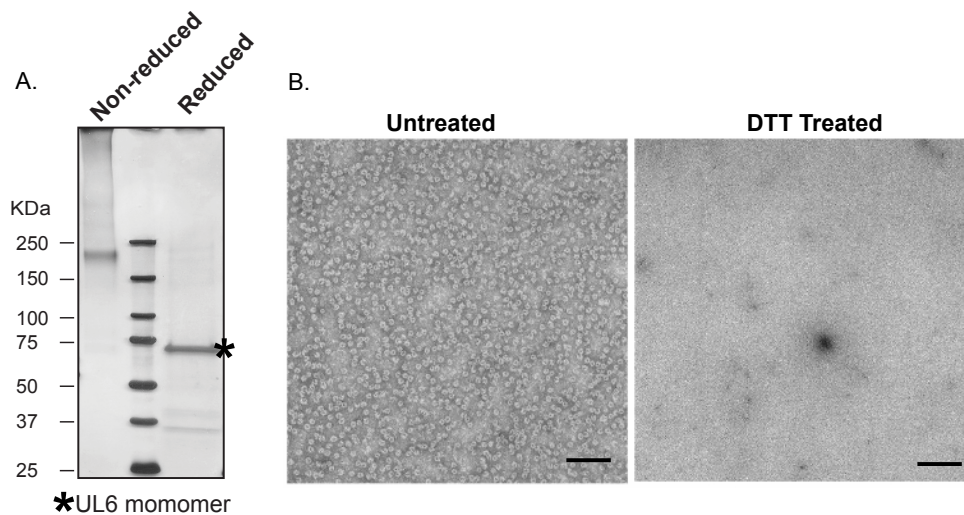


Fig. 2-1 UL6 rings contain disulfide bonds and are destabilized in the presence of reducing agents. UL6 protein was expressed in insect cells and purified by sucrose gradient sedimentation as described in Materials and Methods. A. Fractions containing UL6 were processed for SDS-PAGE under non-reducing and reducing conditions. Molecular weight markers are shown in the middle lane. The gel was silver stained, and the position of molecular weight markers are shown on the left. B. Electron micrographs show preparations of UL6 either untreated (left panel) or treated with DTT to reduce disulfide bonds followed by addition of sodium bisulfite to prevent free sulfhydryl groups from reforming disulfide bonds (right panel). The scale bar in each panel corresponds to 100 nm.

of sodium bisulfite to prevent free sulfhydryl groups from reforming disulfide bonds. Figure 2-1B shows that in the untreated samples, portal rings were readily detected; however, in the DTT-treated samples, portal rings were not detected. These results suggest that disulfide bonds contribute to ring stability.

Disulfide bonds are observed *in vivo*

To determine whether disulfide bonds in the UL6 ring could also be detected in HSV-1-infected cells, cell lysates and capsids were harvested from infected cells at 24 hpi as described in Materials and Methods. Prior to harvest, *N*-ethylmaleimide (NEM) was added to bind and block any free –SH groups. The addition of NEM prior to lysis ensures that any disulfide bonds that are detected are actually present in the infected cell proteins *in vivo* rather than forming once the cells are lysed and the extracts are exposed to an oxidizing environment. Total capsids were harvested as described in Materials and Methods and compared with crude cell lysates. Capsids and lysates were subjected to SDS-PAGE under reducing and non-reducing conditions (Fig. 2-2). A prominent band corresponding to a monomeric unit of UL6 was detected in capsids and lysates under reducing conditions (Fig. 2-2, lanes 3 and 4). Under non-reducing conditions, four slower migrating species (marked a-d) and monomeric UL6 were observed in capsids (Fig. 2-2, lane 1). The proportion of material in bands a-d and monomeric UL6 varies somewhat from preparation to preparation (data not shown), although band b is generally the most prominent of the slower migrating bands. The variability is likely caused by the labile nature of disulfide bonds. Cell lysates contain bands a and b, a weaker signal for band c, no visible band d and monomeric UL6 (Fig. 2-2, lane 2-4).

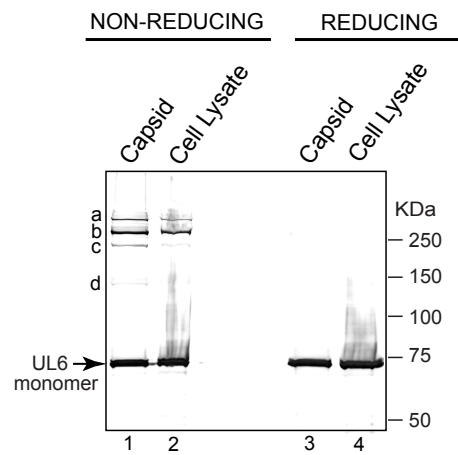


Fig. 2-2 UL6 protein exhibits disulfide linkages in vivo. Total capsids and crude cell lysates from infected cells were isolated and subjected to SDS-PAGE under non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions as described in Materials and Methods. The slower migrating species are labeled a-d. Band d is very faint in this figure but has reproducibly been seen in several experiments. The position of monomeric UL6 is marked with an arrow.

2). Thus, UL6 protein appears to be linked through disulfide bonds in capsids and in cell lysates. The observation that even under non-reducing conditions, UL6 monomer was detected indicates that not all of the subunits in the portal ring were cross-linked; however, since the amount of UL6 in various species is variable, it is not possible to quantify the proportion of cross-linked species in capsids and lysates

Cysteines 166 and 254 are essential for viral infectivity

The predicted sequence of the HSV-1 UL6 gene reveals the presence of nine cysteines. UL6 genes from several alphaherpesviruses were analyzed using the Clustal W program (Thompson, Higgins et al. 1994) revealing that cysteines 145, 166, 254, 283, 293 and 539 are conserved in HSV-1, HSV-2, VZV, PrV and EHV-1 (Fig. 2-3). To investigate which residues are important for the observed disulfide linkages in HSV-1 UL6, each cysteine was mutated to an alanine using site directed mutagenesis as described in Materials and Methods; in addition, one double mutant was made. C to A mutants were tested for their ability to complement the growth of the UL6-null virus, hr74. Vero cells were transfected with plasmids bearing the wild type or mutant UL6 genes and then infected with a UL6 null virus (hr74) at an MOI of 3 PFU/cell. After twenty-four hours, virus was harvested and viral titers were determined on the UL6 complementing cell line, UL6-31. Figure 2-4 shows that most of the substitution mutations were able to complement hr74 as well as the plasmid bearing the wild type gene. An additional substitution mutant C21A was also able to complement hr74 at wild type levels

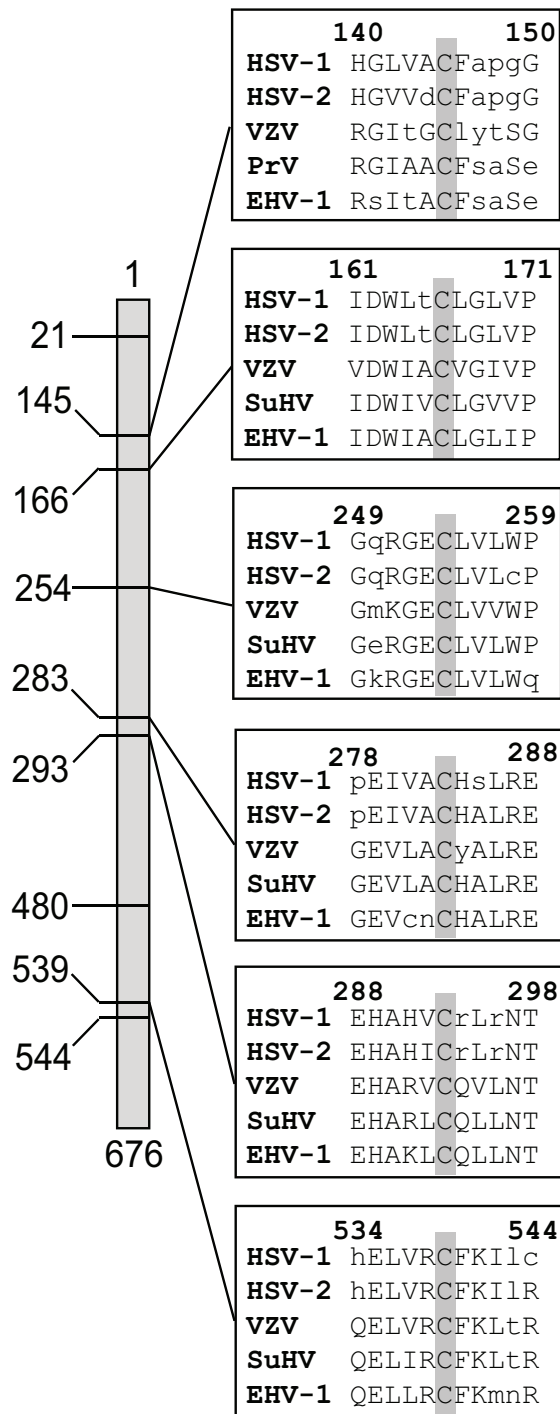


Fig. 2-3. Cysteines 145, 166, 254, 283 293 and 539 are conserved in alphaherpesviruses. The position of all nine cysteines in HSV-1 UL6 are shown on the left. Multiple sequence alignment of the portal proteins from human herpesvirus 1 (HHV-1, HSV-1) gp011, human herpesvirus 2 (HHV-2, HSV-1) gp08, human herpesvirus 3 (HHV-3, VZV) gp55, pseudorabies virus (SuHV-1) UL6 and equine herpesvirus 1 (EHV-1) orf56 reveal that cysteines 145, 166, 254, 283, 293 and 539 are conserved. The numbering reflects the HSV-1 sequence. The sequences were obtained from the NCBI viral genome database, accession numbers NC_001806, NC_001798, NC_001348, NC_006151, NC_001491, respectively. Alignments were performed using Clustal W (Thompson, Higgins et al. 1994).

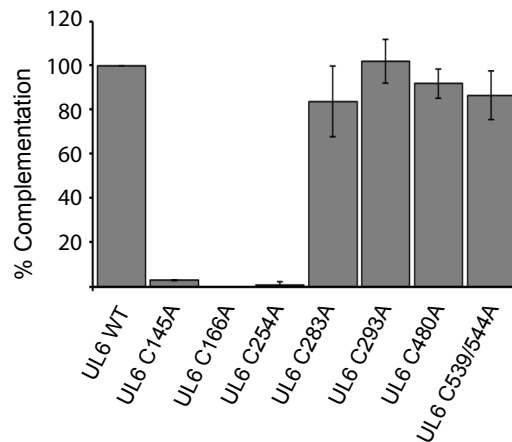


Fig. 2-4 Cysteine to alanine substitutions at positions 145, 166 and 254 abolish complementation ability. Alanine substitution mutations were made at each of the cysteine residues. A transient complementation assay was used to determine the ability of the substitution mutants to complement the null virus hr74. Vero cells were transfected with plasmid DNAs bearing cysteine to alanine mutations and superinfected with hr74 virus, and the resulting progeny virus was titrated on UL6-31 cells. This experiment was repeated three times. Error bars represent the standard deviation.

(data not shown). Three substitutions, C145A, C166A and C254A, were unable to complement hr74.

The lack of complementation could indicate that cysteines C145, C166 and C254 are essential for UL6 function or, alternatively, that mutant proteins are globally or locally misfolded. In order to determine whether the non-complementing mutants were able to synthesize stable UL6 protein, lysates from transfected cells were subjected to SDS-PAGE and Western blotting. C166A and C254A were able to synthesize wild type levels of UL6; however C145A was not, suggesting this mutation may have caused misfolding leading to instability (Fig. 2-5A). Next, to determine whether the mutant proteins could localize appropriately to the nucleus, immunofluorescence microscopy using anti-UL6 antibody was performed on cells transfected with plasmids expressing wild type or mutant UL6 genes. Figure 2-5B shows that as previously reported (Patel, Rixon et al. 1996), wild type UL6 localizes to the nucleus of transfected cells even in the absence of other viral proteins. Cells transfected with plasmids expressing C166A and C254A mutant proteins exhibited nuclear fluorescence similar to wild type; however, the C145A mutant protein signal was less intense and was detected only in the cytoplasm consistent with the Western blot data. This suggests that the C145 mutant protein may be globally misfolded and unable to adopt a conformation that can be recognized by the transport machinery.

Marker transfer was used to introduce the C166A and C254A mutations into the HSV-1 genome as described in Materials and Methods. The global instability of

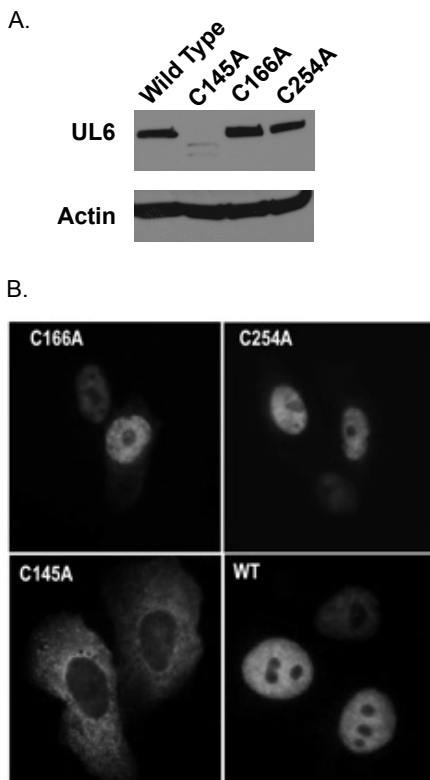


Fig. 2-5 Western blot and Immunofluorescence analysis of wild type and mutant versions of UL6. A) Vero cells were transfected with plasmids expressing wild type or mutant UL6 proteins. LYsates were subjected to SDS-PAGE and blotted with anti-UL6 monoclonal antibody (1C9). Actin was included as an internal loading control. B) Immunofluorescence images of Vero cells transfected with WT, C166A, C254A and C145A plasmids stained with the 4G9 monoclonal antibody.

the C145A mutant prevented analysis of cysteine 145 in the context of infection. In order to determine whether cysteines 166 and 254 are required for viral growth, a viral yield experiment was performed on Vero and UL6-31 cells. Table 1 shows that in Vero cells infected with C166A and C254A mutant viruses, only background levels of infectious virus was produced. C166A and C254A mutants grew to wild type levels on the UL6 complementing cell line, UL6-31, indicating that the defect in virus growth was due to a mutation in the UL6 gene.

The C166A and C254A mutants produce B capsids that contain lower amounts of UL6 than wild type

We next tested whether the C166A and C254A viral mutants could produce portal-containing capsids. Vero cells were infected with wild type or mutant virus, and capsids were separated by sucrose gradient sedimentation. Fractions from across the gradient were subjected to SDS-PAGE and probed with monoclonal anti-UL6 and anti-VP5 antibodies. Figure 2-6 shows that cells infected with wild type virus displayed the expected pattern of A-, B- and C-capsids and that UL6 specifically associated with capsid peaks. In contrast, cells infected with C166A, C254A or the UL6 null virus (hr74) contained only B-capsids suggesting that packaging is defective (Fig. 2-6). Furthermore, mutant UL6 protein exhibited a specific association with viral capsids and was not distributed throughout the gradient. In order to compare the levels of UL6 in mutant and wild type capsids, B-capsids were recovered from sucrose gradients and analyzed by Western blotting with monoclonal anti-UL6 and anti-VP5 antibodies. Figure 2-7 shows serial dilutions of capsids isolated from cells infected with KOS, C166A or C254A.

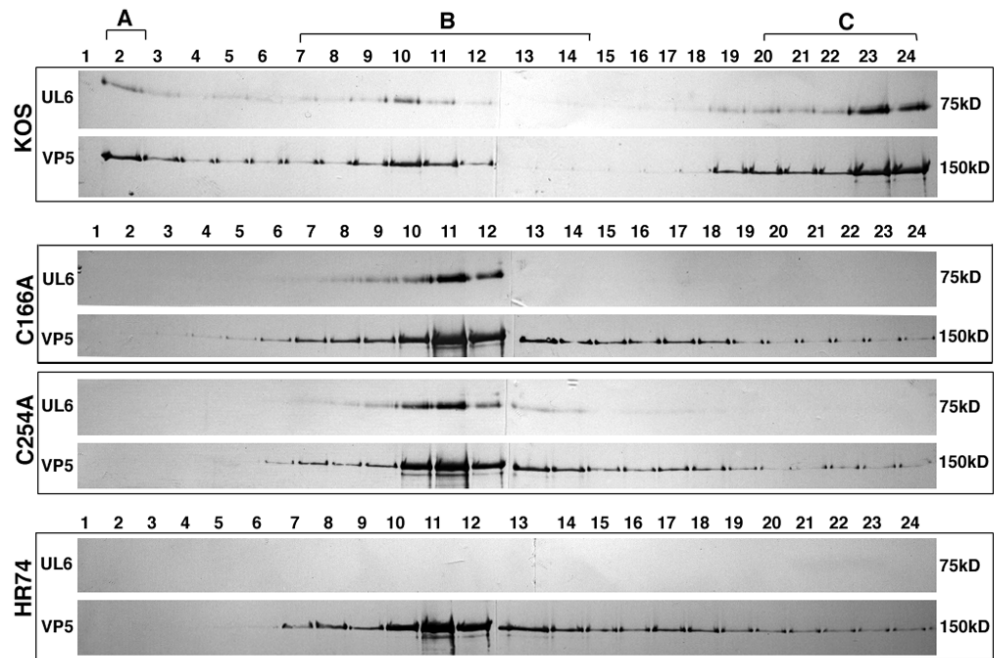


Fig. 2-6 Substitution mutants C166A and C254A produce B- but not A- or C- capsids. Vero cells were infected with KOS (WT), hr74 (null), C166A or C254A as described in Materials and Methods. Infected cell lysates were subjected to sucrose density gradient sedimentation, and fractions (1, top; 24, bottom) were subjected to SDS-PAGE and immunoblot analysis. Blots were divided into strips and probed separately with anti-UL6 and anti-VP5 antibodies. The positions of A, B, and C capsids are shown.

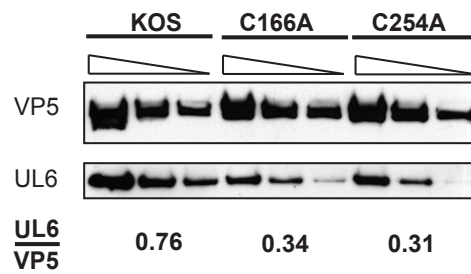


Fig. 2-7 Capsids isolated from cells infected with C166A and C254 mutant viruses contain reduced amounts of the UL6 protein compared to wild type KOS. B- capsids isolated from cells infected with KOS or C166A or C254A were serially diluted (1:1, 1:2 and 1:4) and analyzed by Western blot analysis using anti-VP5 and anti-UL6 antibodies. The images were quantified using Image J software, and results are expressed as a ratio of the relative intensities of the UL6 and VP5 protein bands.

Roughly similar amounts of VP5 were present in wild-type and mutant B-capsids. On the other hand, UL6 was present in reduced amounts in capsids from the C166A or C254A mutant viruses. The relative ratio of UL6 to VP5 was quantified and indicates that mutant capsids contain less UL6 than wild type. The reduced amounts of UL6 in mutant capsids could indicate that each capsid contains fewer than 12 molecules of UL6. Under this scenario, the ability of mutant UL6 protein to form aggregates may be sufficient to nucleate procapsid assembly resulting in capsids each with diminished levels of UL6. Alternatively, it is possible that portal rings are able to form very inefficiently in cells infected with C166A or C254A mutants resulting in a small number of capsids with an intact portal ring and a majority of capsids with none. Under this scenario, however, even if some capsids with an intact portal ring are produced, the absence of C-capsids indicates that packaging is defective in mutant infected cells. Additional experiments will be needed to determine the distribution of UL6 in capsids from mutant-infected cells. We can conclude, however, that cysteines 166 and 254 are essential for efficient portal ring formation and for the production of procapsids that are able to encapsidate viral genomes.

The production of only B-capsids by cysteine substitution mutant viruses indicates that DNA packaging is defective. To verify this, DNA was isolated from cells infected with wild type or mutant viruses, digested with BamH I and subjected to agarose gel electrophoresis and Southern blot hybridization. These experiments indicated that wild type and mutant viruses are able to synthesize similar amounts of viral DNA but that the terminal S and Q fragments indicative of cleavage were

detected only in wild type virus-infected cells (data not shown). Thus C166A and C256A mutants are capable of DNA synthesis and procapsid assembly; however, procapsids are defective for cleavage and packaging and production of infectious progeny.

Cysteines 166 and 254 are necessary for disulfide bond formation

We next asked whether the C166 and C254 mutants displayed the same cross-linked protein bands seen in lysates and capsids from cells infected with wild type virus (Fig. 2-2). Vero cells were infected with KOS, C166A or C254A. After twenty-four hours, cells were treated with NEM, and total intracellular capsids were isolated and subjected to SDS-PAGE under reducing and non-reducing conditions as described in Materials and Method. While slower migrating bands were observed in wild type capsids under non-reducing conditions, no slower migrating bands were seen in mutant capsids under the same conditions (Fig. 2-8). This result implies that cysteines 166 and 254 play a direct role in the formation of the disulfide bonds detected in UL6 in KOS capsids.

To investigate whether cysteines 166 and 254 are involved in ring formation, baculovirus recombinants bearing these mutations were constructed and used to infect insect cells. Solubilized UL6 was isolated from wild type and mutant-infected cells and subjected to sucrose gradient centrifugation. Wild type UL6 protein was able to form a ring structure that could be visualized by light scattering; however, no rings were observed in the expected position in gradients containing the mutant proteins (data not shown). Fractions sedimenting at the position of the wild type rings were collected and analyzed by electron microscopy (Fig. 2-9).

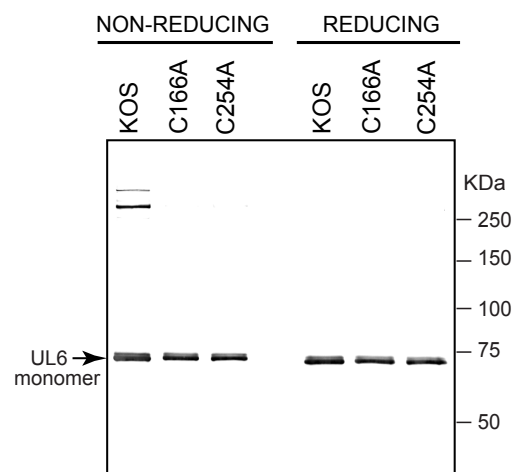


Fig. 2-8 Cysteines 166 and 254 are essential for the formation of the slower migrating species. Total capsids were prepared from cells infected with KOS, C166A or C254A as described in Materials and Methods. For KOS, total capsids include A, B and C capsids; however, the two mutants can only produce B capsids. Monomeric UL6 is labeled with an arrow.

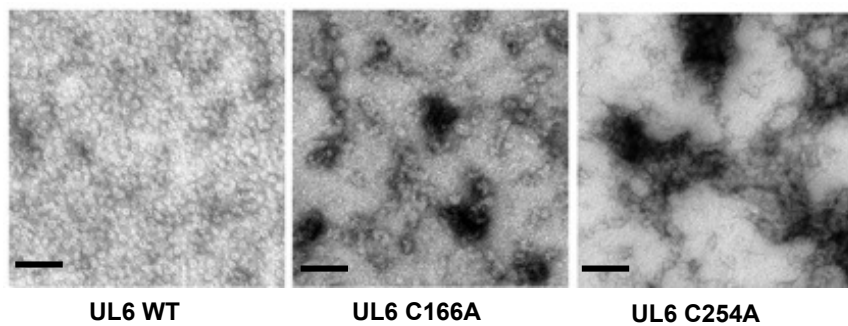


Fig. 2-9 C166A and C254A mutant proteins do not form rings in vitro. Electron micrographs show purified preparations of mutant and wild type UL6 protein expressed in insect cells using a recombinant baculovirus expression system. The photographs show negatively stained rings with dark central channels in the WT and mutant proteins show amorphous particles of variable dimensions and without a distinct central channel. The scale bar in each panel corresponds to 100 nm.

The mutant protein formed aggregates that exhibited variable dimensions and did not contain a distinct central channel. These data demonstrate that cysteines 166 and 254 are necessary for ring formation and/or stability.

Discussion

In this paper we report that UL6 participates in disulfide bonds that are essential for portal ring integrity and for the formation of procapsids that are competent for encapsidation of viral genomes. Slow migrating UL6 bands were observed in purified portal rings that were reduced to monomeric UL6 by the addition of reducing agents, indicating the presence of disulfide cross-links. Since no other viral proteins are expressed in this experiment, it is likely that the slow migrating bands represent inter-subunit disulfide bonds; however, we cannot rule out the possibility that some slow migrating bands result from interactions of UL6 with cellular proteins such as chaperones. The observations that portal rings did not form in insect cells expressing cysteine substitution mutations (C166A and C254A) and that portal rings were destabilized in the presence of reducing agents are consistent with the suggestion that inter-subunit disulfide bonds are present. Slow migrating species of UL6 were also seen in cell lysates and capsids from cells infected with wild type HSV but not in cells infected with mutants C166A and C254A suggesting that these two cysteine residues are required for the disulfide linkages. Mutant C145A was misfolded, and thus we cannot rule out that this residue also participates in disulfide bond formation. The inability of C166A and C254A to produce infectious virus and to package DNA is consistent with disulfide bond formation being essential for the production of procapsids that are competent

for cleavage and packaging. Although it is likely that some of the slow-migrating species seen in cell lysates and capsids (Figs. 2-2 and 2-8) represent inter-subunit interactions within the portal ring, it is also possible that some represent interactions between UL6 and other viral capsid proteins. We are intrigued by the possibility that during capsid formation, encapsidation and the production of mature virus, the disulfide bond profile is dynamic. For instance, it is possible that inter-subunit disulfide bonds play a role in ring formation and that later during capsid assembly some bonds shift such that interactions between UL6 and other capsid proteins form to stabilize the capsid. In fact, some differences were observed in the pattern of slow migrating bands between B- and C- capsids and virions (unpublished results). Further experiments will be required to test this hypothesis and to identify relevant protein-protein interactions in this pathway.

Interestingly, although mutant C166A and C254A proteins cannot form portal rings, they form aggregates when expressed in insect cells. In cells infected with mutant viruses, B-capsids were observed which on average contained UL6 in lower amounts than seen in wild type B-capsids. This behavior is reminiscent of our previous analysis of mutations in the leucine zipper region. UL6 protein expressed in insect cells infected with recombinant baculoviruses bearing mutations in this region were unable to form rings; instead, L429E L436E and D-LZ formed polymorphic aggregates. When studied in the context of viral infection, leucine zipper mutants as well as C166A and C254A assemble B-capsids that contain lower amounts of UL6 than seen in wild type and are not capable of encapsidation (Nellisery, Szczepaniak et al. 2007) and this paper). This may

indicate that it is not necessary to form a complete portal ring for procapsid assembly to occur and that aggregates of UL6 are able to interact with scaffold protein to initiate procapsid assembly. Alternatively, it is possible that a small minority of the B-capsids seen in the mutant-infected cells contain a complete dodecameric ring and that the majority of procapsids contain little or no UL6. Thus ring formation may be extremely inefficient but the few rings that can form may nucleate procapsid formation resulting in a small number of UL6-containing procapsids. Experiments are in progress to distinguish between these alternative explanations. It is also possible that in the B-capsids from mutant infected cells, UL6 is not found at a unique vertex. In any case it is clear that ring formation and/or stability is inefficient in both leucine zipper and the cysteine substitution mutants. Furthermore, the addition of reducing agents to portal rings resulted in disruption of the ring structure. Taken together, these results suggest that portal ring formation and/or stability requires at least two types of inter-subunit interactions driven by disulfide bond formation and coiled-coil interactions typical of leucine zippers. It will be important to understand the assembly principles of this interesting structure because an intact portal appears to be essential for all subsequent steps of capsid maturation. Portal assembly also provides a novel potential target for antiviral intervention.

We report that slower migrating bands of UL6 were observed in capsids isolated from cells that had been pretreated with NEM. The presence of NEM prevents formation of disulfide bonds that could occur upon cell lysis and exposure to oxidizing conditions. This result implies that the disulfide bonds in these capsids

have already formed at the time of lysis. This may be surprising since the nucleus is generally assumed to be a reducing environment (Go and Jones 2008, Jones and Go 2010, Kietzmann 2010). It is possible that the assembly pathway of the UL6 portal occurs in a microenvironment in the nucleus that allows disulfide bond formation. Several observations suggest that the conformation of UL6 may be regulated in infected cells and that the formation of portal rings may take place in an unusual environment. In infected cells, most of the UL6 has been reported to localize to replication compartments where procapsid assembly is believed to occur; however, we have reported that a subpopulation of UL6 could be observed in Virus Induced Chaperone Enriched (VICE) domains which form adjacent to replication compartments during the earliest stages of HSV-1 infection (Burch and Weller 2004, Livingston, Ifrim et al. 2009). VICE domains contain several components of the host protein quality control machinery including molecular chaperones (e.g. Hsc70, the 20S proteasome and ubiquitin) (Burch and Weller 2004, Livingston, Ifrim et al. 2009). We have proposed that HSV-1 infection induces the formation of nuclear protein quality control centers to remodel or degrade aberrant nuclear proteins that would otherwise interfere with productive infection (Livingston, Ifrim et al. 2009). Chaperone proteins in VICE domains may function to aid in the assembly of multimeric complexes. It is of interest that FRAP analysis indicates that the association of Hsc70 with VICE domains is dynamic (Livingston, Ifrim et al. 2009), suggesting movement between VICE domains and replication compartments. It is possible that VICE domains provide a microenvironment conducive for disulfide bond formation and ring formation.

According to this scenario, rings assembled in VICE domains would then enter the replication compartments where they would nucleate procapsid assembly. In any case, the presence of a sub population of UL6 in VICE domains suggests that its conformation is regulated by host cell chaperones.

The observation that the portal ring uses disulfide bond formation for stability is interesting in light of previous observations that HSV capsids contain disulfide bonds potentially involving VP5, triplex and scaffold proteins (Zweig, Heilman et al. 1979, Yang, Yang et al. 2000). We have confirmed the existence of disulfide bonds in capsids and virions and have demonstrated that these bonds contribute to overall capsid stability (Szczepaniak et al, manuscript in preparation). Viral capsids are dynamic structures that must protect viral genomes during the extracellular state while maintaining the ability to disassemble and release viral genomes during the next round of infection. Disulfide bonds may have several advantages for the creation of metastable capsids. In papilloma- and polymoma viruses disulfide bonds have been shown to play an important role in the metastability of capsids (Hare and Chan 1968, Huang, Estes et al. 1972, Walter and Deppert 1975). It is known that redox states are regulated differently in the cytoplasm, the nucleus and the extracellular space (Go and Jones 2008, Jones and Go 2010, Kietzmann 2010). Once papilloma- and polymoma viruses enter the reducing environment of the cytoplasm, it appears that conformational changes occur which facilitate the uncoating and release of the viral genome in order to initiate infection. The observations that disulfide bonds are present in extracellular HSV capsids as well as in portal rings suggest a role for redox state during HSV

infection. Upon entry, capsids and tegument proteins are released into the cytoplasm where rearrangements are possible (Meckes and Wills 2007). Capsids are then transported on microtubules and dock at the nuclear pore where they release their genomes into the nucleus (Ojala, Sodeik et al. 2000) (Sodeik, Ebersold et al. 1997).

In summary, we have now shown that inter-subunit interactions of the portal ring of HSV-1 requires at least two types of protein-protein interactions: coiled-coil interactions and disulfide bond formation. UL6 plays several known roles in infection including nucleation of procapsid assembly, docking of terminase, DNA encapsidation and genome release during the next round of infection. It will be of interest to determine the precise role of disulfide bond crosslinking in portal ring formation, capsid assembly and stabilization and genome release.

TABLE 2-1. Growth of KOS, hr74, C166A, and C254A on Vero and UL6-31 cells

Cell line	Virus (PFU/ml)			
	KOS	hr74	C166A	C254A
Vero	1.9×10^8	1.6×10^3	4.5×10^2	1.2×10^3
UL6-31	1.0×10^8	7.5×10^7	1.6×10^7	3.1×10^7

Virus yields were determined by infecting Vero or UL6-31 cells at an MOI of 0.5 PFU/cell and collecting samples 24 hr post infection. Viral titers were determined on the permissive UL6-31 cell line.

Chapter 3

The putative HSV-1 chaperone protein UL32 modulates disulfide bond formation during infection.

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Abstract

During DNA encapsidation, HSV-1 procapsids are converted to DNA-containing capsids by a process involving activation of the viral protease, expulsion of the scaffold proteins and the uptake of viral DNA. Encapsidation requires six minor capsid proteins (UL6, UL15, UL17, UL25, UL28 and UL33) and one viral protein, UL32, not found associated with capsids. Although functions have been assigned to each of the minor capsid proteins, the role of UL32 in encapsidation has remained a mystery. Using an HSV-1 variant containing a functional HA-tagged UL32, we demonstrated that UL32

was synthesized with true late kinetics and that it exhibited a previously unrecognized localization pattern. At 6 - 9 h post infection (hpi), UL32 accumulated in viral replication compartments in the nucleus of the host cell while at 24 hpi it was additionally found in the cytoplasm. A newly generated UL32 null mutant was used to confirm that although B-capsids containing wild-type levels of capsid proteins were synthesized, these procapsids were unable to initiate the encapsidation process. Furthermore, we showed that UL32 is redox sensitive and identified two highly conserved oxidoreductase-like C-X-X-C motifs that are essential for protein function. In addition, the disulfide bond profiles of the viral proteins UL6, UL25, VP19C and the viral protease, VP24, were altered in the absence of UL32, suggesting that UL32 may act to modulate disulfide bond formation during procapsid assembly and maturation.

Importance

Although functions have been assigned to six of the seven required packaging proteins of HSV, the role of UL32 in encapsidation has remained a mystery. UL32 is a cysteine-rich viral protein that contains C-X-X-C motifs reminiscent of proteins that participate in the regulation of disulfide bond formation. We have previously demonstrated that disulfide bonds are required for the formation and stability of the viral capsids and are also important for the formation and stability of the UL6 portal ring. In this report, we demonstrate that the disulfide bond profiles of the viral proteins UL6, UL25, VP19C and the viral protease, VP24, are altered in cells infected with a newly isolated UL32 null mutant virus, suggesting that UL32 acts as a chaperone capable of modulating

disulfide bond formation. Furthermore, these results suggest that proper regulation of disulfide bonds is essential for initiating encapsidation.

Introduction

The products of Herpes simplex virus 1 (HSV-1) DNA replication are head-to-tail concatemers that are resolved into monomeric genomic units and packaged into a procapsid shell in the nucleus of the infected cell (reviewed in (Brown, McVoy et al. 2002, Baines and Weller 2005, Conway and Homa 2011)). The procapsid is comprised of the major capsid protein (VP5), triplex proteins (VP19C and VP23) and a dodecameric UL6 portal ring. The precursor of the viral protease (UL26) and scaffolding protein (UL26.5) form a scaffold around which the capsid shell assembles (Homa and Brown 1997, Conway and Homa 2011). During DNA encapsidation, the viral protease (VP24) is activated, scaffold proteins are released and viral DNA is taken up (Homa and Brown 1997, Steven 1997, Brown, McVoy et al. 2002, Steven, Heymann et al. 2005, Preston, Murray et al. 2008). HSV procapsid maturation involves dramatic structural remodeling resulting in the conversion of a relatively fragile and porous procapsid into a more stable angular icosahedron filled with DNA (Steven 1997, Steven, Heymann et al. 2005, Roos, Radtke et al. 2009). Three types of capsids designated type A, B or C can be separated by sucrose gradient centrifugation. A-capsids have released the scaffold but contain no DNA as a result of an abortive packaging event. B-capsids resemble procapsids in that they retain the scaffold and contain no DNA. C-capsids contain viral genomes and are destined to mature into infectious virions. We have recently demonstrated disulfide linkages between capsid proteins VP5, VP23 and

VP19C and other components of the mature virion including UL17, UL25 and tegument proteins; furthermore, we showed that disulfide bond formation is required for capsid stability (Szczepaniak, Nellisery et al. 2011). Inter-subunit disulfide bonds also exist between monomeric subunits of the portal ring and contribute to ring formation and/or stability (Albright, Nellisery et al. 2011). The disulfide bonds in the portal ring can be detected in the nucleus. This result was somewhat surprising since this compartment is normally thought to be a reducing environment.

The cleavage and packaging machinery consists of several required viral gene products, six of which are transiently or stably associated with capsids. The UL6 portal ring is found at a unique vertex on the procapsid and acts as a channel through which DNA is taken up (Newcomb, Juhas et al. 2001, White, Stow et al. 2003, Trus, Cheng et al. 2004, Cardone, Winkler et al. 2007, Chang, Schmid et al. 2007). UL15, UL28 and UL33 make up a heterotrimeric complex believed to act as a molecular motor facilitating DNA uptake and cleavage known as the terminase (Yu and Weller 1998, Adelman, Salmon et al. 2001, Beard, Taus et al. 2002, White, Stow et al. 2003). Terminase subunits are transiently associated with procapsids (Yu and Weller 1998, Sheaffer, Newcomb et al. 2001, Beard, Duffy et al. 2004). UL17 and UL25 form a heterodimeric structure referred to as the Capsid Vertex Specific Component (CVSC) that appears to stabilize DNA-containing capsids (Ogasawara, Suzutani et al. 2001, Sheaffer, Newcomb et al. 2001, Thurlow, Rixon et al. 2005, Newcomb, Homa et al. 2006, Thurlow, Murphy et al. 2006, Trus, Newcomb et al. 2007, Conway, Cockrell et al. 2010). A seventh viral protein, UL32, is also essential for encapsidation; however, it is apparently not associated with procapsids or mature virions, and its precise role in

infection remains elusive (Lamberti and Weller 1998). UL32 also differs from the other cleavage and packaging proteins with respect to its intracellular localization. While other cleavage and packaging proteins are found in the nucleus in globular domains known as replication compartments, UL32 has been shown to localize primarily in the cytoplasm with a small fraction observed in replication compartments (Chang, Poon et al. 1996, Lamberti and Weller 1998). In the present study, an HSV-1 virus expressing HA-tagged UL32 was generated and used to demonstrate that UL32 localization is more dynamic than previously reported. At early times during infection UL32 localized predominantly within replication compartments; however, as infection progressed, UL32 was observed primarily in the cytoplasm. This dynamic staining pattern is unique among encapsidation proteins and may provide clues into the function of UL32.

UL32 is a cysteine-rich protein, and fifteen of its twenty-two cysteines are conserved among the herpesviruses family. Cysteines contain a reactive thiol group that can be modified in response to changes in oxidation state. Interestingly, cysteines are involved in many cellular processes including redox signaling and dithiol-disulfide exchange reactions; furthermore, they play important roles at the catalytic site of many enzymes (reviewed in (Netto, de Oliveira et al. 2007)). UL32 contains three C-X-X-C motifs which are reminiscent of highly conserved motifs found in thiol-disulfide oxidoreductases known to catalyze redox reactions essential for maintaining the redox balance within cells through the formation and disruption of disulfide bonds in client proteins (Chivers, Laboissiere et al. 1996, Chivers, Prehoda et al. 1997, Fomenko and Gladyshev 2003, Becerra, Delaye et al. 2007). In this study, we tested the hypothesis that UL32 is a redox-sensitive protein involved in the modulation of disulfide bonds in

HSV-1 proteins. We show that UL32 is redox sensitive and that two of these three conserved C-X-X-C motifs are essential for protein function. Furthermore, the viral proteins UL6, UL25, VP19C and the viral protease, VP24, exhibit an altered disulfide bond pattern in cells infected with a UL32 null mutant supporting the hypothesis that UL32 functions either directly or indirectly to regulate disulfide bond formation. These results also suggest that appropriate disulfide bond formation is required for the formation of procapsids that are competent for encapsidation.

Materials & Methods

Cell lines and viruses. African green monkey kidney cells (Vero CC181; American Type Culture Collection, Rockville, MD) and the UL32-mutant virus complementing cell line, 158, were maintained and propagated in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin. The KOS strain of HSV-1 was used as the wild-type virus. The HSV-1 UL32 null mutant virus *hr64* with an ICP6:LacZ insertion was previously described in Lamberti and Weller (1998). A replication competent virus, HA32, expressing an HA-tagged UL32 protein and a newly isolated true-null UL32 mutant, *hr64FS*, were generated in this study (see below). Cell maintenance and infections were carried out at 37°C.

Construction of HA32. A plasmid (pUL32) capable of expressing the full length UL32 ORF (pUL32) was kindly provided by Dr. A. Patel (CVR). PCR was used to insert a sequence encoding the influenza HA tag (YPYDVPDY) between codons 2 and 3 of the UL32 gene generating pHA-UL32. Plasmid pHA-UL32 and plasmid pUL33 (Beilstein,

Higgs et al. 2009) containing a fragment encoding the adjacent UL33 gene, were then used to generate p33-HA-32, in which a complete UL32-UL33 locus, identical to wt virus except for the sequence encoding the tag, was reconstructed. A fragment spanning the UL32-UL33 region was excised from p33-HA-32 and gel purified. Monolayers of BHK cells (seeded at 1×10^6 per 35 mm Petri dish) were transfected with 0.5 μ g *hr64* DNA and 0.5 μ g linearized plasmid using the calcium phosphate method as previously described (Stow and Wilkie 1976). Cells were incubated in GMEM media supplemented with 5% serum at 37°C for three days. Serial two-fold dilutions were used to infect BHK cells in a 96-well plate and wells containing single plaques were identified and the resultant virus was analyzed for the ability to express HA32 by Western blot. One clone was selected and working stocks of the resulting virus, HA32, prepared.

Construction of *hr64FS*. A UL32 mutant containing a frameshift deletion at nucleotide 166 was generated by two-step sequential PCR mutagenesis using the following primers: Forward 5'-CAGTCACTG**ATAG**GTGTGCCGCCCGTCGGTAC-3' and Reverse 5'-CGGCACAC**CTATC**AGTGACTGGCCGTCAGG-3'. This led to a site-specific deletion of a single nucleotide (between the underlined nucleotides) and the presence of two consecutive in-frame stop codons (bold). The following primers were used to amplify the full-length product containing the frameshift mutation: Forward 5'-CACCATGGCAACTTCGCCCCCGGGGTCC-3' and Reverse 5'-TCATACATAGGTACACAGGGTGTGCTC-3'. The forward primer introduced a NcoI restriction site at the 5' end of the UL32 gene. This PCR product was ligated into pUL32lacZ (Lamberti and Weller 1998) after digestion with NcoI and NheI to create

p32STOPlacZ. p32STOPlacZ was digested with NheI and XbaI and the fragment was inserted in place of NheI-XbaI fragment of p205-PstI (Lamberti and Weller 1998), which contains genes UL31-UL33, resulting in p205-32STOP. Linearized p205-32STOP was co-transfected with infectious *hr64* DNA into 158 cells at a molar ratio of 10:1. Infectious particles were harvested and used to infect 158 cells in a plaque assay, and blue-white screening with X-gal was utilized to select white plaques.

Western blot analysis. Cells were harvested and proteins were separated by reducing SDS-PAGE as previously described and transferred to a PVDF membrane (Albright, Nellisery et al. 2011). Membranes were blocked with 5% milk and incubated overnight with the following antibodies: mouse monoclonal anti-HA (Santa Cruz Sc-7392) 1:1,000; rabbit polyclonal anti HA (Clontech 631207) 1:1,000, rabbit polyclonal anti-UL32 1:1,000 (generated by Open Biosystems to synthetic antigenic peptide); mouse monoclonal anti-ICP8 1:2,000 (abcam ab20194); mouse monoclonal anti-VP5 (EastCoast Bio HA018) 1:1,000; mouse monoclonal anti-VP24 9-2 (Sheaffer, Newcomb et al. 2000) 1,000; mouse anti-Actin (Sigma A5441) 1:10,000.

Growth curves. Confluent Vero or 158 cells were infected with the indicated viruses at an MOI of 0.1. Cells and supernatant were harvested at 0, 6, 12 and 24 hours post-infection. After three freeze-thaw cycles, serial dilutions were plated on Vero cells (Fig. 3-1) or 158 cells (Fig. 3-6), overlaid with methyl cellulose, incubated for 72 h, fixed in 4% formaldehyde, and stained with 1% crystal violet solution. Viral plaques were counted by eye.

Immunofluorescent confocal microscopy. Monolayers of Vero cells were grown on glass coverslips prior to infection. Infected cells were washed with 2X phosphate-

buffered saline (PBS), fixed in 4% paraformaldehyde and permeabilized in ice cold acetone for 2 min followed by blocking in 3% normal goat serum (NGS) diluted in PBS. Cells were incubated in primary antibodies diluted in 3% NGS for 1-2 h at room temperature by inverting coverslips onto a drop of antibody dilution. Dilutions of primary antibodies in 3% NGS were as follows: monoclonal anti-ICP8 1:200; polyclonal anti-ICP8 (367) 1:500; monoclonal anti-VP5 5C and monoclonal anti-VP23 1D2 (both generously provided by Jay Brown, University of Virginia) 1:200; monoclonal anti-VP21/VP22a MCA406 (Serotec Inc.) 1:500; rat monoclonal anti-HA (Roche, cat. 11867423001) 1:200. Cells were washed extensively before incubation in AlexaFluor secondary antibodies (1:200: Molecular Probes) diluted in 3% NGS and mounted onto slides with glycerol gelatin containing 2.5% diazobicyclo-[2.2.2] octane (DABCO; Sigma Chemical Co.). Images were captured using a Zeiss LSM 510 confocal NLO microscope equipped with argon and HeNe lasers and a Zeiss 63X objective lens (numerical aperture, 1.4). Images were processed and arranged using Adobe Photoshop CS3 and Illustrator CS3.

Sucrose gradient sedimentation. Capsids were isolated and separated by sucrose gradient sedimentation as previously described (Albright, Nellisery et al. 2011). Fractions were collected and prepared for Western blot analysis under reducing conditions as described above.

Analysis of conserved residues in UL32. UL32 homologs were identified through a sequence similarity search against the KOS UL32 protein on NCBI Blast. Over one hundred UL32 homologs were identified using NCBI Blast. The twelve homologs shown in Figure 3-3 represent each of the subfamilies of herpesviruses (accession

numbers in parentheses): Human herpesvirus [HHV]-1 KOS (AFE62860.1), HHV-2 (AHG54696.1), HHV-3 (AGL50983.1), HHV-4 (AFY97834.1), HHV-7 (YP_073776.1), HHV-8 (AAC57153.1), Bovine herpesvirus [BOHV]-1 (NP_045326.1), BOHV-5 (YP_003662492.1), Equid herpesvirus 4 [EHV] (NP_045245.1), Suid herpesvirus 1 [PRV] (AGW01108.1), Gallid herpesvirus [GaHV]-2 (YP_001033961.1), GaHV-3 (AEI00238.1).

Generation of substitution mutations. Point mutations were generated in pAPVUL32, where UL32 is driven off of the ICP6 promoter (previously described in (Lamberti and Weller 1998)), by site-directed mutagenesis using the Quickchange II Site-Directed Mutagenesis Kit (Agilent Technologies) per the manufacturer's protocol. Primers are available upon request. Resulting plasmids were sequenced to confirm mutations. Protein expression was confirmed by Western blot analysis.

Transient complementation assay. Vero cells grown to ~75% confluency in 35mm dishes were transfected with 1 µg of empty, wild-type, or mutant plasmid using lipofectamine 2000 according to the manufacturer's protocol. At 4 h post-transfection, cells were superinfected with *hr64FS* at an MOI of 3 PFU per cell. At 24 h post-infection, progeny viruses were harvested and assayed on the 158 cell line for total viral yield. The percent complementation was calculated by dividing the titer obtained for the mutant plasmid by the titer of the WT plasmid and multiplying by 100. The background titers from the empty plasmid samples were subtracted.

DNA isolation and Southern blot analysis. Vero and 158 cells were infected with KOS, *hr64*, or *hr64FS* at an MOI of 5 and harvested 16-20 hpi. Total viral and cellular DNA was isolated, digested with BamHI and resolved by agarose gel electrophoresis.

DNA was transferred to a Gene Screen Plus nylon membrane (New England Nuclear Corp.) and hybridized with ^{32}P -labeled probe. The pKBK plasmid, which contains the 5.9kb BamHI (SQ) junction fragment from KOS (kindly provided by Neal Deluca, University of Pittsburgh), was radiolabeled using the Random Primers DNA Labeling System (Invitrogen).

H₂O₂ treatment. Vero cells were infected with KOS or HA32. At the indicated times before harvest, cells were treated with 5mM H₂O₂ in 2ml serum-free media and harvested in 200µl RIPA buffer (reduced) or 10% trichloroacetic acid (TCA) (non-reduced) at 9 hpi. TCA precipitated samples were resuspended in denaturing n-ethylmaleimide (NEM) buffer (100mM Tris pH 6.8, 1% SDS, 1mM EDTA, 50mM NEM). Samples were subjected to SDS-PAGE in reducing or non-reducing SDS sample buffer.

Silver staining. After SDS-PAGE, gels were fixed for 1 h in a solution containing 30% ethanol and 10% acetic acid and washed twice in 20% ethanol (10 min each wash) and once for 20 min with water. The gel was sensitized in 0.1% sodium thiosulfate for 1 min, briefly rinsed with water, and incubated in cold 0.1% silver nitrate for 30 min at room temperature. Silver nitrate solution was removed; the gel was washed twice with water (1 min each wash) and developed with 3% potassium bicarbonate and 0.05% formaldehyde. Once the protein bands were detected, staining was terminated by replacing the developing solution with 5% acetic acid solution for 10 min.

Cryo-electron microscopy and image reconstruction. Purified B capsids (3 µl) were pipetted onto slightly glow-discharged Quantifoil R2/1 grids (Quantifoil, Jena, Germany), blotted and vitrified in an FEI Vitrobot Mk III (FEI, Hillsboro, OR). Grids were transferred onto a Gatan 626 cryoholder (Gatan, Pleasanton, CA) and imaged in an

FEI Tecnai TF20 microscope operating at 200 kV and 50,000x nominal magnification. Images were collected under low-dose conditions on a Gatan UltraScan CCD camera with an effective pixel size of 2.14 Ångstroms at the sample. Image reconstructions were carried out with the Auto3DEM package (Yan, Sinkovits et al. 2007) using randomly oriented models as the starting point in each case (Yan, Dryden et al. 2007). Resolutions were estimated where the Fourier shell correlation dropped below 0.5. For the control KOS sample of empty capsids, 412 capsid images were collected from 57 micrographs and 370 were used to calculate the final map to 33 Å resolution. The hr64FS sample yielded 1001 capsid images from 104 micrographs, and 900 were included in the final map to a resolution of 21.4 Å.

Thiol modifications. Vero cells were infected with KOS, *hr64FS* or *hr81-1* virus and harvested in 10% TCA at 10 hpi. Samples were centrifuged at 13,000 rpm for 30 min in a bench-top centrifuge. TCA was aspirated and the pellet was washed with 5% TCA followed by another centrifugation. After aspiration, pellets were allowed to dry and resuspended in denaturing buffer (6M Urea, 200mM Tris pH 8.8, 10mM EDTA, 0.5% SDS) containing 20mM NEM to block free thiols. Equal volumes of 20% TCA were added to the samples to precipitate the proteins in a total concentration of 10% TCA. After wash, samples were resuspended in denaturing buffer containing 50mM DTT. DTT was quenched and newly exposed thiols were modified with the addition of 100mM 4-acetamido-4'-maleimidylstilbene (AMS). Reducing loading buffer was added to samples and proteins were separated by SDS-PAGE.

Results

UL32 is a late protein with an unusually dynamic intracellular localization pattern

In this study, we took advantage of an HSV virus expressing HA-tagged UL32 (HA32) to monitor the kinetics of UL32 expression under natural infection conditions. Figure 3-1A shows that in cells infected with the wild-type virus (KOS strain), UL32 was maximally expressed at 24 h post infection (hpi), consistent with late expression kinetics. In cells infected with HA32, a similar pattern was observed (Fig. 3-1A). In order to confirm that this expression pattern reflected true late kinetics, infected cells were treated with the viral DNA polymerase inhibitor, phosphonoacetic acid (PAA) to prevent viral DNA synthesis. In PAA-treated cells infected with KOS or HA32, UL32 expression was prevented, demonstrating that UL32 is a true late protein (Fig. 3-1A). This is the first demonstration that UL32 protein is expressed with true late kinetics, although a previous report suggested that UL32 mRNA was expressed with late kinetics (Holland, Sandri-Goldin et al. 1984). In order to demonstrate that the HA-tagged version of UL32 (HA-UL32) is functional, growth curves of virus produced from Vero cells infected with KOS or HA32 were performed. Figure 3-1B shows that these viruses exhibited identical growth patterns.

UL32 was previously reported to localize predominantly to the cytoplasm of infected cells although a small fraction was observed within replication compartments at 18 hpi (Chang, Poon et al. 1996). A similar pattern was observed in cells transiently expressing UL32 and superinfected with a UL32 null virus (Lamberti and Weller 1998). The predominantly cytoplasmic localization pattern was somewhat surprising considering reports that UL32 is essential for encapsidation, and all of the other viral

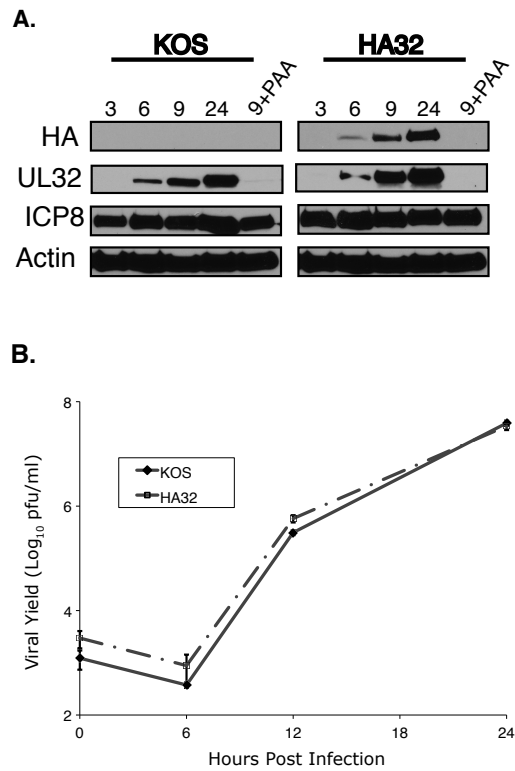


Figure 3-1. HA32 resembles wild type virus in protein expression and viral growth properties. A) Vero cells were infected with KOS or HA32 at an MOI of 3 and collected at the indicated times post infection. Samples were harvested in reducing SDS sample buffer and resolved by SDS-PAGE. Proteins were detected by immunoblotting with the indicated antibodies. B) Growth curves of KOS or HA32 propagated on Vero cells at an MOI of 0.1. Mean values of three independent experiments are plotted and deviations are represented by error bars.

proteins essential for procapsid assembly and encapsidation have been reported to localize to replication compartments in the nucleus (Lamberti and Weller 1998, Taus, Salmon et al. 1998, Higgs, Preston et al. 2008, Scholtes and Baines 2009). To re-examine the intracellular location of UL32, cells were infected with HA32 and processed for immunofluorescence at various times post infection (Fig. 3-2). ICP8 staining served as an infection control and to mark replication compartments (Quinlan, Chen et al. 1984), and cells were counterstained with the DNA dye, TOPRO3, to detect cell nuclei. Interestingly, at 6 hpi UL32 was predominantly detected in replication compartments; however, at later times (24 hpi), a mostly cytoplasmic staining pattern was observed. To examine this phenomenon more quantitatively, we used ImageJ software to analyze the levels of fluorescent intensity in the cytoplasm, nucleus and within replication compartments (illustrated by the yellow box in Fig. 3-2B). At 6 hpi the localization of UL32 strongly correlated with that of ICP8 within the nucleus suggesting that it was present within replication compartments. At 9 hpi UL32 was more diffusely localized within the nucleus although most UL32 was observed in replication compartments. It should also be noted that some UL32 was observed in the cytoplasm at 9 hpi. At 23 hpi UL32 localization no longer correlated with that of ICP8 and was detected in a diffuse pattern throughout the cell. These data reveal that the localization of UL32 progressed during infection from an initial localization within replication compartments to a diffuse localization in both the nucleus and the cytoplasm. Thus, although UL32 is initially observed in replication compartments, in contrast with other viral proteins that play roles in encapsidation, it is not retained there as the infection progresses. We also observed large perinuclear foci containing HA-tagged UL32 at 24

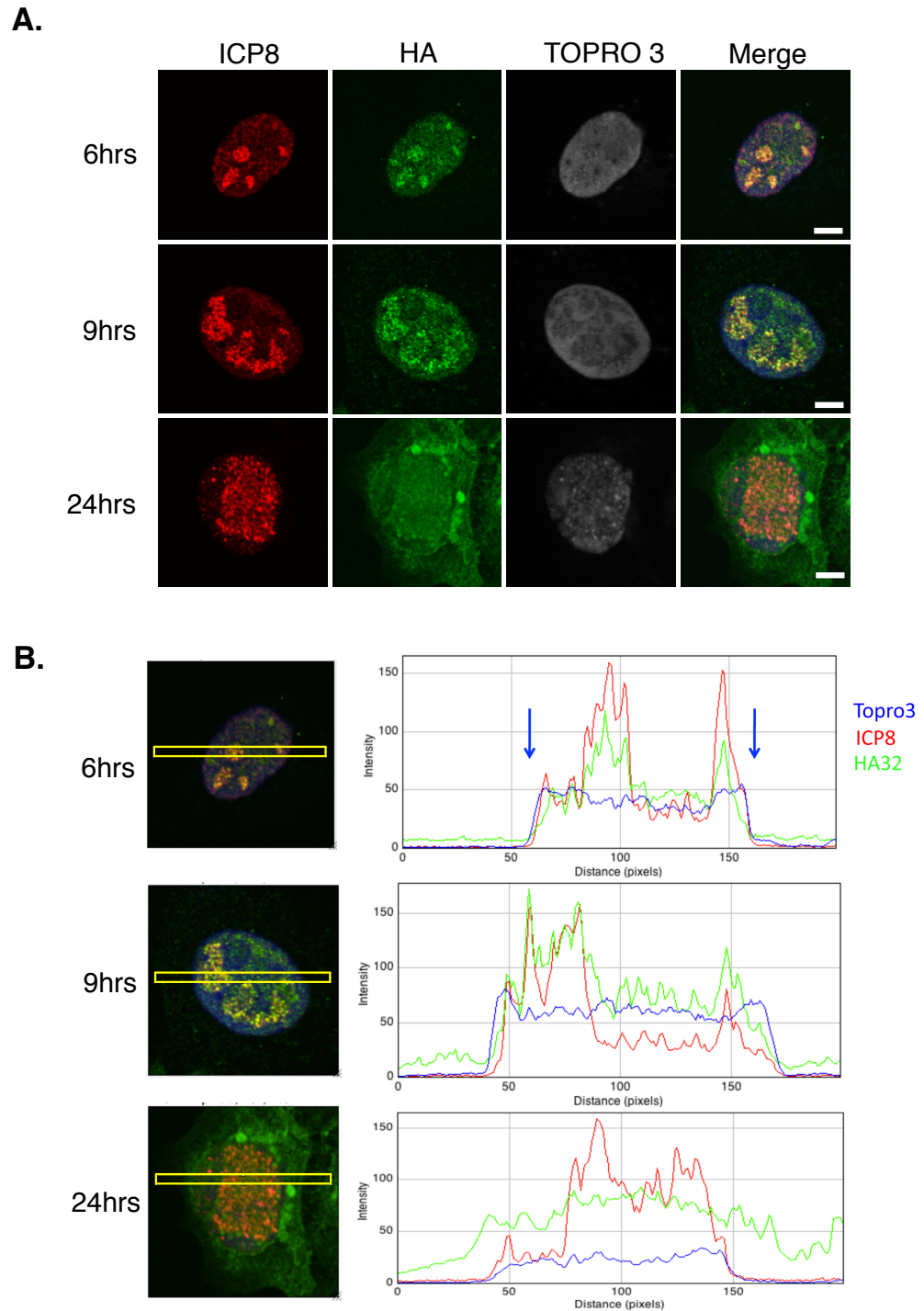


Figure 3-2. UL32 changes localization during infection. A) Vero cells were infected with HA32 at an MOI of 0.1. At 6, 9 and 24 hrs post infection, cells were fixed in 4% PFA and permeabilized in acetone followed by staining for ICP8 (red, mouse antibody) and HA (green, rat antibody). TOPRO-3 staining was performed to visualize the nucleus. Scale bar is 5uM. B) Merged images in A were analyzed by ImageJ software. Yellow boxes indicate the area used to measure fluorescent intensity. Blue arrows mark the edges of the nucleus as indicated by the TOPRO-3 staining. At least 100 cells for each time point were examined, and the staining patterns shown in all three panels represent that seen in nearly all cells on the coverslip.

hpi (Fig. 3-2) or as early as 5 hpi at a higher MOI of 3 (data not shown). Although HA32 and KOS exhibited identical growth curves, we cannot rule out that these foci were caused by the HA tag on UL32; they were not observed in previous reports using a rabbit polyclonal antibody (Chang, Poon et al. 1996) or an EE-tagged UL32 (Lamberti and Weller 1998). We ruled out the possibility that these foci represent aggresomes caused by the accumulation of misfolded proteins since they did not contain either Hsc70 or Hsp70 (data not shown), hallmarks of previously described aggresomes (Muchowski and Wacker 2005). We are intrigued by the possibility that they are related to a second function of UL32 in the cytoplasm, and additional experiments will be required to investigate this possibility.

Conserved C-X-X-C motifs are required for infection

UL32 is cysteine rich and contains C-X-X-C motifs that are reminiscent of cysteine rich motifs present in thiol-disulfide oxidoreductases such as thioredoxin. Oxidoreductases are conserved from bacteria to humans and function as antioxidants involved in redox balance (Chivers, Laboissiere et al. 1996, Chivers, Prehoda et al. 1997, Fomenko and Gladyshev 2003, Becerra, Delaye et al. 2007). For example, thioredoxin is sensitive to oxidation, and its antioxidant activity is coordinated through its C-X-X-C domain (Chivers, Laboissiere et al. 1996, Chivers, Prehoda et al. 1997, Fomenko and Gladyshev 2003, Becerra, Delaye et al. 2007). Figure 3-3 shows that UL32 and homologs from all three subfamilies of herpesviruses contain five conserved cysteine-containing motifs. Sequence alignment using the Clustal W program revealed extensive conservation of cysteines especially within motifs I, III, IV, and V (Fig. 3-3). Motifs I and IV fit the C-X-X-C pattern while motif V contains a well conserved H-X-X-

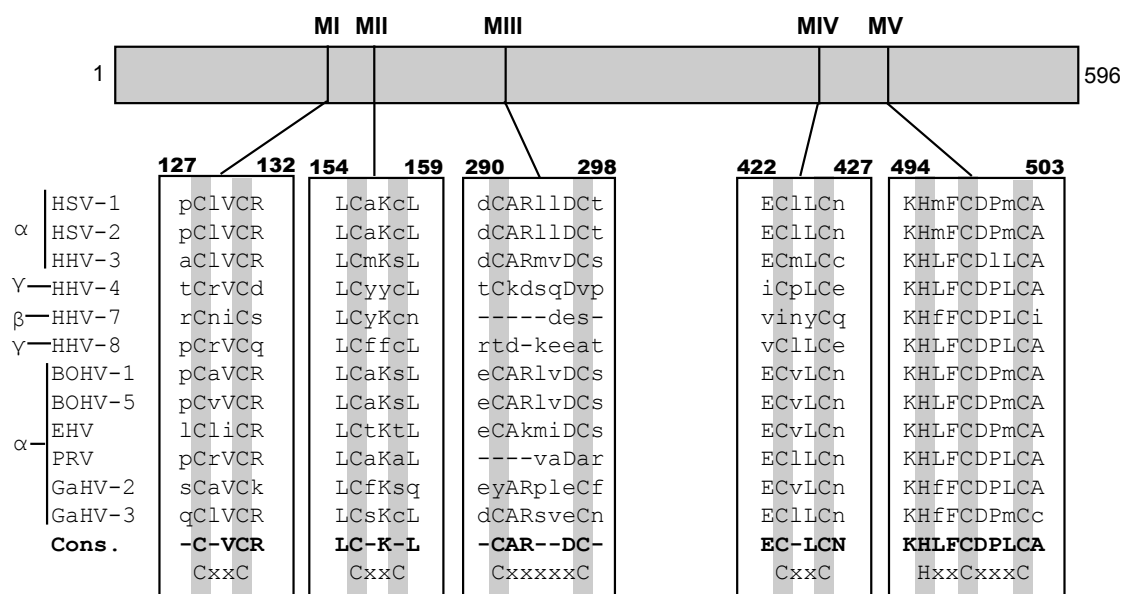


Figure 3-3. UL32 homologs are found in all three subfamilies of herpesviruses. Sequence alignments were performed on UL32 homologs from herpesviruses from the α, β and γ subfamilies. Residues of interest are highlighted in grey and corresponding motifs indicated in the last row. Consensus sequence (Cons.) is represented by the bold lettering. Viruses analyzed are indicated in Materials and Methods.

C-X-X-X-C reminiscent of a motif found in zinc-coordinating proteins responsive to disulfide stress (Kang, Paget et al. 1999, Song, Dove et al. 2003). This motif may be responsible for the observed zinc-binding properties of UL32 (Chang, Poon et al. 1996).

To test whether these conserved motifs are essential for UL32 function, each cysteine was mutated to alanine by site directed mutagenesis to preserve the hydrophobic nature of cysteines, and the histidine in motif V was mutated to the glutamine to preserve the hydrophilic nature at that position. Plasmids bearing these mutations were tested for their ability to complement a UL32-null virus (*hr64FS*) for production of infectious virus (Fig. 3-4). Consistent with the lack of conservation in motif II, mutations in cysteines 155 and 158 resemble wild type UL32 in their ability to complement the null mutant. Most of the other cysteine mutants in motifs I, III, IV and V fail to complement *hr64FS* suggesting that they are essential for UL32 function, although mutants altered at cysteine 291 in motif III and cysteine 498 in motif V are capable of partial complementation. These data suggest that for two of the three most highly conserved motifs (I and IV), both cysteine residues are essential, and for motifs III and V, at least one of the conserved residues is essential. The second cysteine in motif II is not conserved and mutations in both cysteines is tolerated. Thus the degree of conservation correlates well with the results of the mutational analysis.

UL32 is sensitive to oxidation

The sequence similarities between UL32 and redox sensitive proteins led us to ask whether UL32 itself is sensitive to oxidative stress. Redox sensitive proteins, such as Keap1 or thioredoxin-like proteins have exposed reactive thiols that serve as molecular switches that are modified under oxidizing conditions. These modified forms

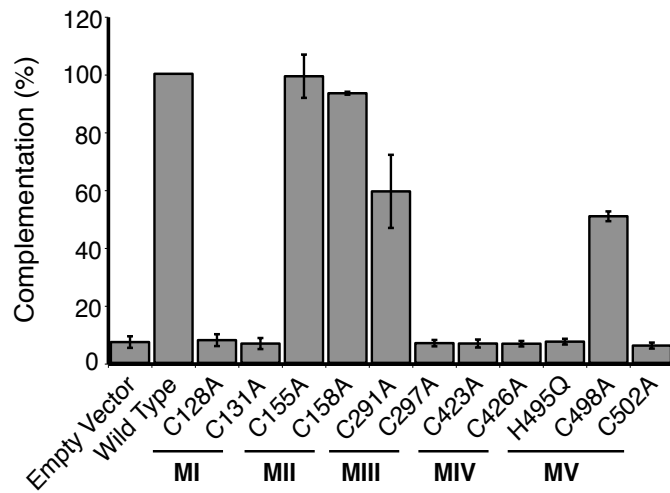


Figure 3-4. Mutagenesis of conserved residues in Motifs I,III, IV and V abolish complementation ability. Alanine substitution mutations were made at every cysteine in Motifs I-V, as well as a histidine-to-glutamine mutation at amino acid 498. A transient complementation assay was used to test the ability for the mutant proteins to rescue the UL32-null virus *hr64FS*. Plasmid DNAs containing each mutation were transfected into Vero cells followed by superinfection with *hr64FS*, and the resulting titer was determined on 158 cells. Error bars represent the standard deviation of three separate experiments.

can be detected by changes in mobility observed by non-reducing SDS-PAGE (Delaunay, Pflieger et al. 2002, Zhang and Hannink 2003, Brown, Day et al. 2013). Vero cells were infected with HA32 at an MOI of 3 for 9 hours, treated with 5mM H₂O₂ for various times and harvested in the presence of NEM buffer to prevent spontaneous formation of disulfide bonds upon lysis (Fig. 3-5 lanes 4-8). Lanes 1 and 2 show control samples of cells infected with KOS or HA32 which were not treated with H₂O₂ and harvested in RIPA buffer. Lysates were subjected to SDS-PAGE under reducing (lanes 1 and 2) and non-reducing conditions (lanes 4-8) and analyzed by Western blot probing with anti-HA antibody. Lysates treated with NEM, but not treated with H₂O₂, contain a prominent 64 kDa band representing NEM-modified monomeric UL32 (Fig 3-5, lane 4). In cells treated with 5mM H₂O₂, slower migrating species, most notably an ~130 kDa band, were observed that became more prominent with time (Figure 3-5, lanes 4-8) and may represent hetero- or homo-dimeric forms of UL32. The slower migrating species were absent in samples treated with H₂O₂ for 30 min followed by treatment with 100mM DTT (data not shown). These results suggest that exposed thiols in UL32 are redox-sensitive and form disulfide-mediated complexes under oxidizing conditions.

Generation and characterization of a new null UL32 mutant

Our previously isolated UL32 mutant, *hr64*, expresses the N-terminal 273 residues of UL32 including the first two of the three C-X-X-C motifs (Lamberti and Weller 1998), raising the possibility that this fragment of UL32 might mask the phenotype of a virus completely lacking UL32. Thus a new UL32 mutant virus (*hr64FS*) containing a frame-shift mutation was generated, resulting in the creation of two

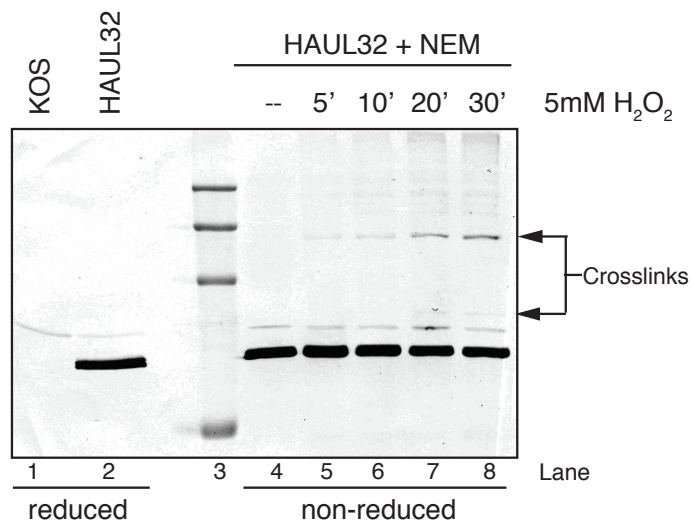


Figure 3-5. UL32 forms slower migrating species in the presence of H₂O₂. Vero cells were infected with HA32 at an MOI of 3. At 9 hpi cells were washed with PBS and overlaid with 2 ml of SFM either with or without 5mM H₂O₂. Cells were washed with PBS and collected in RIPA buffer without NEM (lanes 1 and 2) or TCA precipitated followed by resuspension in NEM buffer (lanes 4-8) at the indicated times. Proteins were separated by reducing or non-reducing SDS-PAGE and analyzed by Western blot using mouse anti-HA antibody. The molecular marker (lane 3) represents from top to bottom 250, 150, 100 and 50 KDa.

tandem premature stop codons at amino acids 56 and 57. This position was chosen to avoid altering the adjacent divergently transcribed UL31 gene. Figure 3-6A shows that *hr64FS* was unable to grow on Vero cells but replicates efficiently on the UL32-complementing cell line, named 158. Figure 3-6B shows that wild type levels of viral DNA were observed in cells infected with *hr64* or *hr64FS*; however, S and Q fragments representing cleavage products were not observed in *hr64*- or *hr64FS*-infected Vero cells consistent with an encapsidation defect. The encapsidation defect was rescued when the virus was propagated on 158 cells. Thus *hr64FS* exhibits the same growth phenotype and encapsidation defect as *hr64*.

Encapsidation of viral genomes requires the assembly of a competent procapsid containing major capsid protein VP5, triplex proteins VP19C and VP23, internal scaffold and protease and a portal ring. In order to determine whether the defect in packaging was due to aberrant capsid composition or morphology, we examined the ability of *hr64FS* to assemble structures that resemble procapsids. Procapsids cannot be isolated directly from infected cells because they are fragile and spontaneously mature into angular B-capsids that have a similar protein composition to procapsids except that the scaffold proteins have been proteolytically processed (Newcomb, Trus et al. 2000). B-capsids from cells infected with KOS, *hr64FS* or *hr81-1* were purified by sucrose gradient sedimentation and analyzed by SDS-PAGE and silver staining to examine their protein composition. *Hr81-1* is a UL15 null virus, and since UL15 is a component of the HSV terminase, this mutant also exhibits a packaging defect. *Hr81-1* is capable of assembling B-capsids and thus serves as a control to ensure that differences between KOS and *hr64FS* are due to the absence of UL32 and not to the

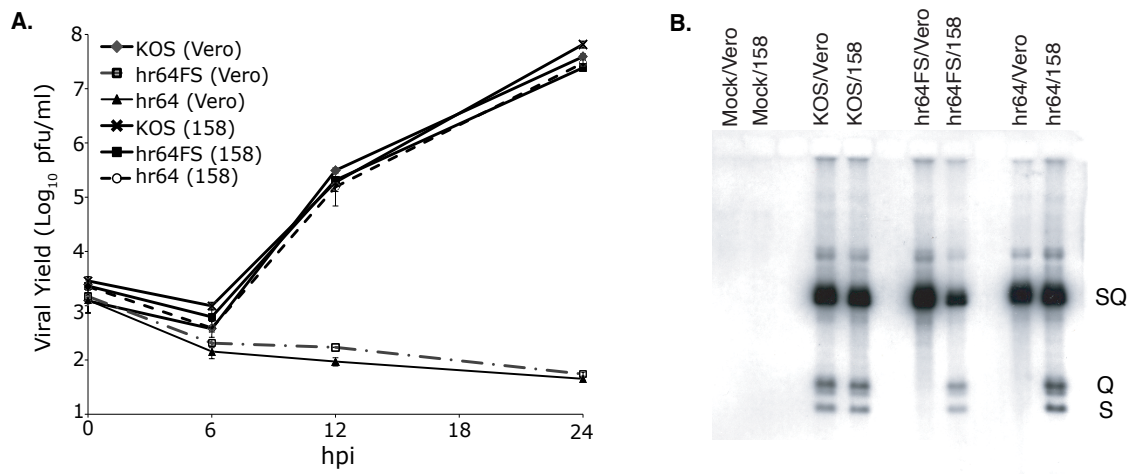


Figure 3-6. UL32 mutants, defective in growth, synthesize wild-type levels of viral DNA but are unable to cleave and package it. A) Growth curves of KOS, *hr64FS* or *hr64* propagated on Vero or 158 cells. Mean values of three independent experiments are plotted and standard deviations are represented by error bars. B) Vero or 158 cells were infected with KOS, *hr64* or *hr64FS* and viral DNA was analyzed by Southern blot as described in Materials and Methods. The blotted DNA was hybridized with ^{32}P -labeled probe containing the BamHI SQ fragment. SQ represents viral DNA junctions and S and Q represent viral DNA termini.

encapsidation defect. Figure 3-7A demonstrates that B-capsids isolated from *hr64FS*-infected cells have a protein composition indistinguishable from capsids isolated from KOS- or *hr81-1*-infected cells. Purified capsids were also analyzed by cryo-EM as described in Materials and Methods, and the cryo-EM reconstructions shown in Fig. 3-7B reveal no differences between the *hr64FS* and KOS capsids. We conclude that capsids assembled in the absence of UL32 resemble those assembled by wild-type virus suggesting that there are no obvious defects in capsid composition or assembly.

We previously reported that *hr64*-infected cells exhibited inefficient localization of the major capsid protein (VP5) to replication compartments (Lamberti and Weller 1998), leading to the hypothesis that UL32 may function as a virally encoded chaperone needed for efficient localization of one or more of the capsid proteins. We analyzed the localization patterns of VP5 and the rest of the viral capsid proteins in cells infected with the new UL32 mutant *hr64FS* (Fig. 3-8). Surprisingly, despite our earlier report that VP5 was mislocalized in cells infected with *hr64*, in cells infected with *hr64FS*, VP5 localized to replication compartments similar to the pattern seen in KOS-infected cells. This experiment has been repeated with three different VP5 antisera, and in each case VP5 was shown to localize to replication compartments in cells infected with either *hr64* or *hr64FS* (data not shown). The reason for this apparent discrepancy is unclear but may be related to differences in culture conditions such as passage number of the cells or levels of glutamine or oxygen in the media. One or more of these differences could alter the folding environment in cells leading to protein mislocalization. We also monitored the behavior of other capsid proteins including VP22a and VP23, and both were found to localize to replication compartments in

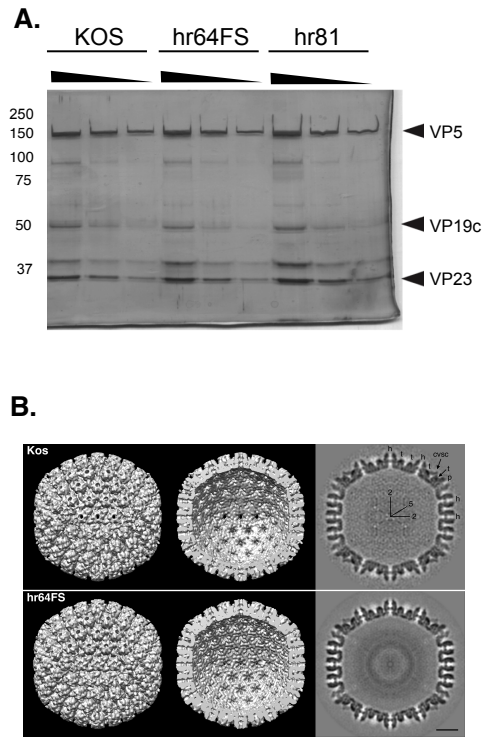


Figure 3-7. *hr64FS* capsids are indistinguishable from KOS capsids. A) Viral capsids were isolated by sucrose gradient sedimentation from Vero cells infected with KOS, *hr64FS* or *hr81*. Dilutions of B-capsids were separated by reducing SDS-PGE and silver stained. B) Cryo-EM analysis and reconstruction of B-capsids isolated from KOS or *hr64FS*-infected cells - surface representations of the capsid exterior (left) and interior (middle) and a grey-scale central section (right). Capsid subunits are indicated as h-hexons, p-pentons, t-triplexes and cvsc-capsid vertex specific component. Icosahedral 2 and 5-fold symmetry axes are also marked. Bar = 200Å.

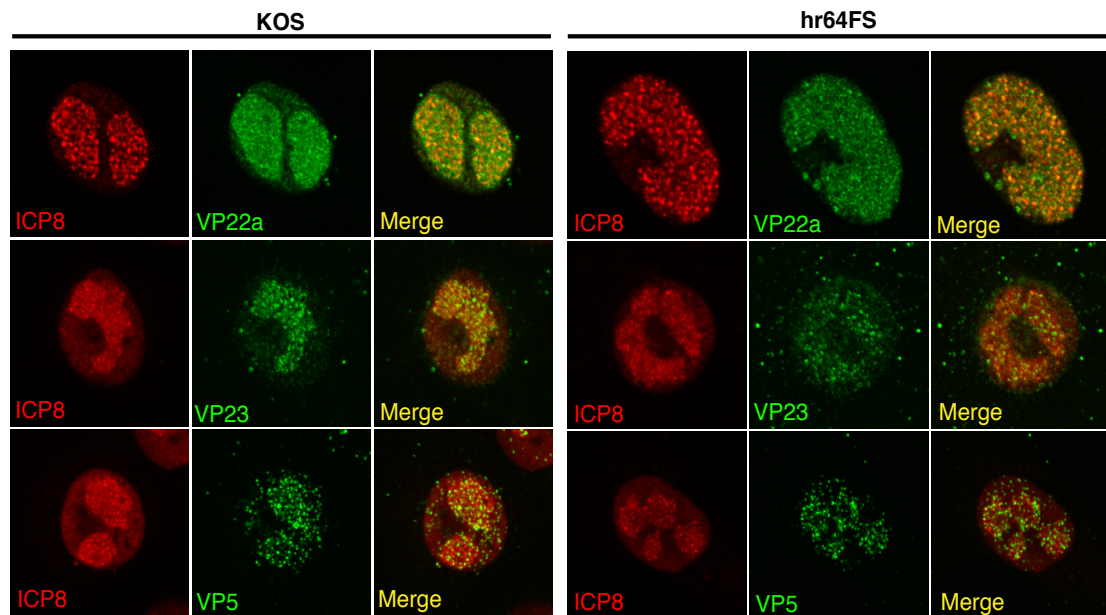


Figure 3-8. Capsid proteins localize to replication compartments in the absence of UL32. Vero cells were infected with KOS or *hr64FS* at an MOI of 3. Infected cells were fixed with 4% PFA and permeabilized in acetone. Cells were stained with antibodies to ICP8 (red channel) plus the capsid proteins indicated (green channel) as described in materials and methods.

hr64FS-infected cells in a similar fashion to their localization in KOS-infected cells (Fig. 3-8). We conclude that UL32 does not influence the localization of capsid proteins, consistent with the observation that protein composition and capsid architecture appears to be normal in the absence of UL32 (Fig. 3-7A and B).

UL32 influences disulfide bond status of viral proteins, including the viral protease (VP24)

We have previously reported that mature HSV capsids are extensively cross-linked by disulfide bonds (Szczepaniak, Nellisery et al. 2011) and that the UL6 portal ring contains inter-subunit disulfide bonds (Albright, Nellisery et al. 2011). This raises the question of how disulfide bond formation in the portal is regulated, especially in a cellular environment that is generally thought to be reducing (Albright, Nellisery et al. 2011). We hypothesize that UL32 acts as a regulator of disulfide bond formation. To test this hypothesis, we took advantage of the thiol-modifying reagent AMS, a maleimide derivative that reacts with free cysteines, adding ~500 Daltons to each cysteine that has a free sulfhydryl. If UL32 were responsible for disulfide bond regulation we would expect a difference in the number of free cysteines available for modification with AMS in cells infected in the presence and absence of UL32. Vero cells were infected with KOS, *hr64FS* or the packaging mutant *hr81-1*. At 11 hpi infected cells were TCA-precipitated, resuspended in denaturing buffer containing NEM to block free thiols, reduced with DTT to free thiols participating in disulfide bonds and subsequently treated with AMS to modify the newly freed thiols as described in Materials and Methods. Figure 3-9 shows that in the absence of UL32, both the viral protease, VP24 and UL6 migrate slower than they do in cells infected with KOS or

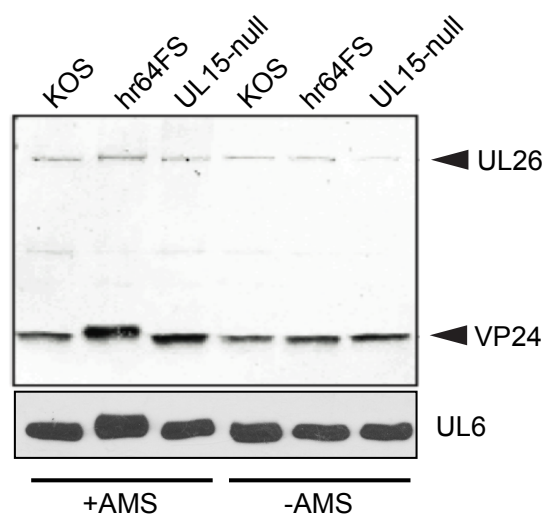


Figure 3-9. UL32 modulates disulfide bond formation in the viral proteins. Vero cells were infected with KOS, *hr64FS* or *hr81* at an MOI of 3 and harvested in 10% TCA at 10 hpi. Free thiols were blocked with NEM, followed by reduction with DTT then treatment with the thiol alkylating agent AMS. Proteins were resolved by reducing SDS-PAGE and detected by immunoblotting with anti-VP24 or anti-UL6 antibody.

hr81-1-infected cells. *Hr81-1* was included in this experiment to show that the disulfide bond alterations seen in *hr64FS*-infected cells were not the result of an encapsidation defect. These shifts were not observed in the absence of AMS and suggest that in *hr64FS*-infected cells, protease and UL6 contained disulfide bonds not observed in cells infected with KOS or *hr81*. Interestingly, similar shifts were observed in the minor capsid proteins UL25 and VP19C (data not shown). These results are consistent with the hypothesis that UL32 regulates disulfide bond formation and that the presence of UL32 may function to prevent premature disulfide bond formation in a number of viral capsid proteins.

Discussion

In this study two new viruses were used to examine the role of UL32 in the encapsidation of HSV-1 DNA: HA32, containing an HA-tagged UL32 protein (HA32) and a null mutant (*hr64FS*) containing a frameshift mutation. HA32 resembles wild type virus in growth and encapsidation while *hr64FS* is defective for both. Several observations were made. (i) *Hr64FS* assembled B-capsids that resemble wild type B-capsids in protein composition and appearance by cryo-EM (Fig. 3-7B); however, they were unable to encapsidate viral genomes. (ii) Although it was previously reported that UL32 is mostly cytoplasmic with some minor staining in the nucleus, a time course experiment (Fig 3-2) revealed a dynamic localization pattern. UL32 was found predominantly in replication compartments early in infection, while later its localization became cytoplasmic/nuclear diffuse. (iii) We confirmed that UL32 is a true late protein. (iv) Two highly conserved C-X-X-C motifs within UL32 were shown to be essential for

viral growth. (v) UL32 was shown to be redox-sensitive. (vi) UL32 influenced the formation of disulfide bonds in the viral proteins UL25, VP19C, UL6 and the viral protease, VP24.

The ability to assemble B-capsids that are indistinguishable from those seen in cells infected with wild type HSV suggests that UL32 is not required for the assembly of procapsids *per se*. On the other hand, we have confirmed that procapsids assembled in cells infected with a UL32 mutant fail to initiate encapsidation. Protease activation marks the initiation of the encapsidation process; however, the mechanism by which protease is regulated is not understood. The formation of inter- and intramolecular disulfide bonds is generally recognized to regulate a wide range of cellular processes. We have previously shown that disulfide bonds are essential for capsid stability and for the formation and or stability of the UL6 portal ring (Albright, Nellissery et al. 2011, Szczepaniak, Nellissery et al. 2011). It is possible that the HSV protease is regulated by redox state. This suggestion may be consistent with previous reports suggesting that the HCMV protease is regulated by disulfide bond formation. Alternatively, initiation of encapsidation may be triggered by redox-stimulated changes in the portal ring. Thus encapsidation may be regulated through a thiol-disulfide exchange mechanism involving protease or portal or both.

The suggestion that UL32 may perform a thiol-disulfide exchange reaction is consistent with the observations that UL32 itself is redox sensitive and that it contains C-X-X-C motifs reminiscent of those found in a number of other thiol-disulfide oxidoreductases. We now report that these conserved C-X-X-C motifs are essential for viral growth. UL32 may act directly on protease or UL6 by catalyzing the formation or

breaking of disulfide bonds; alternatively, UL32 may exert indirect effects by altering the cellular milieu to provide an environment conducive for disulfide bond formation.

The localization of UL32 in infected cells may also provide some clues as to its function. Previous reports that UL32 is predominantly a cytoplasmic protein were hard to reconcile with the phenotype of UL32 mutants that suggested a role in encapsidation. The observation that UL32 was found to localize predominantly to replication compartments early in infection and in the cytoplasm and nucleus at later stages is consistent with additional functions for UL32 at a stage post-encapsidation. Our finding that in the absence of UL32, capsid proteins UL6, UL25 and VP19C exhibited disulfide bonds not seen in wild-type infection is of interest. We and others have found that the cellular environment becomes oxidized during HSV-1 infection (Palamara, Perno et al. 1995, Nucci, Palamara et al. 2000, Vogel, Cinatl et al. 2005, Mathew, Bryant et al. 2010, Gonzalez-Dosal, Horan et al. 2011) (Albright manuscript in preparation), and an oxidized environment may promote disulfide bond formation. Although some disulfide bonds may be beneficial for the virus, it is also possible that an oxidizing environment could lead to aberrant disulfide bond formation between exposed reactive thiols that would be deleterious to the virus. We suggest that UL32 may serve to protect sensitive thiols from aberrant or deleterious disulfide bond formation. Thus, in addition to a possible role in the nucleus regulating protease and/or UL6, UL32 may play additional roles required for capsid maturation in the cytoplasm, possibly by protecting reactive thiols. Alternatively, UL32 may catalyze the formation of disulfide bonds to regulate specific processes, such as protease activation.

This study provides the first evidence that HSV may encode a virally-encoded oxidoreductase. Other large DNA viruses, which require disulfide bonds, have been shown to encode enzymes that function as oxidoreductases (Iyer, Aravind et al. 2001). For instance, vaccinia virus encodes three oxidoreductases (E10R, A2.5L and G4L) which participate in a thiol-disulfide relay responsible for modulating disulfide bonds in the cytoplasm (Senkevich, White et al. 2002). It is becoming increasingly clear that oxidative stress and cellular responses to it play critical roles in modulating a large number of biological processes including pathogenesis by bacterial and viral infections. Many viruses including HSV have been shown to induce oxidative stress, and antioxidants have shown promise in the treatment of viral infection (Palamara, Perno et al. 1995, Garland and Fawzi 1999, Beck, Handy et al. 2000, Nucci, Palamara et al. 2000, Cai, Chen et al. 2003, Abdalla, Ahmad et al. 2005, Vogel, Cinatl et al. 2005, Tian, Jiang et al. 2010). Viral proteins that play roles in sensing and responding to changes in redox status may thus provide targets for therapeutic intervention. The suggestion that UL32 encodes a redox sensitive chaperone responsible for modulating disulfide bond formation in HSV-infected cells indicates that UL32 itself may represent a novel and promising drug target for controlling HSV infections.

Chapter 4

A pro-oxidant environment may be required for efficient early and late gene expression of herpes simplex virus type 1.

Brandon S. Albright, Renata Szczepaniak, and Sandra K. Weller

Abstract

Previous reports have shown the HSV-1 induces the generation of reactive oxygen species (ROS) and requires a pro-oxidant environment for efficient viral growth; however, the role of ROS during HSV-1 infection is not clear. In this study, we confirmed that the intracellular environment becomes oxidized during viral infection using a genetically-encoded redox-sensitive GFP (roGFP2). In addition, we treated HSV infected cells with the antioxidant glutathione (GSH) and investigated viral growth at 6, 12, 24 and 48 hours. GSH treatment reduced HSV growth in Vero and HeLa cells, and to a greater extent, normal human fibroblast (NHF) and human embryonic lung (HEL) fibroblasts. Growth yields were affected at multiplicities of infection (MOIs) of both 0.1 and 5, with a substantial defect in viral growth as early as 6 hours post infection (hpi) at an MOI of 5. Western blot analysis revealed that GSH treatment reduced early (E) and late (L) gene expression but not expression of the immediate early (IE) gene, ICP4. We explored whether the ROS-generating NADPH-oxidase (NOX) family of enzymes was required for HSV infection. We used a pan-NOX inhibitor, diphenylene iodonium (DPI) and found that inhibiting NOX

activity reduced viral growth at early stages. Similar to what we observed with GSH treatment, DPI treatment delayed viral replication compartment formation and E and L gene expression. These data suggest that NOXs may contribute to a pro-oxidant state during infection, which is necessary for an early step in the viral lifecycle.

Introduction

Many viruses, including herpes simplex virus 1 (HSV-1), have been shown to increase the levels of intracellular reactive oxygen species (ROS) during infection (reviewed in (Fraternale, Paoletti et al. 2009, Campagna and Rivas 2010, Bottero, Chakraborty et al. 2013, Paiva and Bozza 2014). ROS are potentially toxic metabolites generated through the incomplete reduction of molecular O₂. Despite the ability of ROS to cause damage to many macromolecules, it is becoming increasingly apparent that they are important secondary messengers involved in a number of biological processes (Sauer, Wartenberg et al. 2001, Valencia and Moran 2004, Collins, Chouchani et al. 2012). The balance of intracellular ROS is crucial to cell physiology and is coordinated by a number of systems that generate ROS (discussed below) or work to clear ROS. The predominant buffer against intracellular ROS is the small tripeptide, glutathione (GSH), which exists in millimolar concentrations within the cell. When ROS are present, GSH serves as a scavenger. Furthermore, a number of enzymes, such as thioredoxin, peroxiredoxin, superoxide dismutase (SOD), etc., work to keep the intracellular redox environment stable. Perturbations in the redox balance can result in either reductive or oxidative stress

(OS), which elicits a response to bring the cell back to homeostasis. When this balance tips in favor of the oxidants, it can cause severe cellular and tissue damage if unresolved.

The two major intracellular sources of ROS are the mitochondria and the NADPH-oxidase (NOX) family of enzymes. Mitochondria generate ROS as an accidental by-product of oxidative phosphorylation, which occurs when electrons escape from the electron transport chain and react with molecular oxygen. NOXs, on the other hand, are a unique class of trans-membrane enzymes designed to intentionally generate ROS (Bedard and Krause 2007). The NOX family comprises seven members (NOXs 1-5 and Duox 1,2) which have specific tissue and cell-type distributions (Bedard and Krause 2007). Although the role of NOX2 has been well established in the production of ROS for pathogen clearance (Sumimoto, Miyano et al. 2005), the roles of the other NOXs are poorly defined (Bedard and Krause 2007).

It has been well documented that many viruses not only tolerate, but also thrive, in a pro-oxidant environment (reviewed in (Peterhans 1997, Fraternale, Paoletti et al. 2009, Paiva and Bozza 2014)). HIV, for instance, induces ROS to trigger activation of the cellular transcription factor NF- κ B, and in turn upregulates viral gene expression (Nabel and Baltimore 1987, Staal, Roederer et al. 1990). In a recent study, NOX4-derived ROS was shown to be important for influenza infection in a cell culture model (Amatore, Sgarbanti et al. 2015). During influenza infection, the production and release of progeny virus requires the nuclear export of viral ribonucleoproteins (vRNPs). This export has been shown to require caspase activation and the subsequent enlargement of nuclear pores (Muhlbauer,

Dzieciolowski et al. 2015). When NOX4 activity was inhibited, vRNPs accumulate in the nucleus, suggesting that NOX4 mediated ROS plays a role in caspase activation during influenza infection. Thus, intentional ROS generation through NOX activation may be a common strategy used by viruses to hijack specific cellular pathways for their own benefit.

Several lines of evidence suggest that HSV-1 induces and requires a pro-oxidant environment: 1) infection has been reported to cause an immediate and sustained drop in intracellular GSH pools (Palamara, Perno et al. 1995, Nucci, Palamara et al. 2000, Vogel, Cinatl et al. 2005), 2) restoration of GSH levels by antioxidant treatment has been reported to inhibit viral growth (Palamara, Perno et al. 1995, Nucci, Palamara et al. 2000, Vogel, Cinatl et al. 2005), 3) intracellular ROS can be detected as early as 1 hpi using the redox-sensitive fluorescent dyes dichlorodihydrofluorescein – diacetate (DCHF-DA) (Schachtele, Hu et al. 2010, Gonzalez-Dosal, Horan et al. 2011, Hu, Sheng et al. 2011) or hydroxyphenyl fluorescein (HPF) (Kavouras, Prandovszky et al. 2007) and 4) oxidized proteins accumulate in HSV-infected cells (Mathew, Bryant et al. 2010). Collectively, these findings are consistent with the idea that HSV induces and requires an oxidizing environment; however, how and why the virus has evolved to tolerate and thrive in an oxidizing environment is unclear.

In this study we set out to define the stage of the viral lifecycle that is impaired by GSH treatment and to gain a better understanding of how oxidative stress is induced by HSV. Here, we confirmed that HSV-1 infection shifts the cellular environment to a pro-oxidant state. We also showed that GSH treatment inhibited

viral growth in Vero cells, HeLa cells, normal human fibroblasts (NHF), and human embryonic lung (HEL) cells. GSH treatment reduced viral growth by over 90% as early as 6 hpi when the infection was carried out at an MOI of 5. Western blot analysis revealed that the IE gene ICP4 was expressed efficiently in treated cells; however, early E and late L gene expression was delayed. Consistent with these findings, we observed that antioxidant treatment resulted in delayed replication compartment formation. We tested the hypothesis that NOX-derived ROS was important for HSV growth and found that inhibiting NOX activity, using the chemical inhibitor DPI, led to similar defects in viral growth, protein expression and replication compartment formation as found during antioxidant treatment. In summary, we suggest that NOX activity is necessary for generating a pro-oxidant environment and that this type of an environment is required for efficient infection. We also propose that the oxidizing environment may be required for efficient expression of E and L viral genes.

Materials and methods

Cell lines and viruses. African green monkey kidney cells (Vero CC181; American Type Culture Collection, Rockville, MD), HeLa cells and human foreskin fibroblasts (HFFs), were maintained and propagated in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% or 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Normal human fibroblasts were

immortalized with hTert, a gift from the lab of Douglas McCarty, and were cultured in MEM supplemented with 15% FBS, 2 mM L-glutamine and 1% P/S. Human embryonic lung cells were cultured in MEM containing 10% FBS and 1% P/S. The KOS strain of HSV-1 was used as the wild-type virus. Cell maintenance and infections were carried out at 37°C.

Detection of ROS with roGFP2. The plasmid carrying roGFP2 was purchased from the University of Oregon. Vero cells were transfected with roGFP2 using Lipofectamine200 according to the protocol. 16 hours post-transfection, cells were treated or infected and harvested at the appropriate times in ice-cold 1% NP-40 buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA, 10 mM NEM). Plates were rocked for 15 min before adding supernatants to wells of a 96-well plate and immediately analyzed. Fluorescence was detected on a Beckman Coulter plate reader (PN 987958) at excitation wavelengths of 405 nm and 488 nm and emission set to 510 nm. Data were processed in excel.

Cell treatments. Reduced L-glutathione (Sigma, #G4251) was dissolved in serum free DMEM or MEM at a stock concentration of 200 mM. Aliquots were stored at -80°C and diluted in media containing 2.5% to 20 mM just before use. Diphenylene iodonium (Sigma, #D2926) was dissolved in DMSO to a stock concentration of 10 mM and was diluted to the appropriate concentration in the cell-type specific media prior to use. DMSO was added to untreated controls in these experiments, but never reached >0.2%.

Growth curves. Confluent cells were infected with KOS at an MOI of 0.1 or 5. Cells and supernatant were harvested at 0, 6, 12, 24 and 48 hours post-infection. After

three freeze-thaw cycles, serial dilutions were plated on Vero cells, overlaid with DMEM supplemented with 2% FBS and 1% human serum, incubated for 72 h, fixed in 4% formaldehyde, and stained with 1% crystal violet solution. Viral plaques were counted by eye.

Western blot analysis. Cells were harvested and proteins were separated by reducing SDS-PAGE as previously described and transferred to a PVDF membrane (Albright, Nellisery et al. 2011). Membranes were blocked with 5% milk and incubated overnight with the following antibodies: 1:1,000, rabbit polyclonal anti-UL32 1:1,000 (generated by Open Biosystems to synthetic antigenic peptide); mouse monoclonal anti-ICP8 1:2,000 (abcam ab20194); mouse monoclonal anti-VP5 (EastCoast Bio HA018) 1:1,000; mouse anti-Actin (Sigma A5441) 1:10,000.

Immunofluorescent confocal microscopy. Monolayers of Vero cells were grown on glass coverslips prior to infection. Infected cells were washed with 2X phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde and permeabilized in ice cold acetone for 2 min followed by blocking in 3% normal goat serum (NGS) diluted in PBS. Cells were incubated in primary antibodies diluted in 3% NGS for 1-2 h at room temperature by inverting coverslips onto a drop of antibody dilution. Dilutions of primary antibodies in 3% NGS were as follows: monoclonal anti-ICP8 1:200; rabbit polyclonal anti-NOX4 (SantaCruz, cat. sc-30141) 1:200. Cells were washed extensively before incubation in AlexaFluor secondary antibodies (1:200: Molecular Probes), diluted in 3% NGS and mounted onto slides with glycerol gelatin containing 2.5% diazobicyclo-[2.2.2] octane (DABCO; Sigma Chemical Co.). Images were captured using a Zeiss LSM 510 confocal NLO microscope equipped with argon and

HeNe lasers and a Zeiss 63X objective lens (numerical aperture, 1.4). Images were processed and arranged using Adobe Photoshop CS3 and Illustrator CS3.

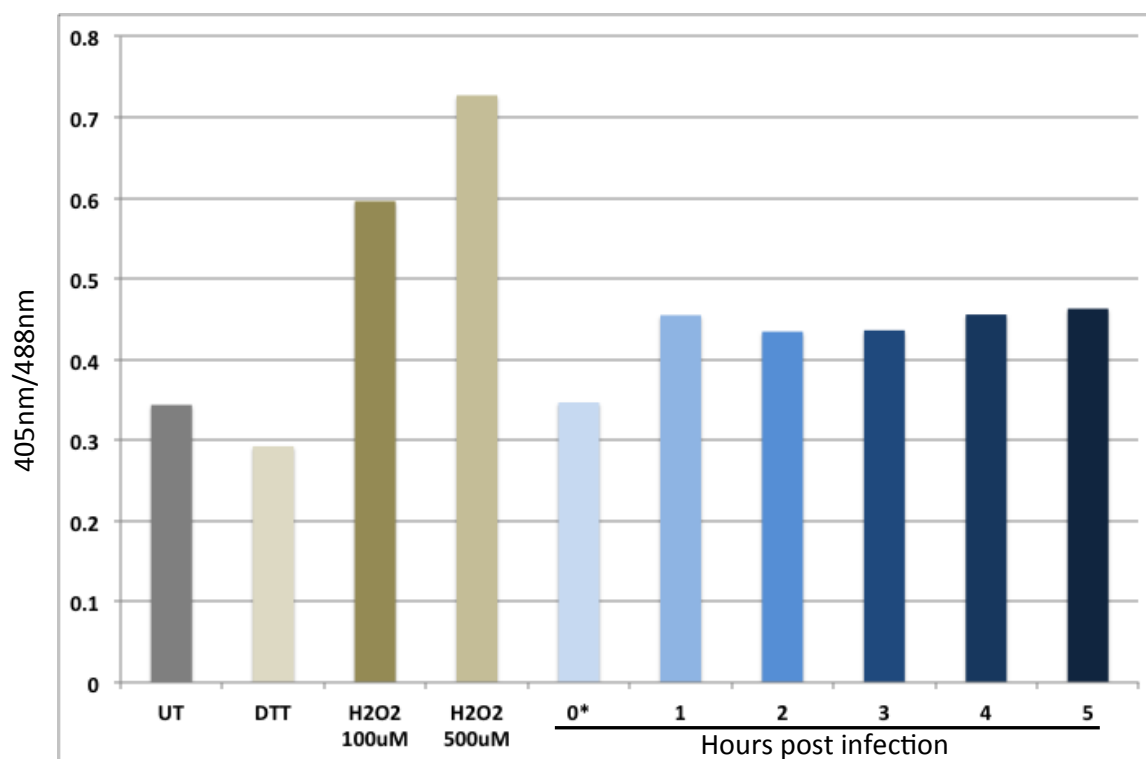
Results

HSV-1 induced ROS can be detected with a redox-sensitive GFP

The goal of our study was to investigate the induction and consequences of ROS by HSV. To this end, we first wanted to confirm that Vero cells were oxidized during HSV infection. Several studies have previously shown that HSV induces intracellular ROS. These ROS were measured directly using redox-sensitive fluorescent dyes such as dichlorodihydrofluorescein – diacetate (DCHF-DA) (Schachtele, Hu et al. 2010, Gonzalez-Dosal, Horan et al. 2011, Hu, Sheng et al. 2011) or hydroxyphenyl fluorescein (HPF) (Kavouras, Prandovszky et al. 2007). These dyes are used ubiquitously as they are cell permeable, allowing for labeling of live cells. Another advantage of using these dyes is that they allow for direct quantification of ROS. Despite these benefits, there are also limitations to the use of these probes. For instance DPH only reacts with highly reactive oxygen species, such as the hydroxyl radical ($\text{OH}\cdot$) or peroxynitrate and thus would not be a reliable detector of other ROS, such as superoxide (O_2^-) or hydrogen peroxide (H_2O_2) (Setsukinai, Urano et al. 2003). Conversely, the mechanism of DCHF-DA oxidation is poorly understood and can react even in the absence of ROS through reactions

with intracellular metals, peroxidases and cytochrome c (Marchesi, Rota et al. 1999, Wardman 2007). Furthermore, the oxidation of DCHF-DA can generate O_2^- , leading to an accumulation of the species it is designed to measure (Wrona, Patel et al. 2005).

For these reasons, we decided to use a genetically encoded redox-sensitive GFP, roGFP2, which is a GFP molecule where two residues on an exposed surface of the protein were mutated to cysteines to serve as a sensitive and rapid redox switch (Dooley, Dore et al. 2004). Upon oxidation, the cysteines form a disulfide bond and induce a conformational change, which causes the protein to preferentially excite at 405 nm, as opposed to the 488 nm when reduced. To test whether HSV induces oxidation, Vero cells were transfected with an expression plasmid carrying roGFP2 as described in materials and methods. 16 hours post transfection, cells were infected with KOS and harvested in 1% NP-40 buffer containing 10 mM n-ethyl maleimide (NEM) to preserve the redox state of the roGFP2. NEM alkylates free thiols and prevents oxidation of the protein during processing. Cells were also treated with 10 mM dithiothreitol or H_2O_2 to serve as the reduced and oxidized controls, respectively. We measured the fluorescence on a plate reader and calculated the ratio of 405 nm/ 488 nm (Figure 4-1). We observed that the fluorescence ratio slightly increased at 1 hpi and this increase was maintained for at least the first 5 hours. Thus, this is the first report using a genetic redox probe to confirm that HSV induces an oxidizing environment.



* Inoculum added for 10min then removed

Figure 4-1 HSV-1 infection induces oxidation of roGFP2. Vero cells were transfected with a plasmid expressing roGFP2. 16 hours post transfection, cells were either treated with the indicated compounds or infected with KOS and harvested at the indicated times in a 1% NP-40 buffer. DTT treatment served as the reduced control and H₂O₂ treatment served as the oxidized control. Emission at 510nm was measured after excitation with 405nm or 488nm on a plate reader.

The effect of GSH treatment on HSV growth varies between cell-types and culture conditions.

We next wanted to confirm previous reports that GSH treatment was inhibitory to viral growth. Unlike other reports that measured viral growth only at 24 and 48 hours (Palamara, Perno et al. 1995, Vogel, Cinatl et al. 2005), we measured viral growth at 0, 6, 12, 24 and 48 hpi to get a more extensive view of when antioxidant treatment inhibited viral growth. Vero cells and HFFs were infected at a multiplicity of infection (moi) of 2 and incubated in media with or without 20 mM GSH (Figure 4-2). Surprisingly, we observed that viral titers at every time point were nearly identical between the treated and untreated in both cell types. We also repeated these experiments using another antioxidant, N-acetylcysteine, and got similar results (data not shown).

These data are in disagreement with the initial work by Palamara, et al, and Vogel, et al, who found GSH to be inhibitory to viral growth in Vero cells and HFFs, respectively (Palamara, Perno et al. 1995, Vogel, Cinatl et al. 2005). Upon examination of the protocols used in these studies, we noticed that we were using Dulbecco's essential modified media (DMEM); whereas, the groups noted above used RPMI 1640 and modified essential media (MEM), which are similar to each other. Since DMEM contains ferric nitrate and is supplemented with double the nutrients as the other two media types, we next asked whether culture media could be a reason for the discrepancy between our results and previously published

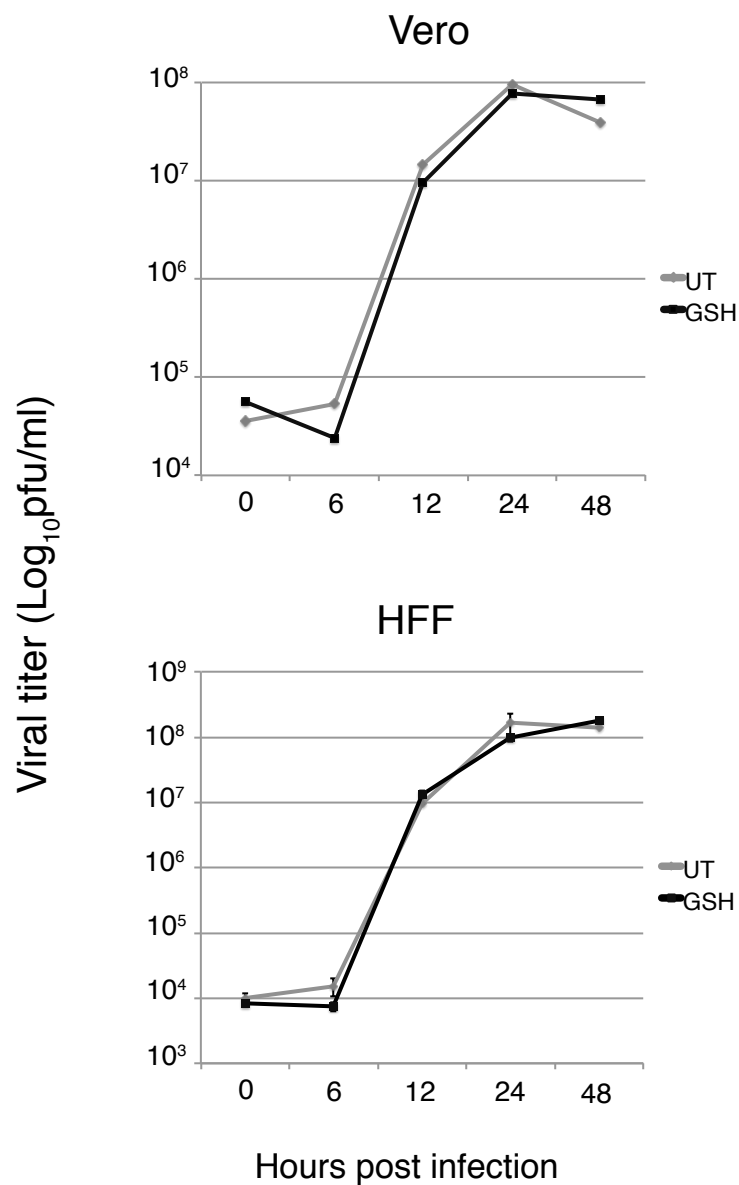


Figure 4-2 KOS infection is not sensitive to antioxidant treatment on Vero cells or HFFs. Vero cells or HFFs were infected at an MOI of 2 and incubated in DMEM containing 2.5% serum without (UT, gray line) or with (GSH, black line) 10 mM NAC. Samples were collected at the specified timepoints and growth was measured by titration on Vero cells. Error bars represent the standard deviation of 3 separate experiments

reports. For this experiment, we cultured Vero cells in MEM for several passages. We then tested the effects of antioxidant treatment during infection of Vero cells under these conditions. Strikingly, we saw attenuated growth beginning at 12 hpi in the presence of antioxidants (Figure 4-3). These data confirm previous reports that the virus is sensitive to antioxidants; however, they illustrate that great care needs to be taken when designing experiments to examine redox systems. How the culture conditions influenced GSH treatment is unclear, but it is possible that culturing Vero cells in DMEM predisposes these cells to stress due to the overabundance of amino acids and vitamins. Alternatively, DMEM is supplemented with ferric nitrate, which is an iron salt, and it is well known that iron in solution can undergo redox cycling. Both MEM and RPMI-1640 contain no iron supplement.

We next asked whether GSH treatment could inhibit viral growth in other cell types. We tested HeLa cells (transformed human cervical epithelial cells), immortalized normal human fibroblasts (stably expressing the hTert gene) and a primary, non-immortal cell type, Human embryonic lung fibroblasts. Cells were infected with KOS at an MOI of 0.1 and incubated in media supplemented with 2.5% FBS with or without 20 mM GSH. Infection was reduced by over ten-fold in HeLa cells at 48 hpi, although the earlier time points displayed a more moderate reduction (Figure 4-4). Strikingly, GSH had a substantially greater impact on viral growth in KOS-infected NHFs and HEL cells (Figure 4-4). Antioxidant treatment resulted in a ~ 2 log decrease in viral yield at 12 hpi and this decrease is sustained for up to 48 hpi. Because infection in NHFs and HEL cells had the greatest response to GSH treatment, these two cell-types were selected for the rest of our studies. Some types

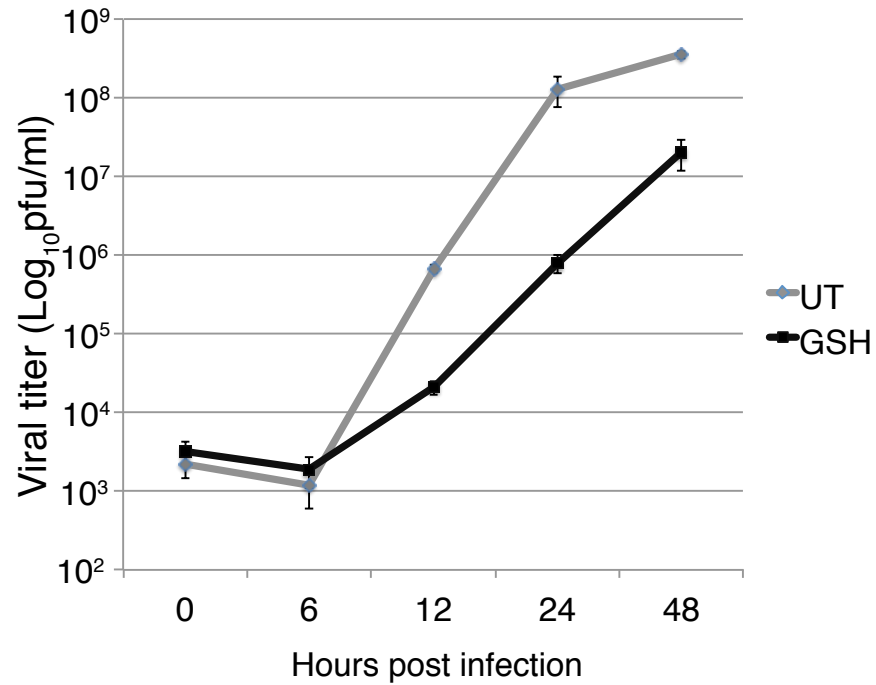


Figure 4-3 Virus grown on Vero cells culture in MEM is susceptible to antioxidant treatment. Vero cells were cultured in MEM containing 5% FBS for several passages before being infected with KOS at an MOI of 0.1. Infected cells were incubated in MEM containing 2.5% serum without (UT, gray line) or with (GSH, black line) 20 mM GSH. Samples were collected at the specified timepoints and growth was measured by titration on Vero cells. Error bars represent the standard deviation of 4 separate experiments

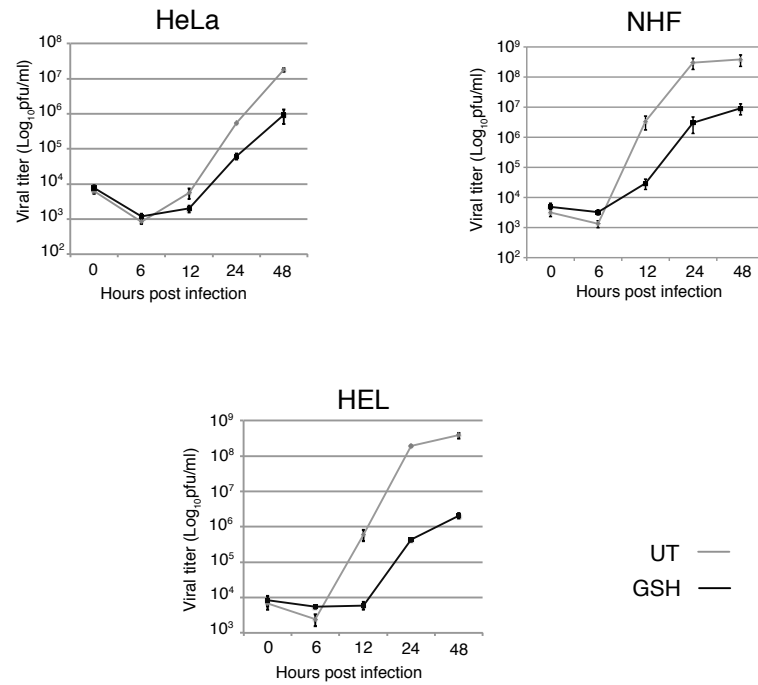


Figure 4-4 Glutathione treatment reduces viral growth on multiple human cell types. HeLa cells, HEL cells or NHFs were infected at an MOI of 0.1 and incubated in MEM containing 2.5% serum without (UT, gray line) or with (GSH, black line) 20mM GSH. Samples were collected at the specified timepoints. Growth was measured by titration on Vero cells. Error bars represent the standard deviation of 3 separate experiments.

of defects can be overcome by increasing the MOI; therefore, we asked whether GSH treatment could block infection at a high MOI. NHFs were infected with KOS at an MOI of 5 and treated as described above. Figure 4-5 shows that GSH treatment reduced viral growth by 10-fold at 6 hours and nearly 100-fold at 12 hours post infection, suggesting that there was a substantial block in the progression of infection at an early time. We also observed a similar defect in growth in GSH-treated HEL cells infected at an MOI of 5 (data not shown). In all, we confirmed that GSH treatment blocked HSV infection in several different cell types and during infection at low (0.1) and high (5) MOIs.

GSH treatment impairs viral replication compartment formation

During HSV-1 infection, the virus hijacks the host-cell nucleus and forms large globular domains called replication compartments. Replication compartments are the sites of viral gene transcription, DNA replication and virus assembly. The formation and growth of replication compartments depends on the expression of IE and E gene products. Thus, replication compartments (RC) serve as a marker for the progression of infection, and we used RC formation as a preliminary step to determine the stage of infection that was impaired during GSH treatment. NHFs were infected with KOS at an MOI of 5, incubate in media with or without 20 mM GSH, harvested at 2, 4, 6 and 12 hpi and processed for immunofluorescence analysis. Cells were stained with antibodies against the viral single-strand DNA binding protein, ICP8, a marker for replication compartments. In the untreated cells

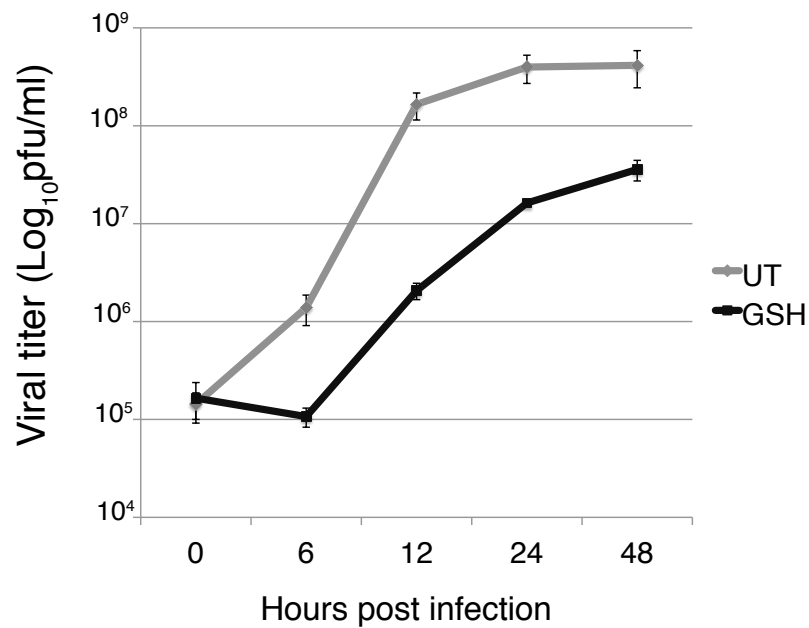


Figure 4-5 GSH inhibits viral growth even at high MOI. NHFs were infected at an MOI of 5 and incubated in MEM containing 2.5% serum without (UT, gray line) or with (GSH, black line) 20mM GSH. Samples were collected at the specified timepoints and growth was measured by titration on Vero cells. Error bars represent the standard deviation of 3 separate experiments.

at 2 hpi, there was a mixture of ICP8 staining patterns; some cells had small foci while others had large globular structures indicative of progressing replication compartments. In the GSH-treated cells, on the other hand, only very small foci were observed at 2 hpi (Figure 4-6). Furthermore, the progression of replication compartment formation was delayed at all time points when compared to the untreated samples. Thus, our data suggest that GSH treatment impairs replication compartment formation.

Viral gene expression is altered in the presence of GSH.

The delay in replication compartment formation led us to speculate that viral gene transcription may be impaired. During infection, there is a tightly regulated cascade of gene expression. IE gene products are required for the expression of E replication proteins which in turn upregulate L gene expression. DNA synthesis is required for true-late gene expression. We set out to assess the expression of viral genes from each of these kinetic classes by Western blot analysis. Cells were infected in the absence or presence of GSH for varying lengths of time, and viral protein expression was analyzed by Western blot. NHFs were infected with KOS (or HA32) at an moi of 5 and harvested at 4, 6, 12 and 24 hpi (Figure 4-7). In the samples treated with GSH, the IE gene ICP4 shows no decrease in expression at 4 hpi when compared to untreated. ICP4 is the major viral transcription factor responsible for activating E and late gene expression as well as repressing the

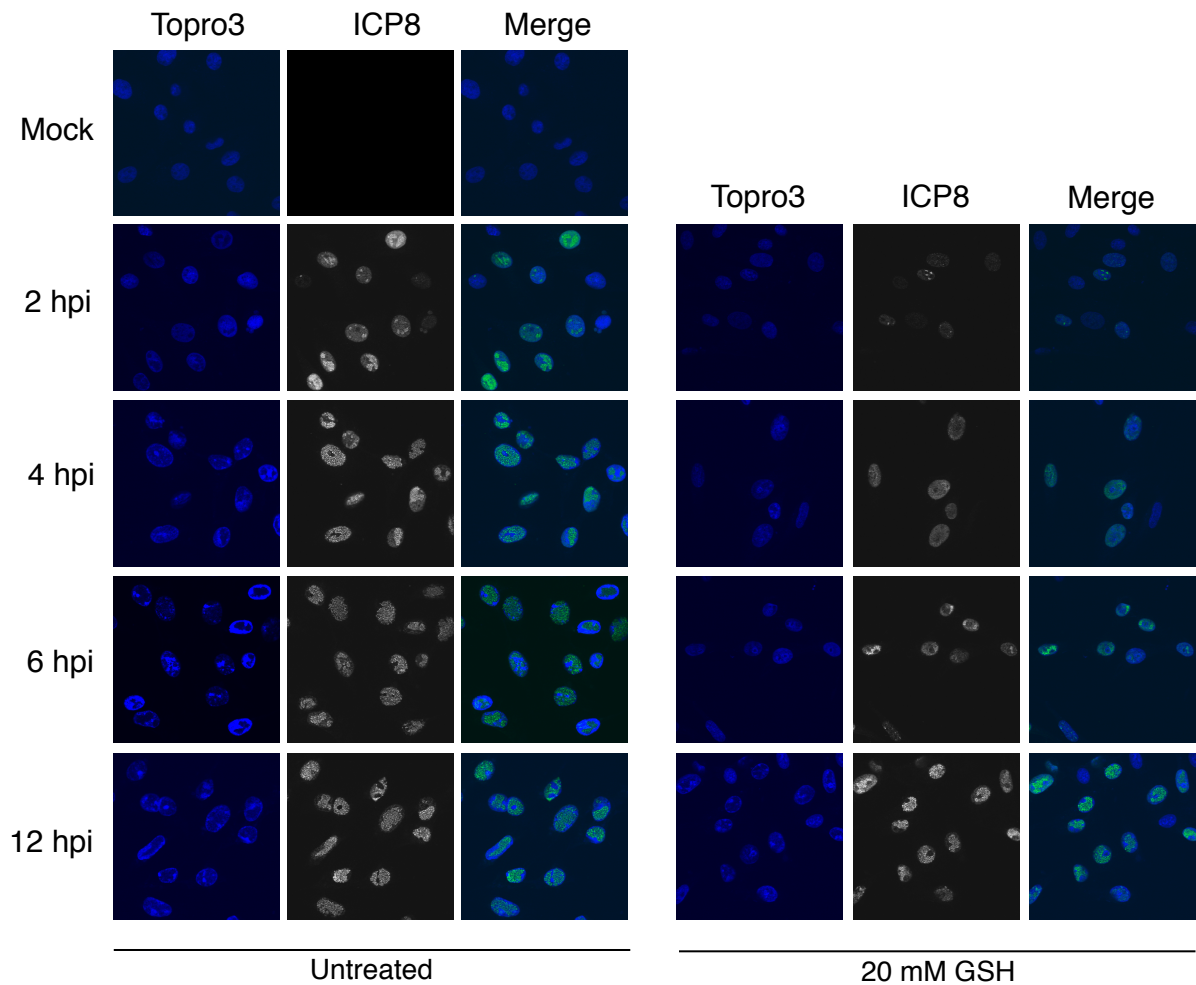


Figure 4-6 Replication compartment formation is delayed in the presence of GSH. NHFs were infected with KOS at an MOI of 5 and incubated in MEM supplemented with 2.5% FBS with or without 20 mM GSH. Samples were fixed in 4% PFA at the indicated times. Cells were permeabilized in 1% Triton-tx and stained with anti-ICP8 antibody (middle panels). The DNA dye, Topro3, was used to stain cell nuclei (blue).

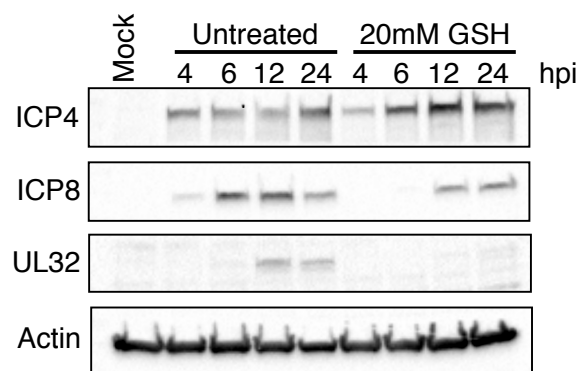


Figure 4-7 Late and true-late protein expression are delayed during GSH treatment. NHFs were infected with KOS at an MOI of 5 followed by incubation in MEM containing 2.5% FBS with or without 20 mM GSH. Samples were harvested in reducing SDS sample buffer and resolved by SDS-PAGE. Proteins were detected by immunoblotting with the indicated antibodies.

expression of IE genes, including itself (DeLuca and Schaffer 1985). Interestingly, at 12 and 24 hpi, ICP4 expression appears more robust in the GSH treated samples than in the untreated samples suggesting its auto-regulatory activity might be impaired in the presence of GSH. The observation that ICP4 is expressed to equal levels at 4 hpi suggests that the viral genome entered the nucleus in GSH-treated cells and is expressing at least one IE gene. On the other hand, the E gene ICP8 showed a delay in appearance and decreased levels, with detectable levels being observed beginning at 12 hpi compared to 4 hpi in untreated cells. Furthermore, the true-late gene, UL32, shows almost no detectable signal even at 24 hpi. In all, these data suggest that in GSH-treated cells the viral genome is able to enter the nucleus, express the first wave of viral genes but that there is a defect in E and L gene expression. Since our results showed that antioxidant treatment blocked replication compartment formation, as well as E and L gene expression, we hypothesized that HSV-induced ROS is important for an early step in the viral lifecycle.

The NOX inhibitor DPI inhibits viral growth

Next, we set out to explore the mechanism behind HSV-1 induced ROS generation. Since NOXs are the main source of deliberate ROS generation and are known to play a major role in other viral infections, we hypothesized that NOXs might be important for HSV infection. To test whether NOXs are responsible for the HSV-induced ROS generation, we used the pan-NOX inhibitor, diphenylene iodonium (DPI). DPI has been reported to attenuate ROS generation in HSV-

infected neural cells; however, this study did not examine the consequences of this treatment on viral growth (Hu, Sheng et al. 2011). NHFs were infected with KOS at an MOI of 0.1 and incubated in media containing 5, 10 or 20 μ M DPI. The equivalent amount of DMSO in the highest (20 μ M) dose was added to a control infection (total concentration of 0.2%). Figure 4-8 shows that at 5 μ M DPI, the viral yield was decreased by over 500-fold at 12 hours with a somewhat less, but still substantial, decrease at 24 and 48 hpi. Similar results were obtained using HEL cells (data not shown). At 10 μ M, the decreases in viral yield are over 1000-fold at every time point and even more substantial in cells treated with 20 μ M. In order to test the effects of MOI on DPI-mediated inhibition, NHFs were infected at an MOI of 5 and treated as described above. Figure 4-9 shows that the effects of DPI treatment were not overcome at high MOI. The decrease in virus production observed in the presence of DPI appears to mimic the growth defects seen in GSH-treated cells at both high and low MOIs.

DPI treatment results in delayed formation of replication compartments

We next wanted to examine replication compartment formation during DPI treatment. NHFs were infected with KOS at an MOI of 5 and treated with media containing DMSO or 10 μ M DPI. We collected the samples at 2, 4 and 6 hpi to monitor early events in the viral lifecycle. Again, ICP8 staining served as the marker for replication compartment formation. In the DMSO control samples, the number of cells that displayed replication compartments progressed from a few at 2 hpi to the

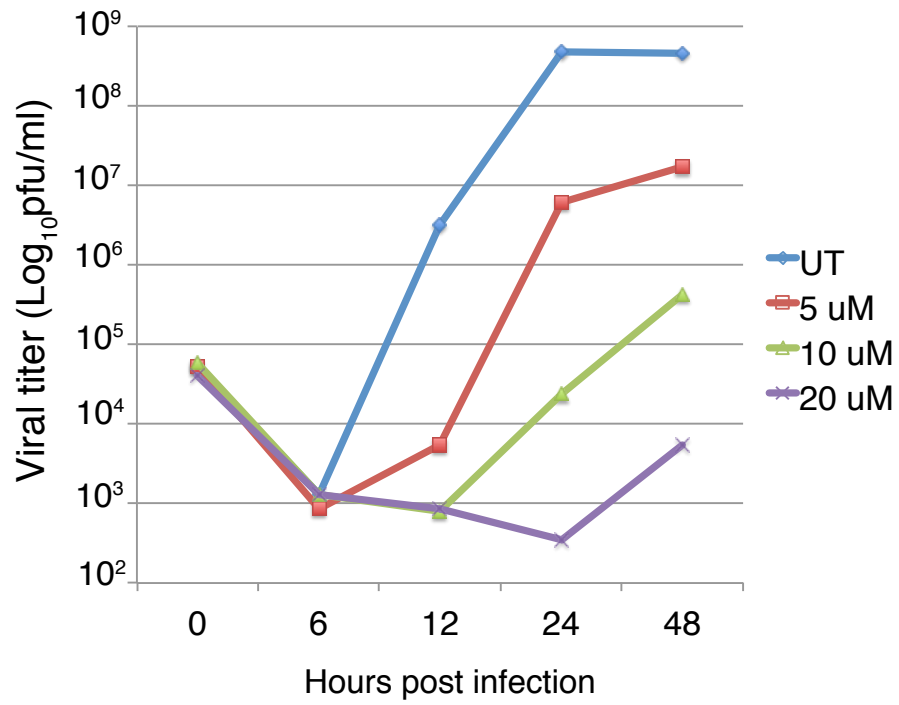


Figure 4-8 DPI treatment impairs growth of KOS on NHFs at low MOI. NHFs were infected with KOS at an MOI of 0.1 and incubated in MEM containing 15% serum with or without varying concentrations of DPI (see legend). Samples were collected at the specified timepoints and growth was measured by titration on Vero cells.

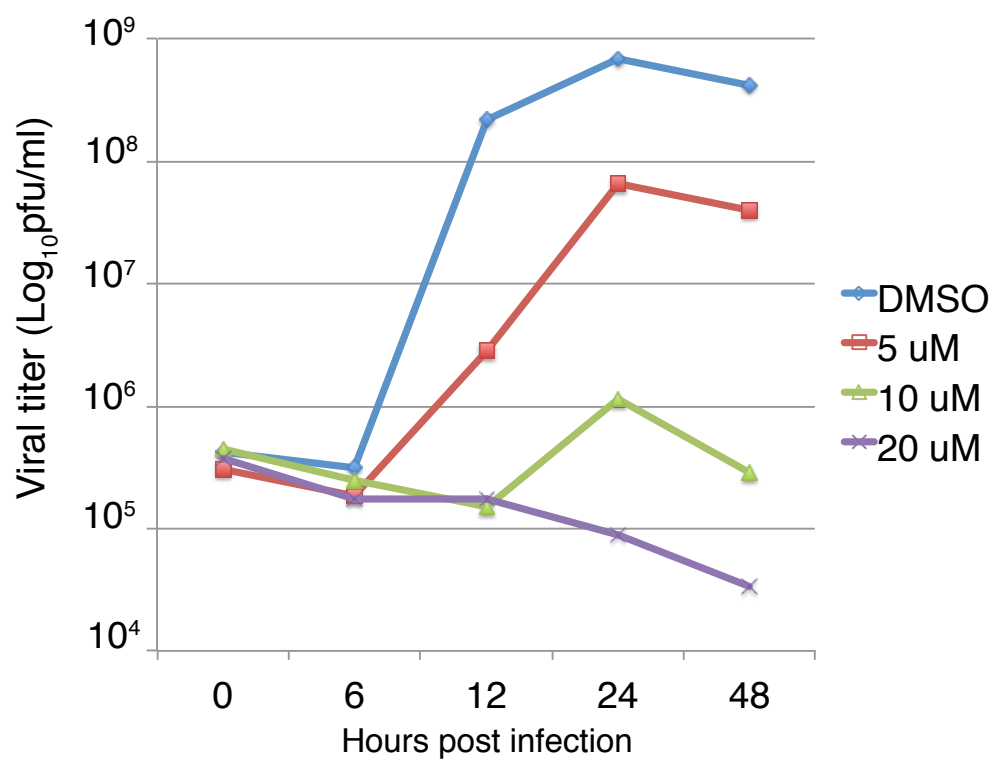


Figure 4-9 DPI treatment impairs growth of KOS on NHFs at high MOI. NHFs were infected with KOS at an MOI of 5 and incubated in MEM containing 15% serum with or without varying concentrations of DPI (see legend). Samples were collected at the specified timepoints and growth was measured by titration on Vero cells.

entire field at 6 hpi. Conversely, in the DPI treated samples, ICP8 staining was very weak compared to the control at each time point. Furthermore, ICP8 fluorescence was found in the cytoplasm in the DPI treated cells, compared to the predominant nuclear staining in the controls. The presence of cytoplasmic ICP8 was surprising and may indicate that either a nuclear import pathway is perturbed, or that ICP8 itself is affected by DPI treatment. Thus, much like the GSH treated samples, replication compartment formation is substantially delayed with DPI treatment.

In this experiment, we also followed NOX4, as a surrogate NOX, to see if it was upregulated during infection. In Figure 4-10, NOX4 staining was very weak in the mock - infected samples, although the fluorescence was slightly brighter in the DPI treated samples. Interestingly, in the untreated cells, NOX4 fluorescence increased at 2 hpi; at 4 and 6 hpi, NOX4 staining was visible in large juxtanuclear bodies. NOX4 also appeared to colocalize with ICP8 within replication compartments. In the DPI treated cells, however, NOX4 staining was mostly diffuse throughout the cell, even when ICP8 staining was nuclear. Since NOX4 has been described as an ER membrane protein (Chen, Kirber et al. 2008), the perinuclear accumulation observed during infection is not surprising and the increasing intensity may just reflect upregulation of the protein. It is striking, though, that we observed NOX4 staining in nuclear replication compartments. We are intrigued by the possibility that the virus hijacks NOX4 and relocalizes it to sites where oxidation might be important. To explore this further, we will first need to confirm that during infection, the antibody is recognizing NOX4 exclusively and not cross-reacting with a viral epitope.

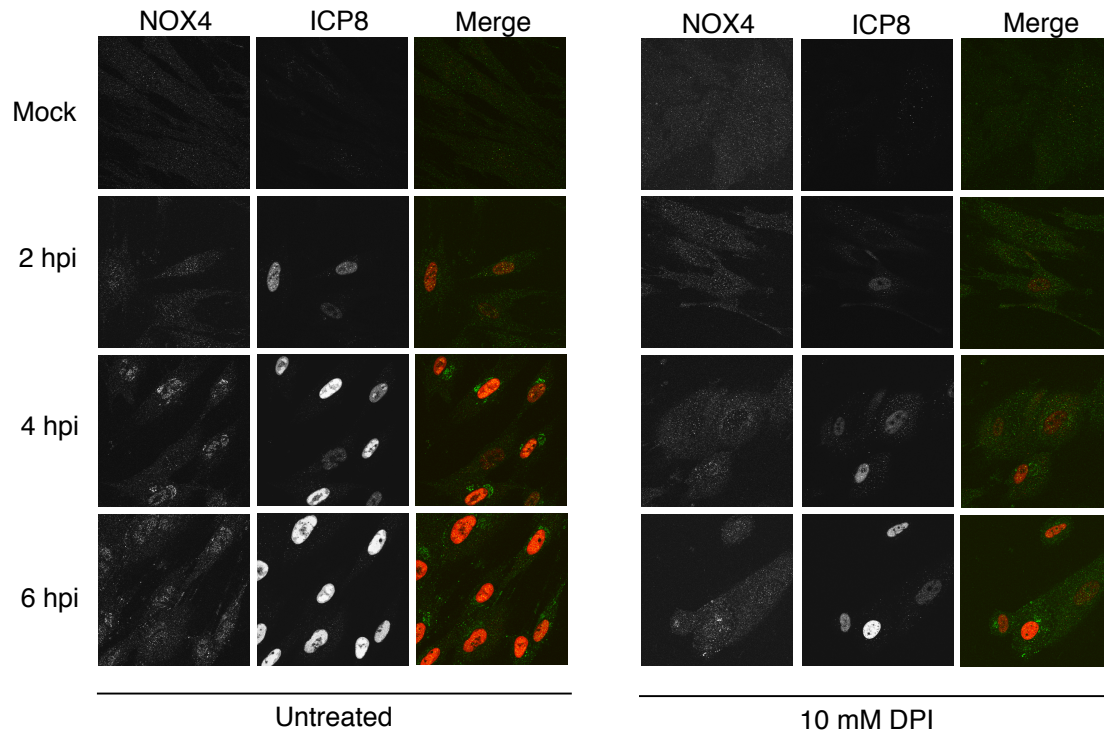


Figure 4-10 DPI treatment delays replication compartment formation. NHFs were infected with KOS at an MOI of 5 and incubated in MEM containing 15% FBS with or without 10 mM DPI. Cells were fixed in 4% PFA at the indicated times, permeabilized in 1% Triton-tx and stained with antibodies against NOX4 and ICP8.

DPI treatment impairs viral gene expression

The defect in viral replication compartment formation led us again to test viral protein expression during DPI treatment. NHFs were infected with KOS at an MOI of 5 and incubated in media containing DMSO, 5 μ M or 10 μ M DPI. Cells were harvested at 3, 6, 9 and 21 hpi in 2x SDS sample buffer. We observed that ICP4 expression could be detected at 3 hpi in both the treated and untreated samples, although the untreated sample appeared more robust (Figure 4-11). ICP8 expression, on the other hand, is severely delayed with no detectable expression until 6 hpi. Although it appears that there is somewhat less ICP4 expression at every time point in the treated samples, this likely wouldn't account for the significant delay in ICP8 expression. What is striking, though, is the difference in the migration pattern of the ICP4 between the untreated and the treated samples. ICP4 is extensively modified during infection (Wilcox, Kohn et al. 1980, Papavassiliou, Wilcox et al. 1991). These modifications, such as phosphorylation and ADP-ribosylation, have been shown to play a role in its regulatory functions. In the untreated samples (Figure 4-11), there is a slight mobility shift beginning at 6 hpi, whereas, the ICP4 in the DPI-treated samples appears to migrate at or near the same position at all of the time points analyzed. These results suggest that inhibiting NOX activity not only attenuates E and L gene expression, but also may alter the phosphorylation status of the major viral transcription factor, ICP4. Collectively, these data show that both antioxidant treatment and inhibition of cellular NOXs inhibits viral growth at an early

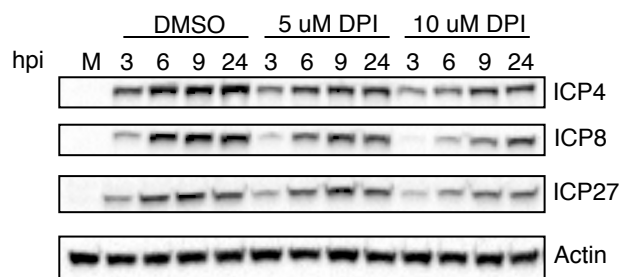


Figure 4-11 DPI treatment delays viral protein expression. NHFs were infected with KOS at an MOI of 5 followed by incubation in MEM containing 15% FBS with or without 5 μ M or 10 μ M DPI. Samples were harvested in reducing SDS sample buffer and resolved by SDS-PAGE. Proteins were detected by immunoblotting with the indicated antibodies.

stage of infection, with both conditions delaying replication compartment formation and altering gene expression.

Discussion

In this study several important and novel findings were reported: 1) a genetically-coded redox probe was used to confirm that the intracellular environment was oxidized in HSV-infected cells; 2) GSH treatment was inhibitory to viral infection in Vero cells, but this was dependent on culture conditions; 3) GSH treatment inhibited viral growth on HeLa cells, and to a greater extent, NHFs and HEL cells at both low and high MOIs; 4) GSH-mediated inhibition was observed at an early stage of infection and correlated with a delay in replication compartment formation and E and L protein expression; 5) treatment with a NOX inhibitor (DPI) reduced viral growth; and 6) DPI treatment severely attenuates replication compartment formation and L protein expression. In summary, these data are consistent with the hypothesis that HSV-1 induces and requires a pro-oxidant environment for efficient infection.

In our initial experiment we used a redox-sensitive GFP system to detect the presence of intracellular ROS. Previous reports showing HSV-induced ROS used the redox-sensitive probes DCHF-DA (Schachtele, Hu et al. 2010, Gonzalez-Dosal, Horan et al. 2011, Hu, Sheng et al. 2011) or HPF (Kavouras, Prandovszky et al. 2007). Although these dyes are routinely used, they suffer from lack of specificity and inadvertent reactions with non-ROS molecules. Even more alarming is that the

oxidation of these probes can lead to ROS as a by-product, thus resulting in an overestimation of intracellular ROS. Redox-sensitive GFPs (roGFPs) were designed to overcome these deficits as they are engineered to react with particular species and they don't create ROS when oxidized. Newer generation roGFPs are linked to specific redox systems. For example, Grx1-roGFP2 is linked to the GSH:GSSG redox couple and has been used to determine the GSH redox potential in subcellular compartments. Another probe, roGFP2-Orp1, is linked to a peroxiredoxin and therefore measures intracellular H₂O₂. In our study, we used roGFP2 to measure the intracellular redox state. We confirmed previous reports that ROS are detected within 1 hpi and are sustained for up to at least 5 hpi (Figure 4-1). Our lab is currently generating stable cell lines expressing roGFP2-Orp1 and Grx1-roGFP2. These will be used to measure HSV-induced H₂O₂ generation and the GSH redox potential, respectively. We are also interested in examining cells expressing these probes during infection using fluorescence microscopy. These data will provide significant insight into the spatial and temporal regulation of ROS during HSV infection.

During this study, we were initially unable to reproduce previous reports that GSH treatment reduces HSV growth in Vero cells and HFFs (Palamara, Perno et al. 1995, Vogel, Cinatl et al. 2005). We then analyzed the differences in our protocols and discovered that while we used DMEM as our basal media, the other reports used MEM and RPMI-1640 for their cell culture. We cultured Vero cells in MEM for several passages and found that GSH treatment inhibited HSV growth under these conditions. One possibility to explain this discrepancy is that DMEM is supplemented

with twice the amount of amino acids and vitamins that are found in MEM or RPMI-1640. It is possible that these extra components induce a robust stress response in Vero cells that is sufficient to abrogate the protection offered by GSH. For instance, if activating a stress response is important for viral infection, and if this activation is prevented by GSH, then it might be possible for the culture conditions to induce activation of that stress response even in the presence of GSH. Our lab has observed that the heat shock protein, Hsc70, accumulates in the nucleoli of our Vero cells cultured in DMEM. Nucleolar accumulation of Hsc70 is a marker of cells that are recovering from stress (Banski, Mahboubi et al. 2010). Thus, under our typical culture conditions, our Vero cells appear to be in a stressed, or near-stressed state.

Another possibility is that DMEM, unlike MEM and RPMI-1640, is supplemented with ferric nitrate, which is a source of iron. Ferric iron can be reduced to ferrous iron in the presence of strong reducing agents in solution. Ferrous iron is then able to undergo Fenton chemistry, which generates the extremely reactive hydroxyl radical. It is possible that the addition of 20 mM GSH could drive this supplemental iron into a free-radical generating redox cycle. This might induce ROS in the media and abrogate the effects of exogenous GSH treatment. It is also possible that GSH uptake is not as efficient in Vero cells cultured in DMEM. Additional experiments will be needed to determine which of these possible reasons can explain the difference in GSH sensitivity observed in cells grown in DMEM vs MEM. Whatever the reason, it is important that issues like these are considered when designing and interpreting redox-related experiments.

Several human cell lines were selected to confirm that GSH treatment was inhibitory for viral growth. For these experiments, we selected two immortalized cell lines, NHFs and HeLa cells, and one primary cell line, HEL cells. We found that viral growth was most significantly inhibited in NHFs and HEL cells. Viral yields were reduced by over 99% in these cell lines at 12 hpi when infection was carried out at an MOI of 0.1. The viral growth defect was even more pronounced during GSH treatment when the cells were infected at an MOI of 5. Viral growth was inhibited by 90% at 6 hpi, which suggests a much earlier defect in growth than the initial report by Palamara, et al (Palamara, Perno et al. 1995). In that study, they reported that GSH inhibited a late stage (24 hrs) of infection based on the following observations: 1) a viral growth defect was detected at 24, 48 and 72 hpi, 2) neither protein nor DNA synthesis were perturbed at 24 hpi in the presence of GSH, 3) EM analysis showed accumulation of intranuclear capsids in GSH treated cells and 4) using a polyclonal anti-HSV antibody, they reported that a band that corresponds to glycoprotein B (gB) is not expressed in GSH treated cells, which would prevent the egress of the capsids from the nucleus. Their overall conclusion was that GSH treatment inhibited viral growth by preventing gB expression.

In addition to the early growth defect, we observed a marked delay in E and L protein expression in GSH-treated cells with the first detectable levels of the E gene ICP8 seen at 12 hpi versus 4 hpi in the untreated cells. Our Western blot data are consistent with our immunofluorescence data showing a dramatic delay in replication compartment formation in GSH-treated cells. Although our results aren't in full agreement with those previously reported, it should be noted that there are major

differences in the designs of these two studies. Palamara, et al only examined viral growth at 24, 48 and 72 hpi, which missed important time intervals that we included in our study (6 and 12 hpi). Furthermore, they looked at viral DNA and protein synthesis, using [³H] thymidine and [³⁵S] methionine, respectively, at 24 hpi. Again, our study included looking at several proteins by WB and IF at much earlier times, giving us a more precise picture of when GSH treatment would exert an inhibitory effect. Thus, the differences in our conclusions are most likely a result of the differences in time points observed and the types of experiments performed. Our data suggest that GSH blocks infection at an early stage of infection.

One of the more interesting revelations in this study is the efficient expression of the immediate early gene ICP4 in GSH-treated cells, but a delay in early proteins and an absence of a true late protein (Figure 4-6). This implies that early gene expression is sensitive to the intracellular redox balance. This finding raises several interesting possibilities. Are viral transcription factors themselves redox regulated? It is interesting to consider that ICP4, the major viral transcription factor, is regulated by redox. To test this, an ICP4 reporter assay could be used to measure its regulation in the presence or absence of GSH. It is also possible that another regulatory gene is regulated by redox. A likely candidate would be the IE gene ICP27. ICP27 has been shown to work in coordination with ICP4 and another IE protein, ICP0 in the activation, as well as the repression of a subset of genes (Sekulovich, Leary et al. 1988, Samaniego, Webb et al. 1995). Interestingly, ICP27 has also been shown to induce oxidative stress (Kim, Choi et al. 2008, Salaun, MacDonald et al. 2010) and to activate the stress kinases NF- κ B, Jnk and p38

(Zachos, Clements et al. 1999, Hargett, McLean et al. 2005, Hargett, Rice et al. 2006). It is possible that ICP27 modulates the activating and repressing activities of ICP4 through inducing cellular kinases. This strategy is not unusual for viruses. For instance, in the case of HIV, ROS induce viral gene transcription by activating the transcription factor NF- κ B. Furthermore, blocking NF- κ B activation with antioxidant treatment prevents viral gene transcription (Nabel and Baltimore 1987, Staal, Roederer et al. 1990). Here, a similar mechanism could be involved where GSH treatment is preventing the activation of a cellular stress kinase that is required for efficient E and L gene transcription.

The most intriguing finding is that inhibiting NOX activity caused cells to be refractory to viral infection. The NOX family of proteins are increasingly being recognized as contributors to the pathogenesis of many viruses, including hepatitis C, HIV and influenza (de Mochel, Seronello et al. 2010, Song, Ju et al. 2011, Vlahos, Stambas et al. 2011, Amatore, Sgarbanti et al. 2015). These viruses are also sensitive to antioxidant treatment (Fraternale, Paoletti et al. 2009). This study is the first, to our knowledge, to implicate a role for NOXs in HSV infection. Using a traditional pan-NOX inhibitor, we showed that viral growth was significantly decreased and that this defect correlated with a decrease in viral replication compartment formation and protein expression. Thus, like cells treated with GSH, early and late gene expression is reduced, RC formation impaired and viral yields decrease. On the other hand, some differences in these experiments should be noted. For instance, the accumulation of cytoplasmic ICP8 in DPI treated cells (Figure 4-10) was surprising and not observed during GSH treatment (Figure 4-6). It

is possible that DPI treatment prevented the efficient intranuclear transport of ICP8. Another possibility is that ICP8 itself is sensitive to a reducing environment. Our lab has observed that ICP8 is sensitive to oxidation and can form disulfide-mediated crosslinks in the presence of H_2O_2 (data not shown). A property of ICP8 is that it forms filaments which appear to be required for the formation of replication compartments (Darwish, A., et al, unpublished). Thus, it is possible that NOX-derived ROS contributes to ICP8 conformation or self-interactions in order for efficient filament formation. This might explain the presence of NOX4 in viral replication compartments. However, we will need to perform a similar study of replication compartment formation in cells that have been depleted of NOX4 (through shRNA knockdown) to confirm that the antibody we used is recognizing NOX4 and not another cellular or viral protein that accumulates in replication compartments.

We also observed that DPI treatment decreased the mobility shift in ICP4 (Figure 4-11) during DPI treatment; whereas, GSH treatment resulted in overexpression of ICP4. The phosphorylation status of ICP4 is important for its regulatory functions (Papavassiliou, Wilcox et al. 1991). This limited shift in mobility suggests the possibility that ICP4 phosphorylation was inhibited when NOX activity was prevented. These results are preliminary, but we are intrigued by the idea that NOX-derived ROS activates stress kinase pathways. Even more exciting would be the possibility that these kinases are responsible for phosphorylating ICP4 to regulate the gene cascade. Although there were some slight differences in our results when comparing the two treatments, it is obvious that facilitating a reducing

environment, either by treatment with antioxidants or by inhibiting ROS generation, is deleterious for viral growth. In fact, it is rather striking that these two treatments exhibit so many similarities.

In this study, we have taken a comprehensive look at the role of ROS during HSV-1 infection. Since Palamara, et al, first reported the relationship between HSV and ROS in 1995, only a few studies have examined this. Several have reported indirect markers of ROS, such as the accumulation of carbonylated (oxidized) proteins (Mathew, Bryant et al. 2010) or decreases in intracellular GSH (Nucci, Palamara et al. 2000, Vogel, Cinatl et al. 2005), while others have directly measured oxidation with fluorescent dyes (Schachtele, Hu et al. 2010, Kavouras, 2007 #247, Gonzalez-Dosal, Horan et al. 2011, Hu, Sheng et al. 2011). Our experiments are consistent with those of others suggesting that HSV-1 requires a pro-oxidant environment. Since similar results have have been reported in many other non-related viruses, regardless of their genome, replicative cycle or other properties, we suggest that evolving to survive and thrive in an oxidizing environment is a common strategy for pathogens. It is likely that ROS were originally generated in cells to combat infection. Since ROS contribute to genetic mutations, this cellular antiviral response could actually be advantageous for the virus. Genetic mutations have been shown to be necessary for increasing the fitness of viruses (Pfeiffer and Kirkegaard 2005). Thus in the interplay of virus and host, ROS, which originally served as a potent antiviral, may now be more beneficial for viruses than for their hosts.

In summary, we conclude that HSV-1 growth is impaired if ROS levels are attenuated, either through the addition of antioxidants or through preventing

intracellular sources of ROS generation. Since early gene transcription and replication compartment formation are impaired when cellular ROS are prevented, we propose that HSV-1 generated ROS are mediators of signaling cascades that allow for the proper regulation of viral gene transcription. Future directions will be aimed at solidifying the role of cellular NOXs during infection. We will need to use inhibitors that target the NOXs more specifically, such as VAS2870 (Wingler, Altenhoefer et al. 2012). Also, a more targeted approach will be needed to define which NOXs, if any, are required for viral infection. Which NOXs are expressed in our cell types and how does their expression change during infection? Quantitative PCR analysis would allow monitoring of the transcription of all of the NOX family members during infection. Thus, candidate NOXs could be identified and investigated more thoroughly. Although not fully complete, the data we have presented has given us an exciting new avenue to pursue.

Chapter 5

Summary and Perspectives

A number of pathogens, viral and bacterial, induce oxidative stress (OS) upon infection (Beck, Handy et al. 2000, Fraternale, Paoletti et al. 2009, Paiva and Bozza 2014). Since reactive oxygen species (ROS) have the potential to cause damage to most macromolecules, OS would be a potent way to combat pathogens. In fact ROS is a common cellular response to infection and are produced by stimulated macrophages and other immune cells to clear pathogens. Interestingly, it now appears that many pathogens have evolved to use increases in ROS for their own benefit. It is surprising that tolerance and even preference for an oxidizing environment has been shown in many organisms including viruses and bacteria (Beck, Handy et al. 2000, Fraternale, Paoletti et al. 2009, Paiva and Bozza 2014).

The interplay between virus and host has gone on through millennia. Understanding how viruses have evolved to use specific cellular pathways not only gives us a snapshot of the biological processes that have shaped the virus and host, but have also proven to be useful in designing therapeutics. For instance, HIV therapies have come a long way since the first reverse-transcriptase inhibitors were developed (Zolopa 2010), but treatment doesn't cure infection due to a reservoir of cells harboring latent virus (Shan and Siliciano 2013). Current research is aimed at reactivating latent HIV, which in combination with anti-retroviral therapy could cure HIV by depleting these reservoirs. Yang, et al, found through a high throughput

screen that the compound 5-hydroxynaphthalene-1,4-dione reactivated latent HIV by generating ROS (Yang, Xing et al. 2009), thus underscoring the importance of understanding the virus-ROS relationship.

The goal of the work presented in this thesis was to provide insight into the induction and consequences of HSV-1 induced ROS. In the process, we uncovered some unexpected, and exciting, new ways in which the virus might benefit from ROS through disulfide bond formation. We also discovered that the viral DNA packaging protein, UL32, is a possible oxidoreductase, which would allow the virus to cope with the increasingly pro-oxidant environment. Lastly, we presented evidence that preventing ROS through inhibition of cellular pro-oxidant enzymes, or treatment with antioxidants, inhibited infection at an early stage and may be linked to a regulatory role in gene expression. Thus this thesis has advanced our understanding of how HSV may use and cope with the intracellular redox balance to establish infection.

Disulfide bond formation is important for HSV-1

In Chapter 2 we showed that disulfide bond formation was essential for the formation and stability of the UL6 portal ring (Albright, Nellissery et al. 2011). Consistent with this, another report from our lab showed that disulfide bond interactions were ubiquitous throughout the capsid (Szczepaniak, Nellissery et al. 2011). Only one other paper showed the presence of disulfide bonds in HSV capsid proteins (Zweig); however, we were the first to illustrate the role these interactions were playing. Furthermore, we were able to map the cysteines responsible for the

UL6 portal ring formation and stability. A surprising observation was that the UL6 portal possibly formed disulfide bonds in the nucleus. Although we knew that HSV-1 had the potential to make the cell more oxidizing, we did not know the extent of the oxidation nor that oxidation occurred in the nucleus. The presence of disulfide bond formation in the nucleus suggests that the nucleus is oxidized to such an extent where these interactions could occur. This work leads to some important questions. How oxidized is the nucleus during infection? How is it being oxidized? How are these disulfide bonds formed? And most importantly, why are these disulfides present? One of the most important characteristics of a virus is that it must be metastable. The virion has to protect the genome from a harsh extracellular environment, but also be able to free the genome at the proper place. We propose that disulfide bonds within the capsid and portal serve as molecular sensors, and that they are destabilized once the virus enters the reducing environment of the host cell cytoplasm (Figure 5-1).

HSV-1 encodes a redox-sensitive chaperone

Prior to the work presented in thesis, the role of UL32 had not been clear. It has always been associated with the DNA packaging step based on the fact that UL32-null mutants have no defect in protein or DNA synthesis, can form capsids but do not cleave package DNA (Schaffer, Aron et al. 1973, Lamberti and Weller 1998, Albright, Kosinski et al. 2015). When we examined the primary peptide sequence, we found a series of conserved motifs we believed could tell us more about possible functions of the protein. A UL32 homologue was identified in every herpesvirus we

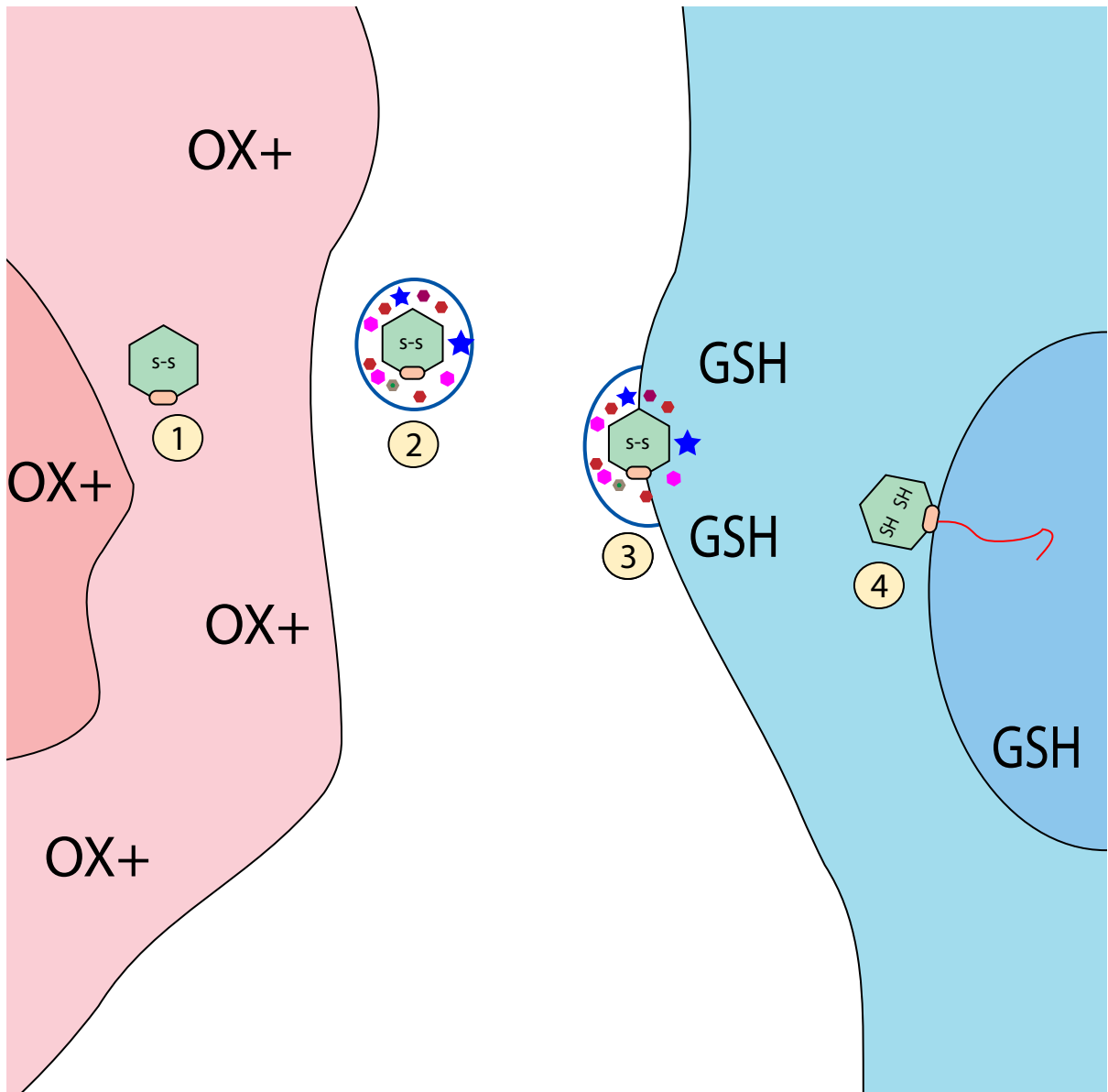


Figure 5-1 Model for disulfide bonds as molecular sensors to trigger genome release. 1) Viral capsids egress from the nucleus of an HSV-1 induced oxidized cell. Disulfide bonds are formed and stabilize the capsid. 2) The capsid remains oxidized in the extracellular environment. 3) Upon entry, the viral capsid is exposed to the reducing environment of the cell and begins to destabilize as it's trafficked to the nucleus. 4) reduction of disulfide bonds within the UL6 portal and/or the viral capsid trigger the release of the genome into the nucleus.

examined, and even more interesting, almost all of these homologues had conserved C-X-X-C motifs (Figure 3-3). C-X-X-C motifs are found in redox-sensitive proteins, such as thioredoxin and other oxidoreductases, and have been shown to be involved in regulating disulfide bond formation through a two-step dithiol-disulfide exchange reaction (Figure 5-2)(Chivers, Laboissiere et al. 1996, Chivers, Prehoda et al. 1997, Fomenko and Gladyshev 2003). This observation led us to test the hypothesis that UL32 is a redox-sensitive protein that influences disulfide bond formation in viral proteins.

If true, the presence of a virally encoded oxidoreductase would be a remarkable evolutionary strategy to survive in a pro-oxidant environment. Reactive cysteines that can form disulfide bonds are found ubiquitously throughout the capsid and it is probable that an oxidizing environment would cause a lot of aberrant disulfide bonding to occur. Thus, we provide an example of a protein that may allow HSV to cope with this problem. We generated a UL32-null virus and found that in the absence of UL32, aberrant disulfide bond formation was observed in several of the capsid proteins. Of all of the proteins that exhibited differential disulfide bond formation in the absence of UL32, the UL6 portal and the viral protease, VP24, were of the most interest. Both of these proteins are absolutely essential for the DNA packaging reaction which is consistent with the defect seen in the UL32 null. The fact that VP24 had a different disulfide bond profile in the absence of UL32 is very intriguing. It has been shown that the viral protease in another human herpesvirus, CMV, contains reactive cysteines and its proteolytic activity is regulated by disulfide bonds (Baum, Ding et al. 1996, Baum, Siegel et al. 1996). Our results suggest that

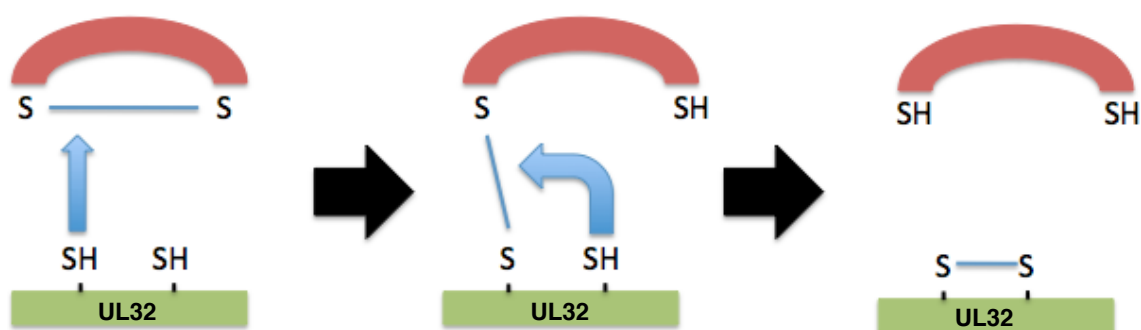


Fig. 5-2 Model for UL32 oxidoreductase activity. In a standard dithiol-disulfide exchange reaction, an oxidoreductase enzyme will interact with a disulfide bonded substrate. One cysteine in the conserved C-X-X-C motif is extremely reactive and forms a disulfide bond with one of the cysteines in the substrate forming a mixed disulfide. This is followed by the formation of a disulfide bond between the two cysteines within the C-X-X-C motif of the oxidoreductase with the complete reduction of the substrate.

VP24 may also be regulated by disulfide bonds which opens up an entire new area of research. During viral DNA packaging, there is a complex series of events that involves the translocation of the genome into the viral capsid and a cleavage and release of the internal scaffold; however, the order of these events and what triggers them is not understood at all. The idea that disulfide bonds regulate proteolysis of the internal scaffold is another example of how the redox environment serves an important role in the lifecycle of HSV. It will be important to determine whether UL32 directly modulates disulfide bonds in VP24 to trigger this event, or if it plays a peripheral role. In 2003, a genome-wide proteomic screen revealed 23 potential viral oxidoreductases in nature, with all of them belonging to dsDNA viruses (Kho, Newman et al. 2003), however the vaccinia virus oxidoreductase has been the only one, to our knowledge, that has been validated and studied. In this system, there are three viral proteins (E10R, A2.5L and G4L) which participate in a thiol-disulfide relay responsible for modulating disulfide bonds in the cytoplasm (Senkevich, White et al. 2002). It would be interesting to see if UL32 is involved in a similar chain. Thus, the work presented in this thesis not only is the first to describe a putative virally-encoded oxidoreductase in HSV, but is also the first to suggest disulfide bond formation as a regulator in viral protease activation.

HSV-1 induced oxidative stress may play a role in gene regulation

In Chapter 4, we follow up on previous work showing that HSV induces the generation of ROS and the virus is sensitive to antioxidant treatment (Palamara, Perno et al. 1995, Nucci, Palamara et al. 2000, Vogel, Cinatl et al. 2005, Schachtele,

Hu et al. 2010, Hu, Sheng et al. 2011, Qiu, Chen et al. 2013). In this study, we showed that antioxidant treatment blocked HSV infection at an early stage of infection. Furthermore we show that the virus is able to express immediately early proteins (the first wave) in the presence of GSH, but that early and late proteins aren't expressed as efficiently. We also observed a similar defect in viral growth by inhibiting ROS-generating enzymes, more specifically the NADPH-oxidases (NOXs). NOX inhibition also led to a delay in the formation of viral replication compartments. In all these data suggest that ROS, possibly generated by NOXs, might be important for an early stage of infection between immediate early gene expression and early gene expression.

The results summarized above suggest that it is possible that HSV-induced ROS stimulate gene transcription through activating redox-sensitive transcription factors. If so, this would be reminiscent of the role ROS play during HIV infection. It has been shown that ROS activate the transcription factor NF- κ B, which upregulates the expression of HIV genes (Nabel and Baltimore 1987, Staal, Roederer et al. 1990). The exciting observation that HSV-1 might induce ROS for regulating its own gene transcription has several implications. It is known that stress reactivates HSV from latency, however, the reasons for this have not been fully worked out. In 2009, it was shown that the viral transcription factor VP16 was required for reactivation from latency and that the VP16 promoter was activated in the presence of hyperthermic stress in the absence of other viral proteins (Thompson, Preston et al. 2009). Furthermore, a much older paper reported that VP16 is responsible for activating the p38/Jnk pathways (Zachos, Clements et al. 1999). Is it possible that

VP16 expression is ROS dependent during lytic infection? It has also been shown that the immediate early protein ICP27 induces ROS and the activation of NF- κ B. ICP27 is also a regulator of late gene expression (Hargett, McLean et al. 2005, Hargett, Rice et al. 2006, Kim, Choi et al. 2008, Li, Johnson et al. 2008, Salaun, MacDonald et al. 2010). Thus, HSV-1 induced ROS might work on multiple levels to facilitate the onset and progression of lytic infection.

Final thoughts and future directions

The work presented here has led us to propose the following model. We suggest that upon infection, the viral capsid is exposed to a reducing cytoplasmic environment, which destabilizes the capsid and possibly the portal, triggering the release of the genome through the nuclear pore. During this time, the virus induces a change to a more oxidizing state, potentially through cellular NOXs, which results in the activation of viral transcription factors to regulate early and late gene expression. During lytic infection, viral structural proteins are made and transported to the nucleus. As these capsid proteins have a lot of reactive cysteines, the viral oxidoreductase, UL32, uses a dithiol-disulfide exchange reaction (Figure 5-2) to prevent aberrant disulfide bond formation and to allow the assembly of capsids that are competent for encapsidation. Furthermore, we propose that UL32 might also regulate the viral protease, triggering its activation at some step of the packaging

process to allow the release of the internal scaffold. This model is preliminary, however, and will require a lot more work to be validated.

The ubiquitous presence of disulfide bonds throughout the capsid and in the portal is interesting but there are significant gaps in our knowledge as to when and where they form. We have observed disulfide bond formation in the portal ring in capsids isolated from the nucleus. The viral capsid proteins, on the other hand, seem to form disulfide bonds only when they are in mature virions in the extracellular environment (Szczepaniak, Nellissery et al. 2011). Interestingly, UL32 has a unique cellular distribution during infection. It is located within replication compartments at early times, consistent with its role in DNA encapsidation, but then accumulates in the cytoplasm at later times. It is then possible that UL32 keeps the egressing capsids in a reducing state until they reach the extracellular environment. Since UL32 is not present in the extracellular virion, this would allow the disulfide bonds within the capsid to form. Since the defect in UL32 is at the packaging step, it is impossible to test this hypothesis using a UL32 null. There is, however, a temperature sensitive UL32 virus that could be used for such a study. For instance, cells could be infected at the permissive temperature until the packaging reaction is complete, and then shifted to the non-permissive temperature, thus abrogating its function. This would allow us to assign a post-packaging role to UL32. If it was involved in keeping the disulfide from forming in the cytoplasm, the predicted result from this experiment is that we would detect disulfide bond formation in capsids earlier than previously published (Szczepaniak, Nellissery et al. 2011). It would also be important to show that UL32 does in fact interact with the viral capsid. Generating

a UL32 mutant virus in which one of the cysteines within the C-X-X-C motif is mutated would be a possible way to test this. As can be seen in Figure 5-2, both cysteines participate in the dithiol-disulfide exchange. If the second cysteine in this reaction was mutated, there would be no resolution of the mixed disulfide, thus trapping UL32 with its partner. The most exciting experiments, however, would be testing whether UL32 was responsible for regulating protease activity. It was previously reported that incubation of the protease in various solvents and anions stimulated its activity by several fold (Hall and Darke 1995). These data suggest that the these conditions allowed for a conformational change in the protease, most likely by allowing a buried hydrophobic pocket to be exposed, which regulated its activity. It would be exciting if a similar protease assay could be used to test the hypothesis that UL32 modulates disulfide bonds within the protease to induce a conformational change, thus leading to its activation.

Another exciting result presented in this thesis is the idea that HSV-1 induced ROS, possibly through NOXs, is important for the proper regulation of gene expression. The results in this thesis are very preliminary, but our results suggest that viral infection is blocked at an early step in the presence of antioxidants or if NOX activity is blocked with a chemical inhibitor. First, we need to establish how HSV induces ROS. Although we showed a block in infection using a pan-NOX inhibitor, it would be important to show that NOXs are in fact inducing ROS. For this line of research, an expression profile should be done on all of the NOX family members during infection. It has been shown that upregulation of NOX expression is correlated with NOX activity (Amatore, Sgarbanti et al. 2015). Thus, this would allow

us to select candidate NOXs for shRNA knockdown studies to confirm their role in HSV infection. Infected NOX knockdown cells could be monitored for ROS generation, viral protein expression and viral growth. It would also be important to test whether mitochondrial ROS contributes to HSV-1 infection. This could be done using Mito-Tempo, which is an antioxidant that is targeted to the mitochondria. Furthermore, it would be exciting to link the ROS to activation of specific pathways that might influence viral gene expression. It would be interesting to identify all of the proteins that are associated with the viral genome during infection with and without antioxidant treatment. It would be exciting if stress-related transcription factors were associated with specific promoters, which could be done using chromatin immunoprecipitation (ChIP), during normal conditions but absent in the presence of antioxidants. ChIP could also be used during inhibition or knockdown of NOXs.

In this thesis, we have explored, and found many novel links between HSV and oxidative stress. As exciting as these results are, they have only set the stage for a much more comprehensive body of work. The role of oxidative stress in viral infection will undoubtedly gain momentum with increasing technologies and it will be exciting to see how the results from these studies contribute to our understanding of virology, cell biology and to diseases in which oxidative stress plays a significant role.

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